THE ENZYMES

CHEMISTRY AND MECHANISM OF ACTION

VOLUME I, PART 1

The Enzymes

CHEMISTRY AND MECHANISM OF ACTION

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VOLUME I, PART I

ACADEMIC PRESS INC., PUBLISHERS

NEW YORK · 1950

First Printing, November 1950
Second Printing, December 1951

PRINTED IN THE UNITED STATES OF AMERICA

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Foreword

Recent years have seen a remarkable advance in every phase of enzymology. Indeed, research in this field has progressed to such an extent that a survey of its findings in comprehensive form has become increasingly imperative. Enzymology has become the central province of biochemistry. Enzymes are the principal tools of the living cell; non-enzymatic substances and conditions known to be important for the functioning of living matter in an ever-increasing number of cases have been shown to exert their action by means of enzymes, sometimes as components of enzyme systems, and in other instances by regulating the rate or mode of action of an existent enzyme. It is therefore self-evident that enzymology must form an essential part of all biological sciences. Equally, the problem of the chemical nature of enzymes and its relation to their catalytic activity is one of the most intriguing questions of organic and physical chemistry. Enzymes have been prepared in a pure state, but we cannot yet explain how they exert their enormous and highly specific catalytic action. All that can be done at the present time is to gather and sift available knowledge, present it in an orderly fashion, and try to utilize it for the advancement of enzymology. It is our aim, therefore, to present systematically the accumulated knowledge in the various phases of enzymology as a comprehensive survey which will be of the most efficient service both to those already working in the field and to those preparing to enter it. In order to accomplish this purpose adequately a great number of our colleagues—each of them an authority on certain aspects of the subjects—have been invited to cooperate in this undertaking. In all, seventy-eight scientists in the United States, Europe, and Australia have united in this endeavor, and it is their sustained interest and effort which has made our project possible.

It is understandable that a treatise by different authors should be less homogeneous than a book written by one, were such a work on enzymology possible in our times. However, the editors have thought it better to give the authors considerable liberty in the composition of their chapters than to try achieve a uniformity which could in any case be only superficial. The editors are well aware that a certain amount of overlapping is inevitable between certain chapters, but they deemed it more helpful to the reader to have the individual chapters as complete as possible without too many cross-references.

In the treatise, an introduction into the special chemistry of the various enzymes is given in a section consisting of eight chapters on general questions of enzymology, such as the formation of enzymes, their role in adapta-

tion, their cytological foundation, their relation to other biologically active substances and to immunochemistry. Sections dealing with the physical chemistry of enzymes, the kinetics of enzyme reactions and the inhibition of enzymes by chemical reagents have also been included here. In a special part of the work chapters have been devoted to all enzymes which can be said with any certainty to possess an individual existence and which are reasonably well known. The editors are aware that reactions, said to be enzymatic, have been reported which have not been mentioned here. The reason for the omission is, in the majority of cases, that the enzymatic nature of the reactions seems very doubtful.

Our general idea has been that this work should be a treatise of enzymology; we did not intend to present to the initiated reader recent developments in the chemistry of certain enzymes, or of certain enzymatic processes, but to present, to any reader, the chemistry of the enzyme or the mechanism of a process as a whole—in other words, to present as far as possible, every fact, either old or new, which has a bearing upon the general understanding of the problem in question. In order to achieve this object, it has not been considered necessary or even appropriate to include a comprehensive survey of practical methods used in enzymology. Therefore, methods have been described in these volumes only to the extent that they are required for a complete understanding of the subject.

When trying to systematize the special chemistry of enzymes, one is confronted with various difficulties. The basis of the classification of enzymes is their specificity, and on the principle of reaction and substrate specificity hydrolytic enzymes and certain others can be classified fairly well. However, in other cases it does not seem possible or suitable to adhere strictly to this scheme. In a few instances, the nature of the prosthetic group of an enzyme can be used as the basis of classification, for example, with enzymes containing iron, copper, etc. But with many enzymes, especially those involved in dissimilations known as fermentation, respiration, etc. it has appeared desirable not only to describe the individual enzymes but also to give comprehensive chapters on the mechanisms as such. In a few cases, so very little is known about the participating enzymes that only an overall description of the complex reactions has been presented. The closing chapters of the work are devoted to tumor enzymology and to enzyme technology.

It must be recognized that a very considerable expenditure of time is involved in the preparation of a work of this kind, and that new material will have inevitably appeared in the literature before the release of the subsequent parts of the work. We hope, however, that most of the pertinent information available at the time of publication will be contained in each volume.

The publication of a work of this scope represents a somewhat venturesome enterprise on the part of the Publishers, and the Editors wish to acknowledge the service which the Publishers are rendering to the cause of our science.

An Author and Subject Index will be included at the end of Volume I, Part 2 and Volume II, Part 2. The Subject Indexes will be prepared by Dr. Martha Sinai.

We regret to report that one of our most illustrious associates, Professor Leonor Michaelis, died a short time ago. This is not the proper place to enter upon an evaluation of his work and its importance for enzymology. May it suffice to state that many of his investigations form a lasting part of the fundamentals of biochemistry—of which the reader will find ample evidence in the subsequent pages. Perhaps the chapter written by Michaelis for this treatise may be considered his last contribution to science.

JAMES B. SUMNER
KARL MYRBÄCK

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Chapter 1

Introduction

By JAMES B. SUMNER and KARL MYRBÄCK

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I. The Role of Enzymes

One way of defining life is as an orderly functioning of enzymes. Disease manifests itself as a disorder, inhibition, or hyperfunction of enzymes. All sorts of chemical changes take place in living matter; most of these changes do not occur spontaneously. On the contrary, these reactions are so slow that many of them by themselves would not take place at all. The reason why the living cell can bring about these reactions is that it possesses an equipment of catalysts, the enzymes. Practically all reactions which occur in organisms can be attributed to the action of enzymes and enzymes and genes can be called the fundamental units of life.

Only within the past fifty years has the significance and importance of enzymes in biology been fully realized. At the present time the enzyme

field has become greatly broadened, and it now interests the bacteriologist and the researcher in natural sciences as well as the biochemist and the physiologist. Enzymic reactions and the quantitative determination of enzymes are acquiring more and more importance in clinical medicine. Enzymic reactions are of immense value in industrial chemistry.

New enzymes are being discovered every year, and there is little doubt that a great many others await detection. Enzyme chemistry and its application in science and technology is in a state of continuous and rapid evolution.

Enzymes are present in all organisms, both in the cells and in such fluids as plant sap, blood plasma, saliva, gastric juice, urine, and milk. Some enzymes seem to occur dissolved in the cytoplasm; many appear to be bound somehow to cell structures such as mitochondria and different types of granules.

Since hundreds of chemical reactions are necessary for the existence, growth and reproduction of organisms it is readily seen that the enzymes which catalyze these reactions must possess a high degree of efficiency in order that they may all be packed into cells. Enzymes are effective in bringing about chemical reactions at relatively low temperatures and in approximately neutral solutions in water, whereas the catalysts frequently employed by the organic or industrial chemist to bring about the same reactions, i.e., strong sulfuric acid, potassium hydroxide, chlorine, are violently corrosive and often require a temperature of 100° or more.

II. Definitions

An *enzyme* or *ferment* is defined as a catalyst of biological origin, possessing a high molecular weight. The term ferment (Latin, *fermentum*) has been used for centuries; the name enzyme (from Greek εγζυμη, literally "in yeast" was introduced by Kühne in 1878.

In the light of our modern conception of the chemical nature of enzymes it seems extremely probable that all enzymes are proteins or contain a protein component. Therefore the definition above can be written: "Enzymes are specific, catalytically active proteins, simple or conjugated." It was supposed earlier, as for instance by Willstätter, that all enzymes are composed of an "active prosthetic group" and a "colloidal carrier." However, it seems clear by now that many enzymes are proteins without any detectable prosthetic groups.

A *catalyst* (Berzelius, Mitscherlich, W. Ostwald) is a substance which influences the velocity of a chemical reaction without being used up in the reaction. The statement, that catalysts act "by their mere presence" is certainly not correct. Catalysts take part in reactions, but they reappear in their original form. They change during the reaction, but they describe a cycle and theoretically a catalyst can convert an unlimited amount of

reacting substance. In practice the amount is limited because the catalyst sooner or later becomes irreversibly changed by side reactions. However, very often the amount of reaction product is extraordinarily large as compared to the amount of catalyst used.

In enzyme chemistry we are, on the whole, concerned only with positive catalysis; the enzymes accelerate reactions, which are, in themselves, thermodynamically possible, i.e., are attended by a loss of free energy. However, in order to make a reaction take place, which is in itself possible, there is a certain resistance to be overcome. In other words, the molecules must be activated; a certain amount of "activation energy" has to be supplied. We can describe enzyme action by stating that the enzyme lowers the amount of activation energy required by the reaction. In reality the "acceleration" of a reaction is often equal to the "bringing about" of the reaction which otherwise does not occur under the prevailing experimental conditions.

Enzymes differ from other catalysts in several respects. These differences are of course due to the chemical nature of enzymes and many of these differences are dependent on properties more or less common in all proteins. Thus, enzymes are thermolabile; they are inactivated at certain temperatures in a manner characteristic for protein denaturation. But the difference between enzymes and other catalysts displays itself most clearly in one respect: enzymes generally have an extremely *specific action*. Whereas metallic nickel, for instance, can be used for the hydrogenation of a great variety of substances, the enzymic hydrogenation of different substances requires a whole series of different enzymes, each specific for one substance, or a small group of substances. The substances on which enzymes act specifically are called the *substrates* of the enzymes. Enzyme action is not only limited to one type of reaction as the hydrolysis of a glycosidic linkage, the desmolysis of a carbon-carbon bond, dehydrogenation, *etc.* (*reaction specificity*), but limited also to a few individual substrates or in many cases to one substrate (*substrate specificity*). This specificity is the basis of enzyme classification.

III. Terminology and Classification

Those enzymes that were the first to be discovered were given names in an unsystematic manner, e.g., diastase, emulsin, ptyalin, pepsin, trypsin. In 1883 Duclaux introduced the custom of naming an enzyme after the substance (substrate) upon which it acts, followed by the ending "-ase," thus peptidase, esterase, and urease. However this principle can not be realized without many exceptions. Sometimes, especially in the case of typical reaction specificity, it has proven necessary to name enzymes after reactions they catalyze. Such names are dehydrases (enzymes removing water), desulfurases (removing hydrogen sulfide), and dehydrogenases

(removing hydrogen). In many cases we are compelled to retain the old unsystematic names or even to find out new unsystematic ones, because the specificity of an enzyme does not always allow a rational nomenclature. The name pepsin is retained for the proteolytic enzyme most active at pH 1.5 and originating from the gastric mucosa of mammals. Its action is certainly a hydrolysis of peptide bonds, but it is limited to peptide bonds between amino acids of definite constitution. In this case the substrate specificity cannot be defined in a simple formula and accordingly no rational name can at the present time be given to this enzyme.

In some cases old unsystematic names have been retained as designation for certain characteristic raw materials, e.g., sweet almond emulsin (β-glycosidase).

A few enzymes have been named after the substances which they synthesize with the ending "-ese" to signify a synthetic action. Thus we have rhodanese, which forms rhodanate irreversibly from hydrocyanic acid and sodium thiosulfate or colloidal sulfur. It would be superfluous and inappropriate to use this designation in the many cases where an enzyme catalyzes degradation of a substrate and its synthesis as well.

The specificity of enzymes is the foundation of the system of classification. First of all it is possible to define a large group of enzymes as hydrolyzing enzymes, or hydrolases. Certain linkages between carbon atoms on one hand and nitrogen, oxygen, or sulfur atoms on the other, are ruptured by these enzymes, and water is taken up. Within the group of hydrolases classification follows the substrate specificity. Thus we have esterases, glycosidases, peptidases and amidases. These groups can be further subdivided: the esterases comprise lipases (hydrolyzing fats), esterases in a limited sense (hydrolyzing simple esters of fatty acids), acetylesterase (acting preferably on acetic acid esters), tannase (hydrolyzing esters of gallic acid and tannins), chlorophyllase (splitting off phytol from chlorophyll) tropinesterase, cholinesterase, phosphatases and sulfatases. A similar classification has been applied to the other groups of hydrolases.

The enzymic degradation of important natural products sometimes involves hydrolysis of more than one type of linkage. In some cases therefore, it may be convenient not to classify enzymes strictly according to reaction specificity, but to collect enzymes of different specificity in special groups which are involved in the degradation or synthesis of certain substances. This has been done here and there in this book, as for instance in the case of enzymes hydrolyzing nucleic acids and their degradation products, or in the case of polysaccharide synthesis.

Some feature of an enzymic reaction, perhaps in itself of secondary importance from the viewpoint of chemistry, may be highly conspicuous or important from a practical point of view, and this may justify a departure from a classification based on reaction specificity. From considerations of

this kind, enzymes which coagulate blood and milk have been treated in separate chapters instead of being included among the proteolytic enzymes, to which group they belong.

Recently an important group of enzymes has been discovered, the action of which is analogous to hydrolysis, with the difference that phosphoric acid has taken the place of water. These enzymes are called phosphorylases. The phosphorylases are the most important agents in the synthesis of oligo- and polysaccharides, and this has occasioned treatment in the same chapter of other enzymes involved in these syntheses.

The action of certain enzymes can be described as a transfer of a radical or group from one substance, the donor, to another substance, the acceptor. Among these enzymes, which may conveniently be called transferases, we have the important groups of aminotransferases (transaminases) and transmethylases. The phosphotransferases (phosphokinases) probably have the same type of action, but since they are deeply involved in carbohydrate metabolism they have been treated in this book along with the enzymes of fermentation, glycolysis and respiration. Certain carbohydrases have an action which gives reason for their designation as "transferases."

A few splitting enzymes cannot conveniently be fitted into the above system of classification of hydrolases. For example, there is penicillinase that splits a peptide linkage in a lactam ring, and thiaminase that splits a carbon in quarternary nitrogen linkage with the uptake of water. Histidase and urocaninase open the imidazole ring. These enzymes have been treated in chapters of their own.

In earlier treatises on enzyme chemistry all enzymes which are not hydrolyzing were sometimes grouped under the title: enzymes of fermentation and oxidation. There are many good reasons for this, but it should not be forgotten that hydrolases also play a role in fermentation and respiration and that among the enzymes of respiration and fermentation there are some with widely differing specificities and modes of action. Neuberg has proposed the name of "desmolases" (Greek $\delta\varepsilon\sigma\mu o\varsigma$) for enzymes that rupture chains of carbon atoms. This designation fits very well one group of enzymes involved in fermentation and respiration, viz., the aldolase and the decarboxylases, but it is not suitable as a designation of oxidizing or dehydrogenating enzymes.

A more detailed classification of the enzymes of fermentation and respiration appears desirable. However it is only partly possible to realize this wish. The classification used in this book is to be regarded as only tentative; it is certainly not logically consistent. It is possible to classify some enzymes according to their chemical nature or, more exactly, according to the chemical nature of their prosthetic groups. Among such are the hematin enzymes, the flavin enzymes, the copper enzymes and those enzymes which contain coenzyme I or coenzyme II.

A group of enzymes with a well definable common reaction specificity comprises those enzymes which remove water (dehydrases) or hydrogen sulfide (desulfarases), or ammonia (desammonases) from certain substrates with the formation of a carbon-carbon double bond. The reaction is reversible, since dehydrases catalyze also the addition of water to certain unsaturated compounds. (In this connection it may not be superfluous to point out that dehydrases are enzymes removing or adding *water* whereas dehydrogenases are enzymes, the action of which can be described as a removal of hydrogen from a substrate or the transfer of hydrogen from a donor to an acceptor. In earlier treatises, especially in the German language, the dehydrogenases were often termed dehydrases.)

A special chapter has been devoted to enzymic syntheses of various substances, which cannot be regarded as simple reversions of the degradation by hydrolases; here we have placed such enzymes as choline acetylase and enzymes involved in the synthesis of urea, peptide bonds and glutamine. Finally there are several highly important phenomena where our knowledge of the participating enzymes or even the reactions themselves is so fragmentary that only a general description of the phenomena as such is possible. Thus chapters have been devoted to subjects as assimilation of CO_2 and N_2 and tumor enzymology.

IV. Enzymes as Systems

The investigation of enzymes has been carried on as a rule using a single enzyme in an unpurified or in partly purified condition and under various conditions of temperature, pH, and substrate concentration. It must be understood, however, that in the living organisms enzymes act together as systems. Thus, in the gastrointestinal tract first one enzyme and then another acts to hydrolyze proteins down to amino acids. In the cells glycogen is broken down to carbon dioxide and water step by step by a large number of enzymic reactions. Hydrogen is removed from various substrates by dehydrogenases. It is next taken up by yellow enzymes and is finally oxidized to water by the cytochromes and cytochrome oxidase.

It can readily be seen that where a system of enzymes is concerned there is little likelihood that intermediate products will accumulate.

V. The Early History of Enzymes

Much of the early history of enzymes is connected with investigations of alcoholic fermentation and putrefaction.[1] In 1659 Willis and in 1697 Stahl tried to explain fermentation as a process whereby a body in a state of internal action communicates this motion to another body that is then fermented. In 1680 van Leeuwenhoeck described the appearance of yeast cells under the microscope but this observation remained unnoticed or

[1] A. Harden, Alcoholic Fermentation, 4th ed., Longmans, Green, London, 1932.

forgotten for more than a century. In 1787 Fabroni defined fermentation as the decomposition of one substance by another substance.

Lavoisier,[2] one of the founders of modern chemistry, in 1789 prepared a balance sheet by which he attempted to show that when sugar undergoes alcoholic fermentation the carbon, hydrogen, and oxygen of the sugar can be accounted for in the alcohol, carbon dioxide, and acetic acid that are formed.

Schwann[3] in 1837 attributed putrefaction to the presence or living bacteria. In 1838 Cagniard-Latour[4] regarded yeast cells as living organisms, probably vegetable in nature and the cause of fermentation. In this same year Kützing[5] stated that alcoholic fermentation is caused by yeast.

In 1839 von Liebig[6] attempted to explain fermentation by means of a theory similar to that of Willis and Stabl. He maintained that a ferment, which is not a living organism, is a substance undergoing a progressive metamorphosis that, as a result of this change, brings about fermentation. Berzelius[7] in this same year explained fermentation as caused by a catalytic force. He maintained that a body by its mere presence could arouse affinities is the fermentable substance which could cause a rearrangement to take place.

Pasteur demonstrated in 1857[8] that lactic acid formation requires the presence of living bacteria and that alcoholic fermentation requires living yeast cells. He observed that during fermentation there occurs a multiplication of the yeast cells. He regarded fermentation as a physiological process. In 1858 M. Traube[9] announced the theory that fermentation is brought about by living organisms and that these organisms act through the ferments which they contain. He regarded ferments as closely related to proteins.

In 1897 Edward Buchner obtained zymase from yeast and showed that this cell-free liquid was able to ferment sugar to alcohol and carbon dioxide.[10] While organized ferments, such as those concerned with fermentation and putrefaction were being investigated, unorganized ferments also were being studied. At one time it was believed that gastric digestion was a grinding process that transferred food into particles fine enough to be absorbed directly. This theory was disproved by de Réamur, who allowed a falcon to swallow a perforated metal tube containing meat. He observed that this meat was dissolved.

[2] A. Lavoisier, Traité Elémentaire de Chimie, Cuchet, Paris, 1789, p. 139.
[3] T. Schwann, *Ann. Physik.* **41**, 184 (1837).
[4] Cagniard-Latour, *Ann. chim. et phys.* **68**, 206 (1838).
[5] F. Kützing, *J. prakt. Chem.* **11**, 385 (1837).
[6] J. von Liebig, *Ann.* **30**, 250 (1839).
[7] J. Berzelius, *Jahresbericht über die Fortschritte der physischen Wissenschaften* **18**, 400 (1839).
[8] L. Pasteur. *Compt. rend.* **45**, 1913 (1857).
[9] M. Traube, Theorie der Fermentwirkungen. Ferd. Dümmlers Verlagsbuchhandlung, Berlin, 1858.
[10] E. Buchner, *Ber.* **30**, 117 (1897).

In 1783 Spallanzani[11] fed animals meat in tiny wire cages and noted that the meat was dissolved in the stomach. He observed also that meat was dissolved by the gastric juice.

In 1810 Planche observed that extracts of plant roots turn tincture of guaiac blue. He noted that the agent that does this is thermolabile and named it "cyanogen." In 1814 Kirchoff observed that the glutinous component of wheat is capable of converting starch into sugar and dextrin. In 1830 Robiquet and Boutron as well as Chalard discovered the hydrolysis of amygdalin by bitter almonds. In 1837 Liebig and Wöhler named the enzyme "emulsin." In 1831 Leuchs described the action of the ptyalin of saliva upon starch. In 1833 Payen and Persoz[12] were able to extract amylase from germinating barley and to precipitate it as a white powder through the addition of alcohol.

In 1835 Faure described sinigrinase, the enzyme (really two enzymes) that decomposes sinigrin into D-glucose, allyl mustard oil and potassium bisulfate. Schwann discovered pepsin in 1836 and in 1856 Corvisart described trypsin.

In 1858 Pasteur discovered that green mould fermented dextro-tartaric acid but not levo-tartaric acid. In 1860 Berthelot obtained solutions of saccharase from yeast. In 1862 Danielewski, using adsorption, separated pancreatic amylase from pancreatic trypsin.

In 1878 Kühne suggested that unorganized ferments, such as pepsin, ptyalin, emulsin, etc. be called "enzymes." In 1883 Duclaux introduced the custom of designating an enzyme by the substrate upon which it acts as the prefix, followed by the ending "ase." Bertrand discovered that certain enzymes require dialyzable substances for their activity and called such substances "coenzymes." Harden and Young in 1906 discovered that yeast zymase contains a coenzyme, subsequently called "co-zymase." In 1898 Croft-Hill demonstrated the synthetic action of yeast maltase.

VI. Some Discoveries and Theories of Fundamental Importance for the Modern Development of Enzyme Chemistry

1. Organized and Unorganized Ferments

At the end of the 19th century many of the reactions now regarded as typically enzymic were thought to be inextricably associated with living organisms as such. Although it was admitted, at least by certain biochemists of those days, that the reactions were "fermentative," it seems to have been supposed that the ferments in question could not be extracted, with intact activity, from the cells or tissues. On the other hand, as we have seen

[11] A. L. Gillespie, The Natural History of Digestion. London, 1898, p. 16.
[12] A. Payen and Persoz, *Ann. chim. et phys.* **53**, 73 (1833).

already, many examples were known where the "ferments" e.g., the amylolytic ferments of malt, could be extracted from cells or tissues or even be spontaneously secreted, as in the case of salivary amylase. These extracts or juices exhibited a typically fermentative power. Hence, it was assumed at that time, that two types of ferments were in existence:

1. Unorganized ferments which were secreted by cells or which could be extracted in active form from cells or tissues.
2. Organized ferments, which were thought to be intrinsically connected with certain structures of the cells in such a manner that a liberation of the enzyme in active form from the structure was impossible.

Zymase, the "ferment" of alcoholic fermentation, was believed to be a typical organized ferment. Existing somewhere in the yeast cell, the "ferment," was supposed to be the immediate cause of fermentation, capable of acting as such only in the living cell. These ideas probably were remnants of the earlier, more general belief that living organisms and substances occurring therein differed in a characteristic but chemically not definable way from "dead" matter, in short the belief in the "vital force."

It was, therefore, the beginning of a new era when, in 1897, Edward Buchner ground yeast cells with quartz powder, squeezed the ground mass in a hydraulic press and obtained a cell-free juice that exhibited a typical alcoholic fermentation, i.e., the disintegration of sugar to alcohol and carbon dioxide according to the Gay-Lussac equation. Buchner's experiments showed that an "organized ferment" could be liberated in active form from its site in or on the structure typical for the living cell and could display its specific action in a homogeneous solution. This conclusion was not immediately accepted by the scientific world; the Buchner yeast-juice was supposed to be "expressed protoplasm" and so on. As late as 1927 Kostytschew[13] tried to explain away the results of Buchner and his innumerable followers.

The experiments of Buchner and other investigations along the same lines paved the way for the general belief that all reactions in living matter are catalyzed by ferments or enzymes which by themselves, without connection with the living cell or any structures thereof, are sufficient for catalytic activity. From a purely enzymological point of view it is immaterial whether an enzyme can be brought into solution or not. Nothing prevents us from assuming that an enzyme, although active in itself, can be insoluble in water or firmly bound to water-insoluble cell constituents. In this last case it may be possible to liberate the enzyme from the insoluble "carrier" by means of a second enzyme, generally a protease. For instance, the saccharase of baker's yeast in most cases is bound firmly to insoluble substances. Simple plasmolysis of the cells does not bring the enzyme into solution, but upon treatment of the cell remnants with proteolytic enzymes (papain) the saccharase goes into solution.

[13] S. Kostytschew, G. Medwedew, and H. Kardo-Sysojewa, *Z. physiol. Chem.* **168**, 244 (1927).

As the result of Buchner's experiment there is, from an enzymological point of view, no difference between organized and unorganized ferments. The terms "ferment" and "enzyme" have an identical significance. But as is evident from the foregoing, the enzymes in cells must not necessarily be thought of as simply dissolved in a homogeneous protoplasm; they can be and often are associated with definite structures of the cells, as for instance with the mitochondria. The questions concerning the cytological foundation of enzymes are of fundamental importance for biology.

2. Dependence of Enzyme Activity on pH

In 1909 Sørensen pointed out that the activity of enzymes depends in quite an orderly way on the hydrogen ion concentration (or more correctly the hydronium ion activity) of the medium. Many contradictory statements about the action of acids or alkali on the activity of enzymes have found a simple explanation in Sørensen's statement, that the determining factor is not the titratable amount of acid or alkali but is the hydrogen ion concentration, conveniently expressed as the pH of the medium.

The activity of any given enzyme depends in a characteristic way on the pH. There exists a more or less narrow region of maximum activity (pH optimum), while on both sides of this region the activity gradually falls off to zero. If the enzyme activity in arbitrary units is plotted against the pH, a more or less bell-shaped curve is obtained, the so-called activity-pH-curve.

Sørensen's discovery in fact lays the foundation of modern experimental enzyme chemistry, and the observance of the pH of the media employed and determination of the pH is now one of the first duties of every worker in the field of enzyme chemistry.

It is not the aim of this book to be a laboratory manual; therefore no detailed information on the methods of pH determination and the instruments used will be given here. The reader is referred to Sørensen's papers, and to textbooks by Michaelis, Clark, Kolthoff, Dole, *The Glass Electrode*, and Bamann-Myrbäck.

Buffer solutions are extensively used in enzyme work. As to the composition and preparation of suitable buffer solutions the reader is referred to the sources mentioned. Some general remarks do not seem out of place here:

In addition to the unspecific action of the pH on enzyme activity buffers may exert specific actions on enzymes or in some cases on substrates. An activity-pH curve of a certain enzyme found when using one type of buffer cannot be said without anything further to be the true pH curve of the enzyme; it may be influenced by some specific action of the buffer substances. In many cases the experiment must be repeated with different types of buffers.

Evidently there are many cases where the ordinary substrates may have considerable buffer capacity. There are also cases where common buffer substances act as substrates, as for instance with the phosphorylases. In such cases it may be difficult to distinguish between the influence of the pH and that of the substrate, especially as the inorganic reaction partner may be an ion, the concentration of which varies with the pH.

It is perhaps unnecessary to point out that many buffer substances may interfere with the methods used for determination of enzyme activity, for example when the enzymic action is determined by means of acidimetric titration. Buffers and indicators have to be chosen with care in such instances. Buffers may interfere with nonenzymic substances present for some reason in the reaction mixtures as with inhibitors or activators of enzymes. The statement that an enzyme is not inhibited by heavy metals has of course no meaning if the experiments have been performed in phosphate or borate mixtures.

3. Enzymes as Electrolytes

In 1911 Michaelis pointed out that the activity pH-curves of many enzymes are rather similar to dissociation curves of weak electrolytes and especially similar to the curves which show the variation of the concentration of the undissociated form of an ampholyte with the pH. Michaelis assumed tentatively (in the case of saccharase) that the fact underlying the activity-pH-curve is that the enzyme is an ampholyte and that only the undissociated, isoelectric form is active.

The idea of the connection of the activity-pH curves and the electrochemical properties of the enzyme has proven most fruitful, even if it must be conceded that the nature of this connection is tolerably clear only in a few cases. Since we know at the present time, that enzymes are proteins, i.e., ampholytes changing their state of electrical charge in the pH regions in question, it seems rather obvious by now that the activity must depend on the electrical charge, or on the state of dissociation of the enzyme.

If a substrate is an electrolyte changing its charge in the pH-range of enzyme activity, it is evidently possible and probable that the dissociation of the substrate is a contributory cause of the pH curve. Northrop has pointed out, for instance, that the pH curves of several proteolytic enzymes, which vary considerably with different substrates, are very similar to the dissociation curves of these substrates.

4. The Enzyme-Substrate Compound and the pH Curve

After several more or less fantastic attempts had been made to explain the action of an enzyme on its substrate, the thought was clearly expressed by Michaelis and Menten that enzymic action is due to the formation of an intermediate compound between enzyme and substrate, which com-

pound, on account of the specific constitution of the enzyme and its mode of attachment to the substrate, is unstable in a manner that enables the "enzymic" reaction to take place. In the case of the hydrolases it may be assumed that in the enzyme-substrate compound the linkage in the substrate to be hydrolyzed is so weakened by attachment of the enzyme molecule to the substrate that it is hydrolyzed at a much lower hydrogen ion concentration than in the absence of the enzyme.

The conception of the enzyme-substrate compound has turned out to be extremely valuable in spite of its being purely hypothetical until recently. Recently however, it has been possible to establish directly the formation of intermediate compounds between certain enzymes and their substrates.

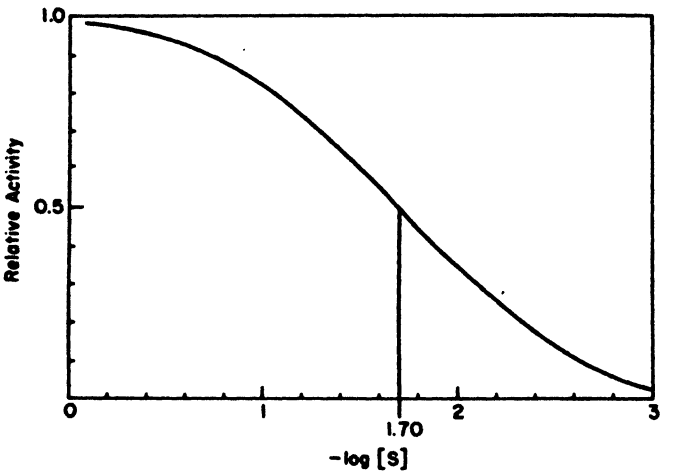

FIG. 1. Dependence of yeast saccharase activity upon the substrate concentration [S]. (R. Kuhn, *Z. physiol. Chem.* **125**, 28 (1923)). The curve corresponds to the Michaelis constant $K_m = 0.020$.

Chance has observed that catalase and peroxidase form compounds with hydrogen peroxide which have distinctive absorption spectra.

It should be mentioned that even recently the necessity of an intermediate enzyme-substrate compound for the enzymic action has been questioned. Rothen, on the basis of experiments on the action of proteases on certain protein films, was inclined to assume an action of the enzymes by long range forces. However, certain assumptions concerning the nature of the films used appear to be unjustified, and today no evidence is available which should make the assumption of enzyme-substrate compounds superfluous.

If the activity of an enzyme is determined at varying substrate concentrations [S] and the activity is plotted against log [S], in most cases an S-shaped curve is obtained similar to the normal dissociation curve. This

led Michaelis and Menten to assume, that an enzyme, the total concentration of which we shall call [Σ], forms with the substrate an intermediate compound, the concentration [ES] of which is determined according to the mass law by the equation:

$$\frac{([\Sigma] - [ES]) \cdot ([S] - [ES])}{[ES]} = K_m \qquad (I)$$

Since [Σ] and [ES] are very small compared to [S], the equation simplifies to:

$$\frac{([\Sigma] - [ES]) \cdot [S]}{[ES]} = K_m$$

The constant K_m is usually called the Michaelis constant. The numerical value of K_m is easily found from curves of the type illustrated in Fig. 1. At half the maximum activity, i.e. when $[ES] = \frac{\Sigma}{2}$ we have: $K_m = [S]$, e.g., we find from Fig. 1 that $K_m = 10^{-1.70} = 0.020$. The "relative activity" of the enzyme is given by the equation:

$$\text{Relative activity} = \frac{[ES]}{[\Sigma]} = \frac{[S]}{[S] + K_m}$$

As mentioned above Michaelis had tried to explain the activity-pH curve of the enzyme saccharase as the dissociation curve of an ampholyte; the alkaline branch of the curve was supposed to be caused by the dissociation of the enzyme as a weak acid HE

$$\frac{[H^+] \cdot ([\Sigma] - [HE])}{[HE]} = K_a \qquad (II)$$

or, since HE was thought to be the active form,

$$\text{Relative activity } \frac{[HE]}{[\Sigma]} = \frac{[H^+]}{[H^+] + K_a}$$

This theory was modified later on by Michaelis and Rothstein. They assumed that enzyme and substrate (sucrose) combine to give a compound which is a weak acid. Only the undissociated molecules [HES] were supposed to disintegrate.

Kuhn pointed out that if both equation (I) and (II) (or the modified equation proposed by Michaelis and Rothstein) are valid, then the alkaline branch of the pH curve must be dependent on the substrate concentration [S]. Experiments by Kuhn and others have shown beyond doubt that this is not the case. On account of these facts Kuhn and Willstätter were inclined to reject the theories of Michaelis. The existence of the enzyme-substrate compound was admitted; the pH-curve was supposed to be caused by a variation of the velocity of disintegration with the pH.

However, the discrepancies between the experiments and the theories of Michaelis do not concern the fundamental thoughts of these theories. If it is assumed that the enzyme is a weak acid 'HE' there is in fact no reason why the attachment of a sucrose molecule should alter the K_a-value of this acid. In other words, it is not probable that in the combination of substrate and enzyme molecules the dissociating acid groups of the enzyme should be mediatory, as implicitly supposed by Kuhn. If it be assumed that the acid group of the enzyme has nothing to do with the binding of the substrate and, therefore, that enzyme and enzyme-substrate compound both are weak acids with the same K_a-value, then a simple calculation shows that the alkaline branch of the pH curve must be independent of [S], and this is, as already mentioned, in accordance with the experiments.

If the acid branch of the pH curve of saccharase is investigated in the same way, it is found to vary with the substrate concentration. With increasing [S] the curve is displaced parallel to itself towards the acid side (Fig. 2). This implies that the dissociating basic groups of the enzyme molecule, which, according to Michaelis, are responsible for this part of the pH-curve, are also involved in the formation of the enzyme-substrate compound. Several reasons favor the assumption that this group is a primary amino group. If it be assumed, (a) that the enzyme is an ampholyte $H \cdot E \cdot NH_2$ with the dissociation constants K_a and K_b, (b) that the attachment of the substrate molecule requires a free amino group without positive charge, and (c) that only the undissociated enzyme-substrate compound $H \cdot E \cdot NH_2 \cdot S$ (and not for instance the ion $^-E \cdot NH_2 \cdot S$) is disintegrating, then we find

$$\frac{([H \cdot E \cdot NH_2] + [^-E \cdot NH_2]) \cdot [S]}{[H \cdot E \cdot NH_2 \cdot S] + [^-E \cdot NH_2 \cdot S]} = K_m$$

$$\frac{([H \cdot E \cdot NH_3^+] + [^-E \cdot NH_3^+]) \cdot [OH^-]}{H \cdot E \cdot NH_2] + [^-E \cdot NH_2]} = K_b$$

$$\frac{([^-E \cdot NH_2 \cdot S] + [^-E \cdot NH_2] + [^-E \cdot NH_3^+]) \cdot [H^+]}{[H \cdot E \cdot NH_2 \cdot S] + [H \cdot E \cdot NH_2] + [H \cdot E \cdot NH_3^+]} = K_a$$

$$\Sigma = [H \cdot E \cdot NH_2 \cdot S] + [^-E \cdot NH_2 \cdot S] + [H \cdot E \cdot NH_2] + [^-E \cdot NH_2] + [H \cdot E \cdot NH_3^+] + [^-E \cdot NH_3^+]$$

or

$$\text{Relative activity} = \frac{[H \cdot E \cdot NH_2 \cdot S]}{\Sigma} = \frac{1}{\left(1 - \frac{K_a}{[H^+]}\right)\left\{1 + \frac{K_m}{[S]}\left(1 - \frac{K_b}{[OH^-]}\right)\right\}}$$

This formula is in good agreement with the experimental data.

Now it is quite clear that this is not the only possible way to look at the problem of the enzyme-substrate compound. In the above treatment, it

has, for instance, been tacitly assumed that the rate of formation of the enzyme-substrate compound is very high compared to that of the decomposition of the substrate. However, if the velocity k_e of the combination of enzyme and substrate is not very great compared to the constant k_d of the decomposition, this will, as shown in detail by Van Slyke,[14] influence the course of the enzymic action. The overall velocity will be

$$-\frac{dS}{dt} = \frac{1}{\frac{1}{k_e[S]} + \frac{1}{k_d}}$$

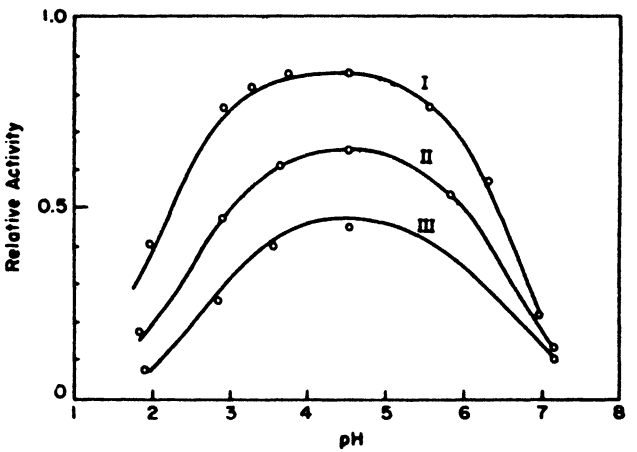

FIG. 2. Activity-pH-curves of yeast saccharase at different substrate concentrations (K. Myrbäck, *Z. physiol. Chem.* **158**, 160 (1926)). Curve I: [S] = 0.220, Curve II: [S] = 0.0722, Curve III: [S] = 0.0361. The curves are calculated from the K_m-value 0.040, which was determined experimentally with the same enzyme preparation. The circles mark the values of the relative activity found in the experiments at different pH's and different substrate concentrations.

Obviously the value of [S] corresponding to half the maximum velocity (the Michaelis constant, formula (I) will be

$$[S] = k_d/k_e$$

Van Slyke points out that variations in k_e, "such as may be caused by pH changes, will cause corresponding variations in the Michaelis constant." Van Slyke mentions that, in the case of urease a drop of one unit in pH will increase the value of the Michaelis constant eleven fold. However, in arriving at this conclusion, Van Slyke implicitly assumes that the number of substrate-binding groups of the enzyme remains constant independent of the pH. This seems improbable, since urease, being a protein, is an ampholyte which must be supposed to change its state of electrical charge

[14] D. D. Van Slyke, *Advances in Enzymol.* **2**, 33 (1942).

in the pH-range in question. Actually the modified Michalis-Menten theory as described above can very well explain the activity-pH curve of the urease at different substrate concentrations. The value of the Michaelis constant remains independent of the pH if it be assumed as in the case of saccharase that the positively charged ions of the enzyme do not bind substrate. When calculated in this way the Michaelis constant may be said to be a value characteristic of a certain enzyme.[15]

It is well known that enzymes having the same action on a substrate but originating from different sources may be quite different proteins. Of course, it cannot be expected in such cases that the value of the Michaelis constant should be the same for the different enzymes. Likewise it cannot be expected that the value of the Michaelis constant for one enzyme should be independent of the degree of purity of the enzyme preparation. If, for instance, a crude enzyme solution contains an enzyme inhibitor with competitive action, this will influence the value of K_m. If the substrate concentration corresponding to half the maximum activity is called $[S_x]$ then $K_m = [S_x]$ if no inhibitor is present, but if the inhibitor has the concentration $[X]$ and the dissociation constant of the enzyme-inhibitor complex is K_x, then

$$K_m = [S_x] \frac{K_x}{[X] + K_x}$$

The pH optimum and the pH curve are, to a certain degree, characteristic of an enzyme. However, it should not be forgotten that the presence of inhibiting substances may distort the true pH curves. If the curve is regarded as the dissociation curve of an ampholyte it can be foreseen that the presence of any substance, such as a metal ion, combining with the acid group of the enzyme will cause a displacement of the alkaline branch of the pH curve towards the acid region. Conversely the presence of inhibiting substances combining with the basic group of the enzyme will cause a displacement of the acid branch of the pH curve towards the alkaline side.

It should also be mentioned that, although the stability maxima of most enzymes are, roughly speaking, the same as the activity optima, there are several cases known where the stability of the enzyme is so low even at pH's near to the pH optimum of activity, that the experimental determination of the activity-pH curve is rather difficult and sometimes yields a distorted curve.

5. Synthesis by Enzymes

In 1898 Croft-Hill attempted to synthesize maltose, allowing yeast extract (α-glycosidase) to act on 40% glucose solution. An enzyme synthesis

[15] K. Myrbäck, *Acta. Chem. Scand.* **1**, 142 (1947).

was observed to occur, since the reducing value decreased and the optical rotation changed. Maltose was one of the products formed by the synthetic action of the yeast enzyme, but was not the only product. This is easily understood, since the yeast α-glycosidase is not a specific maltase and other enzymes may have been present also.

In 1900 Kastle and Loevenhart[16] found that the hydrolysis of fats and other esters by lipases is a reversible reaction and that enzymic synthesis can occur in a dilute mixture of alcohol and acid. The problem of ester synthesis was treated theoretically by Bodenstein and Dietz in 1906–07. The interpretation of experiments with lipases is difficult because of the inevitable pH changes during the reactions. Dietz[17] was able to show that the same equilibrium was reached from both sides and that the equilibrium constant was $K = k_1/k_2$, where k_1 and k_2 are the velocity constants of hydrolysis and synthesis. However, K did not have the same value as in experiments without any enzyme.

Since an ideal catalyst only alters the rate of the reaction, we should expect to find the same equilibrium in the presence of the catalyst as in its absence. This is very often found to be the case. However, the conclusion is correct only when the catalyst does not to any appreciable degree enter into combination with the reacting substances. Therefore, as pointed out by Euler and Josephson[18,19] when calculating the equilibria of enzyme reactions, we have to introduce the affinity (or dissociation) constants of the various enzyme-substrate compounds. In the case of the enzymic hydrolysis and synthesis of a glycoside, we have:

$$[\text{Glycoside-enzyme}] + [\text{Water}] \rightleftarrows [\text{Sugar-enzyme}] + [\text{Alcohol}]$$

supposing that the enzyme has no affinity to the alcohol. The concentrations of the glycoside-enzyme and the sugar-enzyme compounds are determined by the equations

$$\frac{[\text{Glycoside}]\cdot[\text{Enzyme}]}{[\text{Glycoside-enzyme}]} = K_1 \quad \text{and} \quad \frac{[\text{Sugar}]\cdot[\text{Enzyme}]}{[\text{Sugar-enzyme}]} = K_2$$

where K_1 and K_2 are the Michaelis and Menten dissociation constants. The velocity of the enzymic hydrolysis is

$$v_1 = k_1\cdot[\text{Glycoside-enzyme}]\cdot[\text{Water}]$$

and the velocity of the synthesis

$$v_2 = k_2\cdot[\text{Sugar-enzyme}]\cdot[\text{Alcohol}]$$

[16] J. H. Kastle and A. S. Loevenhart, *J. Am. Chem. Soc.* **24**, 491 (1900).
[17] W. Dietz, *Z. physiol. Chem.* **52**, 279 (1907).
[18] H. von Euler and K. Josephson, *Z. physiol. Chem.* **136**, 30 (1924).
[19] H. von Euler and K. Josephson, *Arkiv Kemi Mineral. Geol.* **9**, No. 7 (1924).

Then we have:

$$\frac{dx}{dt} = v_1 - v_2 = k_1 \cdot [\text{Glycoside-enzyme}] \cdot [\text{Water}] - k_2 \cdot [\text{Sugar-enzyme}] \cdot [\text{Alcohol}]$$

and the equilibrium constant will be

$$K = \frac{k_1}{k_2} = \frac{K_1 \cdot [\text{Sugar}] \cdot [\text{Alcohol}]}{K_2 \cdot [\text{Glycoside}] \cdot [\text{Water}]}$$

This formula shows that K will have the same value as in the uncatalyzed reaction only if $K_1 = K_2$.

The decomposition of hydrogen peroxide by catalase, the hydrolysis of urea by urease, of sucrose by saccharase, of starch by amylases and of arginine by arginase are examples of reactions that show no reversibility. Here the position of the equilibrium is thermodynamically unfavorable for synthesis.

Synthetic reactions catalyzed by enzymes can be divided into three types:

I. The synthetic reaction goes to completion or practically to completion. An example is the formation of dextran from sucrose by the enzyme dextran sucrase. The energy necessary for formation of the glycosidic bonds in the dextran is derived from the simultaneous splitting of the glycosidic bonds of the disaccharide; the synthesis can in a way be described as a transfer reaction.

II. A reaction proceeds to a point of equilibrium at which considerable proportions of both substrate and products are present. Examples are the ester syntheses, as described above, or the formation of amylose from the Cori ester by phosphorylase. In this case the energy of the synthesis is derived from the "energy-rich phosphate bond" in the Cori ester. Fundamentally there is of course no difference between the types I and II.

III. The reaction does not take place unless the necessary energy is provided by coupling with another enzymic process. Reactions of this type are in themselves endergonic and require a supply of energy, which is furnished immediately by the breakdown of energy-rich substances, e.g., adenosinetriphosphate and remotely by the oxidation of some metabolite.

Some of the substances that have been synthesized *in vitro* by reactions of type III are:

Acetylcholine
Hippuric acid
Aminohippuric acid
Glutamine
Acetylsulfanilamide
Glucose-6-phosphate

Citric acid
Glutathione
N-Benzoylornithine

VII. The Chemical Nature of Enzymes

The chemical nature of enzymes can be said to have been quite unknown until 1926. It seems that earlier many scientists altogether despaired of the possibility of isolation of enzymes in pure form. The fact that a purer solution of an enzyme often is less stable than a less pure preparation seems to have led to the conviction in certain quarters that the instability would increase with increasing purity to such a degree that the isolation of a pure enzyme would be *eo ipso* impossible.

Nevertheless work of fundamental importance on the purification of enzymes was performed during the first decades of this century. The investigations of Willstätter, von Euler and many others resulted in extensive knowledge of many enzymic reactions, in methods for the determination of enzymes and in enzyme preparations of considerable activity. Nobody knew anything at this time about the actual degree of purity of the enzyme preparations; it seems that many investigators were convinced that the percentage of enzyme even in the best preparations was very low. Nothing could be said with certainty about the chemical nature of the enzymes.

The idea that the enzymes could be protein in nature had, it is true, been expressed by several scientists, possibly from the general belief that proteins are nearer to the so-called life than other substances, but on the other hand, this theory had been decidedly denied by others. Willstätter evidently found his results on the purification of certain enzymes to justify the statement that enzymes do not belong to any of the recognized groups of organic substances. As late as 1925 Oppenheimer spoke of enzymes as substance of unexplained chemical nature belonging to no known group of substances.

However, in 1926, Sumner crystallized urease from jackbean meal and showed it to be a protein. The statement that the isolated globulin crystals were the pure urease was not accepted without further trouble, a fact which may be understood in the light of the prevailing opinions concerning enzymes and their chemical nature. It was assumed that the crystals were in themselves commonplace protein crystals which, for some reason, held the enzyme firmly absorbed. However, the claim of Sumner, after most rigorous examination from several points of view, has proved correct and as far as we can judge at the present time, the protein crystals obtained represent pure urease.

Since 1926 many other enzymes have been isolated in crystalline form. The outstanding work of Northrop and Kunitz should be mentioned. In

every known case the pure enzyme has turned out to be a protein, either simple or conjugated.

When an isolated enzyme has been shown to be a conjugated protein it has been possible in some instances to connect the chemical nature of the enzyme or, more correctly, the chemical nature of the prosthetic group, with the enzyme activity. It seems, for instance, comprehensible that several oxidizing enzymes are chromoproteins with iron porphyrin prosthetic groups. On the other hand, many enzymes, such as urease, pepsin and trypsin, have defied all attempts to establish the presence of a prosthetic group. If we did not know that these substances possess a highly remarkable catalytic action, we should probably classify them along with other more or less ordinary proteins. One would have thought that the clearing up of the question of the chemical nature of enzymes would have automatically thrown light also on the mechanism of enzyme reactions, but expectations in this direction have not been fulfilled. The reason is that too little is known about protein structure.

When we state that many enzymes have proved to be simple proteins without a discernible prosthetic group, this evidently means that no component of another kind than the amino acids is present. Admittedly it is difficult to prove that a protein contains amino acids exclusively, but as far as the amino acid analysis can be used in evidence, these enzymes contain only amino acids and in any case no dissociable prosthetic groups, no metals and no light-absorbing groups other than the amino acids. It seems permissible to assume that these enzymes are in fact simple proteins. However, they must possess some sort of specific group for the combination with the substrate, but it does not seem necessary to assume that these groups should be groups of a kind not present in ordinary amino acids. The current ideas concerning the formation of active compounds between the hydrolases and their substrates suggest that it is not so much the chemical nature of a substrate-binding group as the arrangement of a certain number of such groups with respect to each other on the surface of the enzyme molecule which determines the attachment of the substrate.

The realization has come that the most important function of proteins is enzymic; there can be little doubt that all enzymes are proteins. We may even go so far as to assume that in many cells the plasma consists almost exclusively or perhaps exclusively of enzymes. This idea has been advocated by Virtanen.

The converse statement, namely, that all proteins are enzymes, is probably not correct. It is likely that such food proteins as casein and ovovitellin are elaborated to serve as sources of amino acids for the suckling mammal or for the incubating embryo. It is likely also that seed proteins such as glutelin, gliadin, and edestin serve as sources of amino acids that can be used in the synthesis of enzymes and other cell components.

VIII. General Properties of Enzymes

There are several properties of enzymes which can be said to belong to the proteins as a group whether they are enzymes or not.

1. Heat Inactivation

It has already been mentioned that enzymes generally are destroyed upon heating their solutions to elevated temperatures. It has also been mentioned that this heat inactivation in many respects resembles the changes in protein structure produced both by heat and by certain agents and known as denaturation. Carefully dried preparations are often much more stable than solutions. Dry preparations of saccharase can be heated in boiling toluene without loss of activity, whereas aqueous solutions are rapidly inactivated at temperatures above 55°.

A few enzymes are able to withstand high temperatures even in aqueous solution. Pure ribonuclease can be boiled for a short time and solutions of crystalline trypsin in dilute hydrochloric acid withstand heating to at least 90°. This is quite in accordance with the theory that heat-inactivation of enzymes is caused by protein denaturation. It is interesting to note that a solution of pure trypsin is quite without action on the substrates at 90°, but that its activity is recovered when the solution is allowed to cool. This is an example of reversible protein denaturation. The process is rendered irreversible (with trypsin as well as with other proteins) when a neutral salt is added to the hot solution, resulting in denaturation and flocculation. The denaturation that coincides with reversible loss of activity is probably due to an "unwinding" of the polypeptide chains of the trypsin molecule whereby the orderly pattern of the substrate-binding groups on the surface of the enzyme molecule is disturbed. When the inactive trypsin solution is cooled, a rearrangement of the polypeptide chains into the original molecule must take place.

Inactivation of enzymes, probably of the same type as heat-inactivation can be caused also by vigorous agitation of the solution, by applying high pressure, by radiation, and by many chemical reagents. The stability of an enzyme solution, as already mentioned, is a function of the pH, and every enzyme has its own optimum pH of stability. The position of this optimum varies considerably from enzyme to enzyme. Beyond the optimum enzymes are inactivated, and the inactivating action of hydrogen and hydroxyl ions may be thought to result in denaturation of the protein similar to that caused by high temperature. The inactivation is often irreversible, but cases are known where the activity, after inactivation by acid or alkali, is slowly recovered when the pH of the solution is brought back to the normal value.

2. Antigenic Action

Inasmuch as the enzymes are proteins it would appear that they should be able to act as antigens when injected parenterally into animals to whom they are foreign. This has indeed been shown to be true. The study of the antigenic action of enzymes, the antibodies formed, and the interaction between antigen (enzyme) and antibody (inhibitor) is of great interest not only for enzyme chemistry but for immunology in general, since the catalytic activity of the antigen offers certain possibilities of investigation which are not found with ordinary proteins.

3. Solubility

Most enzymes are readily soluble in water, and others (globulins) are soluble in dilute neutral salt or buffer solutions. The solubilities of enzymes vary greatly: for example, beef liver catalase at its isoelectric point is almost insoluble in distilled water, while horse liver catalase is fairly soluble. The solubility of an enzyme like that of any protein will vary with the pH of the solution, being least at the isoelectric point.

Some enzymes are quite insoluble in water, but this is possibly because they are bound to insoluble cell constituents. In any case the solubility in water is not a prerequisite of enzymic activity. Enzymes may be active even when fixed on the surface of an adsorbent. Yeast saccharase, for instance, is active when adsorbed on aluminum hydroxide.

Determination of solubility has proved to be most valuable for determination of the homogeneity of crystalline enzyme preparations. The crystalline state by itself is not a proof of purity and homogeneity. Different proteins may crystallize together, form solid solutions, etc.

4. Essential Groups in Enzymes

Since certain enzymes contain well defined prosthetic groups it seems reasonable to assume that the prosthetic groups are the mediators of the enzymic action. However, in most cases the action of the prosthetic group by itself is small or nil and the high activity of the enzyme is due to the combination of the prosthetic group with the protein, the "apoenzyme." As already mentioned, there are many enzymes which appear to be devoid of any special prosthetic or other groups which specifically label them as enzymes.

Evidently there are, at least in certain enzymes, parts of the protein moiety which are not, or at least not absolutely essential to the enzyme action. In the case of crystalline chymotrypsin, for instance, it is possible to remove considerable parts of the protein molecule without impairing the enzymic activity. It is also possible to acetylate all the free amino groups (lysine residues) of crystalline pepsin without loss of enzyme activity; the amino groups in this case are nonessential to the activity.

On the other hand there are in the enzyme molecules groups that cannot be removed or changed without loss of the activity; these groups are the essential groups of the enzyme. Some of them are essential because they are involved in the binding of the substrate molecule, others seemingly have nothing to do with the formation of the enzyme-substrate compound but are essential to the decomposition of this compound. The essential groups in some cases may be demonstrated by ordinary chemical methods, in other cases by studies of the inactivation or inhibition of the enzymes by specific reagents.

Sulfhydryl groups have been shown to be essential for the action of many enzymes. Inactivation by oxidants and reactivation by reductants such as cysteine, reduced glutathione, etc. may in many cases be due to oxidation of sulfhydryl or reduction of disulfides. The activity of sulfhydryl enzymes must be dependent on the redox potential of the medium and the thought has been expressed that this may represent a regulatory mechanism for several enzyme actions.

Numerous chemical reagents bring about destruction or inactivation of enzymes by interaction with essential groups in the enzyme molecules. If primary amino groups are essential for the action of a certain enzyme, then nitrous acid under suitable circumstances may bring about a destruction of the activity. Inactivation may be reversible, for instance when ions of heavy metals are bound by groups in the enzyme molecule. Treatment with hydrogen sulfide removes the metal and the enzyme activity is recovered provided that the uptake of the metal has not caused further changes in the enzyme molecule.

It should perhaps be pointed out here that the sensitiveness of highly purified enzyme preparations to certain poisons, especially heavy metals must be remembered in practical work. Minute quantities of copper and certain other metals in water, buffer substances etc. may cause severe poisoning.

5. Coenzymes

Bertrand discovered that certain enzymes require dialyzable substances for their activity and called such substances "coenzymes." The term coenzyme is not used in those cases where inorganic salts, metal ions, etc. are essential to the action of an enzyme.

In 1906 Harden and Young demonstrated that "zymase," the "enzyme of alcoholic fermentation," then thought to be a single enzyme, could be separated by dialysis into two components that were both inactive when alone, but which became active on mixing. The nondialyzable, high-molecular, thermolabile component was thought to be the enzyme proper (later on called the apoenzyme) while the dialyzable, relatively heat-stable component was called the coenzyme of alcoholic fermentation, or later on

cozymase or coenzyme I. It is quite possible that coenzymes as a group can be defined as easily dissociating prostheting groups of enzymes. In the case of the coenzymes I and II they can be even classified as specific substrates of the apoenzymes, since they are hydrogenated by the apoenzymes in the presence of suitable hydrogen donors.

There are several cases known where a vitamin or a simple derivative of a vitamin acts as a coenzyme or a prosthetic group of an enzyme, and perhaps this is the essential action of all vitamins. For examples, the dialyzable and relatively heat-stable coenzyme of yeast carboxylase, cocarboxylase, has been shown to be the pyrophosphoric ester of thiamine.

6. The Activation of Enzymes

Certain enzymes exist naturally in the inactive or zymogen state and can be activated by change of pH or by the action of other enzymes upon them. Thus, pepsinogen is converted to pepsin by a pH more acid than 5.2. Prorennin is also activated by an acid pH. Trypsinogen is converted to trypsin by kinases or by trypsin itself. Chymotrypsinogen is converted to chymotrypsin by the action of trypsin but not by chymotrypsin itself. Procarboxypeptidase is converted to carboxypeptidase by trypsin. Protyrosinase of grasshoppers is converted to tyrosinase by the action of sodium lauryl sulfonate.

Some enzymes, such as arginase, glutaminase, hexokinase, phosphoglucomutase, phosphatases, and certain peptidases are activated by divalent metallic ions, such as Mn, Co, or Mg. Such enzymes probably can be regarded as metalloproteins. It is supposed that the combination of these enzymes and their substrates occurs partly through metallic ions.

Other enzymes, such as the animal amylases, are activated by certain anions, especially by the chloride ion. Little is known about the mechanism of this action; in any case, the action of neutral salts has nothing to do with their action on the solubility of globulins. It has been proposed that the activation should be considered a reactivation of inactivated enzyme. However, in the case of the animal amylases, this explanation is probably not correct, but it is only too true that in many instances the so-called activator has turned out, upon more careful investigation, to be merely a substance which protects the enzyme in question from inactivating agents.

IX. The Determination of Enzyme Activity

In a few cases it is possible at the present time to determine enzymes directly by chemical or physical methods, as for instance when an enzyme has a characteristic absorption spectrum. Determinations of this kind may be possible even if the enzyme has not been obtained in the pure state. Such methods are described in the special chapters of this book. However, in many cases the chemical nature of the enzyme is unknown, or its direct

determination is impossible from a practical point of view. Hence, in most instances it is preferable to determine the concentration of an enzyme by its activity. The rate at which it transforms the substrate is measured and this rate is expressed in arbitrary units. The number of units per gram of the enzyme is a measure of the purity of the preparation. This is possible only if the measured activity *ceteris paribus*, is proportional to the amount of enzyme preparation used in the experiments. Such proportionality is not always found, but it can be said with certainty that for every enzyme, conditions can be found where, at least within definite limits, such a proportionality prevails. If an experiment is performed in order to determine the amount of enzyme in a preparation it evidently is necessary to keep within these limits.

Methods for the quantitative determination of various enzymes will be described briefly in the special chapters of this book. For a detailed description the reader is referred to Bamann-Myrbäck, *Die Methoden der Fermentforschung*. It may suffice to mention briefly here a few conditions that have to be observed in the quantitative determination of an enzyme.

The velocity of an enzymic reaction, like that of all other reactions, varies with temperature. Therefore the temperature should be kept constant, and there are many more or less simple devices for the purpose. Since most enzymes are irreversibly or in a few cases reversibly inactivated by high temperatures, the temperature chosen for enzyme determination should be so low that there is no danger of heat-inactivation.

The pH of the medium should be controlled carefully. The enzymologist should have no superstitious belief in the buffer capacity of the buffer solutions commonly used in enzyme work. Enzyme preparations, substrates (often present in high concentrations) and other substances may change the pH of the buffer seriously. Many erroneous experimental results, sometimes taken as the foundation of far-reaching hypotheses, have been caused simply by uncontrolled changes of the pH of the mixture used for the determination. When controlling the pH of a solution the experimenter should not rely on the value on a scale to which the needle points but check his pH apparatus by testing solutions with known pH.

The stability of an enzyme, as mentioned above, is dependent on the pH. There is a region of maximum stability which may or may not coincide with the region of maximum activity. Even at low temperatures the enzyme may be irreversibly damaged if the pH is far enough from the optimum value.

If the aim is a quantitative determination of an enzyme the substrate concentration should be kept constant in all experiments. The reaction rate generally increases with the substrate concentration but may decrease again at very high concentrations. There are even cases where an enzyme may be irreversibly inactivated by its own substrate (catalase).

If the activity of an enzyme is dependent on a coenzyme, or upon metallic ions, it evidently is also necessary to control the activator concentration. In the quantitative determination of an enzyme of this kind it may be advantageous to work with a large excess of activator.

The activation of an enzyme, as for instance the activation of arginase by manganese ions, may be a slow reaction. If this is the case, the enzyme must be treated with the activator for a sufficient time before the substrate is added.

The rate of the enzymic reaction is measured either by determination of the decrease in the substrate concentration or by the increase in the amount of some reaction product. However the experimentator is warned against the method, unfortunately in common practice, in which a certain reaction time is selected and the degree of transformation at this time is supposed to be the correct value of the reaction rate or the amount of enzyme, respectively. The procedure is correct only when the transformation up to the selected time of assay is proportional to the time. Thus, in every investigation of this kind the limits should be ascertained within which this proportionality prevails. In this range the reaction velocity may be expressed simply and correctly as the substrate transformation per unit of time.

There are a few cases known in which the degree of substrate conversion is proportional to the time until nearly all substrate is used up (zero degree reaction). However, generally the proportionality holds only for relatively low percentages of conversion. In other words, when plotting per cent conversion against time, we do not obtain straight lines but curves which approach asymptotically the limit of 100% conversion. Generally there is no reason why these curves should represent monomolecular reactions. However, many instances are known in which the reaction curve approximately coincides with a monomolecular curve. In such cases a monomolecular reaction constant may be calculated and used as an expression for the reaction velocity or the amount of enzyme.

The monomolecular formula, as is well known, is derived from the assumption that the reaction velocity, i.e., the amount of material changed per unit of time, is always proportional to the concentrations of unchanged material (substrate). If the initial substrate concentration was a and if x is the amount of substrate changed at the time t, then

$$\frac{dx}{dt} = k(a - x)$$

or

$$k = \frac{1}{t} \ln \frac{a}{a - x}$$

Now in the case of an enzymic reaction there is certainly not always a proportionality between the velocity and the substrate concentration.

What can be assumed is that the velocity is proportional to the concentration of the enzyme-substrate compound. Since the substrate concentration practically always is very high compared to the enzyme concentration, the velocity will be proportional to the enzyme concentration but not always to the substrate concentration. If the initial substrate concentration is very high we should expect practically all of the enzyme to be combined with the substrate and to remain so for a considerable part of the reaction. In other words we should expect to find a reaction of zero degree order. On the other hand, if the initial substrate concentration is low ([S] $< K_m$) we should expect to find a first order reaction. However, here we have assumed that the enzyme has an affinity only to the substrate, but it is well known that many enzymes combine also with certain reaction products. Therefore, when the reaction proceeds, more and more of the enzyme will be bound by reaction products; the amount of enzyme-substrate compound and the reaction velocity will decrease; hence it will be something of a coincidence if a monomolecular reaction is found.

Michaelis has derived a formula for the saccharase-saccharose reaction assuming that the enzyme has an affinity not only to saccharose (S) but also to glucose (G) and fructose (F). The corresponding equilibrium constants we shall call K_m, K_g, and K_f. We find

$$[S] \cdot ([\Sigma] - [ES] - [EG] - [EF]) = K_m \cdot [ES]$$

$$[G] \cdot ([\Sigma] - [ES] - [EG] - [EF]) = K_g \cdot [EG]$$

$$[F] \cdot ([\Sigma] - [ES] - [EG] - [EF]) = K_f \cdot [EF]$$

Since [G] is always = [F] we find

$$[ES] = [\Sigma] \cdot \frac{[S]}{[S] + K_m \left\{1 - [G]\left(\frac{1}{K_g} + \frac{1}{K_f}\right)\right\}}$$

If the initial amount of saccharose in an experiment is a and if at the time t the amounts of glucose and fructose are x, then we obtain:

$$\frac{dx}{dt} = \text{const.} \frac{a - x}{a + K_m - x\left\{1 - K_m\left(\frac{1}{K_g} + \frac{1}{K_f}\right)\cdot\right\}}$$

By integration we find the velocity constant to be:

$$k = \frac{1}{t}\left(\frac{1}{a} + \frac{1}{K_g} + \frac{1}{K_f}\right) a \cdot \ln \frac{a}{a - x} + \frac{1}{t}\left(\frac{1}{K_m} - \frac{1}{K_g} - \frac{1}{K_f}\right) \cdot x$$

The function is a superposition of a logarithmic curve and a straight line.

It should be added that the course of the saccharose hydrolysis by saccharase is even more complicated than this, because the affinity of the enzyme is not the same to the α- and β- forms of the reducing sugars.

CHAPTER 2

Physical Chemistry and Chemical Kinetics of Enzymes

BY E. A. MOELWYN-HUGHES

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There are three broad methods of approaching problems in physical chemistry. The thermodynamic method has the advantage of generality and the drawback of being blind to mechanism. The statistical method aims at mathematical rigor and is therefore limited to systems of simple molecules under artificial conditions. The kinetic method employs time as a weapon, and is *par excellence* the method to use in the physical chemistry of enzymes, where we are primarily interested in the study of molecules in

action. It is therefore fitting that the physical chemistry and the chemical kinetics of enzymes should be treated together.

An examination of the movement of enzyme molecules in solution, in an electrostatic field, and in a centrifugal field yields precise information on their dimensions, charge, and mass. The study of such topics reveals the indebtedness of general physical chemistry to early investigations on enzymes. Much of what we know today on interionic attraction in solution, on adsorption, and catalysis had its origin in the study of enzymes. With Sumner's isolation of crystalline urease in 1926 there opened a new period in the study of enzyme chemistry, marked by a heightened precision of experimental work, to which the newer osmotic, centrifugal, and adsorption techniques have greatly contributed.

The hydrolysis, digestion, or general breakdown of organic substances is kinetically easier to tackle than their union, or synthesis, and the present article is consequently limited to them. Organic synthesis by enzymes is still so far from being understood that a review of this aspect of the subject, notwithstanding its biological importance, may well be postponed at present.

I. Determination of Molecular Weights of Enzymes

Enzyme chemistry, like inorganic chemistry, must be firmly based on accurate molecular weights. There has been no lack of ingenuity in devising methods especially adapted for the determination of these constants in aqueous solutions; and it is with the more important of these methods that we are here concerned.

1. Diffusion in a Concentration Gradient. The Exner-Euler Rule

Ordinary diffusion is the natural process whereby equalization of molecular concentration is realized in a system which is initially of uneven concentration. In such a system, more molecules enter one side of an element of volume than leave it on the other side, and the process continues until equilibrium is reached, when the numbers entering and leaving any element of volume in a given time are equal. At this stage, the concentration throughout the system is uniform, and *net* diffusion ceases. It is important, however, to realize that thermal diffusion persists. According to Fick's first law, the number, dN, of molecules, which, in time dt, cross an area O is proportional to the product Odt, and to the concentration gradient $(\partial n/\partial x)$. The proportionality factor is denoted by the symbol $-D$; and D is known as the coefficient of diffusion. Fick's first law therefore is:

$$\frac{dN}{dt} = -DO\frac{\partial n}{\partial x} \tag{1}$$

In this equation, n is the concentration, in molecules per cm.^{3} The coefficient, D, of diffusion is seen to have the dimensions of cm.^{2} sec.$^{-1}$, and to have a value which is independent of the units in which the concentration is expressed. It represents the number of molecules crossing unit area in unit time across a boundary where the concentration gradient is unity. Alternatively, if we imagine two neighboring cubes of volume 1 cm.^{3} each, D is the probability per second that a single molecule in one of the cells shall pass into the other.

It will be convenient to regard the problem from another angle. Quite generally, the number of particles crossing an area O in unit time is equal to the product of the area, O, the concentration, n, and the average velocity, u, of movement in a direction perpendicular to the plane which is being crossed:

$$\frac{dN}{dt} = Onu \tag{2}$$

On comparing these equations, we see that:

$$D = -\frac{nu}{(\partial n/\partial x)} \tag{3}$$

It will be appreciated that the crux of all diffusion problems is the representation of molecular concentration as a function of space and time. In their experimental elucidation, numerous physicochemical properties of the solutions, such as density, refractive index, and absorption of light, have been employed, in addition to the ordinary methods of chemical analysis of samples removed at different times and from different positions of the diffusing system.

Experiment shows that D in the case of nonelectrolytes decreases as the concentration increases. While no complete explanation of this phenomenon has been forthcoming, the fact must be noted, since it is with coefficients of diffusion extrapolated to infinite dilution alone that we shall deal. For a given solute, the coefficient of diffusion increases with the fluidity of the solvent, and with temperature. Some representative data[1] have been brought together in Table I.

Exner made an empirical observation, since confirmed by von Euler[2] and many other workers, that the coefficient of diffusion of a solute is inversely proportional to the square root of the molecular weight, M, as it is in the case of gaseous diffusion. In view of later developments, a proportionality between D and $M^{1/3}$ can be more readily understood, but an in-

[1] L. W. Öholm, *Z. physik. Chem.* 70 370 (1910); Landolt-Börnstein, Physikalish-chemische Tabellen. The datum for deuterium oxide is due to J. A. V. Butler and W. J. C. Orr, *J. Chem. Soc.*, 1273 (1935).

[2] H. von Euler, Chemie der Enzyme, Bergmann, Munich, I (1920); II(i) (1922); II(ii) (1927).

spection of the data leaves no room for doubt that, whatever may be the final interpretation, the original law of Exner and von Euler is the correct one. After omitting some obviously high results, the average value of $DM^{1/2}$ is found to be about 8.17×10^{-6} at 291.1°. Hence:

$$M = 6.66 \times 10^{-9}/D^2_{291.1} \qquad (4)$$

By means of this equation, von Euler[2] measured the molecular weight of saccharase (Table II). With increasing purity, the molecular weight decreases to a limiting value in the neighborhood of 19,000 g. Herzog[3] applied the same method to the determination of the molecular weights of other

TABLE I

COEFFICIENTS OF DIFFUSION OF SOLUTES IN AQUEOUS SOLUTION AT 291.1°

Solute	M, g.	$D \times 10^5$, cm.^{2} sec^{-1}	$DM^{1/2} \times 10^5$	$DM^{1/2} \times 10^5$
H_2	2	4.87	6.14	6.89
D_2O	20	2.07	5.62	9.26
O_2	32	1.98	6.39	11.20
CH_3OH	32	1.40	4.45	7.93
NH_4OH	35	1.90	6.22	11.24
CH_3CN	41	1.38	6.76	8.83
CO_2	44	1.71	6.04	11.35
N_2O	44	1.61	5.69	10.69
C_2H_5OH	46	1.27	4.55	8.64
CH_3COOH	60	1.09	4.27	8.44
$CO(NH_2)_2$	60	1.12	4.39	8.67
$C_3H_5(OH)_3$	92	0.91	4.11	8.73
C_6H_5OH	94	0.93	4.23	9.02
m-$C_6H_4(OH)_2$	110	0.87	4.18	9.17
Arabinose	150	0.64	3.42	7.82
Glucose	180	0.56	3.16	7.51
Maltose	342	0.41	2.87	7.59
Lactose	342	0.41	2.87	7.59
Sucrose	342	0.42	2.94	7.78
Raffinose	504	0.35	2.76	7.78

enzymes, obtaining results of the same order of magnitude. His value for emulsin, for example, is 37,700 g. It seems likely, however, that equation (4) may sometimes lead to molecular weights which are less than the true values by a factor of from 2 to 3.

2. DIFFUSION IN A CONCENTRATION GRADIENT. THE STOKES-EINSTEIN LAW

Before the linear motion of a particle in a viscous medium can be formulated, it is necessary to know the form of the forces which sustain and

[3] R. D. Herzog, *Z. Elektrochem.* **16**, 1003 (1910).

impede its progress. An assumption usually made is that the force opposing the motion is directly proportional to the velocity, u. If we denote by X the driving force, we thus have the following equation of motion:

$$m\frac{du}{dt} = X - Cu \tag{5}$$

Here C is a positive constant. When a steady state is reached, there is neither acceleration nor deceleration. The velocity is constant; hence:

$$\frac{du}{dt} = 0 \tag{6}$$

Denoting by U the velocity of the particle in the steady state, we see that:

$$U = X/C \tag{7}$$

a relation to which we shall frequently refer. If the velocity, u, of the particle initially were less than this limiting value, U, the medium would

TABLE II

APPARENT MOLECULAR WEIGHT OF SACCHARASE OF VARYING PURITIES[3]

Relative purity	$D_{20,1} \times 10^7$, cm.^{2} sec^{-1}	M, g.
4.2	4.9	28,000
9.6	5.4	22,000
58.1	5.6	20,600
210.0	5.8	19,600

bring it up to this value; if initially the velocity were greater than U, the medium would reduce it to this value.

In order to derive an expression for the driving force, X, in the case of thermal diffusion, we make use of the equation for the free energy of a dilute solution containing N solute molecules in a total volume V at a temperature T, which is:

$$A = A^0 + NkT \ln n \tag{8}$$

in which, as before, n is the concentration:

$$n = N/V \tag{9}$$

In equation (8), which is, of course, a somewhat oversimplified one, the term A^0 represents the free energy of the solution when the concentration is unity, and is a complicated function of solute and solvent properties which mercifully need not be evaluated. The linear force is then:

$$X = -\left(\frac{dA}{dx}\right)_{T,N} = -NkT\left(\frac{d\ln n}{dx}\right)_{T,N} \tag{10}$$

An analogous differentiation with respect to the volume yields the osmotic pressure relationship:

$$\pi = -\left(\frac{dA}{dV}\right)_{T,N} = nkT \tag{11}$$

From equation (10), we see that, on an average, the force exerted on *one* solute molecule is:

$$X = -kT\left(\frac{d \ln n}{dx}\right)_{T,N} = -\frac{kT}{n}\left(\frac{\partial n}{\partial x}\right) \tag{12}$$

On substituting in equation (7), the limiting velocity becomes:

$$U = -\frac{kT}{nC}\left(\frac{\partial n}{\partial x}\right) \tag{13}$$

Using the definition of the diffusion coefficient given by equation (3), we thus obtain Einstein's relation[4]:

$$D = kT/C \tag{14}$$

For a spherical particle of radius r moving in a medium of viscosity η the resistance constant is given by the Stokes expression:

$$C = 6\pi\eta r \tag{15}$$

We thus arrive at the Stokes-Einstein equation:

$$D = kT/6\pi\eta r \tag{16}$$

which has been extensively employed to determine the radii of colloid particles, and thence their molecular weights:

$$M = N_0 m = N_0 \tfrac{4}{3}\pi r^3 \rho \tag{17}$$

Here, N_0 is the Avogadro number (6.063×10^{23}), and ρ the density. Its application to the determination of the molecular weights of enzymes is illustrated in Table III, in which, for the sake of comparison, the data for a sugar and a protein have been included.

3. Distribution of Molecules in a Centrifugal Field

The well known work of Perrin has shown how it is possible, by measuring the variation of the concentration of colloid particles with height, to determine the Boltzmann constant, k, and thence the Avogadro number, N_0, provided the mass of the single particle is known. The procedure can, in principle, be reversed; starting with the generally accepted value of k,

[4] A. Einstein, *Ann. Physik* **19**, 371 (1906); Investigations on the Theory of the Brownian Movement. *Trans.* Cowper, Methuen, London, 1926. Recent summaries of theories of diffusion of solutes have been given by H. S. Harned, *Chem. Revs.* **40**, 461 (1947) and by R. B. Dean, *ibid.* **41**, 503 (1947).

which is 1.372×10^{-16} erg per molecule degree, we can measure the mass of a single molecule, and thence its molecular weight. The gravitational field, however, is relatively weak, and the variation of concentration with height is consequently slight. By means of the ultracentrifuge, centrifugal fields of force may be generated which are about one million times as powerful as the gravitational field; the distribution of the molecular population is thus steeper, and molecular weights can be determined with greater accuracy. The method has been extensively employed in the determination of the molecular weight of proteins, particularly by Svedberg,[5] who has also given a thermodynamic derivation of the distribution law. Its statistical derivation may be given as follows.

TABLE III

MOLECULAR WEIGHTS OF GLUCOSE, TRYPSIN, PEPSIN, AND HEMOGLOBIN DERIVED FROM STOKES-EINSTEIN LAW[a]

Solute	T, °K.	ρ, g./cm.^{3}	η, g./cm. sec.	$D \times 10^7$, cm.^{2}/sec.	$r \times 10^8$, cm.	M, g.
Glucose[a]	298.1	1.545	8.94	678	3.58	180
Trypsin[b]	278.1	1.165/	33.3	2.32	26.2/	61,000/
Trypsin		1.333			21.6	34,000
Pepsin[c]	281.1	1.297	17.0	5.44	22.2	36,000
Hemoglobin[d]	278.1	1.33	15.2	4.86	27.3	68,500

[a] L. Friedmann and P. G. Carpenter, *J. Am. Chem. Soc.* **61**, 1745 (1935).

[b] J. H. Northrop and M. L. Anson, *J. Gen. Physiol.* **12**, 543 (1929).
M. Kunitz, M. L. Anson, and J. H. Northrop, *ibid.* **17**, 373 (1934).

[c] J. H. Northrop, *Ergeb. Enzymforsch.* **1**, 318 (1931).

[d] L. G. Longsworth, in H. A. Abramson, L. S. Mayer, and M. H. Gorin, Electrophoresis of Proteins. Reinhold, New York, 1940.

[e] Equations (16) and (17).

/ Hydrated.

We consider a system at constant temperature, T, to contain N molecules in a total and constant volume, V. The energy, ϵ, per molecule may be expressed as a function of the three spacial coordinates, x, y, and z, and of the conjugated momenta, p_x, p_y, and p_z. According to the Maxwell-Boltzmann law, the number, dN, of molecules in a volume element $dxdydz$, possessing components of momenta lying between p_x and $(p_x + dp_x)$, p_y and $(p_y + dp_y)$, and p_z and $(p_z + dp_z)$ bears the following relation to the total number, N, of molecules in the system:

$$\frac{dN}{N} = \frac{e^{-\epsilon/kT}\, dx\, dy\, dz\, dp_x\, dp_y\, dp_z}{\iiiiiint e^{-\epsilon/kT}\, dx\, dy\, dz\, dp_x\, dp_y\, dp_z} \tag{18}$$

[5] T. Svedberg, *Z. physik. Chem.* **121**, 65 (1926); A. Tiselius, *Trans. Faraday Soc.*, **33**, 524 (1937); J. St. L. Philpot, *Nature*, **141**, 283 (1938).

It is convenient to change from Cartesian to cylindrical coordinates, according to which the element of volume is:

$$dxdydz = rdrd\theta dz \tag{19}$$

and the total volume is that of a cylinder of radius R and length L:

$$V = \iiint dx\, dy\, dz = \int_{z=0}^{L} \int_{\theta=0}^{2\pi} \int_{r=0}^{R} r\, dr\, d\theta\, dz = \pi R^2 L \tag{20}$$

The effect of a constant centrifugal field of angular velocity ω can be statistically incorporated as a potential energy, $-(1/2)\, m\omega^2 r^2$; hence, the total

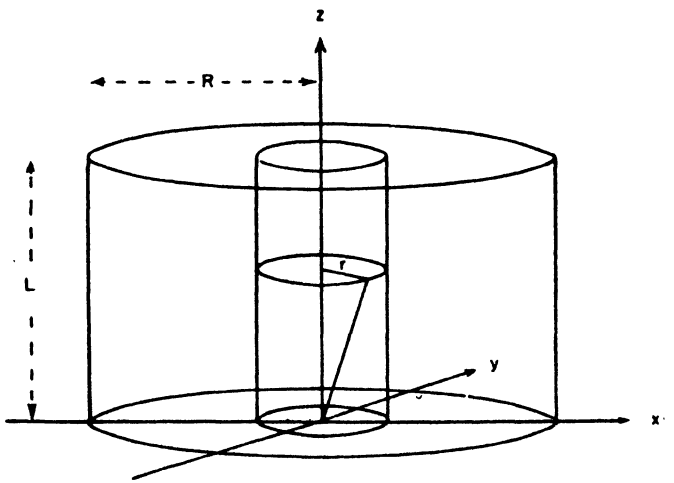


FIG. 1. Transformation from Cartesian to cylindrical coordinates.

energy of a molecule, insofar as it is affected by its translation and by its position, is:

$$\epsilon = \frac{1}{2m}(p_x^2 + p_y^2 + p_z^2) + mgz - \frac{1}{2} m\omega^2 r^2 \tag{21}$$

The p terms are components of momenta; mgz is the gravitational energy. The integral in the denominator, which is $h^3 f$, where h is Planck's constant and f the partition function, must be taken over the whole region of space and energy contemplated (see Fig. 1). We thus have:

$$I = h^3 f = \int_0^{2\pi} d\Theta \iiint_{-\infty}^{\infty} e^{-(1/2mkT)(p_x^2+p_y^2+p_z^2)}\, dp_x\, dp_y\, dp_z$$
$$\int_0^L e^{-mgz/kT}\, dz \int_0^R e^{+(1/2)(m\omega^2 r^2/kT)}\, rdr \tag{22}$$
$$= 2\pi\,(2\pi mkT)^{3/2}(kT/mg)(1 - e^{-mgL/kT})(kT/m\omega^2)(e^{+(1/2)(m\omega^2 R^2/kT)} - 1)$$

In considering the numerator, we note that variations in the angle θ are irrelevant, so that $d\theta$ may be integrated directly, giving 2π. Similarly, we are not concerned with the distribution of velocities or momenta, so that the integration with respect to p_x, p_y, and p_z may be carried out, giving us a term $(2\pi mkT)^{3/2}$ in the numerator also. Hence

$$\frac{dN}{N} = \frac{e^{-mgz/kT}\, e^{+(1/2)(m\omega^2r^2/kT}\, r\, dr\, dz}{(kT/mg)(1 - e^{-mgL/kT})(kT/m\omega^2)(e^{+(1/2)(m\omega^2R^2/kT)} - 1)} \tag{23}$$

But $n_{z,r}$, the concentration of molecules with the specified positions, is $dN/2\pi r dr dz$, and the average concentration is $N/\pi R^2 L$. Consequently:

$$\frac{n_{z,r}}{n} = \left(\frac{mgL}{kT}\right)\left(\frac{e^{-mgz/kT}}{1 - e^{-mgL/kT}}\right)\left(\frac{m\omega^2R^2}{2kT}\right)\left(\frac{e^{(1/2)(m\omega^2r^2/kT)}}{e^{(1/2)(m\omega^2R^2/kT)} - 1}\right) \tag{24}$$

In strong centrifugal fields, the gravitational force, as already stated, is relatively weak, and the appropriate distribution law at all heights thus becomes:

$$\frac{n_r}{n} = \left(\frac{m\omega^2R^2}{2kT}\right) \cdot \frac{e^{(1/2)(m\omega^2r^2/kT)}}{(e^{(1/2)(m\omega^2R^2/kT)} - 1)} \tag{25}$$

Thus the ratio of molecular concentrations at two different distances from the axis of rotation is:

$$\frac{n_1}{n_2} = e^{(1/2)[m\omega^2(r_1^2 - r_2^2)/kT]} \tag{26}$$

which is the desired relation.

Formula (26) has been derived for the distribution of molecular concentration of gases in a centrifugal field. Its application to the distribution of molecular concentrations of solutes in solution gives directly the effective molecular weight, m^*, of the solute as:

$$m^* = \frac{2\,kT \ln (n_1/n_2)}{\omega^2(r_1^2 - r_2^2)} \tag{27}$$

If v_0 denotes the volume of a single dissolved molecule, of density ρ_x, in a solution of density ρ, then:

$$\begin{aligned} m^* &= v_0(\rho_x - \rho) \\ &= v_0\rho_x\left(1 - \frac{\rho}{\rho_x}\right) \\ &= m\left(\frac{\rho_x - \rho}{\rho_x}\right) \end{aligned} \tag{28}$$

The true molecular weight of the solute is thus given by the formula:

$$m = \left(\frac{\rho_x}{\rho_x - \rho}\right)\frac{2\,kT \ln (n_1/n_2)}{\omega^2(r_1^2 - r_2^2)} \tag{29}$$

The consistency of the results obtained by Svedberg and Fåhraeus with carboxyhemoglobin (ρ_H = 1:336 g./ml.) is shown in Table IV, which refers to a solution containing 1 g. protein in 100 ml. solution at 293.3°, centrifuged for 2 days at an angular velocity, ω, of 290.3π radians per second.

4. Rate of Sedimentation in a Centrifugal Field

With very powerful centrifugal fields, the concentration gradient established is so steep as to allow a measurement of the rate of sedimentation. As in the statistical experiments, the concentrations are usually estimated by optical means.

TABLE IV

Centrifugal Determination of Molecular Weight of Carboxyhemoglobin

r_2, cm.	r_1, cm.	c_2[a]	c_1[a]	M	M/16,700
4.61	4.56	1.220	1.061	71,300	4.27
4.56	4.51	1.061	0.930	67,670	4.05
4.51	4.46	0.930	0.832	58,330	3.49
4.46	4.41	0.832	0.732	67,220	4.42
4.41	4.36	0.732	0.639	72,950	4.37
4.36	4.31	0.639	0.564	60,990	3.65
4.31	4.26	0.564	0.496	76,590	4.59
4.26	4.21	0.496	0.437	69,420	4.16
4.21	4.16	0.437	0.308	66.400	3.98
			Average	*67,870*	*4.06*

[a] Grams per 100 ml. solution.

To derive the theoretical formula underlying the method, we refer to the distribution law in the form of equation (25), from which we see that the concentration gradient prevailing at a radial distance, r, is:

$$\frac{dn_r}{dr} = n_r \frac{m^*\omega^2 r}{kT} \tag{30}$$

On comparing with equation (3), we obtain for the diffusion coefficient:

$$D = kT\left(\frac{u}{m^*\omega^2 r}\right) \tag{31}$$

The negative sign does not appear because in this case the transport of matter runs counter to the direction of decreasing concentration. Rearranging, we obtain, for the apparent weight of one molecule:

$$m^* = \frac{kT}{D}\left(\frac{u}{\omega^2 r}\right) = \frac{kTs}{D} \tag{32}$$

and for the molecular weight:

$$m = \left(\frac{\rho_E}{\rho_E - \rho}\right)\frac{kTs}{D} \tag{33}$$

where s stands for the sedimentation constant—a term introduced by Svedberg to denote the linear velocity of sedimentation under unit acceleration. It has the dimensions of time, and, as the data of Table V indicate, can be determined with an accuracy of one in a hundred. From the mean value of s given here, with an independently determined value of the diffusion coefficient, Svedberg obtains a molecular weight of 69,000 for carboxyhemoglobin, in satisfactory agreement with results obtained in other ways.

TABLE V

DETERMINATION OF MOLECULAR WEIGHT OF CARBOXYHEMOGLOBIN BY SEDIMENTATION VELOCITY

Time, min.	$\frac{1}{x}\frac{dx}{dt} \times 10^3$, min.$^{-1}$	Mean x, cm.	Revolutions per min.	$x\omega^2 \times 10^{-7}$	$s \times 10^{+13}$, sec.
30–60	1.23	4.525	39,300	7.66	5.36
60–90	1.30	4.601	39,400	7.96	5.44
90–120	1.30	4.679	39,300	7.93	5.47
120–150	1.28	4.757	39,300	8.02	5.34
150–180	1.33	4.840	39,200	8.16	5.44
				Average	*5.41*

5. OSMOTIC PRESSURE

If a solution obeys Raoult's law, and if the partial molar volumes of solvent and solute are independent of pressure and composition, its osmotic pressure, π, should be given by the relation:

$$\pi = \frac{kT}{v_1}\ln\left(\frac{N_1 + N_2}{N_1}\right) \tag{34}$$

in which v_1 is the molecular volume of the solvent, N_1 the number of solvent molecules, and N_2 the number of solute molecules in the system. This equation is mathematically identical with the following:

$$\pi = \frac{kT}{v_1}\left[\left(\frac{N_2}{N_1}\right) - \frac{1}{2}\left(\frac{N_2}{N_1}\right)^2 + \frac{1}{3}\left(\frac{N_2}{N_1}\right)^3 - \cdots\right] \tag{35}$$

Because of the large molar volumes of enzymes, the molecular ratio, N_2/N_1, with which we have to deal can never be large. In point of fact, it is covered by the range 3×10^{-6} to 3×10^{-4}, the latter figure corre-

sponding roughly to solutions which contain about 50% by weight of enzyme. Unless osmotic pressures can be measured with an accuracy of about one part in 10^5, it is thus pointless to retain any of the terms in equation (35) except the first. Such an accuracy is, of course, not attainable. For systems of interest to us, equation (34) is thus mathematically undistinguishable from the approximation:

$$\pi = kT\left(\frac{N_2}{N_1 v_1}\right) = kTn' \tag{36}$$

where n' is the concentration of solute in molecules per ml. solvent. If we let the term c' stand for the concentration of a solute of molecular weight m, reckoned as grams solute in 100 ml. solvent, we have:

$$n' = c'/100\,m \tag{37}$$

and consequently:

$$\pi = (kT/100\,m)c' \tag{38}$$

Let us now return to the original equation of van't Hoff:

$$\pi = kTn \tag{11}$$

and let the term c stand for the concentration of solute, reckoned as grams solute per 100 ml. solution; then:

$$n = c/100\,m \tag{39}$$

and:

$$\pi = (kT/100\,m)c \tag{40}$$

The osmotic pressure of aqueous solutions of proteins has been investigated experimentally and theoretically by Adair and collaborators,[6] from whose work the data in Table VI have been taken. They refer to the native globin of the ox, prepared in various ways, at 0°, and at the isoelectric point (pH 5.64) of the protein. It is seen that the ratios π/c' and π/c are both reasonably constant. By means of equation (11), the molecular weight ($M = N_0 m$) becomes 37,300, and by means of equation (40) it becomes 36,700. It is, perhaps, worth noting that, over the complete range of protein concentration employed, there seems to be little to choose between van't Hoff's original equation and that derived thermodynamically. The assumptions underlying the latter treatment cannot, therefore, be less plausible than those originally used.

[6] G. S. Adair, *Proc. Cambridge Phil. Soc.* **1**, 75 (1924); *Proc. Roy. Soc. London* **A126**, 16 (1929); J. Roche, A. Roche, G.S. Adair, and M. E. Adair, *Biochem. J.* **26**, 1811 (1932).

With enzymes and other proteins of higher molecular weight, closely similar results are obtained. In buffer solutions not at the isoelectric point, independent estimates must be made of the osmotic pressure, π_i, due to the uneven distribution of ions on the two sides of the membrane. The osmotic pressure is given by the equation:

$$\pi_i = \int_0^{\Psi} nz\epsilon \, d\psi \tag{41}$$

TABLE VI

OSMOTIC PRESSURE OF AQUEOUS SOLUTIONS OF NATIVE GLOBIN OF OX, AT 0°C. AND AT THE ISOELECTRIC POINT (pH 5.64)

Sample	c, g./100 ml. solution	c', g./100 ml. solvent	π, mm. Hg	π/c	π/c'
7	0.84	0.85	4.10	4.88	4.82
6	0.86	0.87	3.96	4.61	4.55
6	0.95	0.96	4.51	4.75	4.70
8	1.01	1.02	4.86	4.81	4.76
6	1.06	1.07	4.46	4.21	4.17
6	1.49	1.51	6.88	4.62	4.56
6	1.53	1.56	7.02	4.59	4.50
7	1.64	1.67	7.59	4.63	4.54
9	2.08	2.13	9.63	4.63	4.52
10	2.50	2.56	10.76	4.41	4.21
6	3.09	3.19	14.62	4.73	4.58
7	3.33	3.42	15.46	4.65	4.52
7	5.79	6.13	28.60	4.95	4.67
7	7.54	8.13	37.40	4.96	4.60
7	10.18	11.30	48.80	4.79	4.32
7	10.18	11.30	53.30	5.23	4.72
			Average	*4.64*	*4.56*
				±0.43	*±0.39*

in which n is the number of enzyme molecules per ml. solution, z the valence of the enzyme ion, ϵ the electronic charge, and Ψ the membrane potential under specified conditions.

It will have been observed that the molecular weight of globin is about one half that of hemoglobin, and, according to Adair and coworkers,[6] is exactly one half if allowance is made for the elimination of the four molecules of hematin which accompany the formation of globin. To this numerical relationship we shall return in a later section.

6. DETERMINATION OF MOLECULAR WEIGHT BY CHEMICAL ANALYSIS

It is well known that the chemical analysis of a pure substance yields only its empirical formula, or its minimum molecular weight. Thus, with

integral values of n, the empirical formula $(CH_2O)_n$ is found for formaldehyde and its polymers, and for the hexoses and starches. Enzymes, however, generally contain small amounts of metals, such as magnesium or iron, or of nonmetals such as sulfur and phosphorus. Accurate physicochemical estimation of the proportion in which these rare constituents are present takes us one step nearer the goal, for the molecule of an enzyme must contain at least one atom of each constituent. Pepsin, for example, prepared from different sources and purified by adsorption and subsequent crystallization, is found by Northrop to contain 0.078 g. phosphorus in 100 g. pure enzyme. Since the atomic weight of phosphorus is 31.024 g., the minimum molecular weight of the enzyme is (31.024/0.078) × 100 =

TABLE VI-A

ELEMENTARY ANALYSIS OF SOME PURE ENZYMES (WEIGHT PER CENT)

Enzyme	C	H	N	S	P	Other elements excluding oxygen
Yellow enzyme[a]	51.5	7.37	15.9	0.48	0.043	—
Trypsin[b]	50.0	7.20	14.9	1.10	0	—
Pepsin[c]	52.4	6.67	15.9	0.86	0.078	0.22 (Cl)
Cytochrome c[d]	49.2	7.33	14.4	1.18	—	0.34 (Fe)
Urease[e]	51.6	7.10	16.0	1.20	—	—
Carboxypolypeptidase[f]	52.6	7.20	14.4	0.47	—	—

[a] H. Theorell, *Ergeb. Enzymforsch.* **6**, 125 (1936).

[b] J. H. Northrop, *J. Gen. Physiol.* **16**, 267 (1932); Crystalline Enzymes. Columbia Univ. Press, New York, 1939.

[c] J. H. Northrop, *Ergeb. Enzymforsch.* **1**, 318 (1931).

[d] D. Keilin, *Ergeb. Enzymforsch.* **2**, 239 (1933).

[e] J. B. Sumner, *Ergeb. Enzymforsch.* **1**, 295 (1932).

[f] M. L. Anson, *J. Gen. Physiol.* **20**, 663 (1937).

39,800 g. This result lies so near that found by other methods as to leave no doubt that the pepsin molecule contains one atom of phosphorus. Similarly, from the percentage of iron in cytochrome c, a minimum molecular weight of 16,500 ± 500 g. is established. Taken in conjunction with the actual molecular weight as determined by the velocity of sedimentation, we conclude that this protein contains one atom of iron per molecule. Hemoglobin, according to the same argument, contains four atoms of iron in one molecule.

The present method of molecular weight determination is clearly applicable only to enzymes which can be prepared with a high degree of purity. A summary of results thus obtained is shown in Table VI-A.

Provided the enzyme preparation may be obtained in a pure, crystalline

state, the molecular weights afforded by the various methods discussed here lead to concordant results, as the data for pepsin (Table VII) indicate. The variation from the mean value is doubtless due in part to the inaccuracy of some of the methods, but it should be remembered that enzymes exist in various states of hydration, and that the bases of various experimental methods of molecular weight determination are not equally trustworthy. The molecular weights of certain enzymes and related proteins are summarized in Table VIII.

TABLE VII

Molecular Weight of Pepsin by Various Methods

Method	Molecular weight
Diffusion coefficient	36,000
Distribution in centrifugal field	39,200
Velocity of sedimentation in centrifugal field	35,500
Osmotic pressure	35,000
Chemical analysis, based on 1 atom P	39,800
Chemical analysis, based on 2 atom Cl	35,000
Chemical analysis, based on 10 atoms S	36,000
Mean	*36,800 ± 3,000*

TABLE VIII

MOLECULAR WEIGHT, M, OF CERTAIN ENZYMES AND OTHER PROTEINS IN WATER

Enzyme	$M \times 10^{-3}$, g.	Protein	$M \times 10^{-3}$, g.
Ribonuclease	14	Cytochrome *C*	14
Saccharase	36(?)	Myoglobin	17
Trypsin	34	Gliadin	27
Pepsin	37	Globin	37
Emulsin	38	Egg albumin	42
Yellow enzyme	76	Hemoglobin	67
Catalase	248	Serum albumin	70
Urease	483	Phyocyanin	272

II. Electrophoresis

Of quite as much importance as the size and shape of enzymes are their electrical properties, in particular the charge on their surface, and the difference in potential between the surface and the solution. These topics have been extensively examined by experimental and theoretical means in recent years.

1. RIGID DOUBLE LAYER OF HELMHOLTZ AND LAMB

The electric potential, ψ, at any point is defined as the electrical work which must be expended in bringing unit positive charge from infinity up

to that point. The potential energy of a charge of magnitude q, at a point where the potential is ψ, is thus ψq, relative to zero potential energy at infinity. If, therefore, an enzyme is a sphere of radius r and possesses a charge q, the electrical potential at any point on its surface is:

$$\psi_1 = q/Dr$$

where D is the dielectric constant of the medium in which the sphere is immersed. This equation implies that the sphere is nonconducting, and that the medium acts as a structureless continuum. Because enzymes in solution are, considered in their entirety, electrically neutral, it seems reasonable to suppose that around each there exists a quantity of electricity of equal magnitude and of opposite sign to that possessed by the enzyme. If such a charge, $-q$, is uniformly spread on the surface of a larger,

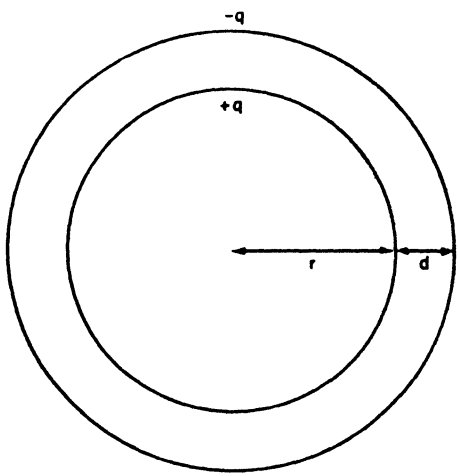


FIG. 2. The rigid double layer.

concentric sphere of radius $r + d$ (Fig. 2), the potential at any point on the surface of this larger sphere is:

$$\psi_2 = -q/(r + d)$$

The potential due to the double layer is thus:

$$\xi = \psi_1 + \psi_2 = \frac{qd}{Dr^2\left(1 + \frac{d}{r}\right)} \tag{42}$$

or, if the thickness, d, of the double layer is small compared with the radius, r:

$$\xi = qd/Dr^2 \tag{43}$$

The surface density of electricity in the present example is:

$$\sigma = q/4\pi r^2 \tag{44}$$

Hence:

$$\xi = 4\pi\sigma d/D \tag{45}$$

A discontinuity of potential thus implies the existence, over the surface of discontinuity, of a double layer of positive and negative electricity, the difference in potential on the two sides being equal to 4π times the electrical moment (per cm.^{2}) of the layer. This is Helmholtz's conclusion, in his own words.[7]

Two extreme accounts may be given in terms of modern theories of the origin of the double layer. The simpler and less probable explanation is that it is due to a set of molecular dipoles, all the positive ends of which give a charge to one sphere, and all the negative ends of which give the charge to the other. The thickness, d, of the double layer in this case is simply the length of the dipoles, which is related as follows to the dipole moment, μ, and the element of charge, e, at either end:

$$\mu = ed \tag{46}$$

If there are n identical dipoles per cm.^{2} of surface, the surface density of electrical charge is:

$$\sigma = ne \tag{47}$$

Hence:

$$\xi = 4\pi n\mu/D \tag{48}$$

If the dipoles are rigid, the concentric spheres possess a net charge of zero, cohere firmly, and do not respond to an external field. To account for the movement known to occur when such a system is placed in a uniform electrostatic field, it is thus necessary to introduce the conception of slip between the layers—a subject examined hydrodynamically by Lamb.[8] From the kinetic-molecular aspect, facility of slip can be thought of as ease of bending of the dipoles from a radial inclination, or of ionization of the polar molecules into cations and anions, one of which is retained by the particle and the other taken up by surrounding solvent molecules. The latter conception is probably nearer the truth insofar as enzymes in aqueous solutions are concerned. Before enlarging on it, we must first deal with the direct experimental measurement of the potential difference between the surface of an enzyme particle and the solution surrounding it.

[7] H. Helmholtz, *Ann. Physik* 7, 337 (1879).
[8] H. Lamb, *Phil. Mag.* (v) 25, 52 (1888).

2. Experimental Determination of Zeta Potential

In addition to the indirect methods afforded by osmosis and electrophoresis, there exists a fairly direct electrostatic procedure by means of which the potential difference between an enzyme surface and its solution may be measured. The enzyme is spread as a unimolecular film on an aqueous solution of known pH in a Langmuir trough kept at constant temperature. One electrode is placed in the bulk of the solution below the film; the other is brought very near the surface from on top. This electrode consists of a copper wire at the tip of which is a radioactive metal, such as polonium; the α-particles emitted by this metal ionize the intervening air gap and establish electrical contact with the film. Working with a sample of pure trypsin provided by Northrop, Schulman and Rideal[9] measured the po-

TABLE IX

Interfacial Potential and Transport Energy for Trypsin-Water Interface[9]

pH	ξ, mv.	$-\xi\epsilon$, cal./g. mole
2	360	8,290
4	306	7,050
6	188	4,330
8	104	2,400
10	80	1,840
12	72	1,660

tentials given in Table IX, the last column of which gives, in calories per gram mole, the energy necessary to bring a single negative ion of unit valence from the bulk of the solution to the surface of the enzyme. The dependence of ξ on pH has the same general form (Fig. 3) in the case of carboxypolypeptidase, pepsin, and egg albumin.

3. Diffuse Double Layer of Gouy

On account of coulomb forces, a positively charged particle repels other positively charged particles, and attracts negatively charged particles. Were these the only forces to be reckoned with, electrically neutral systems formed of ions would consist of an arrangement of ions of one kind surrounded exclusively by ions of the other kind, as in crystalline salts like sodium chloride. In solution, however, the organizing tendency of electrostatic forces is combated by the disorganizing effect of thermal motion, with the result that a positive ion is surrounded predominantly, but not exclusively, by negative ions. Gouy[10] first suggested that the charge, $+q$, on

[9] J. H. Schulman and E. K. Rideal, *Biochem. J.* **27**, 1581 (1933).

a colloid particle may be balanced by a charge $-q$ due to preponderance of negative ions in its vicinity—an idea which has since been applied by Milner, Debye, and others to the simpler systems of solutions of salts. The problem is to determine the average value of the potential, ψ, as a function of the distance, r, from the center of a representative ion. Poisson's equation:

$$\nabla^2\psi = -\frac{4\pi}{D}\Pi \tag{49}$$

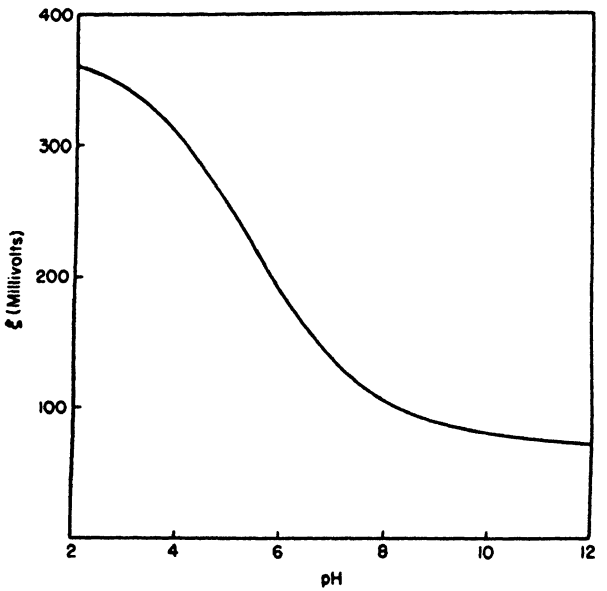


FIG. 3. Interfacial potential of the trypsin-water layer.

relates this potential to the dielectric constant, D, and the charge density, Π, which is the amount of positive electricity per ml. If there are n_+ ions, each with charge ϵ, and n_- ions, each with charge $-\epsilon$, in 1 ml. we have:

$$\Pi = n_+\epsilon + n_-(-\epsilon) \tag{50}$$

By applying of Boltzmann's law to the variation of ionic concentration from the average value, n, and considering only electrostatic energies which are small compared with kT, we have:

$$\frac{n_+}{n} = e^{-\psi\epsilon/kT} \doteq 1 - \frac{\psi\epsilon}{kT} \tag{51}$$

[10] G. Gouy, *J. phys.* **9**, 457 (1910).

and:

$$\frac{n_-}{n} = e^{+\psi\epsilon/kT} \doteq 1 + \frac{\psi\epsilon}{kT} \tag{52}$$

Hence:

$$\nabla^2\psi = \left(\frac{8\pi n\epsilon^2}{DkT}\right)\psi \tag{53}$$

More generally:

$$\nabla^2\psi = \kappa^2\psi, \tag{54}$$

where

$$\kappa^2 = \frac{4\pi\epsilon^2\Sigma n_i z_i^2}{DkT} = \frac{8\pi\epsilon^2 N_0 j}{1000\,DkT} \tag{55}$$

Here, j is the ionic strength, and n_i is the bulk concentration, in ions per ml., of ions of the i'th type, and z_i is the corresponding ionic valence. If the field surrounding the representative ion is spherically symmetrical, the differential equation reduces to:

$$\frac{\partial^2(r\psi)}{\partial r^2} = \kappa^2 r\psi \tag{56}$$

which is known to have the general solution:

$$\psi = \frac{Ae^{-\kappa r}}{r} + \frac{Be^{\kappa r}}{r} \tag{57}$$

The particular solution of interest is that which makes ψ zero when r is infinite, and $d\psi/dr$ equal to the coulombic expression $-q/Dr^2$ when $r = r_0$, the radius of the representative ion, of charge q. It is readily found:

$$\psi = \frac{q}{Dr}\frac{e^{-\kappa(r-r_0)}}{(1 + \kappa r_0)} \tag{58}$$

Making use again of Poisson's equation, we obtain for the electric density

$$\Pi = -\frac{q\kappa^2}{4\pi r}\frac{e^{-\kappa(r-r_0)}}{(1 + \kappa r_0)} \tag{59}$$

This expression will be applied below to the determination of the charge on a protein particle. The total charge of electricity in the whole cloud is:

$$\int_{r_0}^{\infty} \Pi 4\pi r^2\, dr = -q \tag{60}$$

which is equal in magnitude to the charge on the central ion, but of opposite sign.

4. Electrophoretic Mobility

In a field of strength, F, the force acting on a particle of charge, q, is Fq. Equation (5) thus becomes:

$$m(du/dt) = Fq - Cu \tag{61}$$

When a steady state has been reached, both sides of this equation are zero. Letting u now stand for the steady velocity, we have:

$$q = C(u/F) = Cu^0 \tag{62}$$

where u^0 is the electrophoretic mobility, *i.e.*, the velocity in a field of unit strength. If the particle is spherical and subject to Stokes' law (equation 15), then:

$$q = 6\pi\eta r u^0 \tag{62a}$$

These considerations apply only to infinitely dilute solutions, for it has been assumed in deriving them that we can deal with an isolated particle, uninfluenced by the presence of all other solutes. In general, we may write for the mobility at a concentration, n_i, of solute molecules or ions:

$$q = 6\pi\eta r u^0 f(\Sigma n_i) \tag{63}$$

where $f(\Sigma n_i)$ is some function, generally unknown, of all the solute concentrations concerned. In order, therefore, to determine the charge on an enzyme, measurements must be made of the electrophoresis at various ionic and molecular concentrations, and u^0 values thus obtained must be plotted as any convenient function of the total concentration of solute. The concentration itself, its square root, its logarithm, or any other function may be used, provided it affords legitimate extrapolation to zero concentration. In the present state of our knowledge, this method is the only reliable and general one which can be used to measure the charge on a colloid particle from electrophoretic measurements.

Much work has been done in attempting to evaluate $f(\Sigma n_i)$ theoretically. According to Henry,[11] it is explicitly a function of κr, involving seven terms, and varying from unity for zero values of κr (infinite dilution or negligibly small radius) to $\frac{2}{3}$ for infinite values of κr (strong solutions or large radius). The simpler treatment of Hückel[12] may be given to illustrate the nature of the problem.

The effect of the electrostatic field is to push the enzyme ion in one direction and to pull the ion cloud which surrounds it in the opposite direction. Due to the coherence between the enzyme and its cloud, the latter effect has a retarding influence; hence the mobility in real solutions is always less

[11] D. C. Henry, *Proc. Roy. Soc. London* **A133**, 106 (1931).
[12] E. Hückel, *Z. Physik* **25**, 204 (1924).

than that in infinitely dilute solutions. According to equation (59), the amount of electricity in a shell of radius r and thickness dr is:

$$dq = -q \frac{e^{-\kappa(r-r_0)}}{(1 + \kappa r_0)} \kappa^2 r \, dr \tag{64}$$

The force acting on this shell is Fdq. If the movement of the shell can be likened to the movement of a sphere of this radius, which obeys Stokes' law, the increment of velocity due to it is:

$$du' = -\frac{Xq}{6\pi\eta} \frac{e^{-\kappa(r-r_0)}}{(1 + kr_0)} \kappa^2 \, dr \tag{65}$$

The effective total velocity of the whole cloud is obtained by integrating this expression from r_0, the radius of the enzyme, to infinity. It is:

$$u' = -\frac{Xq}{6\pi\eta} \left(\frac{\kappa}{1 + \kappa r_0} \right) \tag{66}$$

The actual velocity of the whole system is thus:

$$u = \frac{Xq}{6\pi\eta r_0} - \frac{Xq}{6\pi\eta} \left(\frac{\kappa}{1 + \kappa r_0} \right)$$

$$= \frac{Xq}{6\pi\eta r_0} \left(\frac{1}{1 + \kappa r_0} \right)$$

and the mobility is:

$$u^0 = \frac{q}{6\pi\eta r_0} \left(\frac{1}{1 + \kappa r_0} \right) \tag{67}$$

This expression we shall use in the next section to determine the charge on a protein ion.

The specific influences of the viscosity of the medium,[13] the shape of the particle,[14] the size of inorganic ions,[15] the conductivity of the sphere, and the deformability of the cloud[16] have been theoretically investigated.[17]

5. Determination of Charge on a Protein Ion

Tiselius and Svensson[18] have measured the mobility of egg albumin in aqueous solutions at 0.5°, keeping the pH constant at 7.10, and varying

[13] M. von Schmoluchowski, *Ann. Physik* **21**, 756 (1906); *Kolloid Z.* **18**, 194 (1916).
[14] J. J. Bikermann, *Z. physik. Chem.* **163**, 378 (1933).
[15] M. H. Gorin, *J. Chem. Phys.* **7**, 405 (1939).
[16] H. A. Abramson, Electrokinetic Phenomena. Reinhold, New York, 1934; H. A. Abramson and L. Michaelis, *J. Gen. Physiol.* **12**, 586(1928).
[17] H. A. Abramson, L. S. Moyer, and M. H. Gorin, Electrophoresis of Proteins. Reinhold, New York (1942). See also the report of a symposium held by the Faraday Society on the Electrical Double Layer, *Trans. Faraday Soc.* **36**, 711 (1940).
[18] A. Tiselius and H. Svensson, *Trans. Faraday Soc.* **36**, 16 (1940).

the ionic strength over a hundredfold. Under these conditions ($D = 87.7$, $T = 273.6°$), it follows from equation (55) that:

$$\kappa = 3.29 \times 10^7 \times \sqrt{j}$$

From diffusion experiments, the radius, r_0, is known to be 2.75×10^{-7} cm. Hence:

$$\kappa r_0 = 8.93 \sqrt{j}$$

In Table X, the first and last columns give the experimental values of the ionic strength and the mobilities. The intervening columns contain the values of κr_0 and the function $(1 + \kappa r_0)$ calculated by means of this relation. On plotting u_0 against $1/(1 + \kappa r_0)$ (Fig. 4) a very satisfactory linear

TABLE X

ELECTROPHORESIS OF EGG ALBUMIN[18c]

j	κr_0	$(1 + \kappa r_0)$	$u^0 \times 10^5$, cm. sec.$^{-1}$/volt cm.$^{-1}$
0	0	1.00	(20.35)
0.00143	0.33	1.33	15.6
0.00442	0.59	1.59	13.2
0.00941	0.87	1.87	11.6
0.0244	1.39	2.39	9.45
0.0494	1.99	2.99	7.60
0.100	2.82	3.82	6.70
0.140	3.34	4.34	6.29
0.200	4.08	5.08	5.70

[a] $T = 273.6°$; $D = 87.7$; pH = 7.10; $r_0 = 27.5 \times 10^{-8}$ cm

relationship is obtained. The value of the ordinate corresponding to unit value of the abscissa gives the electrophoretic mobility at infinite dilution to be 20.35×10^{-5} (cm./sec.)/(volt/cm.), or, expressing the field in electrostatic units:

$$u^0 = 6.105 \times 10^{-2} \text{(cm./sec.)/(e.s.u./cm.)}$$

By means of equation (62), therefore, with $\eta = 1.79 \times 10^{-2}$ g./cm. sec., we find that the charge is:

$$q = 5.67 \times 10^{-9} \text{ e.s.u.}$$

and therefore the valence, obtained by dividing by the electronic charge (4.77×10^{-10} e.s.u.), is:

$$z = q/\epsilon = 11.9$$

which is in absolute agreement with the value found by Adair from his studies of the membrane potential under the same conditions. In point of

fact, Tiselius and Svensson set out by accepting Adair's value for the valence, and use their results to show the agreement with the theory of Henry, which is in all ways satisfactory. The present analysis indicates a comparable agreement with the simpler theory of Hückel, and the impossibility of deciding in favor of either.

6. Isoelectric Point

Though the charge on an enzyme particle is affected by the concentration of all the various ions present in solution, it is most sensitive to the concentration of hydrogen ions and hydroxyl ions. If, for example, we add a

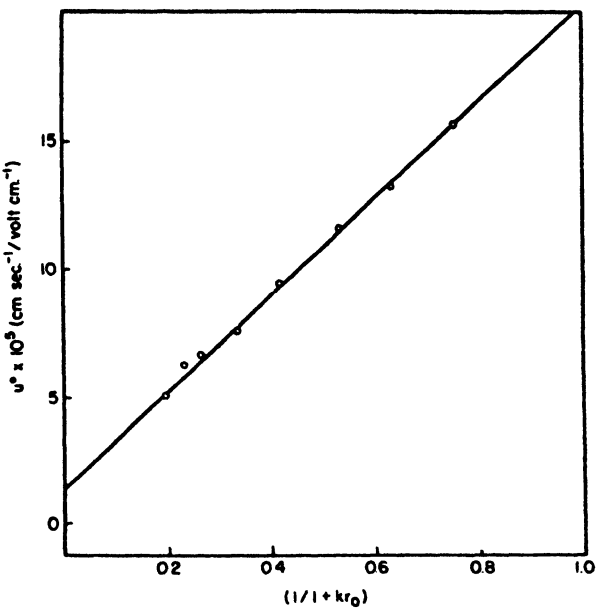


Fig. 4. Effect of ionic strength on electrophoretic mobility of egg albumin at constant pH.

strong acid to a solution containing a negatively charged enzyme, its charge is reduced, and consequently the velocity with which it moves toward the anode is diminished. With further increases in the concentration of hydrogen ion the charge on the enzyme is reduced to zero and the particles are motionless in an electrostatic field. A still further increase in the hydrogen ion concentration endows the enzyme with a positive charge, and a consequent motion in the opposite direction, that is, toward the cathode. The isoelectric solution is that in which the enzyme has zero charge and mobility. A typical curve showing the dependence of u^0 on pH is to be seen in Fig. 5, to which the data in Table XI refer.

From the data of Longsworth on the mobility of egg albumin at various

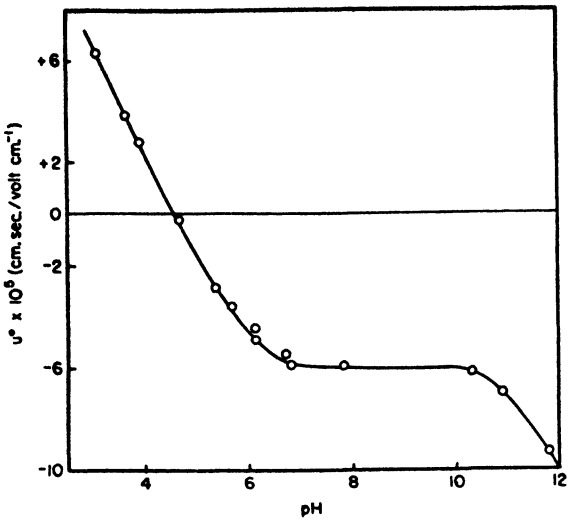


FIG. 5. Effect of pH on electrophoretic mobility of egg albumin at constant ionic strength.

TABLE XI

EFFECT OF pH ON MOBILITY AND VALENCE OF EGG ALBUMIN AT 0°, AND IONIC STRENGTH OF 0.1[19]

Buffer	pH	$u^0 \times 10^5$, cm. sec^{-1}/volt $cm.^{-1}$	z Spherical model	z Cylindrical model
Glycine	3.05	+6.25	+11.06	+13.13
Acetate	3.62	+3.89	+6.89	+8.17
"	3.91	+2.79	+4.94	+5.86
"	4.64	−0.20	−0.35	−0.42
"	5.33	−2.82	−4.99	−5.91
"	5.65	−3.53	−6.24	−7.40
Cacodylate	6.12	−4.46	−7.89	−9.34
Phosphate	6.12	−4.94	−8.74	−10.40
"	6.71	−5.46	−9.68	−11.4
"	6.80	−5.92	−10.5	−12.4
Barbiturate	7.83	−5.92	−10.5	−12.4
Glycine	10.28	−6.21	−11.0	−13.0
"	10.88	−7.00	−12.4	−14.7
"	11.81	−9.31	−16.5	−19.6

[19] L. G. Longsworth, *in* H. A. Abramson, L. S. Moyer, and M. H. Gorin Electrophoresis of Proteins, New York, 1942.

pH values, Abramson, Moyer, and Gorin[17] have calculated the valences of the proteinate ion, using Henry's theory[11] as modified by Gorin,[15] to take account of the finite mean radius, r_i, of the buffer ions. The charge on a spherical particle is given by the expression:

$$q = 6\pi\eta r_0 u^0 \frac{(1 + \kappa r_0 + \kappa r_i)}{(1 + \kappa r_i) f(\kappa r_0)} \tag{67a}$$

If we were to omit the correcting term, κr_i, we would obtain Henry's equation of electrophoresis. Henry's function, $f(\kappa r_0)$, in the present instance is 1.097. If that function was taken as unity we would, of course, obtain equation (67). The valences of the protein, based on a cylindrical model, have been computed by adopting a resistance factor differing from Stokes'

TABLE XII

ISOELECTRIC POINTS OF CERTAIN ENZYMES AND SIMPLE AMPHOLYTES

Enzyme	pH at isoelectric point	H ion concn., g. mole/liter
Pepsin	2.85	1.41×10^{-3}
Urease	5.05	8.91×10^{-6}
Catalase	5.58	2.63×10^{-6}
Trypsin	7.50	3.16×10^{-8}
β-Fructosidase	8.25	5.62×10^{-9}
Flavase	9.85	1.41×10^{-10}
p-Aminobenzoic acid	2.14	7.24×10^{-3}
Aspartic acid	2.96	1.10×10^{-3}
Alanine	6.20	6.31×10^{-7}
Oxyhemoglobin	6.75	1.78×10^{-7}
Arginine	10.50	3.16×10^{-11}

expression for spheres. Within the range considered the valence of the egg albumin particle varies from about +12 in acid solution to about −18 in alkaline solution. These are not necessarily the maximum valences, nor is there any reason to expect them to be equal.

A summary of the concentrations of hydrogen ions for isoelectric solutions of enzymes and simpler amino acids is given in Table XII.

7. MULTIPLE IONIZATION OF COLLOIDAL AMPHOLYTES

The charge on an enzyme particle has its origin in the ionization of the (usually weak) acidic and basic organic groups of atoms which form an essential part of its structure. In order to gain a pictorial representation of the phenomenon, let us suppose that these groups are all situated on the

enzyme surface, and that one enzyme has a number, a, of acidic groups and a number, b, of basic groups. It will be necessary also to assume that the groups are sufficiently far apart to ionize independently, so that we are virtually dealing with the ionization of a weak acids and b weak bases. The rate of increase in the concentration, n_{H}, of hydrogen ions in solution becomes, by Langmuir's method:

$$+\frac{dn_{\mathrm{H}}}{dt} = n_E a\,[\nu_{\mathrm{H}}\theta_{\mathrm{H}} - k_{\mathrm{H}} n_{\mathrm{H}}(1 - \theta_{\mathrm{H}})] \tag{68}$$

where n_E is the number of enzyme molecules per ml., and θ_{H} is the fraction of acidic groups undissociated. At equilibrium, this rate is zero, and consequently:

$$\theta_{\mathrm{H}} = \frac{k_{\mathrm{H}} n_{\mathrm{H}}}{\nu_{\mathrm{H}} + k_{\mathrm{H}} n_{\mathrm{H}}} \tag{69}$$

Similarly, for the fraction of basic groups undissociated, we obtain:

$$\theta_{\mathrm{OH}} = \frac{k_{\mathrm{OH}} n_{\mathrm{OH}}}{\nu_{\mathrm{OH}} + k_{\mathrm{OH}} n_{\mathrm{OH}}} \tag{70}$$

The constants k_{H}, ν_{H}, k_{OH}, and ν_{OH} in these expressions can readily be shown to be related as follows to the ionization constants of the acidic and basic groups:

$$K_a = \nu_{\mathrm{H}}/k_{\mathrm{H}} \tag{71}$$

$$K_b = \nu_{\mathrm{OH}}/k_{\mathrm{OH}} \tag{72}$$

The charge of electricity on the enzyme surface is thus:

$$q = a(-\epsilon)(1 - \theta_{\mathrm{H}}) + b\epsilon(1 - \theta_{\mathrm{OH}}) \tag{73}$$

On substituting the expressions for θ and K, using the ionic product of water:

$$K_w = n_{\mathrm{H}} n_{\mathrm{OH}} \tag{74}$$

we obtain for the valence of the enzyme:

$$\frac{q}{\epsilon} = b\frac{n_{\mathrm{H}}}{(K_w/K_b) + n_{\mathrm{H}}} - a\frac{K_a}{K_a + n_{\mathrm{H}}} \tag{75}$$

In strongly acid solutions, the valence is seen to be b, the number of basic groups per enzyme particle; in alkaline solution, the valence is $-a$, the number of acidic groups per enzyme particle. At the isoelectric point, when the valence is zero, the concentration of hydrogen ions is given by the quadratic equation:

$$n_{\mathrm{H}}^2 + \left(1 - \frac{a}{b}\right) K_a n_{\mathrm{H}} - \frac{a}{b}\frac{K_w K_a}{K_b} = 0 \tag{76}$$

which reduces, when $a = b$, to:

$$n_H^2 \text{(isoelectric)} = K_w K_a / K_b \tag{77}$$

This well known relationship can, of course, be derived in other ways. The present derivation has been given in order to illustrate Langmuir's method, which will be used in other sections of this article.

The increase in heat content attending a reaction for which the equilibrium constant is K is given by the van't Hoff isochore:

$$\Delta H = RT^2(d \ln K/dT) \tag{78}$$

The temperature variation of the isoelectric pH is thus given by the relation

$$-4.606\, RT^2 \frac{d(\text{pH})}{dt} = \Delta H_w + \Delta H_a - \Delta H_b \tag{79}$$

in which ΔH, with the subscripts w, a, and b are the increases in heat content associated with the ionization of one gram mole each of water, acid, and base, respectively. If the two latter are equal, $d(\text{pH})/dT$ is determined by the heat of ionization of water (13,450 cal.), and is -0.0331 at 25°. On the other hand, solutions of proteins may be buffered at concentrations of hydrogen ions remote from the isoelectric point. Application of equation (78) then gives a composite heat effect in terms of the mole fractions of acid and base that are ionized. By this means, Adair, Cordero, and Shen[20] have determined ΔH_a as 6,500 ± 700 cal. in the case of hemoglobin, and have reconciled previously conflicting results derived from titration and calorimetric sources.

III. Catalysis

Detailed aspects of catalysis by enzymes are to be found in any of the standard works on this subject.[21] Recent advances have also been the subject of review.[22] A brief account will here be given of the principal features of enzyme catalysis, outlining, as it were, the trunk and branches of the tree, but omitting reference to the twigs and foliage.

As in earlier passages, the concentration in molecules per cm.^{3} is denoted by the letter n, which is related as follows to the concentration, c, expressed in gram moles/liter:

$$n = \frac{N_0}{1000} c = 6.06 \times 10^{20} c \tag{80}$$

[20] G. S. Adair, N. Cordero, and T. C. Shen, *J. Physiol.* **67**, 288 (1929).

[21] E. Bamann and K. Myrbäck, Methoden der Fermentforschung. Thieme, Leipzig, 1941; J. B. S. Haldane and K. G. Stern, Allgemeine Chemie der Enzyme. Steinkopff, Dresden and Leipzig, 1932; E. Waldschmidt-Leitz, Enzyme Action and Properties. London, 1929. See also reference 2; J. B. Sumner; *Ergeb. Enzymforsch.* **1**, 295 (1932); J. B. Sumner and G. F. Somers, Chemistry and Methods of Enzymes. 2nd ed. Academic Press, New York, 1947.

[22] E. A. Moelwyn-Hughes, *Ergeb. Enzymforsch.* **2**, 1 (1933); **6**, 23 (1937); Handbuch der Enzymologie. Akademische Verlagsgesellschaft, Leipzig, 1939.

Subscripts, E, S, P, C, and I will be used to denote, respectively, enzyme, substrate, product, complex, and inhibitor.

1. Dependence of Reaction Velocity on Concentration of Substrate. Michaelis Law

Let us consider an enzyme–substrate system described by the following scheme:

$$\underset{(n_E - n_C)}{\text{Enzyme}} + \underset{n_S}{\text{substrate}} \underset{k_1}{\overset{k_2}{\rightleftarrows}} \underset{n_C}{\text{complex}} \xrightarrow{k_3} \underset{n_P}{\text{products}}$$

in which k_1 and k_3 are unimolecular velocity coefficients, and k_2 is a bimolecular velocity coefficient. By equating the rate of disappearance of substrate molecules to the rate of appearance of product molecules:

$$-\frac{dn_S}{dt} = +\frac{dn_P}{dt} = k_2(n_E - n_C)n_S - k_1 n_C = k_3 n_C$$

we see that:

$$\frac{(n_E - n_C)n_S}{n_C} = \frac{k_1 + k_3}{k_2} = K \tag{81}$$

Similarly, by equating to zero the sum of the rates of appearance and disappearance of complex molecules:

$$+\frac{dn_C}{dt} = k_2(n_E - n_C)\, n_S - k_1 n_C - k_3 n_C = 0$$

we obtain, for the stationary concentration of intermediate complex:

$$n_C = \frac{k_2 n_E n_S}{(k_1 + k_3) + k_2 n_S}$$

By eliminating n_C, the rate of reaction becomes:

$$r = -\frac{dn_S}{dt} = \frac{k_3 n_E n_S}{K + n_S} \tag{82}$$

Hence, at all concentrations of substrate, the rate of reaction is directly proportional to the concentration of enzyme molecules. This is the first law of enzyme catalysis, to which there are but few exceptions. At relatively low concentrations of substrate, the rate equation becomes that of a bimolecular reaction:

$$-\frac{dn_S}{dt} = k_2\left(\frac{k_3}{k_1 + k_3}\right) n_E n_S \tag{83}$$

Because, however, the concentration of catalyst is constant, the unimolecular law is obeyed, namely:

$$\frac{1}{t} \ln \frac{n_s^0}{n_s} = k_{uni} = \frac{k_1 k_2 n_E}{k_1 + k_2}$$

Here, n_s^0 represents the initial concentration of substrate, and n_s its concentration after a time, t, from the start of the reaction. This relation also has found wide applicability, by von Euler, Van Slyke, Nelson, Northrop, and others. Returning to equation (82), we note that the rate of reaction increases as the initial concentration of substrate is raised, until it reaches, asymptotically, a limiting rate, which is given by the relation:

$$R = -\left(\frac{dn_s}{dt}\right)_{limit} = k_3 n_E \tag{84}$$

The constant, k_3, thus stands for the upper limit to the number of substrate molecules which one enzyme can decompose in one second. Warburg[23] has called it the turnover number. On comparing equations (82) and (84), we have:

$$\frac{r}{R} = \frac{n_s}{K + n_s} \tag{85}$$

a relation which is illustrated by Fig. 6. Clearly, when the rate, r, equals one half the limiting rate, R, the concentration of substrate is numerically equal to K:

$$K = n^*_s \tag{86}$$

It is by this means that the Michaelis constant has been evaluated for a large number of enzyme–substrate systems (see Table XIII).

The increase in free energy attending the dissociation of the complex into free enzyme and substrate molecules is given by the van't Hoff isotherm as:

$$\Delta G^0 = -RT \ln K \tag{87}$$

Numerical values are included in Table XIII. If the Michaelis constants were known at various temperatures, we could, on applying equation (78) determine the increase in heat content, ΔH, attending the dissociation of the complex, and the increase in standard entropy, using the definition:

$$\Delta S^0 = \frac{\Delta H - \Delta G^0}{T} \tag{88}$$

Only in a very few cases are such data available. From von Euler's work on

[23] O. Warburg, *Ergeb. Enzymforsch.* 7, 210 (1938). The time unit used by Warburg is, however, the minute.

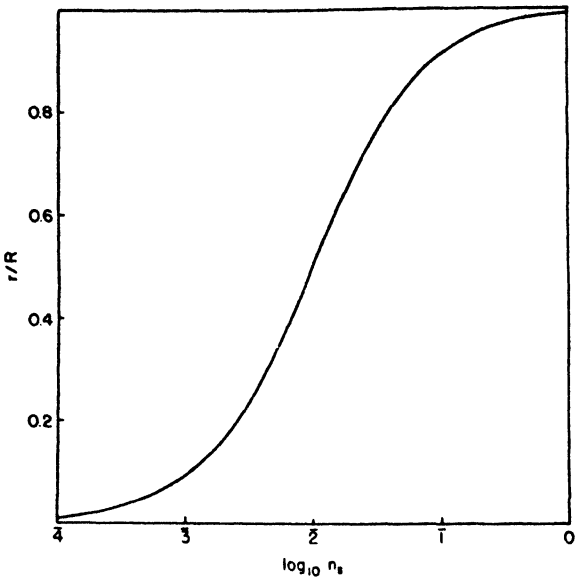


FIG. 6. Influence of substrate concentration on the rate of enzymatic reactions.

TABLE XIII

MICHAELIS CONSTANTS FOR ENZYME-SUBSTRATE SYSTEMS AT 298.1°

Enzyme	Substrate	K, g. moles/liter	$\Delta G°$, cal./g. mole
Maltase	Maltose	2.1×10^{-1}	900
Maltase	α-Methylglucoside	5.6×10^{-2}	1700
β-Glucosidase	Helicin	1.6×10^{-2}	2400
"	Arbutin	4.2×10^{-3}	1900
"	Salicin	2.6×10^{-3}	2200
"	β-Methylglucoside	8.6×10^{-1}	100
Saccharase	Sucrose	2.8×10^{-2}	2100
"	Raffinose	4.5×10^{-1}	500
Zymase	Glucose	6.0×10^{-3}	3000
Lipase	Ethyl D-mandelate	5.0×10^{-4}	4500
"	Ethyl L-mandelate	1.6×10^{-3}	3800
Phosphatase	Glycerylphosphoric acid	3.0×10^{-3}	3400
Urease	Urea	2.5×10^{-2}	2200
Oxygenase	Oxygen	1.5×10^{-6}	7900
Oxidase	Citrate	2×10^{-4}	5000
Dehydrogenase	Methylene blue	7×10^{-6}	7000
Xanthine oxidase	Adenine, xanthine	3×10^{-6}	6200

the sucrose–saccharase system, it is found that ΔH is 2000 cal., and ΔS^0 is 0.3 cal./mole degree. His results and those reproduced by the equation:

$$K = e^{\Delta S^0/R} \cdot e^{-\Delta H/RT} \qquad (89)$$

are given in Table XIV. The magnitude and sign of the entropy term indicate that the complex is slightly more orderly than the free enzyme and substrate.

Hitchcock, Weidenhagen, and others have shown how an equation of the Michaelis type can be derived on the basis of Langmuir's adsorption theory. Let us suppose a single enzyme to have on its surface a number, n_0, of active centers on which substrate molecules can be adsorbed. At equilibrium, let a fraction, θ_S, of these centers be occupied. Then:

$$+\frac{dn_S}{dt} = \nu_S n_0 \theta_S - k_S n_S(1 - \theta_S) = 0$$

where ν_S is the probability per second that an adsorbed molecule evaporates, and $k_S n_S$ is the total number of collisions made per second by substrate

TABLE XIV

MICHAELIS CONSTANTS FOR SACCHARASE-SUCROSE SYSTEM AT VARIOUS TEMPERATURES[a]

	$K \times 10^2$, g. moles/liter	
$T°$	Observed	Calculated
274	21	20
288	25	24
298	26	27
312	32	31

[a] After von Euler.[2]

molecules on the active portion of the surface. Then:

$$\theta_S = \frac{k_S n_S}{k_S n_S + \nu_S n_0} \tag{90}$$

which agrees formally with equation (85). When the substrate is present at very high concentrations, the fraction, θ_S, becomes unity, that is, all the active centers are occupied. When the velocity has reached half its limiting value, $\theta_S = \frac{1}{2}$, and $K = n_0 \nu_S / k_S$.

The treatment can be taken a stage further by using theoretical expressions for ν_S and k_S. For the former, we have in terms of Planck's constant, h:

$$\nu_S = (kT/h)e^{-E_1/RT}$$

and for the latter we have the kinetic theory expression:

$$k_S = \sigma n_0 \sqrt{\frac{kT}{2\pi m}}\, e^{-E_2/RT}$$

where o is the area of an active spot, and k is Boltzmann's constant. Hence:

$$K = \frac{(2\pi mkT)^{1/2}}{ho} e^{-(E_1-E_2)/RT} \tag{91}$$

Similar calculations have led to the conclusion that there are from 1 to 8 active centers on a saccharase molecule.[22] Sumner and Myrbäck[24] had, by another method, previously estimated the number as 7 in the case of urease.

2. Dependence of Reaction Velocity on Concentration of Inhibitor

Molecules other than the specific substrates can be adsorbed on the enzyme surface. They are termed inhibitors, or, if strongly adsorbed, poisons. The divalent cations of copper, zinc, mercury, and lead, the univalent cations of silver and ammonium, the univalent anions cyanide and hydrosulfide, and molecules such as cysteine and glutathione are typical examples. The adsorption of inhibitors is generally reversible, so that, by kinetic experiments, the equilibrium constant governing the formation of the inhibitor–enzyme complex can be investigated in the same way as the equilibrium governing the formation of the substrate–enzyme complex (equation 81). Its value in the saccharase–aniline and the saccharase–silver examples are 2.5×10^{-4} and 4.0×10^{-8} moles/liter, respectively.[25] For the complete inhibition of one molecule of urease, 7 atoms of silver are required.[24]

In applying the adsorption theorem to the problem of inhibition, we may denote by θ_I the fraction of the enzyme surface covered by inhibitor molecules at equilibrium. In the presence of substrate, the fraction of the surface which is unoccupied is now $(1 - \theta_S - \theta_I)$. On solving two simultaneous equations of the Langmuir form, we thus obtain, for the fraction, θ_S, covered by adsorbed substrate molecules:

$$\theta_S = \frac{k_S n_S}{k_S n_S + \nu_S n_0 + k_I n_I(\nu_S/\nu_I)} \tag{92}$$

Since the rate of reaction is proportional to θ_S, we see that it is adversely affected by the inhibitor. The fraction of the surface covered in the uninhibited reaction system is given by equation (90). Denoting it by the symbol θ_S^0, we obtain the following ratio between the velocity of the free and the inhibited reaction:

$$\frac{\theta_S^0}{\theta_S} = 1 + \frac{k_I(\nu_S/\nu_I)n_I}{k_S n_S + \nu_S n_0} \tag{93}$$

This relation is illustrated in Fig. 7 with reference to the classical work of von Euler and Svanberg[2] on the inhibitive action of mercuric chloride on

[24] J. B. Sumner and K. Myrbäck, *Z. physiol. Chem.* **189**, 218 (1930).
[25] K. Myrbäck, *ibid.* **158**, 160 (1926).

the invertase–sucrose system ($n_s = 1.70 \times 10^{20}$ molecules/cm.^{3}). From the gradient of the curve, we have:

$$\frac{(k_I/v_I)}{(k_S/v_S)} = 6.9 \times 10^4 = \frac{K_{\text{enzyme–substrate}}}{K_{\text{enzyme–inhibitor}}}$$

Taken in conjunction with the known heat effect already discussed, we find that 8500 cal. are given out during the formation of one mole of com-

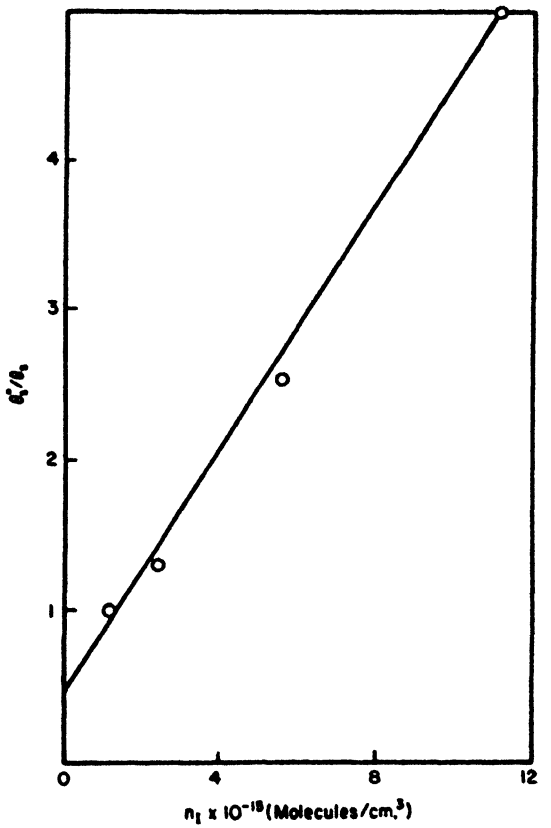


FIG. 7. Inhibiting effect of mercuric chloride on the rate of the sucrose–saccharase reaction.

plex. That this is greater than the heat evolved during the formation of the enzyme–substrate complex is consistent with the greater stability of the inhibitor complex.

By setting θ_s^0/θ_s of equation (93) equal to 2, we obtain the following expression for the concentration of inhibitor necessary to reduce the velocity of reaction to one half of the inhibited value:

$$n_I = \frac{k_S n_S + v_S n_e}{k_I(v_S/v_I)} \tag{94}$$

Enzymes can often be vigorously heated in the presence of the products of reaction without loss in catalytic activity, though similar heating in the absence of these products leads to inactivation, or loss of activity. This well known protective action finds a ready explanation in terms of the adsorption theory. Inhibition by the products of reaction immediately results if the products are more strongly adsorbed than the reactants. This aspect of enzyme chemistry is one which Langmuir specifically considered in his fundamentally important paper.[26] The differential rate equation is obtained by replacing the term n_I by n_P in equation (92) and by noting that n_P is proportional to $n_S^0 - n_S$. The integrated expression shows that the time required for half completion increases linearly with respect to the initial concentration of substrate. There can be little doubt that the sensation of satiety has its origin in the covering of enzyme surfaces progressively with the course of reaction.

In terms of the chain theory, inhibition may be due to the interruption of the reaction mechanism in the solution surrounding an enzyme, and need not be connected with the interfacial effect.

3. Dependence of Reaction Velocity on Concentration of Hydrogen Ions

The variation of the velocity with respect to pH in the case of two typical enzyme-catalyzed reactions is illustrated in Fig. 8, which has been plotted from the data of Michaelis and Davidsohn. The ordinate represents not the actual velocity, but the ratio of the actual velocity to the maximum value measured at the pH optimum. The occurrence of this optimum concentration of hydrogen ion is one of the most characteristic features of enzyme reactions, differentiating them sharply from other catalyses in solution. Experiment shows that the value of the pH optimum is to some extent dependent on the concentration and nature of both substrate and buffer (see Table XV). Reported differences in the optimum pH for the same enzyme derived from different sources, but investigated under identical conditions, may probably be attributed to incomplete purification.

A comparison of the data of Tables XII and XV shows that there is a parallelism between the pH of the isoelectric point and the pH of optimal catalytic activity. Though not identical, isoelectric pH and pH optimum seldom differ by more than 2, the difference being negative in acid solutions and positive in alkaline solutions. These facts suggest that the most effective catalytic state of the enzyme is the uncharged state. This is the classical interpretation of Michaelis,[27] who showed, by means of mass action that the fraction of unionized ampholyte varies with respect to pH in the same way as the rate of reaction varies. In modern terminology, it may be said

[26] I. Langmuir, *J. Amer. Chem. Soc.* **38**, 2221 (1916).
[27] L. Michaelis, *Biochem. Z.* **33**, 182 (1911)

that the rate of reaction is approximately proportional to the fraction of the enzyme surface which is uncharged. This interesting conclusion is by no

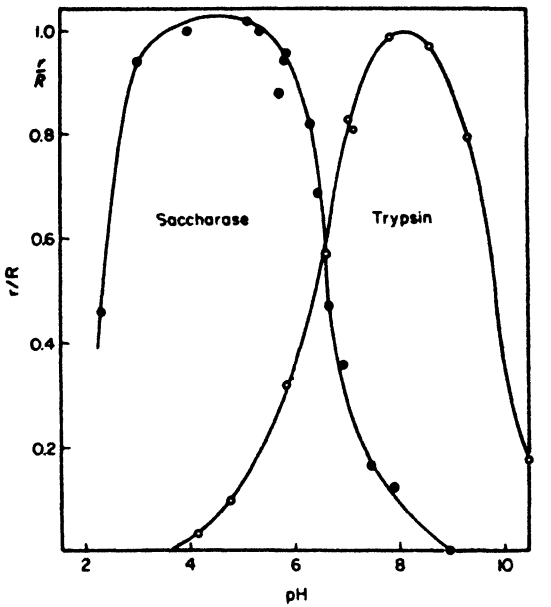


FIG. 8. Dependence of rate of enzymatic reactions on pH.

TABLE XV

OPTIMAL pH OF CERTAIN ENZYME-SUBSTRATE SYSTEMS

Enzyme	Substrate	Optimal pH
Pepsin	Egg albumin	1.5
"	Casein	1.8
"	Hemoglobin	2.2
"	Gelatin	2.2
Yellow enzyme	Proteins	5.3
α-Glucosidase	α-Methylglucoside	5.4
"	Maltose	7.0
Lactase	Lactose	5.7
β-h-Fructosidase	Sucrose	5.7
Amylase	Starch	6.2
Zymase	Glucose	6.2
Urease	Urea	6.6
Trypsin	Proteins	7.8

means an easy one to understand. If nature has made an enzyme in such a way that it can exist in many valence states, from a highly charged cation to a highly charged anion, why should its principal function, which is

catalysis, ultimately depend on the absence of electrical charge? The answer cannot be given until we have more precise knowledge than we now possess. Two aspects of the problem may be briefly discussed here.

The primary effect of the charge on the enzyme is to orient all polar molecules within its reach so that they lie with their polar axes in radial directions, and as near as possible to the enzyme surface (Fig. 9). Most substrate molecules, though polar, are not as polar as water, or as compact. A charged enzyme, therefore, preferentially draws water molecules to its surface, and strives to arrange them in a radial pattern. If catalysis entails contact of the substrate with the naked enzyme, a high charge on the enzyme is thus disadvantageous. When the enzyme is electrically neutral, the water molecules lying nearest the surface, though possibly still not as free as water molecules in the pure liquid, are more capable of free rotation, and thus can be more readily dislodged by the advancing substrate.

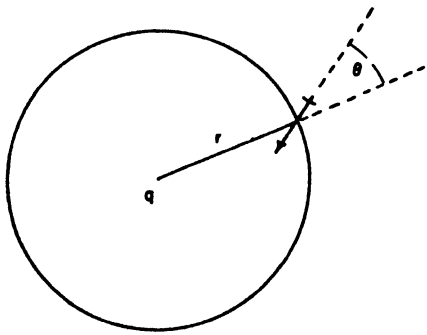


FIG. 9. Orientation of a water molecule on an enzyme surface.

Finally, a net charge of zero on an enzyme may result while its surface still holds positive and negative charges, such as H^+ and OH^-, or positive and negative groups, such as —COOH and —NH_2, provided they are present in equal numbers. It may well be that the peculiarity of enzyme catalysis is the necessity for the simultaneous contact of a substrate molecule on two sites of opposite polarity. The rate of reaction in that event is proportional to the product $\theta_A\theta_B$, where θ_A and θ_B have the form given by equations (69) and (70). It can readily be shown that this product has a maximum value at the isoelectric point.

4. SCHÜTZ LAW

It was discovered by Schütz that, when the concentration of substrate is high compared with that of the enzyme, the amount, x, of substrate transformed in time t is proportional to the square root of the product of the time and the concentration of enzyme:

$$x = n_P = k\sqrt{n_E t} \qquad (94a)$$

There have been many attempts at deriving such an equation theoretically.[26, 28] Its form, and the conditions under which it applies suggest that the governing process in this case is diffusion, and the proportionality of n_P to $t^{1/2}$ can be directly proved in terms of Einstein's theory of Brownian movement.[4] It can be shown that, using Einstein's general argument, the average value of the square root of the square of the displacement in space is given by the equation:

$$s = \sqrt{(\bar{s})^2} = \left(\frac{kTt}{\pi\eta r} \tanh \frac{3\pi\eta r t}{m}\right)^{1/2} \tag{95}$$

where m is the mass of a spherical particle of radius r, moving at temperature T, in a medium of viscosity η. When only short times are considered, expansion of the hyperbolic tangent gives the classical result:

$$\frac{1}{2}m\left(\frac{s}{t}\right)^2 = \tfrac{1}{2}mc^2 = \tfrac{3}{2}kT \tag{96}$$

For long time intervals, however, we obtain:

$$s = \sqrt{\frac{kTt}{\pi\eta r}} \tag{97}$$

The volume swept through by one enzyme molecule in time t is $\pi r_E^2 s$, and the number of collisions made with substrate molecules is $n_S \pi r_E^2 s$. If each encounter leads to chemical change, we obtain, for the total number of product molecules found in a volume, V, after time t:

$$N_P = Vn_P = n_E n_S \sqrt{\pi k T r_E^3 t/\eta} \tag{98}$$

There have been numerous other, but still incomplete, interpretations.[28]

5. Dependence of Reaction Velocity on Temperature

The effect of temperature on reaction velocity is most conveniently given in terms of the apparent energy of activation, which is defined by means of the Arrhenius equation:

$$E_A = RT^2 \frac{d \ln k}{dT} = -R \frac{d \ln k}{d(1/T)}. \tag{99}$$

From the second form of this equation, it is seen that E_A may be evaluated by plotting the logarithm of the velocity constant as a function of the reciprocal of the absolute temperature. Experiment shows that enzymes reduce the energy of activation of a chemical reaction to some value below that of the uncatalysed reactions, and thus fall in line with other catalysts.

[28] S. Arrhenius, Immunochemie. Leipsig, 1907; J. H. Northrop, *J. Gen. Physiol.* **2**, 471 (1920); E. A. Moelwyn-Hughes, J. Pace, and W. C. McC. Lewis, *ibid.* **13**, 323 (1930).

Enzymes, however, bring about a greater lowering in E_A than do inorganic catalysts (see Table XVI).

As a rule, the apparent energy of activation decreases with an increase in temperature for enzyme reactions as for many other catalyzed and uncatalyzed reactions in aqueous solution. To determine the temperature coefficient of the energy of activation correctly, however, requires data of greater precision than are usually available. On account of the many factors which can influence the velocity of enzyme reactions, its measurement is intrinsically difficult. Too much emphasis must, therefore, not be laid on the points of disparity which appear from an inspection of Table XVII.

TABLE XVI

COMPARISON OF ENZYMATIC AND NONENZYMATIC CATALYSES[a]

Reaction	Catalyst	E_A, cal./mole
Decomp. of hydrogen peroxide	None	18,000
	Colloidal platinum	11,700
	Liver catalase	5,500
Hydrol. of ethyl butyrate	Hydrogen ion	16,800
	Hydroxyl ion	10,200
	Pancreatic lipase	4,500
Hydrol. of casein	Hydrogen ion	20,600
	Trypsin-kinase	14,400
	Trypsin (pure)[b]	12,000
Hydrol. of sucrose	Hydrogen ion	25,560
	Invertase	8,700
	Yeast invertase[c]	11,500
	Malt invertase	13,000
Hydrol. of benzoylglycine	Hydrogen ion	22,100
Hydrol. of benzoyl-L-arginine	Trypsin (pure)	15,500

[a] From E. A. Moelwyn-Hughes, Kinetics of Reactions in Solution. 1st ed., Oxford Univ. Press, London, 1933, p. 225.

[b] J. A. V. Butler, *J. Am. Chem. Soc.* **36**, 2971 (1941); H. Lineweaver, *ibid.*, **61**, 403 (1937).

[c] I. W. Siser, *Ezymologia* **4**, 215 (1938).

On the whole, it is the striking parallelism with some of the simplest reactions of nonenzymatic systems which chiefly impresses one. To a first approximation, the apparent energies of activation decrease linearly with respect to temperature, so that we may write:

$$E_A = E_0 - FRT \tag{100}$$

where R is the gas constant, and F an integer. According to the law of the distribution of energy, in the general form given by Berthoud, the fraction of the total number of molecules in a classical system which possess an energy exceeding E is:

$$\frac{N^*}{N} = e^{-E/RT}\left[\frac{(E/RT)^{s-1}}{(s-1)!} + \frac{(E/RT)^{s-2}}{(s-2)!} + \cdots + 1\right] \tag{101}$$

where $2s$ is the number of quadratic terms required uniquely to define the energy of a single molecule. If the rate of reaction is proportional to this fraction, an equation of the form of equation (100) ensues. F, or $s - 1$, stands for the number of internal oscillators, or pairs of quadratic terms, contributing to the energy of activation. In terms of Fischer's lock-and-key analogy, F may be regarded as a measure of the size of the key. If, as now appears probable, F is smaller for enzymatic catalysis than for nonenzymatic catalysis, we may conclude that the key used by the enzyme is smaller than that used by the inorganic catalyst.

6. Dependence of Reaction Velocity on Chain Length

When a single act of activation leads to the chemical conversion of many molecules, the reaction is said to proceed by a chain mechanism. The number of substrate molecules decomposed per unit activation is termed

TABLE XVII

Variation of Apparent Energy of Activation with Respect to Temperature

Reaction	Catalyst	E_0, cal./mole	F
Mutarotation of sugars	None	22,800	10.3 ± 6
Hydrolysis of sucrose	Hydrogen ion	58,200	47 ± 2
	Invertase	39,500	52[a]
	Invertase	14,300	9[b]
Hydrolysis of urea	Urease	22,000	20[c]
	Urease	37,600	49[d]

[a] von Euler
[b] pH 5.3. Nelson and Bloomfield
[c] Van Slyke
[d] Poland

the chain length. The chain may be propagated by a single activated molecule, distributing its excess energy to many substrate molecules, or, as more frequently happens, by generating a second active molecule, which, in turn, can generate a third. A chain mechanism is characterized, in thermal systems, by exothermicity, and, in photochemical systems, by a high quantum yield. In both systems, there is a general sensitiveness to surface conditions and a failure of the simple kinetic laws. There is considerable evidence to show that certain oxidative and reductive reactions, as catalyzed by enzymes, proceed by a chain mechanism. In terms of the chain theory, as advanced by Christiansen and Kramers and by Semenoff it is possible to interpret the low, and even the negative, values found for the Michaelis constant. In enzymatic catalysis, however, as in nonenzymatic catalysis in solution, chain reactions, though interesting, are not prevalent.[28]

[28] F. Haber and R. Willstätter, *Ber.* **64**, 2844 (1931); E. A. Moelwyn-Hughes, *Acta Physicochim. U.R.S.S.* **3**, 593 (1935).

7. Molecular Statistics

The comparison of the observed rate of an enzyme reaction with that predicted by some reasonable theoretical formula appears to have been first attempted in the sucrose – saccharase and hydrogen peroxide – catalase systems.[30] In both cases, the concordance between experiment and theory was sufficient to justify the treatment, which has subsequently been found to apply equally well to catalysis by carbonic anhydrase,[31] cholinesterase,[32] and trypsin.[33] The simplest formulation of the theory is as follows.

According to equation (2), the total number of collisions made per second between the enzyme and substrate molecules in 1 cm.^{3} is:

$$Z = onu = 4\pi r_E^2 n_E n_S \sqrt{\frac{kT}{2\pi m_S}} = n_E n_S r_E^2 \sqrt{\frac{8\pi kT}{m_S}} \qquad (102)$$

This expression may be regarded as a special case of the formula:

$$Z = n_E n_S (r_E + r_S)^2 \sqrt{8\pi kT\left(\frac{1}{m_S} + \frac{1}{m_E}\right)} \qquad (103)$$

which reduces to equation (102) when r_E is much greater than r_S, and when m_E is much greater than m_S. To obtain the fraction of these collisions which the substrate makes on the catalytically active portion of the surface, we must multiply by the fraction θ, which is the ratio of the active surface to the total surface. Finally, in the case of simple activation, only the fraction $e^{-E/RT}$ of all the colliding pairs has the requisite energy. Hence, the theoretical rate of reaction:

$$-\frac{dn_S}{dt} = n_E n_S \theta r_E^2 \sqrt{\frac{8\pi kT}{m_S}}\, e^{-E/RT} \qquad (104)$$

But the bimolecular velocity coefficient (equation 83), under the conditions imagined, is defined by the equation:

$$-\frac{dn_S}{dt} = k_2 n_E n_S .$$

Hence, using equation (80), the bimolecular velocity coefficient, in liters per mole second, is:

$$k_2 = \theta \frac{N_0 r_E^2}{1{,}000} \sqrt{\frac{8\pi kT}{m_S}}\, e^{-E/RT} \qquad (105)$$

[30] J. B. S. Haldane, *Proc. Roy. Soc. London* B108, 569 (1931); E. A. Moelwyn-Hughes, *Trans. Faraday Soc.* 25, 503 (1929).
[31] F. J. W. Roughton, *Ergeb. Enzymforsch.* 3, 295 (1934).
[32] L. H. Easson and E. Stedman, *Proc. Roy. Soc. London* B121, 142 (1936).
[33] J. A. V. Butler, *J. Am. Chem. Soc.* 63, 2971 (1941).

Applied to the case of benzoylarginine ($m_s = 278/N_0$), hydrolyzed by trypsin ($r_E = 2.62 \times 10^{-7}$ cm.), we obtain the theoretical formula:

$$k_2 = \theta \times 11.4 \times 10^{10} \times \sqrt{T} \times e^{-E/RT}$$

Butler's experimental results may be summarized in the form:

$$k_2 = 9.2 \times 10^{10} \times \sqrt{T} \times e^{-E/RT}$$

where E is 15,000 cal. Hence the estimated value of θ is 0.8. A slightly improved calculation, based on equation (101), leads to a lower value for the fraction of the total surface which is catalytically effective. Of greater interest, however, than any immediate numerical agreement is the utility of the kinetic method for exploring the enzyme surface.

IV. Structure and Stability

Within the last 20 years, information has gradually been won concerning the thermal stability of enzymes, as measured by the activation energy required for their destruction, and concerning the extent of hydration, as indicated by careful density determinations on the crystals and their solutions. Contemporaneously, new techniques in X-ray analysis of crystals have been increasingly applied to the problem of structure, with the result that the enzyme framework can now be seen by the mind's eye, though as in a glass darkly.

1. Hydration

By careful treatment at high temperatures, proteins may be obtained in an anhydrous state. Under ordinary conditions, however, they exist as hydrates, exerting, in the crystalline state, definite pressures of water vapor, like hydrates of inorganic salts. Density measurements afford a means of determining the extent of hydration, a convenient measure for which is the weight of water of hydration per gram anhydrous protein.

Let us consider a system of specific volume, v, which contains a weight fraction, x_E, of enzyme, or protein generally, a weight fraction, x_S, of substrate, and a weight fraction, x_w, of water. Then

$$v = v_E x_E + v_S x_S + v_w x_w$$

where v_E, v_w, and v_S are, respectively, the partial specific volumes of enzyme, substrate, and water. Only experiment can decide whether these partial specific volumes are functions of the composition of the solution. When we have only the protein in solution, x_S is zero; and we may denote x_E by x, without a subscript, and x_w by $1 - x$. Then:

$$\begin{aligned} v &= v_E x + v_w(1 - x) \\ &= v_w - (v_w - v_E)x \end{aligned} \qquad (106)$$

This formula we shall now apply, first to a solution of a protein in water, and secondly to a hydrated protein crystal.

If an aqueous solution of enzyme contains c grams of anhydrous enzyme per ml. solution, the weight fraction of enzyme is $c/\rho = cv$. Hence:

$$v = v_w - (v_w - v_E)cv$$

or:

$$v/v_w = [1 + v_w(1 - v_E\rho_w)c]^{-1}$$

On converting into densities, and rearranging, we find:

$$\rho = \rho_w + (1 - v_w\rho_E)c \tag{107}$$

For aqueous solutions containing up to 50 g. anhydrous protein in 100 ml. solution, a linear relationship has been established between ρ and c. The term in brackets is thus a constant:

$$K = 1 - v_w\rho_E \tag{108}$$

It has been called the density increment,[34] and has a value of 0.2527 in the case of hemoglobin. The value is independent of temperature and the presence of buffers, within the region examined. The density increment equation can be rewritten to give the specific volume of the protein:

$$v_E = (1 - K)v_w$$

In the present example, v_E becomes 0.7473 ml./g. Since the molecular weight, M, is 67,000 g., the molar volume is 50.07 liters, and the molecular volume is 8.26×10^{-20} ml.

We next apply equation (106) to hydrated crystals, in order to obtain the ratio:

$$\frac{\text{weight fraction of water}}{\text{weight fraction of anhydrous protein}} = \frac{1 - x}{x} \tag{209}$$

Substituting the value for x given by equation (106), the hydration ratio can be expressed in terms of specific volumes or densities by the relations:

$$\frac{1 - x}{x} = \frac{v - v_E}{v_w - v} = \frac{\rho_w(\rho_E - \rho)}{\rho_E(\rho - \rho_w)} \tag{110}$$

The density, ρ, of the hydrated crystal is found by buoyancy experiments with small specimens in aqueous media, synthetically brought to the required density by the addition of ammonium sulfate and sucrose. For hemoglobin in a phosphate buffer at pH 6, it is found that $\rho = 1.225$.

[34] G. S. Adair and M. E. Adair, *Proc. Roy. Soc. London* **A190**, 341 (1947); **B120**, 422 (1936). See also C. N. Riiber and N. A. Sørensen, *Kgl. Norske Videnskab. Selskabs Skrifter* **1** (1938).

With $\rho_w = 0.998$ and $\rho_s = 1.332$, we have a hydration ratio of 0.353. The molecular weight of the hydrated protein is thus 67,000 × 1.353 = 90.700 g., and its molar volume is 90,700/1.225 = 74.0 liters, an increase of 48% over the molar volume of the anhydrous protein.

Some interesting conclusions can be drawn from these results on the assumption that both anhydrous and hydrated molecules are spherical. For example, the increase in the radius, due to hydration, is 3.75 Å, which lies near the layer thickness in ice (3.7 Å). We note also that one protein molecule requires 23,700/18 = 1320 water molecules to hydrate it.[35] If all these merely cover the surface, without entering the protein structure, the average area of the water molecule is 6.95 Å² on the protein surface, and

TABLE XVIII

DIMENSIONS OF HEMOGLOBIN MOLECULE FROM DENSITY DATA

Measurement	Anhydrous	Hydrated
Volume of protein molecule, × 10^{20} cm.³	8.26	12.22
Area of protein molecule, × 10^{12} cm.²	9.17	11.9
Radius of protein molecule, × 10^{8} cm.	27.02	30.77
Difference between radius of hydrated and anhydrous molecules	3.75	
Thickness of layers in ice	3.70	
Area, o, per water molecule, × 10^{16} cm.²	6.95	9.01
$v^{1/3}$, × 10^{8} cm.	2.64	3.00
Distance apart of neighboring water molecules in liquid water, × 10^{8} cm.	2.68	

9.01 Å² on the outside of the water layer. If the water molecules are arranged in a plane cubic lattice, their average distance apart is $\sqrt{6.95}$ = 2.64 Å, which is very near the average distance apart (2.68 Å) of the centers of neighboring water molecules in ordinary water, as determined from the density, using the Bernal and Fowler tetrahedral arrangement. It may be said, therefore, that the hydration of the protein considered takes place by the formation of a unimolecular layer of water molecules over the complete protein surface, and that these water molecules have spacings indicating a character intermediate between liquid water and ice (see Table XVIII).

Many anhydrous proteins have densities, ρ_s, of about 1.34 g./ml. The nearness of this figure to the density of solid urea (1.336) and its divergence from the density of crystalline glycine (1.75) suggest that the strong electrostatic forces at work in the "zwitterion" crystal do not play a prominent part in determining the structure of large protein molecules[36]

[35] Compare the value of 1500 molecules, which, according to Northrop's diffusion data (Table III) are attached to one molecule of trypsin.

[36] E. J. Cohn, *Ann. Rev. Biochem.* **4**, 93 (1935).

2. Bound Water

The precise state of the water molecules which hydrate an enzyme plays an important, and possibly even a determining, role concerning which the following considerations are pertinent.

The energy of a molecule with moment of inertia, I, and dipole moment μ, placed at a distance, a, from a charge, q, in a medium of dielectric constant, D, is:

$$\epsilon = \tfrac{1}{2}I\left(\frac{d\theta}{dt}\right)^2 + \frac{q\mu}{Da^2}(1 - \cos\theta). \tag{111}$$

where θ is the angle at which the electrical dipole is inclined to the radial direction, about which motion is assumed to take place (Fig. 9). For small displacements from the equilibrium position, the vibration of the molecule is a simple harmonic motion, with frequency:

$$\nu^0 = \frac{1}{2\pi a}\sqrt{\frac{q\mu}{DI}} \tag{112}$$

The general solution can be given in terms of elliptic functions. Of more immediate interest are the conditions governing the change of the motion from an oscillation to a free rotation. They are, for rotation:

$$\frac{\epsilon Da^2}{2q\mu} > 1 \tag{113}$$

and for vibration:

$$\frac{\epsilon Da^2}{2q\mu} < 1 \tag{113a}$$

Giving the energy, ϵ, its equipartition value of $(1/2)kT$, we obtain for the critical distance, a, which corresponds to the transition in motion, the expression:

$$a = 2\sqrt{\frac{q\mu}{DkT}} \tag{114}$$

Thus the water molecule ($\mu = 1.83 \times 10^{-18}$ e.s.u.) at 298.1°, under the influence of unit charge, alters its motion when:

$$a = 29 \times 10^{-8}\ \text{cm.}$$

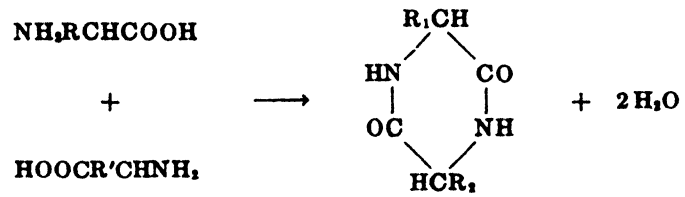
It has been assumed that D for the interaction of the ion and the dipole is unity. Though the assumption cannot be quite true, it is known that D must lie nearer unity than the value (*ca.* 80) for the solvent. In any event, it appears that the water molecules surrounding an enzyme are in a somewhat delicately balanced electrostatic state, being not as free as ordinary water molecules or as clamped as those which hydrate elementary ions.

3. X-Ray Evidence

Enzymes are known, from chemical analysis, to consist of various amino acids, linked as polypeptide chains or as closed rings of the diketopiperazin type.[37] Substituted glycines undergo both kinds of condensation:

$$NH_2RCHCOOH + NH_2R'CHCOOH \rightarrow NH_2RCHCONHR'CHCOOH + H_2O$$

and:



Moreover, the numbers of the various amino acid residues obtainable from one molecule of enzyme stand in integral ratios to one another. The yellow enzyme, for example, contains the following amino acids in the relative proportions given by the figures in brackets: histidine (3), tryptophan (4), phenylalanine (6), tyrosine (8), glutamic acid (8), arginine (8), and lysine (16). Similar analyses are available for other proteins.[38] It seems plausible, therefore, to regard the yellow enzyme as built up of four equal portions, each of which contains at least 1 molecule of tryptophan, 2 of tyrosine, glutamic acid, and arginine, and 4 of lysine. Between these four portions there can be three layers, each layer containing at least 1 molecule of histidine and 2 molecules of phenylalanine. On this supposition, the enzyme has the form of a multiple sandwich, which is the configuration of the egg albumin molecule predicted by Astbury and recently established for other proteins by X-ray analysis.[39] Myoglobin (M = 17,000) is thought[40] to consist of parallel polypeptide chains lying in a layer the thickness of which is about 10 A, and the area of one of the "flat" surfaces of which is about 2000 A^2. If the disc were circular, its radius would be 26.5 Å. In appearance, it must resemble a very fat coin. The X-ray analysis of the hemoglobin molecule reveals it as of roughly cylindrical shape, the radius being 28.5 A, and the height 34 A. The height is due to four layers each of rather less than 9 Å in thickness (Fig. 10). The hemoglobin molecule is thus probably formed by the union of four myoglobin molecules which, on the whole, retain their shape. Insulin (M = 12,000) is known to polymerize reversibly into a protein of molecular weight 48,000.

[37] W. T. Astbury, *Nature*, **137**, 803 (1936); and *in* Bamann-Myrbäck, Methoden der Fermentforschung. Vol. 1, Thieme, Leipzig, 1941, p. 498.

[38] T. W. J. Taylor, *Ann. Repts. Progress Chem. Soc. London*, **34**, 302 (1937).

[39] M. F. Perutz, *Proc. Roy. Soc. London* **A195**, 474 (1949); J. C.Kendrew and M. F. Perutz, *ibid.* **A194**, 375 (1948).

[40] J. C. Kendrew, *Acta Cryst.***1**, 336 (1948).

These findings, while being of the highest importance to enzyme chemistry, are insufficient to form the basis of generalizations. It would certainly be unscientific to conclude that every enzyme, or even a given enzyme under all conditions, has a cylindrical shape, or that all proteins are built up of a common unit.

4. Inactivation

What little knowledge there is concerning the thermal stability of enzymes has accrued from the kinetic study of their inactivation, which is a relatively slow, and sometimes reversible, transformation suffered by them in solutions at high temperatures. In its response to changes in pH, and in

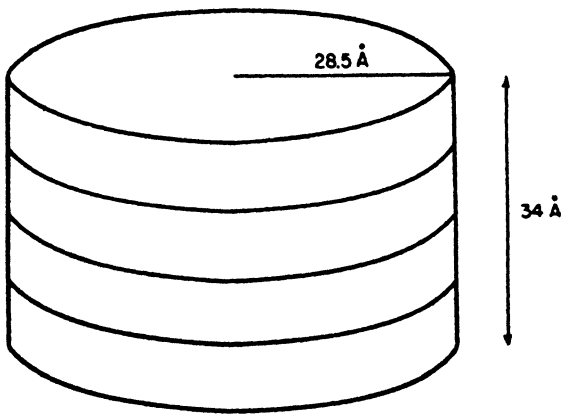


Fig. 10. Structure of the hemoglobin molecule.

many other features, inactivation resembles denaturation. Generally, the velocity of inactivation is unimolecular:

$$-\frac{dn_x}{dt} = kn_x \tag{115}$$

but examples are known of fractional kinetic orders, such as:

$$-\frac{dn_x}{dt} = kn_x^{3/2} \tag{116}$$

As for so many other chemical changes in solution, hydrogen and hydroxyl ions act as catalysts. The unimolecular constant for the inactivation of trypsin kinase in a solution containing 24% glycerol at 50° is found to be given by the relation:

$$k = 4.5 \times 10^{-6} + 1.5\,[H^+] + 0.4\,[OH^-]$$

which corresponds to an optimum stability at pH 6.9. Occasionally, the pH corresponding to optimum stability is identical with the pH of optimum catalytic effect. For pepsin, however, the former is 4 and the latter 2. The rate of inactivation in neutral solution can be neglected in the case of pepsin. We are then left with two catalytic coefficients, which indicate great sensitivity toward hydroxyl ion concentration. The unimolecular velocity coefficient for the catalyzed inactivation has been given as:

$$k = k_{H}[H^+] + k_{OH}[OH^-]^z$$

where z has been ascribed the values[41] 3, 4, and 5. As Steinhardt points out, it appears that inactivation is suffered chiefly by the z-valent pepsinate ion.

In view of the difficulties attending the problem, only approximate values can be found for the apparent energy of inactivation. They are summarized in Table XIX. There can be little doubt that these E_A values, when measured with greater precision, will be found in general to decrease with rise in temperature. The most that can be said at present is that an equation of the very approximate form:

$$E_A = 80{,}000 - 42\,RT \qquad (117)$$

fits many of the data. The order of magnitude of the energy required to deprive an enzyme of its catalytic power is thus 80 kcal. How this energy is expended is not yet known. Its magnitude is not inconsistent with the view that a single, firm covalent bond is broken. On the whole, however, the evidence suggests that a distribution of the energy among a number of weaker bonds is more probable. Thus two peptide links (E_A = 22,100) may have to be broken simultaneously, or ten hydrogen bridges (E_A = 5000) of the kind formed between two carboxylic acids, or a still greater number of the weak bonds holding together specially oriented water molecules.

The kinetics of the inactivation of trypsin by X-rays has been investigated experimentally and theoretically.[42]

5. Molecular Statistics

Attempts have been made, despite the complexity of the problem, to formulate theoretically expressions for the extent and rate of enzymatic inactivation.[22, 43] We shall consider here certain simple mechanisms which may well form part of the whole process.

[41] R. Ege, *Z. physiol. chem.* **143**, 159 (1925); L. Michaelis and K. Rothstein, *Biochem. Z.* **105**, 60 (1920); J. Steinhardt, The Stability of Crystalline Pepsin. Levin and Munksgaard, Copenhagen, 1937.

[42] G. L. Clark and J. H. Northrop, *J. Gen. Physiol.* **9**, 87 (1925).

[43] H. Eyring and A. E. Stearn, *Chem. Revs.* **24**, 253 (1939); A. E. Stearn, *Ergeb. Enzymfors.* **7**, 1 (1938); S. Glasstone, K. J. Laidler, and H. Eyring, The Theory of Rate Processes. McGraw-Hill, New York, 1941. In deriving equation 125, I have used the method of the imaginary frequency (E. A. Moelwyn-Hughes, Physical Chemistry. 3rd. revision, Cambridge Univ. Press, London, 1950, p. 566).

If an enzyme molecule is a cylinder, denoted by the symbol P_3, one mode of breakdown is obviously a splitting into two smaller cylinders, which we may denote by P_1 and P_2:

$$P_3 \rightleftarrows P_1 + P_2$$

To calculate the equilibrium constant:

$$K = \frac{n_1 n_2}{n_3}$$

we note that the molecular partition function of a regular cylinder of mass m, of length L, and of radius R may be taken as:

$$f = \frac{(2\pi mkT)^{3/2}}{h^3 n} e \frac{2\pi(2\pi I_a kT)^{1/2}}{h} \frac{8\pi^2 I_b kT}{\sigma h^2} e^{-\epsilon/kT} \tag{118}$$

where I_a is the moment of inertia for rotation about the cylindrical axis, and I_b the moment of inertia about a diameter parallel to the base and passing through the center of gravity. By taking the mass of the molecule to be evenly distributed, we find that:

$$I_a = \frac{1}{2} mR^2 \tag{119}$$

and:

$$I_b = \frac{m}{4}\left(R^2 + \frac{L^2}{3}\right). \tag{120}$$

The long cylinder, P_3, may be thought of as the two short cylinders bound by three doubly degenerate vibrators, which enable it to execute a kind of concertina motion. By familiar methods, taking σ as 2, it is then found that

$$K = \frac{(2\pi kT)^3}{h^6}\left(\frac{2\pi m_1 m_2}{m_3}\right)^{3/2}\left(\frac{2\pi I_1 I_2}{I_3}\right)_a^{1/2} \left(\frac{I_1 I_2}{I_3}\right)_b \prod^{6} (1 - e^{-h\nu/kT})\, e^{-(\epsilon_1+\epsilon_2-\epsilon_3)/kT} \tag{121}$$

Provided the breakdown does not involve a change in hydration, we can simplify this expression, since, under these conditions:

$$m_3 = m_1 + m_2 \qquad \text{and} \qquad I_{a,3} = I_{a,1} + I_{a,2}$$

Hence, by taking the binding frequencies as firm, we have:

$$K = \frac{1}{\pi\sqrt{2}}\left[\frac{4\pi^2 RkT}{h^2}\left(\frac{m_1 m_2}{m_3}\right)\right]^3 f\left(\frac{L_1}{R}, \frac{L_2}{R}\right) e^{-(\epsilon_1+\epsilon_2-\epsilon_3)/kT} \tag{122}$$

where:

$$f(L_1/R, L_2/R) = \frac{(1 + L_1^2/3R^2)(1 + L_2^2/3R^2)}{[1 + (L_1 + L_2)^2/3R^2]} \tag{123}$$

This function, when the breakdown is into two halves ($L_1 = L_2$), amounts to 0.84 in the case of hemoglobin. Using Adair's value of the molecular weight and Perutz's values of L and R, it is found that the standard entropy change is:

$$\Delta S^0 = 100.3 \text{ cal./mole degree}$$

at 298.1°. From the magnitude of ΔS^0, there is much to differentiate the union of two proteins from other dimerizations in solution.

A simpler mechanism can be suggested as the first stage in the breakup of an enzyme, namely, the gaining of one degree of internal rotation about

TABLE XIX

APPARENT CRITICAL ENERGIES OF INACTIVATION

Enzyme	E_A, kcal. mole
Insulin	36
Trypsin	41
Trypsin-kinase	42
Enterokinase	43
Amylase (malt)	43
Catalase (mussel)	45
Lipase (pancreatic)	46
Saccharase	60
Pepsin	61
Cozymase	70

the cylindrical axis. The ratio of the equilibrium concentrations of molecules possessing such an internal rotation and those not possessing it is then given, in the simplest case imaginable, by the expression:

$$K = \frac{[2\pi(2\pi I_{a,1}kT)^{1/2}/h][2\pi(2\pi I_{a,2}kT)^{1/2}/h](kT/h\nu^*)e^{-\Delta\epsilon/kT}}{[2\pi(2\pi I_{a,3}kT)^{1/2}/h](1 - e^{-h\nu/kT})^{-1}}$$

$$\doteq \frac{(2\pi kT)^{3/2}}{h^2\nu^*}\left(\frac{I_1I_2}{I_3}\right)_a e^{-\Delta\epsilon/kT} \quad (124)$$

where ν is the frequency of the rapid oscillation executed between the two parts of the molecule in its rigid state, and ν^* the slow oscillation frequency between them when they are capable of independent rotation about a common axis. Considering the uncoiling to be midway along the length of the molecule, the expression simplifies to:

$$K = \frac{\pi RkT(2\pi mkT)^{1/2}}{h^2\nu^*} e^{-\Delta\epsilon/kT}$$

The unimolecular constant for such an isomerization is obtained by multiplying by ν^*, that is:

$$k_{uni} = \frac{\pi RkT(2\pi mkT)^{1/2}}{h^2} e^{-\Delta\epsilon/kT} \tag{125}$$

Applied to the case of pepsin, the pre-exponential term becomes 10^{17} sec.$^{-1}$. From the experimental value of k_{uni} at 50°, this formula leads us to expect an energy of activation of 32,000 cals. for the inactivation process. (See Table XIX.)

Acknowledgements

In dealing with such a wide subject as the physical chemistry of enzymes, it is inevitable that I should have to treat many topics concerning which I have no direct experimental familiarity. I have therefore relied upon the written word generally, and in particular upon the friendly advice of my colleagues Mr. G. S. Adair, Mr. J. C. Kendrew, and Dr. F. M. Perutz.

Chapter 3

Enzyme Specificity*

By BURCKHARDT HELFERICH

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I. Introduction

History, Definition, Classification

In 1837, Berzelius recognized that alcoholic fermentation is an enzymatic process. The process is catalyzed by the enzymes of yeast which were later collectively called zymase. This conception was progressively adopted for other enzymes and enzymatic processes as well. At the present time, the idea that enzymes are the catalysts of definite enzymatic reactions is generally accepted; it constitutes the basic fact in enzyme chemistry.

In their catalytic role, enzymes are distinguished in a very characteristic manner from other, less complex catalysts, especially those of inorganic nature. Hydrogen ions catalyze the hydrolysis of esters, acetals, acid amides, and so on. Many unsaturated compounds can be hydrogenated with a platinum catalyst. The rate of the reaction depends upon the conditions of the reaction and the properties of the unsaturated compounds, but less intimately upon the catalyst itself and particularly upon its gross chemical composition.

* Translated by Erich Hirschberg, McArdle Memorial Laboratory, The University of Wisconsin, Madison, Wisconsin.

In contrast, enzyme catalysts are not only limited to a special reaction which is generally characterized by a simple inorganic reactant, such as hydrogen in hydrogenation, water in hydrolysis, oxygen in peroxide decomposition, phosphoric acid in phosphorolysis. Enzymes as catalysts of enzymatic reactions are tied to a much larger extent to the organic reactant, to a specific class of organic compounds, or even to a particular organic molecule. They are *specific* catalysts of specific reactions; they attack specific organic compounds and the latter are the *specific substrates* of the particular enzyme. Carbohydrases hydrolyze only the acetal-type bond of glycosides, oligosaccharides, and polysaccharides, but not ester linkages or the peptide bonds in proteins. Moreover, a particular carbohydrase is capable of catalyzing the hydrolysis of only a small group of glycosides, oligosaccharides, or polysaccharides or even of a single compound of this type.

This limitation of enzyme catalysts to a specific reaction, a simple inorganic reactant *and* a definite group of (usually) organic substances or even a single such substance is called enzyme specificity.

The degree of specificity varies greatly with the different classes of enzymes and with the individual enzymes. Lipases, for example, usually can hydrolyze not only fats but also simpler esters of carboxylic acids that differ in their alcohol component as well as in their acid component. The reaction rate may differ greatly for different substrates. In contrast to this low specificity of many lipases and other esterases, a particular carbohydrase usually hydrolyzes only specific glycosidic derivatives of a single sugar. This enzyme specificity towards certain substrates was recognized a long time ago in a few striking instances. Pasteur observed in 1858 that D-tartaric acid is more rapidly "fermented," i.e., used up by molds than the L-form. According to Emil Fischer,[1] only three of the sixteen possible aldohexoses, D-glucose, D-mannose, and sometimes D-galactose, are converted to alcohol and carbon dioxide in alcoholic fermentation by yeast; the other aldohexoses, especially the optical antipodes, are not attacked. This specificity in alcoholic fermentation by yeast enzymes appears to be due to the fact "that the yeast cells with their asymmetric agent can attack only those carbohydrates the geometry of which is not too different from that of D-glucose."[2] Sweet almond emulsin splits principally β-D-glucopyranosides, without attacking the α-form or the stereoisomeric antipodal derivatives of L-glucose. "The reason for these facts most probably lies in the asymmetric structure of the enzyme molecule" (Emil Fischer[1]).

A very clear and convincing comparison bearing on enzyme specificity was formulated by Fischer in 1894: "To use a comparison, I should like

[1] E. Fischer, *Z. physiol. Chem.* **26**, 60 (1898).
[2] E. Fischer and H. Thierfelder, *Ber.* **27**, 2031 (1894).

to say that enzyme and glucoside must fit each other like the key and the lock in order to be able to react with each other."[3] The concept expressed by this statement has now been extended to almost all fermentations and other enzymatic processes. It constitutes the conscious or unconscious basis for many investigations on enzyme action. It is still most fertile and it probably approaches the correct explanation of enzyme specificity.

Enzyme specificity may be classified according to the various reactants. Reaction specificity is the term employed when the general reaction, i.e., the simple, usually inorganic reactant such as water, phosphoric acid, hydrogen, oxygen, and so on, is considered. The dependence of the enzyme reaction upon the other, usually organic reactant, i.e., the enzyme substrate, is called substrate specificity.

If a particular enzyme affects only one substance or a group of related substances in a reaction, without having any measurable effect on others, we speak of absolute specificity. As far as we know at the present time, the reaction specificity with few exceptions is absolute.

If, on the other hand, an enzyme acts on various but usually related compounds and if these compounds are attacked by the enzyme at varying speeds, relative specificity is said to be involved.

In some instances, this relative specificity may be transformed into an absolute one, at least for practical purposes. It has been observed, for example, that several members of a homologous series are attacked by an enzyme at a rate that decreases with increasing molecular weight. One of the higher members is bound to be the first that is no longer affected by the enzyme to a measurable extent: relative specificity has changed to absolute. The boundary between absolute and relative specificity in this instance depends upon the sensitivity of the analytical test of the enzyme reaction and is therefore somewhat arbitrary. A different chemical composition of the substrates, a different molecular weight of polymers, a different structure of isomers, and a different stereochemistry may be conditions determining absolute or relative substrate specificity. If stereochemical specificity involves diastereoisomers as the two different substrates, there is no fundamental difference from structural isomerism since diastereoisomers react more or less differently with every different substance. This point is often overlooked and should be emphasized. In contrast, the absolute or relative specificity toward optical antipodes (true stereochemical specificity) is a special case of great importance.

Sometimes specificity in enzyme chemistry may also be considered from a different point of view. It is often found that enzymes of different origin, for example, from different organs of the same animal or from different species, while catalyzing the same reaction of the same substrate (iso-

[3] E. Fischer, *Ber.* **27**, 2985 (1894).

dynamic enzymes), do so at very different rates, i.e., with a different relative specificity. The terms organ specificity and species specificity are then employed.

In principle, it is quite conceivable that isodynamic enzymes exhibit not only relative but also absolute specificity. While a few substrates may perhaps be attacked by two isodynamic enzymes of different origin, others may be susceptible to only one of the two enzymes.

Any treatment of an enzyme may, under certain conditions, change the relative specificity of the enzyme toward various substrates. This shift may go so far that the relative specificity actually becomes absolute towards some substrates which were attacked by the enzyme prior to the treatment.

II. Typical Examples of Specificity

1. Reaction Specificity

Hydrolysis and the reverse process of condensation may be catalyzed by hydrogen ions in almost all instances, but at different rates. In principle it does not matter whether esters, acetals, glycosides, or peptides are being hydrolyzed (or synthesized). On the other hand, certain enzymes are capable of catalyzing only one of these reactions. Esterases and lipases can split only esters or fats, without hydrolyzing glycosides or peptides. Carbohydrases act specifically on glycosides, oligosaccharides and polysaccharides and cannot attack ester or peptide bonds in fats or proteins. Proteins are split only by proteolytic enzymes, not by the other hydrolases.

"Oxidative enzymes" catalyze hydrogen transfer but are without hydrolytic activity. Catalases cleave hydrogen peroxide into oxygen and water but catalyze no other reactions. In all these instances, the particular enzyme, especially if it has been isolated and obtained pure, is limited in its action (with very rare exceptions) to one type of reaction to the exclusion of other types. It exhibits an absolute reaction specificity in regard to the type of reaction. Even enzyme preparations which are still far from pure often catalyze only one type of reaction and are devoid of enzymes having a different reaction specificity.

Reaction specificity may be further subdivided. Some enzymes catalyze the hydrolysis of carboxylic acid esters; others, the phosphatases, attack only esters of phosphoric acid; still others, the sulfatases, deal with esters of sulfuric acid, and so on.

Only very few exceptions to this absolute reaction specificity have been observed. However, there have been instances of an overlapping with other reactions of a similar type. Crystalline trypsin and crystalline chymotrypsin not only fulfill their main task, the hydrolysis of peptide bonds, but can

also split and synthesize simple esters of acetylated α-amino acids.[4] It has been shown that this is a true specific enzyme action. Crystalline chymotrypsin hydrolyzes tryptophan methyl ester into methanol and free tryptophan. When the enzyme is allowed to act on the racemic ester, it hydrolyzes only the derivative of L-tryptophan, leaving the D-ester unchanged.[5]

2. Substrate Specificity

The boundary between reaction and substrate specificity is somewhat arbitrary. The more specifically an enzyme is limited in its action to a particular class of organic compounds or to a single substance, the more appropriately we speak of substrate specificity. The real significance of this term lies in the most intimate dependence of the action of an enzyme upon the particular molecule or part of the molecule of its substrates.

The following section presents typical examples of this substrate specificity: a) toward optical antipodes; and b) toward diastereoisomers, other isomers, and substances of different composition. The examples are taken from various classes of enzymes, in the order in which the enzymes appear in the special part of this book.

a. Typical Examples of Substrate Specificity toward Optical Antipodes. Stereochemical Enzyme Specificity in the True Sense

A separate treatment of substrate specificity toward optical antipodes is justified in view of its importance for enzyme chemistry. Optical antipodes show entirely the same behavior toward physical agents and chemicals which are not asymmetric. There are no qualitative or quantitative differences, no differences in the type or rate of reaction between the D- and the L-form if the reaction involves any non-asymmetric reactant or if it is catalyzed by a non-asymmetric catalyst.

When enzymes are the catalysts, the situation in all known instances is fundamentally different.

The first observation of a stereochemical enzyme specificity was made by Dakin[6] on esterases exposed to optical antipodes. If the hydrolysis of racemic D,L-mandelic acid ester by pig liver esterase is interrupted before the reaction has gone to completion, the mandelic acid which has been liberated is dextrorotatory. Under the conditions of the experiment, the D-ester is hydrolyzed more rapidly than the L-ester. This result is not possible when hydrogen ions are the catalyst.

[4] G. W. Schwert, H. Neurath, S. Kaufmann, and J. E. Snoke, *J. Biol. Chem.* **172**, 221 (1948).
S. Kaufmann, G. W. Schwert, and H. Neurath, *Arch. Biochem.* **17**, 203 (1948).
[5] M. Brunner, E. Sailer, and V. Kahn, *Helv. Chim. Acta* **31**, 1908 (1948).
[6] H. D. Dakin, *J. Physiol. (London)* **30**, 253 (1904).

Later experiments demonstrated that the reaction is much more complicated than it appeared at first glance. Pig pancreatic lipase hydrolyzes L-mandelic acid ester more rapidly than the D-ester when the esters are studied separately or in a racemic mixture.[7] In contrast to this enzyme, pig liver esterase exhibits a very unusual behavior.[7,8] It splits the L-ester more rapidly than the D-ester if these substrates are investigated in separate solutions. If, however, the racemic mixture of the two esters is offered to the enzyme, the D-ester is more rapidly hydrolyzed than the L-ester, as mentioned above. The explanation for this unexpected behavior is given in the special part of this book. It involves a different affinity of this enzyme for the two esters together with a different decomposition rate of the two enzyme-substrate compounds.[9] This stereochemical selection by the enzyme of one of the two optical antipodes is influenced by the addition of several other substances, especially asymmetric ones,[10] by different substrate concentrations[11] and even by a different treatment of the enzyme preparations themselves.[12] Such additions or changes may even cause the preference to be reversed.

The stereochemical specificity of esterases towards optical antipodes is generally only relative, although the difference in the rate of hydrolysis may be considerable. Only a few specific esterases exhibit absolute specificity toward optical antipodes, e.g., a cholinesterase in its action on acetyl-β-methylcholine chloride.[13]

$$H_3C \cdot CO \cdot O \cdot CH(CH_3) \cdot CH_2 \cdot N(CH_3)_3 \cdot Cl$$

The racemic D,L-compound as well as the D-ester alone are hydrolyzed by this enzyme, but the L-ester is not.

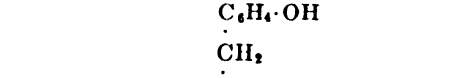
It has been established that the relative stereochemical specificity of esterases towards optical antipodes in a homologous series of closely related compounds is not dependent on the direction of optical rotation.[14] In a study of the esters of secondary active alcohols, the preferred esters all belonged to one stereochemical series.

The usual low, i.e., relative, specificity of esterases was encountered with esters of asymmetric acids and symmetrical alcohols as well as with esters of optically active alcohols with inactive acids.

[7] R. Willstätter and F. Memmen, *Z. physiol. Chem.* **138**, 216 (1924).
[8] R. Willstätter, R. Kuhn, and E. Bamann, *Ber.* **61**, 886 (1928).
[9] P. Rona and R. Ammon, *Ergeb. Enzymforsch.* **2**, 50 (1933).
[10] E. Bamann and P. Laeverenz, *Ber.* **63**, 394 (1930); *Z. physiol. Chem.* **193**, 201 (1930).
R. Ammon and H. Fischgold, *Biochem. Z.* **234**, 54 (1931).
[11] E. Bamann, *Ber.* **62**, 1538 (1929).
[12] E. Bamann and P. Laeverenz, *Ber.* **63**, 2939 (1930).
[13] D. Glick, *J. Biol. Chem.* **125**, 729 (1938).
[14] P. A. Levene and R. E. Marker, *J. Biol. Chem.* **97**, 379 (1932).
P. Rona and E. Chain, *Biochem. Z.* **258**, 480 (1933).

Stereochemical specificity plays an important role in the field of the carbohydrases. In all known cases, specificity is absolute when two isomeric substrates are derivatives of two enantiomorphic sugars. Glucosides of L-glucose are not split by enzymes which act on the corresponding glucosides of D-glucose.[15] Phenol-β-D-xyloside, but not the optical antipode derived from L-xylose, is hydrolyzed by sweet almond emulsin.[16] Similarly, phenol-L-arabinosides, but not the corresponding D-arabinosides, are split by enzymes of almond emulsin.[17]

With proteins, polypeptides, and oligopeptides, the peptide bonds of the amino acids belonging to the natural or L-series are usually hydrolyzed preferentially or exclusively. Crystalline chymotrypsin splits carbobenzoxy-L-tyrosylglycinamide, but not the corresponding derivative of D-tyrosine.



Carbobenzoxy-L-tyrosylglycinamide

In this instance, even the racemic derivative of D,L-tyrosine is not hydrolyzed, probably because the affinity between the stereoisomers is greater than the affinity of the enzyme for the L-form.[18] Papain-HCN catalyzes the synthesis of benzoylleucinanilide from benzoylleucine and aniline only when L-leucine is involved.[19]

Benzoylleucine Aniline

D-Amino acid oxidase oxidizes only D-amino acids, i.e., the antipodes of the natural amino acids and not the members of the natural L-series.[20] Enzyme specificity is absolute in this instance. *Acetobacter suboxydans* oxydizes only D-arabitol to D-xylulose but does not attack L-arabitol.[21]

Liver histidase decomposes only L-histidine, the natural amino acid, not the D-form.[22]

Concerning the enzymes of alcoholic fermentation (desmolases), the oldest and most important example has already been mentioned. L-Glucose,

[15] E. Fischer, *Ber.* **28**, 1152 (1895); *Z. physiol. Chem.* **26**, 60 (1898).
[16] B. Helferich, E. Günther, and W. W. Pigman, *Ber.* **72**, 1953 (1939).
[17] B. Helferich, H. Appel, and R. Gootz, *Z. physiol. Chem.* **215**, 277 (1933).
[18] M. Bergmann and J. S. Fruton, *J. Biol. Chem.* **124**, 321 (1938).
[19] M. Bergmann and J. S. Fruton, *Advances in Enzymol.* **1**, 63 (1941); *J. Biol. Chem.* **133**, 703 (1940).
[20] K. Felix and K. Zorn, *Z. physiol. Chem.* **258**, 16 (1939).
[21] R. M. Hann, E. B. Tilden, and C. S. Hudson, *J. Am. Chem. Soc.* **60**, 1201 (1938).
[22] S. Edlbacher and J. Kraus, *Z. physiol. Chem.* **191**, 240 (1930).

L-mannose, L-fructose, and L-galactose are not fermented by yeast enzymes[1]; only the D-form of these sugars is used. Here too specificity is absolute.

b. Typical Examples of Substrate Specificity toward Diastereoisomers, Other Isomers, and Substances of Different Composition

In this section, typical examples of substrate specificity toward all the substances mentioned above are presented, since all these substances react differently towards different chemical reagents even when the difference is only a structural or diastereoisomeric one. The separation between structural isomers and diastereoisomers is not fundamental, as has been pointed out.

(1) *Esterases.* Many esterases are characterized by a low, merely relative specificity. They usually hydrolyze a whole series of esters of different carboxylic acids with different alcohols, even with polyhydroxy compounds such as glycerol, i.e., fats. This relative specificity permits the determination of lipases on the basis of their influence on the hydrolysis of simple esters like ethylbutyrate. The rate of enzymatic hydrolysis may vary greatly. Under favorable conditions, the splitting of benzylstearate by pancreatic lipase is almost quantitative in less than an hour, while *n*-propylstearate under the same conditions is hydrolyzed only to 80% in 14 hours.[23] But there are also esterases capable of splitting only a few closely related esters; an example is cholinesterase, which hydrolyzes acetylcholine and a few other acylcholines to free choline and acid.[24] This high specificity has been questioned in the case of several cholinesterases.[24,25] The rate of hydrolysis increases with the number of carbon atoms in the acid from two (acetyl-) to four (butyryl-) although the saponifiability by alkali decreases with an increase in the number of carbon atoms.[13]

Chlorophyllase can split only esters of pigments having a phorbide structure, e.g., chlorophyll a or chlorophyll b.[26]

Phosphatases with a strictly absolute reaction specificity toward the phosphoric acid esters exhibit only relative specificity toward the esters of various alcohols or phenols. The velocity of hydrolysis of the various esters may vary a great deal. The boundary between phosphomonoesterases and phosphodiesterases appears to be very sharp. The former hydrolyze only monoesters, the latter only diesters of phosphoric acid. The specificity of these two groups of phosphatases toward the two types of phosphoric acid esters is absolute.

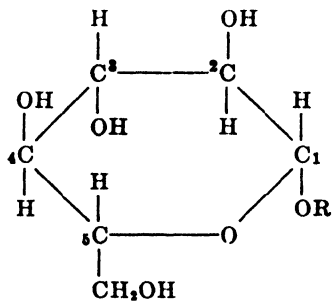
Special esterases with a rather high specificity are known, the substrates of which may be changed only in a few details without abolishing the ac-

[23] A. K. Balls and M. B. Matlack, *J. Biol. Chem.* **123**, 679 (1938).
[24] L. H. Easson and E. Stedman, *Biochem. J.* **31**, 1723 (1937).
[25] E. A. Zeller and D. C. Utz, *Helv. Chim. Acta* **32**, 338 (1949).
[26] H. Fischer and R. Lambrecht, *Z. physiol. Chem.* **253**, 253 (1938).

tivity of the enzyme. Tannase, an enzyme splitting tannins or depsides, requires two free phenolic hydroxyl groups on the benzene ring that carries the carboxyl group; moreover, none of the hydroxyl groups may be in the ortho position to the carboxyl group.[27]

(2) *Carbohydrases.* The specificity of carbohydrases has been investigated and discussed intensively for many years.[28] These studies have been greatly complicated by the fact that, with the exception of the crystalline amylases prepared recently,[29] no carbohydrases have been isolated in pure form until the present time. Therefore, it is difficult to ascertain whether a single carbohydrase or a mixture of enzymes is being employed.

The best example of a rather highly purified carbohydrase, the specificity of which has been extensively studied, is β-D-glucopyranosidase from sweet almonds, one of the oldest known enzymes. It is the main constituent of sweet almond emulsin, a mixture of various carbohydrases. It catalyzes the saponification of amygdalin and of β-D-glucopyranosides; the general formula of these sugars is as follows:



Studies on the specificity of this enzyme are aided by the fact that several methods are available for the synthesis of β-D-glucopyranosides and for clearly definable changes of the molecule in the carbohydrate portion.

A change in the aglucone (R in the formula) is almost always connected with no more than a change in the rate of hydrolysis; i.e., in the relative specificity of the enzyme toward almost all β-D-glucopyranosides.[30] Opinions to the contrary are not sufficiently convincing.[31] Changes in the carbohydrate portion exert a much more pronounced influence upon the specificity of the enzyme and will be discussed first.

If the structure is changed, e.g. to the furanoside ring, the enzyme is

[27] J. B. Sumner and G. F. Somers, *Chemistry and Methods of Enzymes.* Academic Press, New York, 1947, p. 86.

[28] A. Gottschalk, *Nature* **160**, 113 (1947).
W. W. Pigman and R. M. Goepp, Jr., Chemistry of the Carbohydrates. Academic Press, New York, 1948, p. 474.

[29] K. H. Meyer, E. H. Fischer, and P. Bernfeld, *Helv. Chim. Acta* **30**, 64 (1947).

[30] B. Helferich, *Ergeb. Enzymforsch.* **7**, 85 (1938).

[31] B. Helferich, *Ergeb. Enzymforsch.* **9**, 80 (1943).

unable to act. Neither methyl-β-D-glucofuranosides nor the α-derivatives are hydrolyzed by sweet almond emulsin,[32] although acids split glucofuranosides more rapidly than glucopyranosides. The "ring specificity" of this enzyme, as of all carbohydrases, is absolute.

The influence of changes in configuration of a single or several centers of asymmetry were also investigated. Experimental results are available in the following instances (the numbers in brackets indicate the carbon atom at which the configuration has been changed in comparison to the above formula of the D-glucopyranoside ring):

Methyl-α-D-glucopyranoside [1][1]
Methyl-α-L-glucopyranoside [2, 3, 4, 5][1]
Methyl-β-L-glucopyranoside [1, 2, 3, 4, 5][1]
Phenol-α-D-galactoside* [1, 4][33]
Phenol-α-D-mannopyranoside* [1, 2][34]
Phenol-β-D-mannopyranoside [2][34]
Methyl-α-D-gulopyranoside [1, 3, 4][35]
Methyl-β-D-gulopyranoside [3, 4][35]
Phenol-α-D-talopyranoside [1, 2, 4][36]

* These substrates are also hydrolyzed by sweet almond emulsin; this action, however, is not carried out by the β-D-glucopyranosidase but rather by another enzyme.

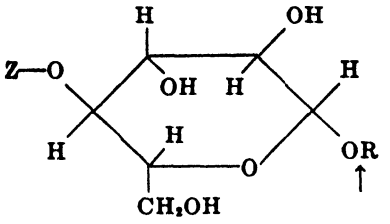
The 16 stereoisomeric aldohexoses yield 32 stereoisomeric glycopyranosides. One series of these, β-D-galactopyranosides, will be discussed below. Of the remaining 30 pyranosides which are isomeric with β-D-glucopyranoside, 9 have been tested with sweet almond β-D-glucosidase and failed to be hydrolyzed. The specificity of this enzyme is absolute toward changes in configuration in the carbohydrate portion of the glucoside; this is probably true of all cases including those not yet investigated. The only exceptions, probably, are the β-D-galactopyranosides. These substrates are split by sweet almond emulsin.[37] Until the present time, a separation of the effect of this enzyme on β-D-glucopyranosides and β-D-galactopyranosides has not been achieved. Moreover, the two effects are very similar in regard to their relative specificity when the aglycone portion is changed. It is therefore easily possible, though by no means definitely proven, that both

[32] E. Fischer, *Ber.* **47**, 1980 (1914).
W. N. Haworth, C. R. Porter, and A. C. Waine, *J. Chem. Soc.* **1932**, 2254.
[33] B. Helferich, S. Winkler, R. Gootz, O. Peters, and E. Günther, *Z. physiol. Chem.* **208**, 94 (1932).
[34] B. Helferich and S. Winkler, *Z. physiol. Chem.* **209**, 269 (1932).
B. Helferich, H. Heyne, and R. Gootz, *Z. physiol. Chem.* **214**, 139 (1933).
[35] B. Helferich, W. W. Pigman, and H. S. Isbell, *Z. physiol. Chem.* **261**, 55 (1939).
[36] W. W. Pigman, *J. Research Natl. Bur. Standards* **26**, 197 (1941).
[37] E. Fischer, *Ber.* **28**, 1155 (1895).

effects are due to the same enzyme and not to two distinct enzymes.[38] This possibility cannot be assumed to exist with other enzyme preparations or with other carbohydrases of different origin. β-D-Glucopyranosidases without action on β-D-galactopyranosides are known; the converse situation also exists.[39]

Besides changes in the structure and configuration of the β-D-glucopyranoside ring, other chemical changes and substitutions in this ring were tested for their effect on saponification by the β-D-glucopyranosidase of sweet almond emulsin. It was established that changes in the carbon atoms and hydroxyl groups in positions 1 through 5 exerted a much greater influence on enzyme activity than changes in the carbon atom or hydroxyl group in position 6. The two cases are therefore discussed separately.

If the hydroxyl groups in positions 2,3, or 4 of vanillin-β-D-glucopyranoside, a substrate subject to very rapid hydrolysis by the enzyme, are esterified with *p*-toluenesulfonic acid, enzyme activity is abolished.[40] The same result was achieved with the methyl ether of the hydroxyl groups in position 3 [41] and with the 2,4,6-trimethyl ether of phenol-β-D-glucopyranoside.[42] Replacement of the hydroxyl group in position 2 by hydrogen also prevents enzyme action on this methyl-β-D-2-desoxygluco-(=manno-) pyranoside.[43] *N*-Acetyl-β-D-glucosaminepyranoside, where the hydroxyl group in position 2 is replaced by an *N*-acetylamino group, is hydrolyzed by sweet almond emulsin; this effect is, however, probably not caused by the β-D-glycopyranosidase but rather by another enzyme of this preparation.[44] And, finally, if the hydrogen atom of the hydroxyl group in position 4 is replaced by another sugar, as in the β-D-maltosides, or -cellobiosides, or -lactosides,



Z = $C_6H_{11}O_5$
= α-D-glucosyl- or
β-D-glucosyl- or
β-D-galactosyl-

the glucosidic linkage (cf. arrow) is not hydrolyzed by the β-D-glucopyranosidase of sweet almond emulsin until the bond between the two sugars (at Z) is broken.[45]

[38] B. Helferich and H. Scheiber, *Z. physiol. Chem.* **226**, 272 (1934).
[39] E. Hofmann, *Biochem. Z.* **285**, 429 (1936); *Naturwissenschaften* **22**, 406 (1934).
[40] B. Helferich and S. Grünler, *J. prakt. Chem.* N.F. **148**, 107 (1937).
[41] B. Helferich and O. Lang, *Z. physiol. Chem.* **216**, 123 (1933).
[42] W. W. Pigman and N. K. Richtmyer, *J. Am. Chem. Soc.* **64**, 369 (1942).
[43] E. Fischer, M. Bergmann, and H. Schotte, *Ber.* **53**, 516 (1920).
[44] B. Helferich and A. Iloff, *Z. physiol. Chem.* **221**, 252 (1933).
[45] S. Petersen, *Ber. Verhandl. Math.-phys. Klasse sächs. Akad. Wiss.* **85**, 154 (1933). B. Helferich and E. Weber, *Ber.* **69**, 1411 (1936).

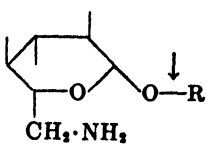
These and all other known examples lead to the conclusion that esterification, ether formation, or substitution in the hydroxyl groups in positions 2,3, and 4 prevent enzyme action. The three free hydroxyl groups in the pyranose ring having the normal stereochemical position and also stereochemical configuration of carbon atoms 1 and 5 are essential for the action of the β-D-glucopyranosidase of sweet almonds. The enzyme is absolutely specific for this portion of its substrates.

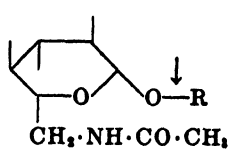
A number of changes on carbon atom 6 and its hydroxyl group, i.e., outside the pyranoside ring, are compatible with the action of this enzyme. Hydrolysis has been shown to occur in all the β-D-glucopyranosides of phenol or vanillin in which the hydroxyl group in position 6 is replaced by H, F, Cl, Br, OCH_3, or I. These substituents are listed in the order of decreasing velocity of enzymatic hydrolysis and in order of increasing substituent volume. The hydroxyl group itself stands between H and F in this list. The rate of hydrolysis by the enzyme of the compounds with the two largest substituents approaches the lower limit of analytical detection.[46,47]

If one of the hydrogen atoms at carbon atom 6 is replaced by CH_2OH, enzymatic hydrolysis can probably still be detected. This assumption is based on the fact that in a comparable case, with the β-D-galactopyranoside ring in phenyl-β-D-α-mannoheptoside, hydrolyzability can just be detected.[46]

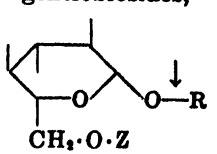
Even larger substituents, e.g., those produced by esterification of the hydroxyl group in position 6 with methanesulfonic acid or with *p*-toluenesulfonic acid, cause the rate of hydrolysis to become so slow as to escape detection. The specificity has become "absolute."[40,47]

The size of the substituent is not the only decisive factor. Replacement of the hydroxyl group in position 6 not only by NH_2 but also by $NH \cdot CO \cdot CH_3$ does not render the glucoside quite stable toward the enzyme.[48]





If the hydrogen of the hydroxyl group in position 6 is replaced by a second sugar, as in gentiobiosides,

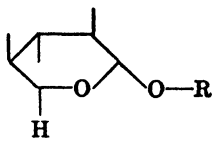


Z = $C_6H_{11}O_5$
= β-D-glucosyl-

[46] B. Helferich, S. Grünler, and A. Gnüchtel, *Z. physiol. Chem.* **243**, 85 (1937).
[47] W. W. Pigman, and N. K. Richtmyer, *J. Am. Chem. Soc.* **64**, 374 (1942).
[48] B. Helferich, A. Iloff, and H. Streeck, *Z. physiol. Chem.* **226**, 258 (1934).

hydrolysis of the bond between the disaccharide and the aglucone by the enzyme is prevented. The glucosyl residue is too large a substituent. Amygdalin, when acted upon by β-D-glucopyranosidase, is split primarily between the two sugars and only secondarily between glucose and benzaldehyde cyanhydrin.[49]

On the other hand, the glycosidic linkage of a β-D-glucopyranoside carrying hydrogen in place of the CH_2OH in position 6 is split by β-D-glucopyranosidase of sweet almonds. β-D-Xylosides



have been shown to be hydrolyzed by the same enzyme in sweet almond emulsin as the β-D-glucopyranosides,[50] provided a sufficiently pure enzyme preparation and a suitable aglucone such as phenol are employed.

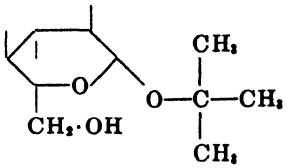
The effect of a change at carbon atom 6, outside the pyranoside ring, is limited to the rate of enzymatic hydrolysis and therefore to relative specificity, unless the change is too drastic and especially unless the new substituent is too large. If it takes up too much space, relative specificity is transformed into an absolute one.

Among the cases which have been mentioned above, two are remarkable in a particular sense. The term "sugar specificity" of a carbohydrase is sometimes understood to mean that at least one distinct enzyme is required for the hydrolysis of the glycosides of each sugar, particularly among the natural ones. However, the β-D-glucopyranosidase of sweet almonds is capable of hydrolyzing not only the derivatives of β-D-glucopyranose but also those of β-D-xylopyranose and of β-D-isorhamnopyranose [D(+)-glucomethylose, D-chinovose, D-epirhamnose]. It is not necessary to postulate the existence in sweet almond emulsin of special enzymes for the β-pyranosides of these other two sugars since the pyranose ring in all three glucosides has the same structure and configuration. The hydrolysis of β-D-isorhamnosides even proceeds with a remarkable velocity.

The effect of various aglucone groups in the β-D-glucopyranosides on hydrolysis by β-D-glucopyranosidase has been very thoroughly investigated from various points of view.[30,51] It became possible to establish certain rules. For example, the rate of hydrolysis increases with increasing number

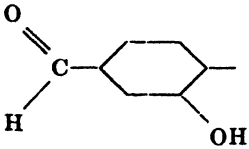
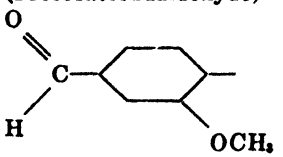
[49] Auld, *J. Chem. Soc.* **1908**, 1280.
Armstrong and Horton, *Proc. Roy. Soc.* (*London*) **B80**, 330 (1908).
R. Weidenhagen, *Z. ver. deut. Zuckerind.* **79**, 591 (1929).
[50] B. Helferich and H. Appel, *Z. physiol. Chem.* **205**, 231 (1932).
[51] Summary: S. Veibel *in* Bamann-Myrbäck, Die Methoden der Fermentforschung. Thieme, Leipzig, II, 1941, p. 1778.
W. W. Pigman, *Advances in Enzymol.* **4**, 41, (1944).

of carbon atoms from 1 to 7 in the series of *n*-alkylglucosides and then decreases in the glucosides of the higher normal alcohols.[42] The hydrolysis of cetyl-β-D-glucopyranoside is no longer detectable, probably because of its low solubility in water.[52] Branching of the alkyl groups sometimes decreases the rate of hydrolysis. A remarkably low rate is exhibited by trimethylcarbinol-β-D-glucoside,[53]



probably on account of steric hindrance.

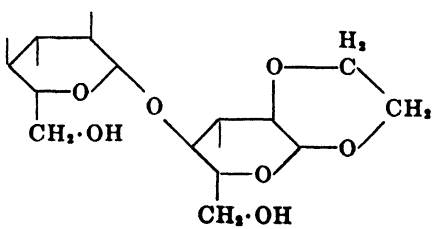
The rate of enzymatic hydrolysis may also vary greatly with aromatic aglucone groups as in phenol-β-D-glucopyranosides. The high velocity of hydrolysis of *o*-cresol-β-D-glucopyranoside is especially characteristic for the β-D-glucopyranosidase of sweet almonds. The corresponding derivatives

Aglucone group	Enzymatic hydrolysis $k \times 10^3$ (30°)	Acid hydrolysis $k \times 10^5$ (60°)
CH_3—	1.0	0.35
$CH_3 \cdot CH_2 \cdot CH_2$—	8.7	0.46
$(C_2H_5)_2CH$—	84.0	1.18
$(CH_3)_3C$—	0.011	9.97 (40°)
Aglucone group	**Enzyme efficiency (30°)**	**Acid hydrolysis $k \times 10^4$**
C_6H_5—	0.34	23
$C_6H_4 \cdot CH_3$-(ortho)	4.3	18
$C_6H_4 \cdot CH_3$-(para)	0.12	21
 (Protocatechualdehyde)	10	13
 (Vanillin)	13	35

[52] E. Fischer and B. Helferich, *Ann.* **383**, 70 (1911).
[53] S. Veibel and H. Lillelund, *Compt. rend.* **203**, 126 and 692 (1937).

of *m*-cresol and *p*-cresol are split at a lower rate comparable to that of the phenol derivative itself,[38]

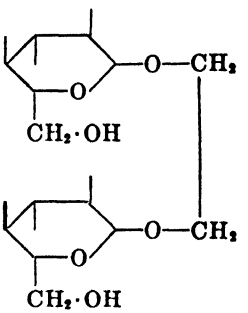
In regard to this relative enzyme specificity toward various β-D-glucopyranosides it is of particular interest that the sequence of increasing velocity of hydrolysis by the enzyme does not coincide with the capacity to be hydrolyzed by hydrogen ions; this is demonstrated in the following table with some typical examples.[54] The action of the enzyme must involve a particular effect which differs at least in part from that of acid hydrolysis. This table indicates that the rate of saponification of β-D-glucopyranosides by β-D-glucosidase may vary widely. The vanillin derivative is split 40,000 times faster than the trimethylcarbinol derivative. But changes in the aglucone group do not cause a disappearance of enzymatic hydrolysis, except in a few "extreme" cases. Therefore, it is easily understood that the same β-D-glucopyranosidase is capable of hydrolyzing β-D-glucopyranosides which have a second sugar as their "aglucone." Gentiobiose, cellobiose, and other disaccharides and oligosaccharides with a terminal unsubstituted β-D-glucopyranoside ring are split by the β-D-glucopyranosidase of sweet almonds (see the formula on p. 91, R = monosaccharose or oligosaccharose). This hydrolysis is not prevented by a substitution or other change in the second (reducing) sugar. Neither oxidation to bionic acid or biosone[55] nor reduction to the corresponding hydrobial,[56] nor the transformation of the reducing group to the oligosaccharide[45] nor even the simultaneous ether formation with the hydroxyl group in position 2 as in the glycololigosaccharide anhydrides[57]



prevents enzyme action. These changes affect the activity of the enzyme only in the manner of a change in the aglucone group. β-D-Glucopyranosidase does not require the presence of a specific structure, configuration, or composition of the second reducing sugar or of the entire bioside in order to hydrolyze substrates such as gentiobiose or cellobiose. To assume the existence in sweet almond emulsin of a special cellobiase or gentiobiase introduces an unnecessary complication not required by any experimental

[54] W. W. Pigman, *Advances in Enzymol.* **4**, 56 a. 61 (1944).
[55] E. Fischer and G. Zemplén, *Ann.* **365**, 1 (1909).
C. Neuberg and S. Saneyoshi, *Biochem. Z.* **36**, 44 (1911).
[56] M. Bergmann and W. Freudenberg, *Ber.* **62**, 2783 (1929).
[57] B. Helferich and K. Thiemann, *Z. physiol. Chem.* **281**, 126 (1944).

data obtained until the present time. β-D-Glucopyranosidase is even capable of removing by saponification the terminal, nonreducing glucose of a longer chain of β-D-glucopyranoside rings as in cellotriose or cellotetrose. But this relative specificity becomes absolute if the chain is lengthened further. Cellopolyoses with more than about six glucose units are not hydrolyzed by almond β-D-glucopyranosidase.[31,58] The enzymes that hydrolyze other higher cellopolyoses and cellulose itself are distinct entities. This phenomenon may be connected with the following finding: if the second hydroxyl group in glycol-β-D-glucopyranoside is also bound to a β-D-glucopyranoside ring,



the rate of enzymatic hydrolysis is markedly decreased, although the enzyme now has two β-D-glucoside linkages at its disposal.[59] The two neighboring sugar groups inhibit the action of the enzyme (formation of an intermediate), perhaps by mutual affinity.

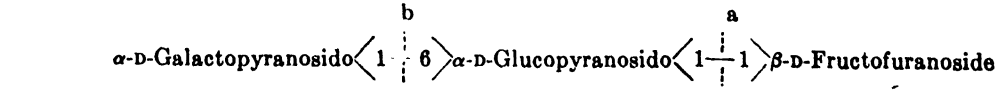
The specificity of the β-D-glucopyranosidase of sweet almonds may be summarized as follows: this enzyme requires in absolute specificity the presence of the β-D-glucopyranoside ring, without substitution and without change in structure and configuration. The β-D-galactopyranosides constitute a possible exception. Enzyme activity is not abolished by replacement of the CH_2OH group in position 6 with hydrogen, or by a change at carbon atom 6 and its hydroxyl group, as long as this change is not too extreme and particularly as long as the new substituent does not take up too much space. Changes in the "aglucone," the portion of the substrate molecule linked by a glucosidic bond, do not prevent enzyme action, but influence the rate of hydrolysis. This relative specificity is transformed into an absolute one only in a few instances, as with cetylglucoside or the cellopolyoses.

It is not permissible to carry these conclusions about a single carbohydrase—a β-D-glucopyranosidase prepared in a definite way from a particular source, i.e., sweet almonds—over to other β-D-carbohydrases or, in

[58] W. Grassmann, L. Zechmeister, R. Bender, and G. Tóth, *Ber.* **67**, 1 (1934).
W. Grassmann, L. Zechmeister, G. Tóth, and R. Stadler, *Ann.* **503**, 167 (1933).
[59] B. Helferich and R. Hiltmann, *Ann.* **531**, 160 (1937).

general, to other carbohydrases. Even the relative specificity of different β-D-glucosidases (snail emulsin, takadiastase, etc.) varies more or less. The range of the absolute specificity may also be different. The relative and absolute specificity of other carbohydrases of different origin may vary even more widely, as shown, e.g., by the behavior of other β-D-galactosidases and other α-D-glucosidases.[38,39,60]

An attractive hypothesis postulated by Weidenhagen[61] assumes the existence of a single glucosidase for all α-glucosidic derivatives and similarly for all β-glycosidic derivatives of a sugar. According to this concept, there is only one β-D-glucopyranosidase, only one α-D-glucopyranosidase, only one β-D-galactopyranosidase, etc., all of which are capable of splitting not only the corresponding glycosides (heterosides) of the particular sugar but also the corresponding oligosaccharides and even polysaccharides. Differences in relative specificity between the various isodynamic enzymes, i.e., carbohydrases of different origin, are based on the impurities in the enzyme or in its colloidal "carriers," according to this hypothesis. However, there is no experimental basis for the assumption that only colloidal carriers and impurities are responsible for the very numerous and often very incisive differences in the relative and even absolute specificity of isodynamic enzymes, as long as no one has succeeded in separating these entities from the enzyme or from its prosthetic group. Moreover, some enzymes are known that do not conform with Weidenhagen's hypothesis, although in many other instances the latter appears to be close to the true situation. Some enzymes that hydrolyze cane sugar are characterized as β-D-fructofuranosidases, others as α-D-glucopyranosidases.[61,62] The first type of saccharase is capable of splitting the saccharose linkage between fructose and glucose even if it (linkage a) is blocked by a third sugar, as in raffinose:

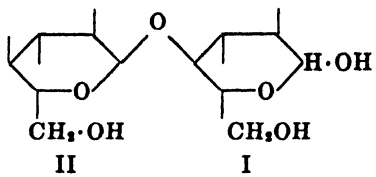


The linkage in raffinose indicated by b is hydrolyzed by α-D-galactosidases, e.g., that in sweet almond emulsin.[63] The α-glucopyranosidase type of saccharase (or invertase) can hydrolyze other simple α-D-glucopyranosides like phenol- or methyl-α-D-glucopyranoside as well.[61]

However, there is no doubt that other enzymes exist that have a specific-

[60] K. Hill, *Ber. Verhandl. Math.-phys. Klasse sächs. Akad. Wiss.* **86**, 115 (1934).
[61] R. Weidenhagen, *Ergeb. Enzymforsch*, **1**, 168 (1932).
[62] R. Kuhn, *Z. physiol. Chem.* **129**, 57 (1923).
R. Kuhn and H. Münch, *ibid.* **163**, 1 (1927).
[63] E. Fischer and E. F. Armstrong, *Ber.* **35**, 3144 (1902).
C. Neuberg, *Biochem. Z.* **3**, 519 (1907).

ity different from that required by Weidenhagen's hypothesis. Carbohydrases derived from a number of bacteria or molds hydrolyze maltose but not methyl-α-D-glucoside, although both substrates contain a terminal α-D-glucopyranoside ring.[39,64,65] In this case, some investigators postulate the existence of a glucomaltase which has a special affinity for the reducing glucose portion (I) in maltose,



II I

in contrast to a glucosidomaltase that cleaves off the glucosidic portion (II) of maltose and is also capable of hydrolyzing other α-D-glucopyranosides.[65]

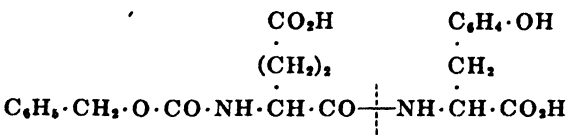
Other findings which were not in agreement with Weidenhagen's hypothesis have been published; the hypothesis is not as all-inclusive as was assumed at first. It should be restricted to those enzymes for which it has been provided with experimental proof.

(3) *Proteolytic Enzymes.* For some time, proteolytic enzymes have been found to be even more suitable for specificity studies than the carbohydrases. The older synthetic methods of Emil Fischer and the newer procedures of M. Bergmann and his collaborators have yielded a large number of well defined oligopeptides which can be used as enzyme substrates. On the other hand, a whole series of proteolytic enzymes has been isolated in pure crystalline form. The use of these crystalline enzymes presumably assures the investigator that he is dealing with individual catalysts and not with mixtures. These possibilities have been successfully exploited in the last few years for investigations of specificity, especially by Bergmann and his collaborators. Their results have initiated a remarkable change in our knowledge of the proteolytic enzymes and their classification. A few examples may be given.[66]

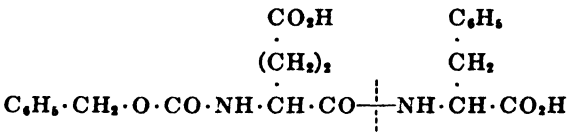
For a long time, it was believed that pepsin and trypsin attack only proteins of higher molecular weight. However, it was established that crystalline pepsin, chymotrypsin, and papain are capable of hydrolyzing oligopeptides and even dipeptides, if these substrates are substituted in a manner suitable for the enzyme. The following table lists a number of substrates which were studied along these lines.[66]

[64] K. Myrbäck, *Z. physiol. Chem.* **196**, 196 (1931).
[65] J. Leibowitz and P. Mechlinski, *Z. physiol. Chem.* **154**, 64 (1926).
J. Leibowitz and S. Hestrin, *Nature* **141**, 552 (1938).
[66] M. Bergmann and J. S. Fruton, *Advances in Enzymol.* **1**, 63 (1941).
M. Bergmann, *ibid.* **2**, 49 (1942).

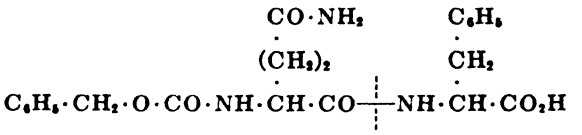
1. Carbobenzoxy-L-glutamyl-L-tyrosine



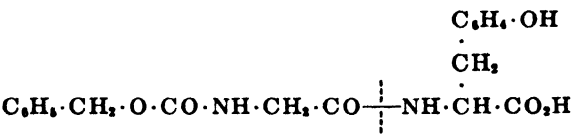
2. Carbobenzoxy-L-glutamyl-L-phenylalanine



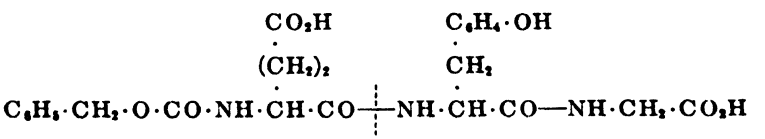
3. Carbobenzoxy-L-glutaminyl-L-phenylalanine



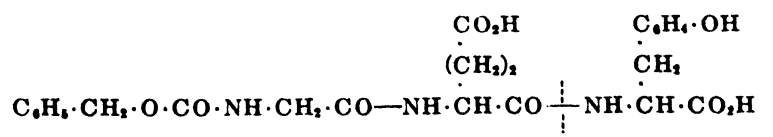
4. Carbobenzoxyglycyl-L-tyrosine



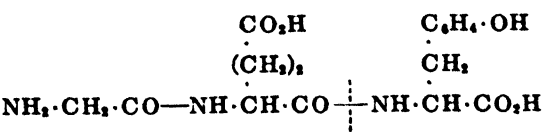
5. Carbobenzoxy-L-glutamyl-L-tyrosylglycine



6. Carbobenzoxyglycyl-L-glutamyl-L-tyrosine

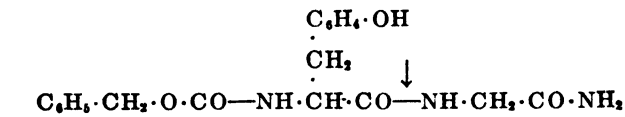


7. Glycyl-L-glutamyl-L-tyrosine



8. L-Glutamyl-L-tyrosine

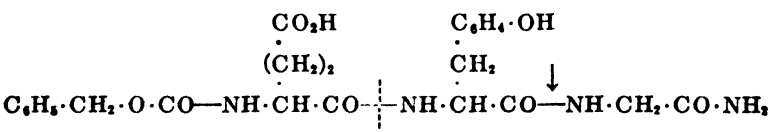
9. Carbobenzoxy-L-tyrosylglycinamide



Crystalline hog pepsin—and pepsin from other animals—splits compounds Nos. 1, 2, 6, and 7, i.e., derivatives of L-tyrosine with two free carboxyl groups, at the linkage indicated with | ; this reaction proceeds at a rather high rate. Substrates 5 and particularly 3 and 4, in which one of these carboxyl groups is blocked or absent, are split more slowly. If both carboxyl groups are blocked or absent, enzymatic hydrolysis does not take place; the same is true if a free amino group is too close to the point at which hydrolysis would take place (No. 8). In every instance, L-tyrosine or L-phenylalanine is hydrolyzed off, and this reaction always involves the peptide bond which includes the amino group of these two amino acids.

Crystalline chymotrypsin also hydrolyzes oligopeptides of these two aromatic amino acids, but with this enzyme the reaction involves the peptide bond which includes their carboxyl group; this bond has been marked with an arrow (No. 9). A further prerequisite for hydrolysis by chymotrypsin is that no free amino or carboxyl group may be too close to the bond to be hydrolyzed. This is well illustrated by the following substrate (No. 10):

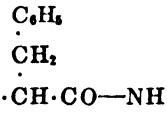
10. Carbobenzoxy-L-glutamyl-L-tyrosylglycinamide



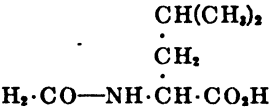
This compound is hydrolyzed by both pepsin and chymotrypsin, but at different loci. Pepsin splits the peptide bond which includes the amino group of tyrosine; chymotrypsin hydrolyzes the peptide bond which includes the carboxyl group of this amino acid. If in this substrate there is no carbobenzoxy group (left end of the formula) blocking the amino group, then pepsin is without effect. On the other hand, if the terminal amide group (right end of the formula) is absent, i.e., if the carboxyl group of glycine is free, then chymotrypsin is unable to attack the substrate.

Derivatives of the "unnatural" D-tyrosine are attacked neither by pepsin nor by chymotrypsin.

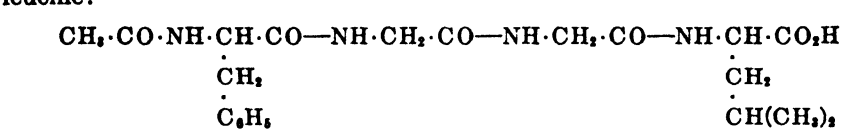
The difficulties which beset the interpretation of studies on specificity may be illustrated by the following example.[66] Cysteine-papain hydrolyzes neither acetyl-L-phenylalanylglycine



nor glycyl-L-leucine



If both substrates are simultaneously placed in contact with the enzyme, the latter synthesizes a tetrapeptide, acetyl-L-phenylalanylgl[illegible]ylglycyl-L-leucine:



If the same enzyme now acts on this substrate, it can split off L-leucine and then glycine. Thus with the aid of the first dipeptide as "co-substrate," the second dipeptide is hydrolyzed by cysteine-papain.

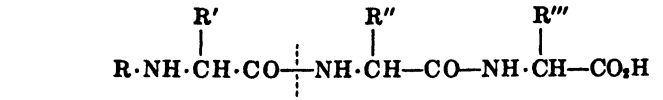
The specificity of proteinases can also be classified according to another point of view. Some can attack only terminal peptide bonds and, therefore, split off only terminal amino acids from a larger molecule (exopeptidases). Other peptidases are capable of hydrolyzing more centrally located peptide bonds as well (endopeptidases).

In regard to the stereochemical specificity of proteolytic enzymes it may be stated that most of them can hydrolyze only the peptide bonds of the "natural" amino acids, i.e., the peptides of glycine or the L-amino acids. The stereochemical specificity of these enzymes is absolute. Others, e.g., papain, can also split peptides of D-amino acids, but at a lower rate than those of the L-series.

Striking examples of substrate specificity, i.e., the relation between enzyme specificity and the structure and configuration of the substrate, have been found among the peptidases, which split a peptide bond near a polar group such as $-NH_2$ or $-COOH$.

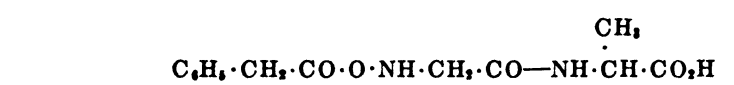
Aminopolypeptidase hydrolyzes peptide bonds adjacent to a free amino group,[67] or, more generally, to a basic nitrogen carrying at least one hydrogen atom.[68] Therefore, this enzyme hydrolyzes peptide bonds—at least tripeptides—starting from the basic end (left end of the formula).

[67] W. Grassmann and H. Dyckerhoff, *Z. physiol. Chem.* **179**, 41 (1928).
[68] M. J. Johnson, *J. Biol. Chem.* **122**, 89 (1937).

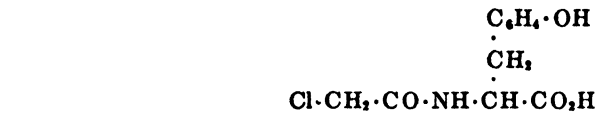


On the other hand, carboxypeptidase (earlier named carboxypolypeptidase) splits peptide bonds adjacent to a free carboxyl group:[69,70]

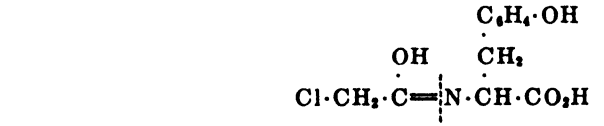
Carbobenzoxy-glycyl-L-alanine



It is not necessary for the acid substituent of the amino group to be an amino acid. Chloracetyl-L-tyrosine



is also split by this enzyme. But an essential condition appears to be that the hydrolyzed peptide bond be able to change to an enol (lactim) form:

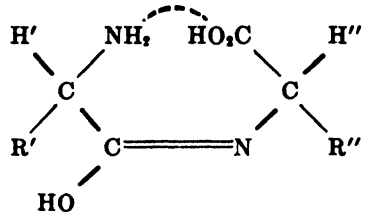


It has been stated that only the *trans*-form is split.[71] If the carboxyl group is too far removed from the point of cleavage, the enzyme is without effect. Glycyl-ε-amino-*n*-heptanoic acid is not hydrolyzed by this enzyme;[72] no hydrolysis occurs if the amino acid to be split off belongs to the "unnatural" D-series.

Very careful investigations have been devoted to the substrate specificity of dipeptidase, an enzyme which is capable of hydrolyzing only dipeptides. Both the amino group of one amino acid and the carboxyl group of the other must be unsubstituted to permit this enzyme to act.[73] Further-

[69] E. Waldschmidt-Leitz, W. Grassmann, and H. Schlatter, *Ber.* **60**, 1906 (1927).
E. Waldschmidt-Leitz and A. Purr, *Ber.* **62**, 2217 (1929).
[70] M. Bergmann and J. S. Fruton, *J. Biol. Chem.* **117**, 189 (1937).
K. Hofmann and M. Bergmann, *J. Biol. Chem.* **134**, 225 (1940).
[71] M. Bergmann, L. Zervas, and H. Schleich, *Z. physiol. Chem.* **224**, 45 (1934).
[72] E. Abderhalden and F. Broich, *Fermentforsch.* **14**, 115 (1933).
[73] W. Grassmann, *Ergeb. Enzymforsch.* **5**, 79 a. 103 (1936); Habilitationsschrift, Univ. of Munich, 1928.

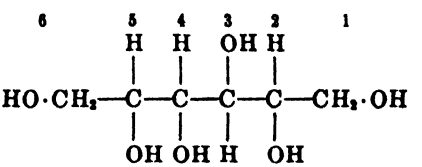
more, only the *cis*-enol form (*cis*-iminohydrin) is hydrolyzed. In the substrate,



both hydrogen atom H′ and H″ may be replaced by methyl groups without loss of enzyme activity. Indeed, a dipeptide of the "unnatural" D-alanine is hydrolyzed by the enzyme at a low rate; on the other hand, the corresponding derivative of D-leucine is not hydrolyzed because in the latter the C_4H_9 group replacing the two hydrogen atoms H′ and H″ is too large and prevents enzyme action by steric hindrance.[74] In contrast to these data, the L-leucine dipeptide is hydrolyzed; the large R′ shown in the above formula then lies on the other side of the ring, away from the locus of enzyme action, and therefore does not prevent the enzyme from acting. On the contrary, a longer chain length of R′ or R″ increases the rate of enzymatic hydrolysis.[75]

(4) *Oxidative enzymes.* Recent investigations and the progressive elucidation of the oxidative enzymes have in many instances led to a better understanding of their substrate specificity.

One of the oldest examples in this field is the rule established by Bertrand in studies of the oxidation of alcohols by certain bacteria. He found that in polyalcohols of the sugar series a secondary hydroxyl group is oxidized (dehydrogenated) only if adjacent to a primary hydroxyl group and only if the secondary hydroxyl group is in the *cis*-position to a secondary hydroxyl group on the next carbon atom. The reduction product of D-glucose, D-sorbitol,



[74] M. Bergmann, L. Zervas, J. S. Fruton, F. Schneider, and H. Schleich, *J. Biol. Chem.* **109**, 325 (1935).
Schneider, Dissertation, Univ. of Munich, 1934.
P. A. Levene, L. W. Bass, and R. E. Steiger, *J. Biol. Chem.* **81**, 221 (1929); **82**, 155 (1929).

[75] W. Grassmann, L. Klenk, and T. Peters-Mayr, *Biochem. Z.* **280**, 307 (1935).

is oxidized at carbon atom 5, not at carbon atom 2, and therefore yields L-sorbose and not D-fructose.[76]

The stereochemical specificity of the oxidative enzymes of these bacteria is determined by the configuration of this one portion of its substrate.

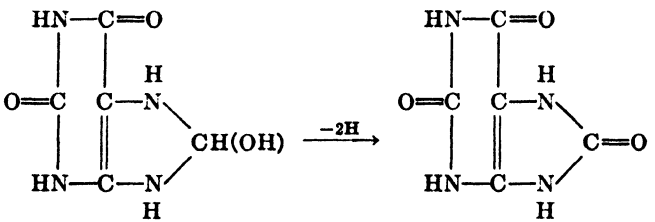
A less particular enzyme is alcohol dehydrogenase of plants[77] and animals,[78] which oxidizes various primary alcohols to aldehydes.

A very specific action is that of β-hydroxybutyric dehydrogenase, which dehydrogenates only L-β-hydroxybutyric acid,[79] and malic dehydrogenase, which converts L-malic acid to oxalacetic acid.[80] α-Glycerophosphate dehydrogenase exhibits strict specificity in the oxidation of L-α-glycerophosphate to 3-phosphoglyceraldehyde:

$$(HO)_2PO\cdot O\cdot CH_2\cdot CH(OH)\cdot CH_2OH \xrightarrow{-2H} (HO)_2PO\cdot O\cdot CH_2\cdot CH(OH)\cdot CH{:}O$$

Neither the optical antipode nor the β-ester are attacked.

In contrast to these enzymes, the so-called Schardinger enzyme or xanthine oxidase[81] has a much wider range of action, a lower substrate specificity. It oxidizes various aliphatic and aromatic aldehydes and also some purines, as illustrated by the oxidation of xanthine to uric acid:[82]



Hydrated xanthine Uric acid

In all these cases a secondary alcohol group is dehydrogenated. But a high substrate specificity is observed also in some instances when hydrogen is removed from a secondary amino group. D-Amino acid oxidase,[83] an enzyme that occurs very frequently in animal tissues, exhibits strict specific-

[76] G. Bertrand, *Ann. chim. et phys.* **3**(8), 275 (1904).
[77] B. Andersson, *Z. physiol. Chem.* **210**, 15 (1932).
[78] F. Batelli and L. Stern, *Compt. rend. soc. biol.* **61**, II, 419 (1931).
[79] D. C. Harrison, *Ergeb. Enzymforsch.* **4**, 309 (1935).
I. Banga, K. Laki, and A. Szent-Györgi, *Z. physiol. Chem.* **217**, 43 (1933).
[80] D. E. Green, *Biochem. J.* **30**, 2095 (1936).
A. Hahn, *Z. Biol.* **92**, 355 (1931–32).
[81] F. Schardinger, *Z. Untersuch. Nahr. u. Genussm.* **5**, 1113 (1902).
[82] E. J. Morgan, C. P. Stewart, and F. G. Hopkins, *Proc. Roy. Soc.* (*London*) **B94**, 109 (1922–23).
[83] H. A. Krebs, *Z. physiol. Chem.* **217**, 191 (1933); *Klin. Wochschr.* **11**, 1744 (1932); *Biochem. J.* **29**, 1620 (1935).
K. Felix and K. Zorn, *Z. physiol. Chem.* **258**, 16 (1939).
P. Holz and H. Büchsel, *ibid.* **272**, 201 (1942).

ity in oxidizing the "unnatural" D-amino acids (not the L-isomers) to imino acids and then hydrolyzing the latter to form α-keto acids:

$$\begin{array}{l} \mathrm{H} \\ | \\ \mathrm{R{\cdot}C{\cdot}CO_2H} \\ | \\ \mathrm{NH_2} \end{array} \xrightarrow{-2\mathrm{H}} \mathrm{R{\cdot}C(=NH){\cdot}CO_2H} \xrightarrow{+\mathrm{H_2O}} \mathrm{R{\cdot}CO{\cdot}CO_2H}$$

The rate of this enzymatic reaction varies with the different D-amino acids. With regard to these substrates, the enzyme possesses only relative substrate specificity.

The reversible dehydrogenation at two adjacent carbon atoms in a saturated compound, to yield an unsaturated one, sometimes exhibits a very high, absolute specificity. For example, succinic dehydrogenase oxidizes succinic acid to fumaric acid,[84]

$$\begin{array}{l} \mathrm{HO_2C{\cdot}CH_2} \\ | \\ \mathrm{H_2C{\cdot}CO_2H} \end{array} \longrightarrow \begin{array}{l} \mathrm{HO_2C{\cdot}CH} \\ \| \\ \mathrm{HC{\cdot}CO_2H} \end{array}$$

but only a few derivatives of succinic acid, e.g., methylsuccinic acid, are subject to the same reaction.[85]

Some phenol oxidases, like the monophenol oxidases and the polyphenol oxidases, are relatively unspecific; others, like dopa oxidase which oxidizes only L-3,4-dihydroxyphenylalanine to melanin,[86] exhibit a high specificity.

Among the enzymes concerned with oxidative metabolism, mention should be made of the catalases, which, with a high specificity, cause the decomposition of hydrogen peroxide to water and oxygen, and of the peroxidases, which catalyze the oxidation of many phenols and amines in the presence of hydrogen peroxide.

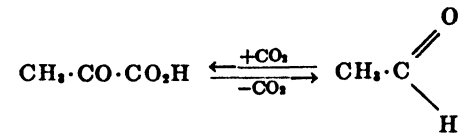
The important role of the coenzymes in connection with the specificity of oxidative enzymes is discussed below.

(5) *Miscellaneous.* Among the hydratases, a word should be said about fumarase that is strictly specific for the reversible transformation of fumaric acid into L-malic acid;[87]

$$\begin{array}{l} \mathrm{HO_2C{\cdot}CH} \\ \| \\ \mathrm{HC{\cdot}CO_2H} \end{array} \underset{-\mathrm{H_2O}}{\overset{+\mathrm{H_2O}}{\rightleftharpoons}} \begin{array}{l} \mathrm{OH} \\ \cdot \\ \mathrm{HO_2C{\cdot}CH} \\ | \\ \mathrm{HC{\cdot}CO_2H} \\ \mathrm{H} \end{array}$$

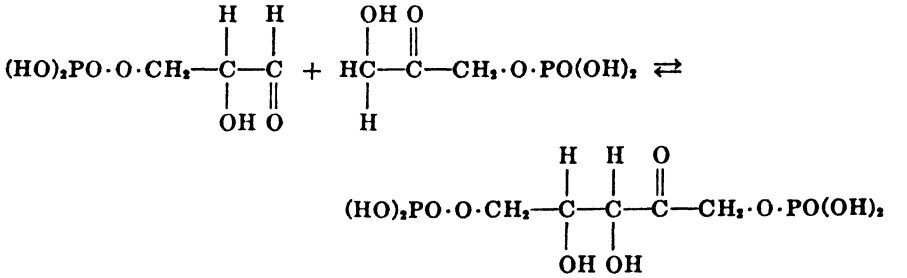
[84] T. Thunberg, *Skand. Arch. Physiol.* **22**, 430 (1909).
[85] T. Thunberg, *Biochem. Z.* **258**, 48 (1933).
[86] B. Bloch, *Z. physiol. Chem.* **98**, 226 (1917).
B. Bloch and F. Schaaf, *Biochem. Z.* **162**, 181 (1925).
[87] F. Batelli and L. Stern, *Biochem. Z.* **31**, 478 (1911).
H. Einbeck, *ibid.* **95**, 296 (1919).

Carboxylase decarboxylates pyruvic acid to acetaldehyde



but can also react with other α-keto acids in the same way.

Among the desmolases, an important enzyme is aldolase, which combines dihydroxyacetone phosphate with aldehydes and especially, reversibly, with phosphoglyceraldehyde to yield fructose-1,6-diphosphate:[88]



This enzyme is highly specific. In one direction, it requires dihydroxyacetone phosphate for the reaction and in the other direction fructose-1,6-diphosphate.

The oxidative enzymes, hydratases, desmolases, and other related enzymes have a varying but often quite strict specificity, as demonstrated by the examples cited above. It is therefore hardly surprising that the enzyme systems made up of these enzymes exhibit a much higher specificity in catalyzing their reactions. Alcoholic or aerobic fermentations, assimilation processes, and similar reactions are restricted to a few substrates, usually even to a single one. Among the hexoses, only the epimeric sugars D-glucose, D-mannose, and D-fructose are fermented by yeast enzymes. In view of their tautomerism, these three sugars to some extent constitute a single substance. In addition, fermentation proceeds with D-galactose, usually after a certain period of adaptation.

The same may be said of other enzymatic processes that probably involve systems of different individual enzymes, e.g., the formation of anti-enzymes and of the Abderhalden protective enzymes whose specificity can be used for strictly specific tests of definite substances and conditions.

III. Specificity of Enzymes as a Basis for their Classification

The specificity of enzymes is of decisive importance. The action of an enzyme as catalyst for a certain reaction makes it possible to subdivide

[88] O. Meyerhof and K. Lohmann, *Biochem. Z.* **271**, 89 (1934); **273**, 413 (1934). O. Meyerhof, K. Lohmann, and P. Schuster, *Biochem. Z.* **286**, 301 a. 319 (1936).

enzymes first of all into groups of those concerned with similar reactions. Insofar as enzymes have not been isolated in pure form and their chemical composition and structure have not been elucidated, this classification has been nearly the only one possible. Today it is still the most important one, even for isolated crystalline enzymes of which the coenzyme portion has a well-understood structure. The protein portion is still a mystery in many ways. This unknown part of an enzyme, according to present knowledge, is responsible for substrate specificity as well as for species and organ specificity. This specificity remains fundamental for the classification and identification of enzymes, for the apoenzymes as well as for the holoenzymes. The qualitative determination of enzyme specificity (activity of the enzyme toward different substrates) does not suffice for this purpose. If a different quantitative behavior of isodynamic enzymes toward different substrates is observed, if a different substrate specificity is established, then these enzymes must be considered to be different, especially if they are available in the pure crystalline state.

But this possibility has its limits. The situation becomes more difficult if the enzyme under investigation is accompanied by other enzymes or by various substances such as carbohydrates or proteins. These contaminants of different types may have a more or less well-recognized influence on the action of the enzyme and on relative as well as absolute specificity. Even different treatments of an enzyme preparation may affect its specificity in different ways and may change its absolute and relative behavior toward various substrates. In all these instances, the decision whether the enzymes in question are identical or not is somewhat dubious and arbitrary. The lack of recognition of these facts introduced into the literature many disagreements that cannot be resolved until the enzymes have been isolated in the pure state. Proof that two enzymatic reactions with different specificity of a single enzyme preparation are mediated by two distinct, separable enzymes can be offered only if the two enzyme effects are successfully separated. Proof of a real separation is established only if the total yield of enzyme units after separation, expressed in per cent of original enzyme units before treatment, is greater than 100.[31]

Of course, the investigation of enzyme specificity is not the only method of classification. It is complemented and supported by other properties of the enzyme, e.g., affinity for various substrates, dependence on various added substances, etc. The decisive importance of a more thorough knowledge of enzyme specificity for their classification has been aptly illustrated by the proteolytic enzymes. Until a few years ago, these enzymes were classified mainly according to their pH optimum and their distribution in various species and tissues, and only secondarily according to their then recognized substrates, most of which had a high molecular weight and an unknown structure. Crystallization in the pure state of some of the most

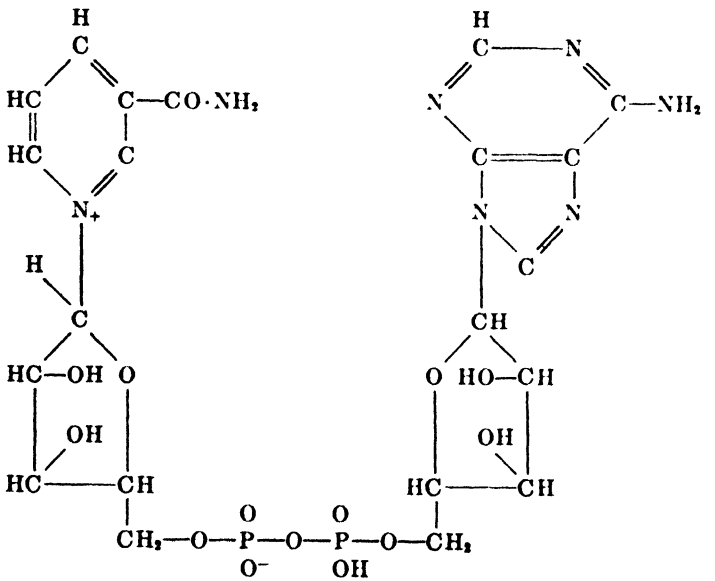
important proteolytic enzymes brought about only small changes in this respect. A clear and well-founded classification of these enzymes became possible only upon study of their specificity toward well-defined, low molecular weight substrates.[66]

IV. Localization of Specificity in the Enzyme Molecule

A few enzymes have been separated into two parts: a coenzyme, heat-stable and of low molecular weight, and an apoenzyme, a substance endowed with the characteristics of a protein. Some examples are provided by various dehydrogenases and a few desmolases. Only the mixture or combination of coenzyme and apoenzyme, called holoenzyme, exhibits enzyme activity.[89]

In these instances, the question may be posed whether the coenzyme or the apoenzyme is responsible for the specificity of the holoenzyme. The following discussion, taking the dehydrogenase involved in alcoholic fermentation as an example, provides the answer to this question.

The coenzyme of this holoenzyme is cozymase or coenzyme I of yeast; it is well recognized to be a diphosphopyridine nucleotide with the following structure[90]:



This coenzyme, by reduction of its pyridine ring, takes two hydrogen atoms from the substrate and is transformed into dihydrocoenzyme I.

[89] H. Theorell, *Biochem. Z.* **278**, 263 (1935).
[90] K. Myrbäck, *Ergeb. Enzymforsch.* **2**, 139 (1933).
K. Myrbäck and H. v. Euler, *Z. physiol. Chem.* **198**, 236 (1931); **203**, 143 (1931).
K. Myrbäck, H. v. Euler, and H. Hellström, *ibid.* **212**, 7 (1932).

Coenzyme I by itself has no dehydrogenase activity. Combination with the apoenzyme, an enzyme protein of apparently definite but still unknown structure, is required to restore enzyme activity in the holoenzyme, and a specific dehydrogenase is then obtained. These facts characterize the role of the coenzyme in determining the specificity of this enzyme. The coenzyme is the deciding factor in reaction specificity but not in substrate specificity.

This concept is in agreement with the observation that coenzyme I is the coenzyme of not only one but a whole series of holoenzymes. Approximately 35 enzymes with different substrate specificities are known to be associated with the same coenzyme I. In all these enzymes, the coenzyme appears to be the carrier of reaction specificity.

The differences in absolute and relative substrate specificity of these enzymes reside in their different apoenzymes or in a portion of their molecules, i.e., the enzyme proteins. The latter are responsible for the more or less pronounced substrate specificity. Even in cases where the holoenzyme is known to be separable into apoenzyme and coenzyme, the essential and significant property of an enzyme, its absolute and relative substrate specificity, is connected with the enzyme protein.

If no separation into coenzyme and apoenzyme has been achieved, it is believed, nevertheless, that a certain portion or portions of the enzyme protein is responsible for absolute and relative substrate specificity.[74,91] It is therefore inaccurate and misleading to speak of the enzyme protein only as the "carrier," the "pheron" of enzyme activity since the decisive property of an enzyme, namely its substrate specificity, resides in the protein portion.

On the contrary, the coenzyme, as far as we know now, lies on the same level as many other substances, often very simple ones, like salts or ions which are obligatory or effective activators of enzyme action. On the basis of these considerations it is quite reasonable to think of enzymes as proteins of which a portion possesses a definite structure and configuration and is responsible for substrate specificity.[92] A great step forward is made whenever it becomes possible to resolve a holoenzyme into apo- and coenzyme and to elucidate the structure and configuration of the latter. But to speak of the coenzyme as *the* active group goes too far since the secret of absolute and relative specificity toward definite substrates is preponderantly contained in the enzyme protein.

Disregarding certain metal ions (as with many peptidases, see p. 107),

[91] H. v. Euler, *Z. physiol. Chem.* **143**, 79 (1925).
W. W. Pigman, *J. Research Natl. Bur. Standards* **27**, 1 (1941); *Advances in Enzymol.* **4**, 41 (1944).
[92] J. H. Northrop, *J. Gen. Physiol.* **13**, 739 (1930); *ibid.* **13**, 767 (1930); *Ergeb. Enzymforsch.* **2**, 104 (1933).

the existence of coenzymes of the type discussed above in hydrolyzing enzymes appears rather improbable. For in water, the usual solvent employed in enzyme reactions, there is an overwhelming quantity of water molecules or ions. There is little reason to assume the existence of a special coenzyme as acceptor or donor of water molecules or ions until such a coenzyme is actually proved to be present.

The fact that substrate specificity in its fine shadings depends on the enzyme protein, clarifies yet another observation. Isodynamic enzymes often exhibit organ or species specificity (cf. p. 81). Corresponding to their different origin in different organisms or different organs of the same organism, the enzyme proteins despite their very similar action do not have exactly the same absolute or relative specificity. They differ more or less in this regard just as proteins differ generally from species to species and from organ to organ.[92]

The specificity of carbohydrases and the existence of individual oligosaccharases may be considered as an example of this concept.

The carbohydrases are among the enzymes that have not been separated into coenzyme and apoenzyme. The locus of enzyme specificity must lie in the enzyme protein. It may be assumed that one or more adsorption areas in the enzyme protein are responsible for the specific adsorption of the substrates, i.e., the glucosides and oligosaccharides.[91] These adsorption areas may be influenced by neighboring groups. The same or nearly same adsorption area may occur in proteins of different origin. On the basis of these differences in the tissue or organism which is the source of the protein, the same or nearly same adsorption area may cause a different relative substrate specificity and even a different absolute specificity toward some substrates. This assumption corresponds to the tendency toward simplification expressed by the hypothesis of Weidenhagen but without requiring that all isodynamic enzymes (e.g., all α-glucosidases, all β-D-galactosidases, all saccharases, or all maltases) be identical.

It is well known that catalysts and similar reagents found in nature may have a qualitatively similar but quantitatively more or less different action, with a different "relative specificity." The various vitamins D or the different types of penicillin are good examples of this fact. In both of these, a part of the molecule of the "isodynamic" compound is identical. But the quantitative effect is not the same since other neighboring parts of the molecule are different and by their different character cause the relative differences in vitamin D activity or disinfecting power.

The identity of the most important portion of isodynamic enzymes is particularly plausible in view of the structural and stereochemical conception of enzyme specificity.

[92] P. Holz and H. Büchsel *Z. physiol Chem.* **272**, 201 (1942).

V. Inferences about Enzyme Structure Drawn from Enzyme Specificity

The investigation of enzyme specificity and the availability of exact information on the structure and configuration of the substrates attacked by the enzyme allow us to draw some conclusions about the enzyme molecule and its structure and configuration. To use Emil Fischer's metaphor: the investigation of the lock, the substrate, which is opened by the key, the enzyme, yields valuable information about the structure of the key itself, i.e., the enzyme.

A fundamental conclusion of this type was drawn a long time ago. Enzymes act differently on optical antipodes (cf. p. 83); they exhibit true stereochemical specificity. Regardless of whether this difference becomes manifest in terms of relative or absolute specificity, it is necessary that the enzyme itself have an asymmetric structure, at least in a part of the enzyme system or molecule. All enzymes are optically active substances; none of them are symmetrical or racemic mixtures or racemic compounds.

It is quite generally assumed that each enzymatic process consists of at least two consecutive reactions. First, an intermediate compound of enzyme and substrate is formed. It is not particularly important or significant whether this intermediate represents a definable and specific adsorption complex, a definite molecular compound, a compound involving a type of salt linkage or a compound formed by means of covalent bonds, hydrogen bonds, dipoles, or van der Waal's forces; this is true especially since the boundaries between some of these binding forces are uncertain and indistinct.

The second reaction involves a decomposition of this enzyme-substrate complex into the products of enzyme action, whereby the enzyme is released for the next combination with substrate.

Both of these reactions may have a substantial effect on enzyme specificity. The enzyme-substrate complex between a particular enzyme and different substrates as well as that between different but isodynamic enzymes and a single substrate may be formed with different velocities. The rate of decomposition of this complex may vary with the different structure and configuration of the enzyme as well as of the substrate.

More exact knowledge of substrate specificity suggests in many instances that the essential part of substrate specificity is involved in the formation of the enzyme-substrate complex.

If, for example, the study of a well-defined carbohydrase such as the β-D-glucopyranosidase of sweet almonds indicates that the unchanged β-D-glucopyranose ring is required for the reversible hydrolysis of the β-D-glucosides (cf. p. 94), then it is obvious that this β-D-glucopyranoside ring must fit a certain portion of the enzyme molecule in some physical or chemical way, in structure and stereochemistry, in some manner which

favors the hydrolysis of the glucoside linkage. Following up this thought, Lettré has discussed the possibility that the corresponding part of the enzyme molecule may be an optical antipode of the β-D-glucopyranoside ring. The enzyme-substrate complex would then be a racemic or partially racemic compound of the two corresponding parts of the enzyme and the substrate.[94] The fact that L-glucose or derivatives of L-glucose have been encountered only very rarely in natural products argues against this assumption. Another concept proposed that the β-D-glucopyranoside ring itself or a closely related ring may be present in the enzyme molecule. This thought is supported by the finding that even highly purified preparations of this enzyme contain approximately 3% carbohydrate.[95] The combination of enzyme and substrate in a form suitable for the hydrolysis would be produced by the same forces which hold the molecules together in a crystal. It is well known that these forces possess a high specificity; one need only mention the seeding of supersaturated solutions, particularly of the sugar group. Only a crystal of the same substance, identical in structure and configuration, can initiate crystallization in such a supersaturated solution. Even the concept of at least two different adsorption areas in different but isodynamic enzymes[95,96] can be envisaged since a glucopyranoside ring has at least two sides which may combine with the enzyme. This assumption rationalizes the different relative or absolute specificity of two isodynamic enzymes.

This view may perhaps be applied to other enzymes. The adsorption area in the enzyme must fit the adsorption area of the substrate as the cast fits the template. If at equilibrium the adsorption area is more or less occupied by another compound, then this compound inhibits to a greater or lesser degree the formation of the enzyme-substrate complex and therefore the action of the enzyme. The effect of the adsorption area in the enzyme as well as in the substrate may be more or less inhibited and even completely nullified by neighboring groups in the enzyme molecule or in the substrate molecule; this inhibition may take place to a different degree for different enzymes or different substrates.

Since the formation of the enzyme-substrate complex in some manner involves electrical forces, it is quite obvious that ionic charged groups in the substrate molecule, e.g., carboxyl or amino groups, usually have a

[94] H. Lettré, *Z. angew. Chem.* **50**, 581 (1937).

[95] B. Helferich, W. Richter, and S. Grünler, *Ber. Verhandl. sächs. Akad. Wiss. Math.-phys. Klasse* **89**, 385 (1937).

B. Helferich and W. W. Pigman, *Z. physiol. Chem.* **259**, 253 (1939).

P. Edman and E. Jorpes, *Acta Physiol. Scand.* **2**, 41 (1941).

[96] T. Miwa, C. Cheng, M. Fujisaki, and A. Toishi, *Acta Phytochim. (Japan)* **10**, 155 (1937).

CHAPTER 4

Enzymes in Relation to Genes, Viruses, Hormones, Vitamins, and Chemotherapeutic Drug Action*

BY M. G. SEVAG, J. S. GOTS, AND E. STEERS

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* This investigation was supported, in part, by a research grant from the Division of Research Grants and Fellowships of the National Institute of Health, U. S. Public Health Service.

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I. Genes and Enzymes

When the final phenotypic expression of a gene is a morphogenetic one, the process is beyond chemical interpretation and must be expressed in chemically vague terms. If, however, the gene change results in the modification of a chemical process, the action of genes leading to it may be made in terms of definite chemical reactions. The relationship of genes and enzymes was early recognized through the suggestions that inheritable chemical differences controlled by genes might be accomplished through the control of enzymes. This subject has been treated in comprehensive detail in several recent reviews.[1–6] We shall consider briefly the various examples

[1] S. Wright, *Physiol. Revs.* **21**, 487 (1941).
[2] G. W. Beadle, *Chem. Revs.* **37**, 15 (1945).
[3] G. W. Beadle, *Am. Scientist* **36**, 69 (1948); **34**, 31 (1946).
[4] G. W. Beadle, *Ann. Rev. Physiol.* **10**, 17 (1948).
[5] D. M. Bonner, *Science* **108**, 735 (1948).
[6] J. B. S. Haldane, New Paths in Genetics. Harper, New York, 1942.

of chemical situations which appear to be gene controlled, with particular emphasis on evidences of enzymatic involvement.

1. Gene Controlled Abnormal Enzymatic Activities

a. Formation of Abnormal Products

Perhaps the earliest recognized type of difference in congenital chemical activity is the presence of abnormal products as demonstrated by accumulation or excretion. Since these chemical analogues of structural malformations are inheritable as simple recessives, they were attributed to a transformation of the gene to one of its recessive allelomorphs which no longer could perform its normal function, or performed it more slowly. In effect, this represents a "genetic block" analogous to the determination of intermediates in a normal chain of enzymatic functions by classical chemical blocking. The normal products are not produced and intermediates accumulate or are excreted.

(1) *Phenylalanine-tyrosine metabolism in man.* In the phenylalanine-tyrosine metabolism of man,[6,7] several such genetically acquired metabolic dysfunctions have been recognized. By feeding experiments, the precursors of the accumulated intermediates have been determined. The condition known as *alcaptonuria* is characterized by a blackening of the urine on standing. This is due to the oxidation of homogentisic acid (2,5-dihydroxyphenylacetic acid), which is not present in the urine of normal individuals, to CO_2 and H_2O via acetoacetic acid through normally functioning metabolic paths. Gross (cited by Garrod[7]) recorded one of the first cases of a failure in performing a chemical process due to a lack of enzymatic activity. The enzymatic ability to destroy homogentisic acid was present in blood serum of normal persons but not in blood of alcaptonurics.

Another type of metabolic abnormality of this group has been found in *phenylpyruvic oligophrenia* (*amentia*); *phenylketonuria*.[8,9] This is a condition characterized by imbecility and the excretion of phenylpyruvic acid. It is apparently connected with a disturbance in the metabolism of phenylalanine as determined by feeding experiments.

Through an as yet unknown process tyrosine also acts as a precursor in melanin production. Tyrosinase and dopa (3,4-dihydroxyphenylalanine) oxidase are the enzymes involved in its formation. The absence of melanin (albinism) may be considered as another type of genetic block in this group.[7] The general problem of pigmentation difference as a type of inheritable chemical difference will be considered later.

The following chart shows the paths of phenylalanine-tyrosine metab-

[7] A. E. Garrod, Inborn Errors in Metabolism. 2nd ed., Oxford Univ. Press, London, 1923.

[8] A. Fölling, *Z. physiol. Chem.* **227**, 169 (1934).

[9] L. S. Penrose, *Lancet* **2**, 192 (1935).

olism and the location of genetic blocks responsible for the above disorders. Tyrosinosis is included in the chart, but since only one case is on

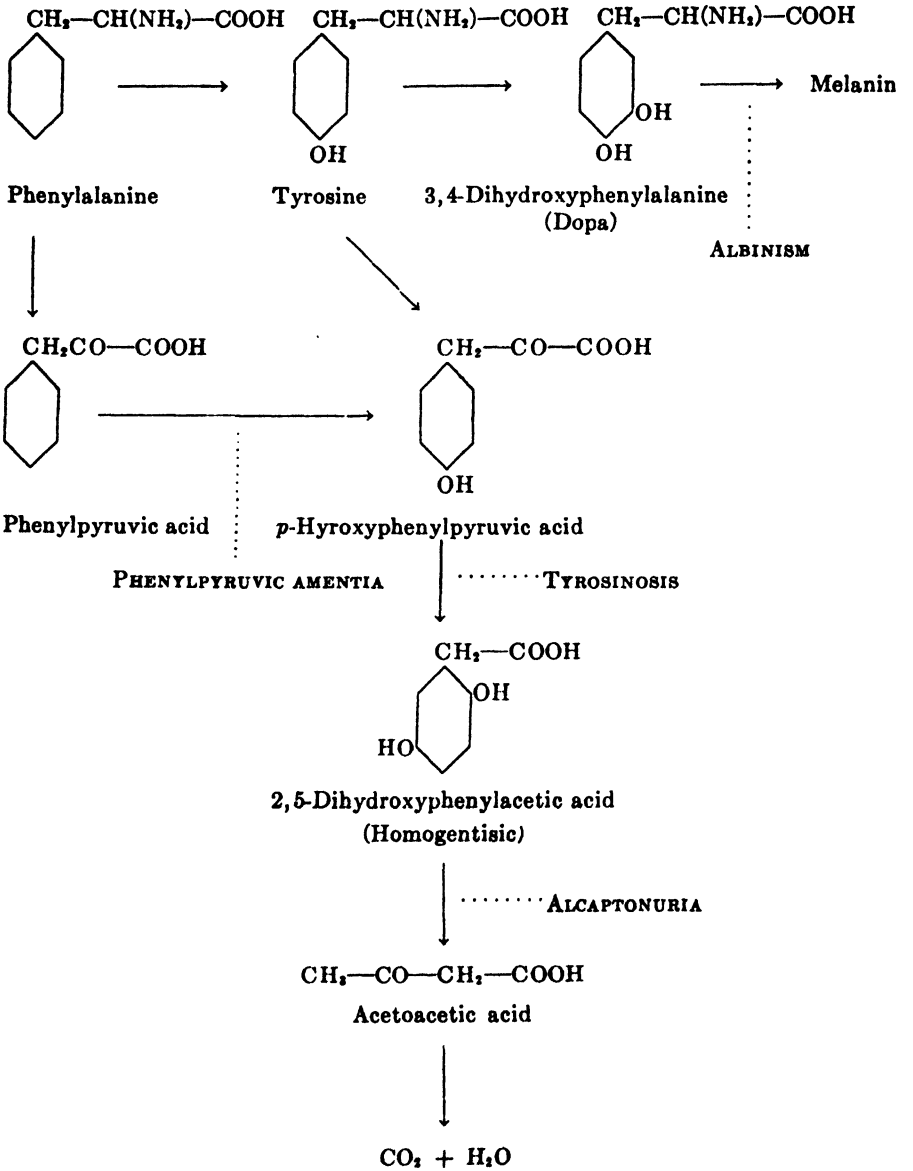

FIG. 1. Phenylalanine-tyrosine metabolism in man and associated "genetic blocks" (based on Haldane[6]).

record its genetics has not been determined. It is characterized by the excretion of *p*-hydroxyphenylpyruvic acid.[6]

(2) *Other chemical anomalies in man.* A number of other disorders of man, the chemistry of which is either vague or complex, have been attributed to genetically acquired disorders of metabolism and characterized by accumulation or excretion of abnormal products (intermediates).[6, 7, 10] These are: (a) *Cystinuria*, a condition in which appreciable amounts of cystine appear in the urine. It has been alternately considered as an error in general protein metabolism of which cystine is only one factor, or as an error of deamination in which the amino acid is excreted as such. (b) *Hematoporphyria congenita* (congenital porphyrinuria), which is characterized by red urine due to deposition of excess porphyrin. (c) *Pentosuria*, pentose in the urine. (d) *Congenital steatorrhea*, an error in fat utilization characterized by fatty stools and presumably associated with a disorder of the pancreas. (e) *Amaurotic idiocy* (*Tay-Sach's disease*) which is probably a defect in lipide metabolism characterized by excess deposits of lipides in the ganglia and glia cells of the brain and often associated with abnormal deposition of phospholipides (Niemann-Pick's disease). (f) *Xeroderma pigmentosum*, a dermatological manifestation of congenital hypersensitiveness to the action of light. (g) *Glycogen storage* (*von Gierke's disease*), a congenital anomaly in which there is an accumulation of glycogen. Though its mechanism has not been determined it is probably due to a defect in the glycogenolysis sequence.

(3) *Uric acid excretion in the Dalmatian dog.* A recessive characteristic exists in the Dalmatian coach hound whereby more nitrogen is excreted as uric acid (2–3%) than normal (0.2–0.4%).[11–13] This type of uric acid excretion is normally the main product of nitrogenous metabolism of reptiles and birds. The other non-primate mammals can oxidize uric acid to allantoin by the enzyme uricase. In accordance with the developed concept of abnormal excretion as a result of an inheritable error in metabolism, the failure of the liver of the Dalmatian dog to convert uric acid to allantoin would be expected. However, Wells[14] and Klemperer *et al.*[15] could find no differences in enzyme concentration in the liver of normal and Dalmatian dogs and thus concluded that there was no quantative relationship between uric acid excretion of the Dalmatian and uricase content.

(4) *Are all abnormal products inborn errors of metabolism?* In those cases in which the chemical expression is one of an increased urinary excretion of an intermediate metabolite we must consider the possibility that the

[10] M. Bodansky and O. Bodansky, Biochemistry of Disease. Macmillan, New York, 1940.

[11] S. R. Benedict, *Harvey Lectures* **10**, 346 (1915–16).

[12] H. Onslow, *Biochem. J.* **17**, 334, 564 (1923).

[13] H. C. Trimble and C. E. Keeler, *J. Heredity* **29**, 281 (1938).

[14] H. G. Wells, *J. Biol. Chem.* **35**, 221 (1918).

[15] F. W. Kemperer, H. C. Trimble, and A. B. Hastings, *J. Biol. Chem.* **125**, 445 (1938).

situation is not a chemical defect but rather an anomaly of kidney excretion. It is possible that the difference may not be due to lack of an enzyme but may be caused by a genetically inherited abnormality in the secretory function of the kidney. Such a high rate of renal secretion would remove the intermediate from the body before it could be metabolized. Neuberger, *et al.*[16] and Friedman and Byers[17] have considered this in the cases of alcaptonuria in humans and uric acid excretion in the Dalmatian, respectively, and offer experimental data to indicate that this may indeed be the fundamental anomaly.

Aside from this possibility, abnormal products of excretion or accumulation may arise from mechanisms other than accumulation of the precursor to a blocked reaction. Glucuronates may be found in detoxification mechanisms. Metabolic perversions such as Bence-Jones protein may occur under pathological conditions. Structural changes in the thyroid and pancreas may be associated with functional metabolic disorders. Several products are the result of bacterial action (indole, urobilin, indoxyl). On this basis a survey on a broader scale of the other cases of abnormal excretory products would be required.

b. Pigmentation

Pigmentation is one of the few of several morphogentic characters which may be expressed in chemical terms. The types of pigmentation which have been extensively studied from this point of view are (1) melanin production, (2) flower pigment, and (3) eye color in insects.

(1) *Melanin production.* Melanin is a pigment of unknown chemical structure formed from the precursors, tyrosine and 3,4-dihydroxyphenylalanine (dopa). The principle enzymes involved are tyrosinase and dopa oxidase. Differences in tyrosinase or dopa oxidase content of skin extracts from pigmented and albino laboratory mammals have been demonstrated and reported by many.[18–22] Since the presence or absence of pigment involves not only the presence or absence of enzyme, but also formation and presence of chromogen (tyrosine?), temperature, pH, secondary catalysts, chemical inhibitors, and other chemical elements that cannot be clearly described, the mechanism remains beyond simple definition. Several comprehensive critiques deal with the complexities of this problem.[1,23–25]

[16] A. Neuberger, C. Rimington, and J. M. G. Wilson, *Biochem. J.* **41**, 438 (1947).
[17] M. Friedman and S. O. Byers, *J. Biol. Chem.* **175**, 727 (1948).
[18] F. M. Durham, *Proc. Roy. Soc. London* **74**, 310 (1904).
[19] H. Onslow, *Proc. Roy. Soc. London* **B89**, 36 (1915).
[20] B. Bloch, *Z. physiol. Chem.* **98**, 226 (1917).
[21] P. Koller, *J. Genetics* **22**, 103 (1930).
[22] D. C. Charles, *Genetics* **23**, 523 (1938).
[23] S. Wright, *Biol. Symposia* **6**, 337 (1942).
[24] C. E. M. Pugh, *Biochem. J.* **27**, 475 (1933).
[25] R. Goldschmidt, Physiological Genetics. 1st ed., McGraw-Hill, New York, 1938.

The work of Danneel and Schaumann (reviewed by Danneel[26]) has contributed greatly towards the clarification of this problem. Working with the Himalayan rabbit (white with black extremities) they related melanin formation to a chain of three reactions which are interdependent. These are: 1) a reaction that is suppressed by x-rays and is involved in the formation of the Golgi apparatus and lipochondria of cells. 2) An aerobic formation of dopa oxidase that occurs in black rabbits even under high temperatures, but is temperature sensitive in Himalayan rabbits and completely absent in albinos. 3) A reaction following this of pigment production that requires oxygen and is inhibited by cyanide.

(2) *Flower Pigmentation.* The chemistry of flower pigmentation has been extensively analyzed. The conditions that determine the existence of a particular color are rather complex; the details may be found in several excellent reviews.[6,25,27,28] In general, the color depends on the existence of either one pigment, where it depends on the type of pigment and pH (indicator), or several pigments where it depends on combination effect, background effect, or copigment effect. The pigments involved are plastid pigments (carotin, xanthophyll), anthoxanthins (flavone derivatives, e.g., quercetin), anthocyanins (anthocyanidin glycosides, e.g., pelargonidin (1OH), cyanidin (2OH), delphinidin (3OH)), chalcones (e.g., butein) and flavocyanins. The particular pigment derivatives involved depend on OH position, glycoside position and type, methylation of OH, pH control, organic acid additions, and oxidations. Genes exercise highly specific action upon these synthetic chemical processes as determined by the prevention of the above reactions by the mutated genes. Though it is thought that the mutated genes may operate to control the rates of the processes concerned with the specific enzymes involved, it is difficult to draw conclusions on the exact mechanism since the particular catalysts involved exist by assumption only. Enzymatic differences between wild and mutant varieties have, as yet, not been determined.

(3) *Eye color in insects.* A complex analysis of a pigment system has evolved through studies of eye color variation in mutants of such insects as Drosophila, Ephestia, and Bombyx. The red eye color of the wild type *Drosophila melanogaster* (fruit fly) is controlled by two pigments; a water-soluble red (basic side) pH indicator and a brown water-soluble pigment. Both of these may act as O/R indicators in that they may be reversibly oxidized and reduced to leuco forms. The nature and absolute amount of the pigments and the equilibrium between oxidized and reduced forms are genically controlled by way of enzymes. The many variations of eye color in Drosophila are expressions of a disorganization of the complex con-

[26] R. Danneel, *Ergeb. Biol.* **18**, 55 (1941).
[27] R. Scott-Moncrieff, *J. Genetics* **32**, 117 (1936).
[28] W. J. C. Lawrence and J. R. Price, *Biol. Revs. Cambridge Phil. Soc.* **15**, 35 (1940).

tinuity of the necessary prerequisites leading to the normal eye pigment of the wild type. The details of the chemistry, physiology and genetics of these processes may be found in several reviews by Ephrussi,[29,30] Beadle and Tatum,[31] and Caspari.[31a]

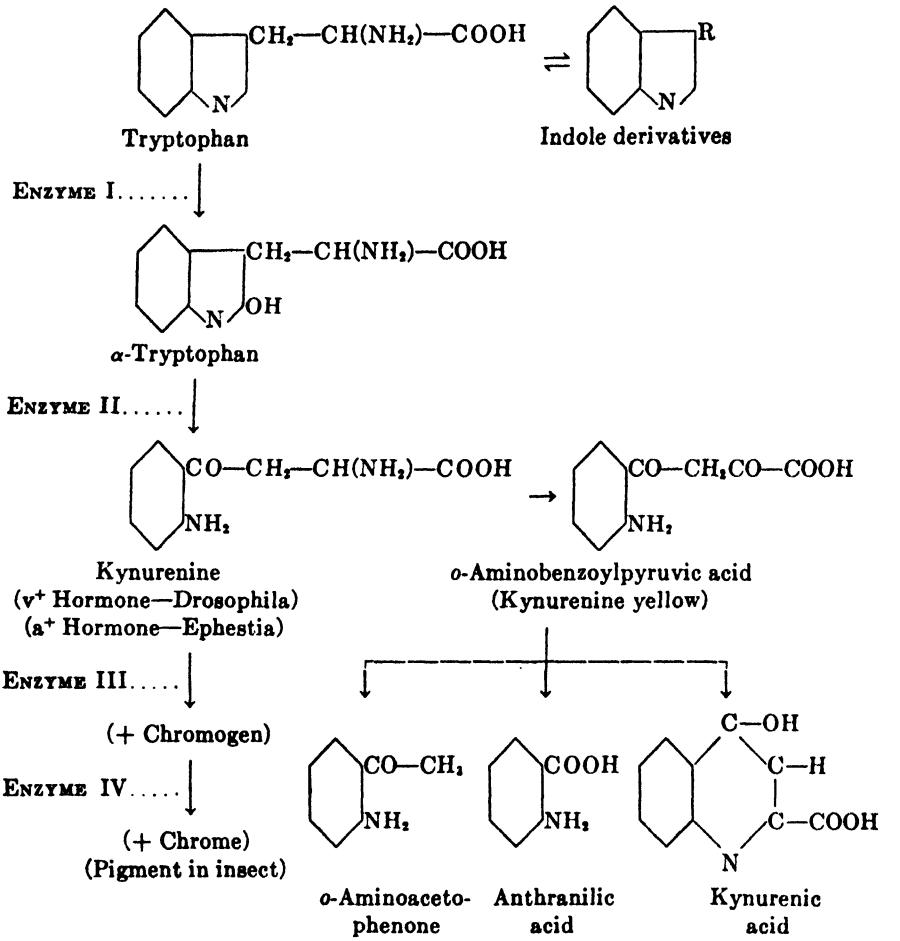

FIG. 2. Tryptophan pathways in the formation of eye pigment in insects (based on Kikkawa[34]).

One very important precursor is a diffusible substance of hormonal nature required to release a process which can then continue in its absence. It has been identified as kynurenine and is identical with both the "v⁺

[29] B. Ephrussi, *Cold Spring Harbor Symposia Quant. Biol.* **10**, 40 (1942).
[30] B. Ephrussi, *Quart. Rev. Biol.* **17**, 327 (1942).
[31] G. W. Beadle and E. L. Tatum, *Am. Naturalist* **75**, 107 (1941).
[31a] E. Caspari, *Quart. Rev. Biol.* **24**, 185 (1949).

hormone," the essential defect of the vermillion Drosophila mutant, and the "a^+ hormone" absent in the red eye mutant of Ephestia (meal moth). Kynurenine is formed by the oxidation of tryptophan. The actual block in mutants not containing it appears to be the initial oxidation of tryptophan to α-oxytryptophan since the latter can produce pigment when injected into the Ephestia mutant.[32] Caspari[33] has made an extensive study of the nature of kynurenine lack in Ephestia mutants and has correlated it with increased tryptophan accumulation, inhibition of tryptophan oxidation and general protein changes with profound concomitant biochemical alterations.

The complexities of eye color variation in insects has been reduced by Kikkawa[34] to four main reactions controlled by four enzymes dependent on the presence or absence of the genes controlling them. Several side reactions may also be present which may influence the concentrations of the intermediate substrates and precursors. For example, kynurenine may be diverted from the pigment producing sequence to the formation of *o*-amino acetophenone, anthranilic acid, or kynurenic acid. Likewise, tryptophan availability or diversion is influenced by its indole derivatives.

In conclusion, since we have no knowledge of the enzymes involved, the entire analysis of the relation of the genetics of eye color in insects to enzymes must, at best, be based on assumptions. However, since enzymes must be concerned in the catalysis of the various steps and since the specificity of genes is of the same order as the specificity of enzymes, the assumptions seem probable.

c. Differences in Enzymatic Reactions

(1) *Xanthophyllase in rabbits.* Another type of pigment variation is found in the yellow fat of a mutant rabbit.[35,36] Fat soluble xanthophyll pigments in foods are responsible for the color. The fat of the ordinary rabbit is colorless due to the enzyme, xanthophyllase, which destroys the pigment by oxidation. The liver of the yellow-fat rabbit is lacking in this enzyme. The failure to inherit the enzyme is controlled by a simple recessive, genetically linked with skin and hair pigmentation. The lack of fat pigmentation, however, may be independent of gene control since the rabbit lacking xanthophyllase will not develop yellow fat when kept on a carotene and xanthophyll deficient diet.

This demonstration of a specific enzyme lack introduces us to a type of

[32] A. Butenandt, W. Weidel, and E. Becker, *Naturwissenschaften* **28**, 447 (1940).
[33] E. Caspari, *Genetics* **31**, 454 (1946).
[34] H. Kikkawa, *Genetics* **26**, 587 (1941).
[35] S. G. Willimot, *Biochem. J.* **22**, 1057 (1928).
[36] W. E. Castle, *Proc. Natl. Acad. Sci. U. S.* **19**, 947 (1933).

genetically acquired chemical difference characterized by the presence or absence of specific enzymatic reactive systems. In general, all chemical differences have been explained on the basis of enzyme reaction changes, but only a few have been experimentally demonstrated to be a result of a definite lack of enzyme activity.

(2) *Atropinesterase in rabbits.* Some rabbits possess in their blood serum an enzyme "atropinesterase" capable of hydrolyzing atropine and monoacetylmorphine.[37,38] This peculiarity is inherited in a gene which is incompletely dominant and associated with the gene for the extension of black pigment in the coat.

(3) *Amylase.* Strong amylase activity of digestive juice and body fluid of silkworm larvae has been shown to be genetically dominant over weak activity (cited by Wright[1]).

A difference in amylase activity is also reported to be responsible for differences in starch make-up of waxy and normal corn.[39]

(4) *Cyanogenesis in white clover.* Certain strains of white clover (*Trifolium repens*) contain HCN derived from cyanogenic glucosides and the enzyme necessary to hydrolyze them. The glucosides have been identified as lotaustralin and linamarin, which yield HCN, a glucose, and a ketone (ethyl methyl ketone for the former and acetone for the latter) under the hydrolytic action of the enzyme, linamarase. Various strains exist which contain the glucosides alone, the enzyme alone, neither, or both. The differences among the strains are controlled by two independent dominant factors, one determining the ability to produce the cyanogenic glucosidic substrates and the other determining the presence of the enzyme. This offers one of the best evidences for gene-enzyme control in that *in vitro* cyanogenesis could be produced by the addition of extracts from acyanogenic plants, one providing the substrates and the other the enzyme.[40,41]

(5) *Genetically controlled fermentations in yeast.* Lindegren and Spiegelman have reported that the ability of yeasts to ferment melibiose[42] and galactose[43] after having been grown in their presence is genetically controlled. The details and further genetic implications of these relationships may be found in resumés by Lindegren[44] and Spiegelman.[45]

[37] D. Glick and S. Glaubach, *J. Gen. Physiol.* **25**, 197 (1941).
[38] P. B. Sawin and D. Glick, *Proc. Natl. Acad. Sci. U. S.* **29**, 55 (1943).
[39] R. A. Brink, *Quart. Rev. Biol.* **4**, 520 (1929).
[40] S. S. Atwood and J. T. Sullivan, *J. Heredity* **34**, 311 (1943).
[41] S. S. Atwood, *Advances in Genetics* **1**, 1 (1947).
[42] C. C. Lindegren, S. Spiegelman and G. Lindegren, *Proc. Natl. Acad. Sci. U. S.* **30**, 346 (1944).
[43] C. C. Lindegren, *Ann. Missouri Botan. Garden* **32**, 107 (1945).
[44] C. C. Lindegren and G. Lindegren, *Cold Spring Harbor Symposia Quant. Biol.* **11**, 115 (1946).
[45] S. Spiegelman, *Cold Spring Harbor Symposia Quant. Biol.* **11**, 256 (1946).

d. Mutations Involving Growth Factor Requirements in Neurospora

The final type of inheritable chemical variation to be considered involves the analysis of mutants that require various chemical agents for essential physiological functions. The extensive work with the bread mold, Neurospora, by Beadle and his associates, has been the most fruitful in providing further foundation for the concept of genic control of enzyme activity[2–5,46–49] Similar work has been done recently with the mold, Ophiostoma.[50] The chemical mutations involved in these molds are reminiscent of the earlier and much criticized work with the green flagellate, Chlamydomonas.[51,52] In this case, the mutants obtained required specific "sex hormones" identified as carotenoid pigments and their derivatives, which were found to be necessary for motility, mating, and copulation activities of various mating types. This has been subjected to much criticism and its significance requires and must await confirmation by independent investigations.

By various mutagenic agents (x-ray, ultraviolet, mustard gas) a vast number of Neurospora mutants, as referred to above, have been obtained by Beadle and his associates which require for their existence preformed essential metabolites not required by the wild type. It has been determined, by classical genetic methods, that these nutritive-requiring mutants differ from the wild type by only one gene and the inheritance of the specific synthetic deficiency is associated with the mutation of this gene. Several mutants have been demonstrated to operate in a given chain of reactions, each mutant being unable to perform a specific reaction in the chain. Since biochemical synthesis is a series of sequential reactions each catalysed by a specific enzyme, the production of the enzyme appears to be gene controlled.

Thus, at the present time Neurospora presents the best material for support of the one to one relationship of gene to enzyme. One gene is said to control one enzyme. The strength of this hypothesis rests with the fact that the data so far obtained are not incompatible with the concept. It does not preclude the possibility of incompatibilities which have not yet been recognized because of difficulties inherent in the isolation and maintenance of mutants which might, for example, require non diffusible nutrilites. As Delbruck[53] points out, the demostration of a one-gene control of several

[46] G. W. Beadle and E. L. Tatum, *Proc. Natl. Acad. Sci. U. S.* **27**, 499 (1941).
[47] N. H. Horowitz, D. Bonner, H. K. Mitchell, E. L. Tatum, and G. W. Beadle, *Am. Naturalist* **79**, 304 (1945).
[48] E. L. Tatum and G. W. Beadle, *Ann. Missouri Botan. Garden* **32**, 125 (1945).
[49] D. Bonner, *Cold Spring Harbor Symposia Quant. Biol.* **11**, 14 (1946).
[50] N. Fries, *Nature* **155**, 757 (1945); **159**, 199 (1947).
[51] F. Moewus, *Ergeb. Biol.* **18**, 287 (1941).
[52] T. M. Sonneborn, *Cold Spring Harbor Symposia Quant. Biol.* **10**, 111 (1942).
[53] M. Delbruck, *in* discussion of D. Bonner[49].

enzymes might necessitate the isolation and maintenance of such mutants, a technically difficult feat. A mutant requiring both isoleucine and valine has been analyzed and shown to be deficient in only one reaction.[54] The double requirement was a reflection of competitive antagonism between the accumulated precursor for the one end product, isoleucine, and the precursor for the other, valine. The possibility that more than one gene can control only one enzyme exists in the isolation of several mutants which appear to be blocked in identical reactions. For example, seven mutants are involved in the ornithine-arginine cycle, which consists of only three known stages. Indeed, each mutant may be involved in an unknown intermediate reaction, but until such reactions are established, this apparent incompatibility still exists. It has been emphasized that an enzyme found to be lost or incapacitated as a result of a given mutation by no means proves that it has been directly derived from a corresponding gene. Further work may show that in some of these cases mutations in other genes may effect the given enzyme. It is too early to conclude that the one-to-one relationship really exists. Additional evidence is necessary.

Whatever the genetic implications, the Neurospora material has presented the biochemist with several practical tools. Thus, the number of different mutations affecting a particular biosynthetic series is a reflection of the minimum number of steps involved. The use of this method in unraveling the mysteries of biosynthetic mechanisms is quite impressive. From this analysis, we have gained information in the biosynthesis of tryptophan,[55,56] arginine,[57] methionine,[58] nicotinic acid,[59] purines and pyrimidines,[60–63] and choline.[64] The specific requiring mutants have been used for the bioassay of pyridoxine,[65] *p*-aminobenzoic acid,[66] choline,[67] inositol,[68] leucine,[69] lysine,[70] adenine,[61] cytidine and uridine[62] (see also review by

[54] D. Bonner, *J. Biol. Chem.* **166**, 545 (1946).
[55] E. L. Tatum and D. Bonner, *J. Biol. Chem.* **151**, 349 (1943).
[56] E. L. Tatum, D. Bonner, and G. W. Beadle, *Arch. Biochem.* **3**, 477 (1944).
[57] A. M. Srb and N. H. Horowitz, *J. Biol. Chem.* **154**, 129 (1944).
[58] N. H. Horowitz, *J. Biol. Chem.* **171**, 255 (1947).
[59] G. W. Beadle, H. K. Mitchell, and J. F. Nyc, *Proc. Natl. Acad. Sci. U. S.* **33**, 155 (1947).
[60] H. S. Loring and J. G. Pierce, *J. Biol. Chem.* **153**, 61 (1944).
[61] H. K. Mitchell and M. B. Houlahan, *Federation Proc.* **5**, 370 (1946).
[62] H. S. Loring, G. L. Ordway, and J. G. Pierce, *J. Biol. Chem.* **176**, 1123 (1948).
[63] H. K. Mitchell, M. B. Houlahan, and J. F. Nyc, *J. Biol. Chem.* **172**, 525 (1948).
[64] N. H. Horowitz, *J. Biol. Chem.* **162**, 413 (1946).
[65] J. L. Stokes, A. Larsen, C. R. Woodward, Jr., and J. W. Foster, *J. Biol. Chem.* **150**, 17 (1943).
[66] E. L. Tatum and G. W. Beadle, *Proc. Natl. Acad. Sci. U. S.* **28**, 234 (1942).
[67] N. H. Horowitz and G. W. Beadle, *J. Biol. Chem.* **150**, 325 (1943).
[68] G. W. Beadle, *J. Biol. Chem.* **156**, 683 (1944).
[69] F. J. Ryan and E. Brand, *J. Biol. Chem.* **154**, 161 (1944).
[70] A. Doermann, *J. Biol. Chem.*, **160**, 95 (1945).

Ryan[71]). The analysis of several mutants has also led to a system whereby the differences of exogenous and endogenous metabolism can be studied.[48]

2. Critique on the Assumed Direct Relationship between Genes and Enzyme Synthesis

At present, only two cases exist of the direct demonstration of an enzymatic process that is lacking in the mutant but present in the wild Neurospora. The pantothenic acid-requiring mutant has been shown by Wagner and Guirard[72] to be unable to couple the two precursors, beta-alanine and pantoyl lactone, to form pantothenic acid. The wild strain could. Mitchell and Lein[73] have shown that whereas the wild type could convert indole to tryptophan in the presence of the proper coenzyme (pyridoxal phosphate), the tryptophan-requiring mutant could not. Later reports showed that neither of these two mutants were indeed lacking in specific enzyme activity. Wagner[73a] found that with variation in temperature conditions, the pantothenicless Neurospora mutant could produce pantothenic acid from pantoyl lactone and beta-alanine. He concludes that the enzyme is not lacking and that the mutant must possess a mechanism which prevents this enzyme from functioning. Gordon and Mitchell[73b] in investigating the inability of the extracts from the tryptophanless mutants to perform the indole-serine conversion to tryptophan found that these extracts could indeed demonstrate such activity when dialyzed, fractionated with ammonium sulfate or when the mycelia were thoroughly washed with distilled water. Since extracts from normal cells, as well as a number of naturally occurring amino acids, could inhibit these activated mutant preparations, the lack of activity *in vivo* may also be due to inhibited processes. These findings serve to emphasize the fact that genetic blocks can be expressed only as a lack of enzymatic ability and not loss of enzyme, *per se*.

Several other cases of direct demonstration of the incapacitation of an enzymatic reaction in mutants as compared with the wild type have been recorded above. In all cases, the interpretation is that the absence of an activity is due to the lack of the enzyme. Its synthesis has not occurred. It must be emphasized at this point that there is no evidence that the failure to perform the particular reaction is definitely a factor of enzyme lack *per se*.

Consideration must be taken of the various factors which might influence optimal enzymatic activities in these systems other than the absence of the

[71] F. J. Ryan, *Federation Proc.* **5**, 366 (1946).
[72] R. P. Wagner and B. M. Guirard, *Proc. Natl. Acad. Sci. U. S.* **34**, 398 (1948).
[73] H. K. Mitchell and J. Lein, *J. Biol. Chem.* **175**, 481 (1948).
[73a] R. P. Wagner, *Proc. Natl. Acad. Sci. U. S.* **35**, 185 (1949).
[73b] M. Gordon and H. R. Mitchell, *Rec. Genetics Soc. Am.* No. 18, 90 (1949).

specific enzyme. The latter may indeed be present, but for various reasons it may be unable to manifest itself.

(1) *The presence of inhibitors* has been suggested as an important consideration in the prevention of the enzymatic process. Inhibitors of melanin-producing enzymes have been found in skin extracts of albino laboratory mammals.[19, 21, 74] The inability of the red-eye Ephestia mutant to transform tryptophan to kynurenine has been associated with an inhibition of this reaction.[33] The inability of the two Neurospora mutants to perform their respective biosynthesis as discussed above may also be considered as possible inhibited reactions.

(2) The difference in activity as a result of *coenzyme lack* has been ruled out in at least one case,[73] but it may be involved as the missing factor in other instances. Particularly related to this problem is the demonstration by Kidder and Dewey[75] of the inability of several protozoa and fungi to synthesize thiamine. This deficiency was associated with the lack of "S factor" which serves as the prosthetic group for the enzyme coupling pyrimidine and thiazole to give thiamine. It is also apparent that the enzymes responsible for the production of the coenzyme may be the ones affected. It becomes necessary, therefore, not to limit the analysis of a synthetic system to one reaction and concluding, *a priori*, therefrom that the inability to perform such a reaction is the reflection of a specific enzyme deficiency.

(3) The availability of a precursor of a blocked reaction depends on the velocities of the alternate reactions involved in its synthesis and efficient utilization. Several reactions may be proceeding at various controlled rates, one of which leads to the precursor. A diversion of velocities by alteration of rate-regulating mechanisms may accelerate another reaction along other channels so that the normal precursor is not formed or formed more slowly. Furthermore, its utilization may be likewise diverted along other paths to form new products or cause an ineffecient rapid breakdown. Caspari[33] has considered these possibilities as an explanation for the nonavailability of kynurenine for the production of eye pigment in insects. Either tryptophan may be diverted through accelerated deamination processes thus preventing kynurenine formation or the kynurenine formed may suffer a shift toward greater velocities in the formation of anthranilic acid, kynurenic acid, and other nonpigment-forming substances (Fig. 2). A Neurospora mutant requiring adenosine accumulates an abnormal purple pigment.[61] This has been explained on the basis of the precursor utilization being diverted from its normal formation of adenosine through inosine to the formation of the abnormal pigment. That the pigment can be formed indicates the presence of a potential system whose rate is normally suppressed in favor of the more economical formation of adenosine. The

[74] B. Ginsburg, *Genetics* **29**, 176 (1944).
[75] G. W. Kidder and V. C. Dewey, *Growth* **6**, 405 (1942).

velocities of these many potential reactions must be under strict control of some rate-regulating mechanism. Barron[76] has suggested that glutathione may act as such a rate-regulating substance. Spiegelman *et al.*[77] have demonstrated the inhibition of rate determining reactions with sodium azide which resulted in uncoupling of anaerobic oxidative phosphorylations with increased utilization of substrate. McElroy[78] and Clifton[79] have recently reviewed the aspects of this subject. At any rate, the observed impaired activity may be a factor of an as yet ill-defined system of velocity balance between several possible reactions. The role of hormones in regulating enzymatic activity will be considered later.

(4) A final consideration may be the formation of an enzyme protein altered in specificity or otherwise inactive. Thus, the mutated gene may not prevent the synthesis of the enzyme but may lead to the production of an impaired form or one of more restricted stability, for example, as regards temperature or pH. An example of the inheritability of the impairment of the enzyme protein was described by us.[80] Pneumococci rendered resistant to atabrine showed impaired dehydrogenase activity resulting from an alteration of the protein moiety of the flavoprotein enzyme. This enzyme could readily be inactivated by temperature and dilution, and reactivated in the presence of high concentration of riboflavin. Riboflavin contents of the susceptible and resistant cells were quantitatively alike. The impairment is due to a loss of affinity between the protein and its coenzyme. In the atabrine-resistant cells the flavoprotein was greatly dissociable, leading to enzyme inactivation or impairment. High concentrations of preformed riboflavin were required to shift the equilibrium from right to left and thus restore the impaired activity. Many other factors are necessary for optimal enzymatic activity and it would be superfluous to list them all in a treatise of this sort.

(5) The mutation may be other than an effect on an enzymatic process as evidenced by abnormal function of excretory mechanisms (alcaptonuria and uric acid excretion). This may be more logically applicable to the higher animals where secretory functions may be impaired. However, a similar analysis may be applied to the lower organisms through alterations in cell permeability or lack of ability to concentrate and/or assimilate required substances.

The influence which the gene exerts on the enzyme is unknown. The relation between gene and observed character must of necessity be rather indirect. How the gene acts still remains undetermined and its unraveling must await further information on protein synthesis and gene replication

[76] E. S. G. Barron, L. Nelson, and M. I. Ardao, *J. Gen. Physiol.* **32**, 179 (1948).
[77] S. Spiegelman, M. D. Kamen, and M. Sussman, *Arch. Biochem.* **18**, 409 (1948).
[78] W. D. McElroy, *Quart. Rev. Biol.* **22**, 25 (1947).
[79] C. E. Clifton, *Advances in Emzymol.* **6**, 269 (1946).
[80] M. G. Sevag and J. S. Gots, *J. Bact.* **56**, 737 (1948).

II. Viruses and Enzymes

The obligate parasitism of viruses is the most fundamental characteristic and property that serves best in distinguishing them from bacteria. Very little is understood concerning mechanisms of invasion and proliferation of viruses in host cells or what part the host plays in their propagation. The synthesis of the virus particles must involve catalysts provided by the virus or host, or both. So far, studies with analytical procedures known in enzymology have failed to indicate the possession by isolated viruses of enzymatic abilities, or at best very restricted ones. One possibility is that the viruses possess partial enzymatic systems that must be complemented and completed by host factors. The final alternative is that viruses are completely devoid of independent metabolism and the enzymes required for their increase in number must be provided by the host cells. Two general approaches to this problem have been made: (1) an analysis of virus particles for enzymatic ability, and (2) a comparison of metabolism of normal host cells with infected cells.

1. Enzyme Activities of Free Virus Particles

In order to determine conclusively whether virus particles can perform enzymatic reactions, pure uncontaminated suspensions must be assured. Those plant viruses which have been crystallized in the form of nucleoproteins have been found to be completely devoid of independent metabolism. However, MacFarlane and Dolby[81] have reported slight phosphatase activity in tobacco mosaic virus and in bushy stunt virus. The primary difficulty encountered in determining whether virus particles have metabolic activities is the inability of obtaining suspensions free from host materials in concentrations sufficiently high to permit study. Vaccinia elementary bodies and bacteriophage have been obtained in comparatively pure form by methods primarily involving differential ultracentrifugation.

a. Vaccinia Virus

Parker and Smythe[82] found no evidence of oxygen uptake or acid production by vaccinia elementary bodies in the presence of glucose, hexose monophosphate or methylene blue. MacFarlane and Salaman[83] using 2:6-dichlorophenolidinophenol in place of methylene blue as a hydrogen acceptor, could demonstrate no dehydrogenase activity by elementary bodies in the presence of glucose, hexose monophosphate, succinate, α-glycerophos-

[81] M. G. MacFarlane and D. E. Dolby, *Brit. J. Exptl. Path.* **21**, 219 (1940).
[82] R. F. Parker and C. V. Smythe, *J. Exptl. Med.* **65**, 109 (1937).
[83] M. G. MacFarlane and M. H. Salaman, *Brit. J. Exptl. Path.* **19**, 184 (1938).

phate, lactate, acetate, formate, malate, glycine, alanine, leucine, and peptone. However, the virus suspensions did exhibit marked activity for both catalase and phosphatase. Since the lack of dehydrogenase activity may have been a factor of coenzyme removal by repeated washings, MacFarlane and Dolby[81] later repeated the experiments for determination of dehydrogenase ability in the presence of the proper coenzymes. Negative results were still obtained for lactate, triosephosphate, hexose monophosphate, and alanine dehydrogenases in the presence of coenzymes I and II, flavin-adenine-dinucleotide and yellow enzyme of Warburg. The preparations were also negative for zymohexase, enolase, alpha- and beta-glucosidases and nucleosidase activities. However, demonstrable activity was exhibited for phosphomono- and diesterases, and ability to hydrolyze ribonucleic acid and adenosine-5-phosphate. The latter was the most actively hydrolyzed in comparison with beta-glycerophosphate and adenosine-3-phosphate.

Hoagland *et al.*[84] confirmed the absence of dehydrogenase activity in the presence of malate, succinate, lactate, and pyruvate and were also in agreement with the previous report that vaccinia elementary bodies possess phosphatase and catalase, as well as lipase, activity. The obvious interpretation of possible host contamination or activity adsorbed from detritus was considered. When lipase and catalase were added to elementary body suspensions, marked adsorption of the enzymes occurred as determined by great increase in activity after adsorption. Thus, the virus can effect an enormous concentration of enzymes out of dilute solutions. This indicates that the demonstrable activity could have come from cell detritus by adsorption which could not be removed by repeated washing. Some selective adsorption does exist since urease could not be adsorbed. Hoagland *et al.*[85] also showed the absence of cytochrome oxidase and any demonstrable cytochrome system.

Purified vaccinia bodies were found to contain copper,[85] flavin-adenine-dinucleotide,[86] and biotin.[87] The copper was present in concentrations of 0.05% of the dry weight of virus. The flavine-adenine-dinucleotide was identified by use of D-amino acid oxidase and it could participate in the oxidative deamination of D-alanine; it was present in concentrations comparable to that found in most cells but less than that found in yeast. The presence of these substances, which can be linked with enzyme and respira-

[84] C. L. Hoagland, S. M. Ward, J. E. Smadel, and T. M. Rivers, *J. Exptl. Med.* **76**, 163 (1942).

[85] C. L. Hoagland, S. M. Ward, J. E. Smadel, and T. M. Rivers, *J. Exptl. Med.* **74**, 69 (1941).

[86] C. L. Hoagland, S. M. Ward, J. E. Smadel and T. M. Rivers, *J. Exptl. Med.* **74**, 133 (1941).

[87] C. L. Hoagland, S. M. Ward, J. E. Smadel, and T. M. Rivers, *Proc. Soc. Exptl. Biol. Med.* **45**, 669 (1940).

tory systems, suggested that vaccinia virus possesses incomplete metabolic systems necessary for its survival but relies in part, or even in the main, upon its intracellular environment for substituents allowing their completion. Thus, the lack of dehydrogenase activity would be expected if only a partial make-up is present. The presence of FAD, however, is no proof that it exists as an integral part of the virus organization and that its function is metabolic. Furthermore, it is difficult to conceive of the existence of only one portion of a system to the exclusion of the many other coenzymes and substances necessary for its metabolic completion.

In general, there is no conclusive evidence that the enzymatic activities recorded are initimately associated with the functions of the virus particles. The observations that vaccinia particles are able to adsorb enzymes from solution suggests that the demonstrated activities were derived from the host cells and associated with readily sedimented components. Schmitt[88] has stated that, in view of their great surface and relatively mild treatment of isolation, it is not surprising that enzymes have been found associated with particulates. Whether the enzyme is an integral part of the particulate, whether it has been adsorbed nonspecifically, and whether it bears certain specific enzymes to the exclusion of other particulates, are questions whose answers must be awaited. It is interesting that the enzymes found are known to be in high concentrations in the host cells, in detritus, and in leucocytes. No enzyme has been found that is not present in normal host cells and that could not be accounted for on the basis of adsorption.

b. Bacterial Viruses (Bacteriophage)

The action of bacterial viruses on their hosts produces lysis and a presumable removal or dilution of bacterial substances by the process of lysis, and subsequent proteolysis. The lyzates are, in effect, phage suspensions of fairly high concentration. Further purification can be accomplished by differential ultracentrifugation. To date, it has not been conclusively demonstrated that bacterial viruses, in lyzates or centrifuged suspensions, have any marked demonstrable enzymic activity of their own.

As early as 1923 Fejgen and Supniewski[89] reported the absence of catalase and reductase activity in dysentery phage lysates. They, however, erroneously concluded that since all living material must possess these enzymes, the bacteriophages were therefore nonliving and nonparticulate. Bronfenbrenner[90] was unable to determine respiration in active (5×10^{10} to 1×10^{15}) Staphylococcus phage filtrates or in substance precipitated from

[88] F. O. Schmitt, *Advances in Protein Chem.* **1**, 25 (1944).
[89] B. Fejgen and J. Supniewski, *Compt. rend. soc. biol.* **89**, 1385 (1923).
[90] J. J. Bronfenbrenner, *Proc. Soc. Exptl. Biol. Med.* **22**, 81 (1924).

these filtrates. Bachman and Wohfield[91] and Wohfield[92] also found coliphage unable to utilize oxygen or evolve CO_2. Though McKinley and Coulter[93] found slight evolution of CO_2 by coliphage, they attributed it to increased growth rate of the bacteria rather than phage activity.

Eaton[94] reported that Staphylococcus cultures lysed by bacteriophage could continue to utilize oxygen and give off CO_2 for some time and the rate of oxygen consumption was greater than could be accounted for by the number of intact host cells present. Though the oxygen uptake may have been due to autoxidation, the CO_2 evolution indicated to him a metabolism of either the phage itself, or some product of the action of the phage in bacteria, or a result of a combination of factors. Wynd and Bronfenbrenner[95] were unable to confirm this on Staphylococcus lyzates with microrespiration techniques sensitive to 0.0002 mg. of oxygen.

The latter workers also found that lysis does not destroy bacterial catalase. Lominski[96] also demonstrated catalase activity in Staphylococcus lyzates and found it proportional to the number of cells that undergo lysis.

Phosphatase activity, using hexosediphosphate as substrate, was demonstrated in centrifuged phage suspensions by Schüller.[97] This activity could not be related to lytic action of the phage since bone and kidney phosphatase had no lytic activity. He was unable to find trypsin, papain, lipase, amylase, maltase, catalase, arginase, and urease activities in the phage suspensions.

From the above presented data it can be seen that no clear-cut evidence of metabolic activity of either purified phage preparations or concentrated lyzates has as yet been demonstrated.

c. Virus Hemagglutination

The possibility that viruses may indeed possess a unique enzyme system has found recent support in the study of the phenomenon of hemagglutination by various viruses. Hirst[98] and McClelland and Hare[99] first demonstrated the ability of influenza virus to bring about the agglutination of erythrocytes of several species, particularly human and chick. This phenomenon has also been demonstrated with Newcastle disease virus,[100]

[91] W. Bachmann and T. Wohlfield, *Zentr. Bakt. Parasitenk. Abt. I., Orig.* **104**, 256 (1927).
[92] T. Wohlfield, *Z. Hyg. Infecktionskrankh.* **108**, 733 (1928).
[93] E. B. McKinley and C. B. Coulter, *Proc. Soc. Exptl. Biol. Med.* **24**, 685 (1927).
[94] M. D. Eaton, Jr., *J. Bact.* **21**, 143 (1931).
[95] F. L. Wynd and J. J. Bronfenbrenner, *J. Bact.* **38**, 659 (1939).
[96] I. Lominski, *Compt. rend. soc. biol.* **116**, 839 (1934).
[97] H. Schüller, *Biochem. Z.* **276**, 254 (1935).
[98] G. K. Hirst, *Science* **94**, 22 (1941).
[99] L. McClelland and R. Hare, *Can. J. Pub. Health* **32**, 530 (1941).
[100] F. M. Burnet, *Australian J. Exptl. Biol. Med. Sci.* **20**, 81 (1942).

fowl plague,[101] mumps,[102] vaccinia,[103] and variola.[104] Hirst[105] postulated the enzymatic nature of this reaction on the basis that the virus can be eluted from the agglutinated cells rendering them inagglutinable by fresh virus, yet the eluted virus is still capable of agglutinating fresh red blood cells. This would imply a substrate site (receptor) on the erythrocyte which can be acted upon by a virus enzyme that changes it to a nonreceptor form. The virus enzyme is then easily dissociated from the complex in unchanged and still active form. Hirst[106] demonstrated similar receptors in ferret lung cells and postulated the enzymatic destruction of these receptors as a necessary preliminary of infection by the virus.

An enzyme essentially similar to the virus enzyme was found to be present in soluble form in culture filtrates of *Vibrio cholerae*.[107, 108] This enzyme is capable of removing receptors from erythrocytes in the same manner as the virus and has thus been designated as the Receptor Destroying Enzyme or RDE. Stone[109] demonstrated a close correspondence between the virus and RDE and concluded that they are essentially similar in nature. RDE has also been shown to be able to remove receptors for influenza virus from mouse lung.[110] Burnet[110a] produced antibody against RDE which inhibited the biological functions of RDE.

If the substrate were available in free form separate from red blood cells, the study of the action of both virus and RDE would be greatly facilitated. De Burgh *et al.*[111] extracted and purified from human erythrocytes, as well as human lung, a substance containing 2.6% nitrogen and 50% polysaccharide which could inhibit hemagglutination by virus. Francis[112] demonstrated the presence in human serum of an inhibitor of the hemagglutination by heated influenza virus. Anderson[113] then showed that this "Francis inhibitor" could be inactivated by the action of RDE as well as by the active viruses of the mumps-influenza group, which suggested that it may be the same substrate as the red cell acceptors. A noncellular alternate

[101] D. Lush, *J. Compt. Path. Therap.* **53**, 157 (1943).
[102] J. H. Levens and J. F. Enders, *Science* **102**, 117 (1945).
[103] F. P. O. Nagler, *Med. J. Australia* **1**, 281 (1942).
[104] E. A. North, *Australian J. Exptl. Biol. Med. Sci.* **22**, 105 (1944).
[105] G. K. Hirst, *J. Exptl. Med.* **76**, 195 (1942).
[106] G. K. Hirst, *J. Exptl. Med.* **78**, 99 (1943).
[107] F. M. Burnet, J. F. McCrea, and J. D. Stone, *Brit. J. Exptl. Path.* **27**, 228 (1946).
[108] F. M. Burnet and J. D. Stone, *Australian J. Exptl. Biol. Med. Sci.* **25**, 227 (1947).
[109] J. D. Stone, *Australian J. Exptl. Biol. Med. Sci.* **25**, 137 (1947).
[110] S. Fazekas de St. Groth, *Australian J. Exptl. Biol. Med. Sci.* **26**, 29; 271 (1948).
[110a] F. M. Burnet, *Australian J. Exptl. Biol. Med. Sci.* **27**, 217 (1949).
[111] P. M. de Burgh, Pen-Chung Yu, C. Howe, and M. Bovarnick, *J. Exptl. Med.* **87**, 1 (1948).
[112] T. Francis, Jr., *J. Exptl. Med.* **85**, 1 (1947).
[113] S. G. Anderson, *Australian J. Exptl. Biol. Med. Sci.* **26**, 347 (1948).

source of substrate was thus made available. Serum mucoprotein and glandular mucins from ovarian cysts as well as the purified blood group O and purified receptor substances were shown to possess the same properties as the "Francis inhibitor."[114-116] Egg white was recently shown to contain a similar inhibitor.[116a, 116b] Thus, the substrate was characterized as a mucopolysaccharide protein complex (mucin) which can be inactivated by heat, proteases, and periodates; which can inhibit hemagglutination by heated virus but can not do so after having been acted on by active virus and RDE; and which is found on the erythrocytes of several species, mouse and ferret lung, cyst fluids, human serum, blood group O substance, egg white, and purified receptor substance. It is probably similar to the polysaccharide from Klebsiella which inhibits mumps hemagglutination[117]; and to apple pectin and the other polysaccharides which Green and Wooley[118] showed could inhibit both hemagglutination and mouse infection by influenza virus. Wooley[119] later purified a water-soluble supposed polysaccharide from human red blood cells which could antagonize the inhibition of hemagglutination by apple pectin. This ability to antagonize could be inactivated by active virus.

Thus, the above data may indicate the existence of a mucinase type enzyme in those viruses which are capable of agglutinating erythrocytes. Since the evidence has been derived from biological rather than chemical tests, the true nature of this system must await further chemical and enzymological analysis. The more direct action of swine influenza virus on the egg-white inhibitor of hemagglutination was studied by Eckert *et al.*[119a] and shown to have a marked effect on reducing the viscosity of semipurified inhibitor substance. Though these reactions appear to be definitely involved in the adsorption mechanisms of viruses, their role in the propagation of the viruses within the host and the injury suffered by the host must be indirect if related at all. Excellent discussions of the general mechanism of hemagglutination have been published by Burnet and his collaborators.[120, 121]

[114] J. F. McCrea, *Australian J. Exptl. Biol. Med. Sci.* **26**, 355 (1948).
[115] F. M. Burnet, *Australian J. Exptl. Biol. Med. Sci.* **26**, 371 (1948).
[116] F. M. Burnet, *Australian J. Exptl. Biol. Med. Sci.* **26**, 389 (1948).
[116a] F. Lanni and J. W. Beard, *Proc. Soc. Exptl. Biol. Med.* **68**, 312, 442 (1948); **17**, 116 (1949).
[116b] P. H. Hardy, Jr. and F. L. Horsfall, Jr., *J. Exptl. Med.* **88**, 463 (1948).
[117] H. S. Ginsberg, W. F. Goebel, and F. L. Horsfall, *Proc. Soc. Exptl. Biol. Med.* **66**, 99 (1947).
[118] R. H. Green and D. W. Wooley, *J. Exptl. Med.* **86**, 55 (1947).
[119] D. W. Wooley, *J. Exptl. Med.* **89**, 11 (1949).
[119a] E. A. Eckert, F. Lanni, D. Beard, and J. W. Beard, *Science* **109**, 463 (1949).
[120] F. M. Burnet, *Lancet* **1**, 7 (1948).
[121] S. G. Anderson, F. M. Burnet, S. Fazekas de St. Groth, F. M. McCrea, and J. D. Stone, *Australian J. Exptl. Biol. Med. Sci.* **26**, 403 (1948).

2. Comparison of Activity of Virus Infected Cells with Normal Host Cells

A promising approach towards an understanding of the enzymatic nature of virus action and its propagation within the host is offered by an examination of normal and infected host cells. If an activity could be demonstrated in infected cells which was greater in magnitude or even peculiar to the infected cells, this could be a reflection of a diversion of normal metabolism of host cells towards an increased activity in one or more systems required for virus synthesis. Conversely, if the infected cell metabolism were markedly diminished, or even absent, this could be interpreted in terms of the nature of virus infection and mechanism of destruction of host cells, or, it might be another manifestation of diversion of activities, such as certain normal metabolisms being diminished in favor of increased abnormal processes necessary for virus propagation.

a. Poliomyelitis and Western Equine Encephalitis (WEE)

Brodie and Wortis[122] studied excised brain and spinal cord of normal monkeys and those experimentally infected with poliomyelitis virus. They could find no difference in RQ or lactic acid content. Racker and Kabat,[123] however, found the anaerobic glycolysis of the brain tissue of mice infected with poliomyelitis to be inhibited 5–50%, though no change in oxygen consumption could be demonstrated. The endogenous dehydrogenase activity was higher in infected tissue, but no difference was evident in the presence of substrates (glucose, lactate and succinate). The phosphatase activity of infected brain was high with nucleic acid and ATP as substrates but not with beta-glycerophosphate. Kabat *et al.*[124] and Kabat[125] added additional evidence of interference with carbohydrate metabolism by demonstrating decreased lactic acid, phosphocreatine and residual organic phosphate content, and ATP increase in infected brain tissue. Wood *et al.*[126] and Utter *et al.*[127] were unable to confirm an interference with anaerobic glycolysis in brains of experimentally infected mice. They criticized the previous findings as inadequate and emphasized the difficulty in finding measurable differences in tissue of which only a small portion of the total cells are damaged. Racker and Krimsky[128, 129] later demonstrated 45–85% inhibition of glucose and hexose monophosphate utilization by infected brain and localized the inhibition in the glucose phosphorylation mecha-

[122] M. Brodie and S. W. Wortis, *Arch. Neurol. Psychiat.* **32**, 1159 (1934).
[123] E. Racker and H. Kabat, *J. Exptl. Med.* **76**, 579 (1942).
[124] H. Kabat, D. Erickson, C. Ekland, and M. Nickle, *Science* **98**, 589 (1943).
[125] H. Kabat, *Science* **99**, 63 (1944).
[126] H. G. Wood, I. I. Rusoff, and J. M. Reiner, *J. Exptl. Med.* **81**, 151 (1945).
[127] M. F. Utter, J. M. Reiner, and H. G. Wood, *J. Exptl. Med.* **82**, 217 (1945).
[128] E. Racker and I. Krimsky, *Federation Proc.* **5**, 252 (1946).
[129] E. Racker and I. Krimsky, *J. Exptl. Med.* **84** 191 (1946).

nisms since hexose diphosphate utilization was not disturbed. The inhibition could also be demonstrated by the *in vitro* addition of virus to normal mouse brain. The inhibition of glycolysis could be prevented by the addition of DPN and glucose. Since both tobacco mosaic and influenza virus could also inhibit normal mouse brain metabolism in like manner, the interpretation of these findings as to the nature of specific virus action becomes difficult. These workers later localized the inhibited site to be at the glyceraldehyde phosphate dehydrogenase step in the anaerobic breakdown of glucose since the crystalline dehydrogenase could restore the inactivation. Ferrous salts and proteolytic enzymes could also cause inhibition which could be relieved in like manner by the crystalline enzyme. It was therefore postulated that inhibition of anaerobic glycolysis was due to the presence of nondialyzable iron in purified virus preparations which activated a proteolytic enzyme (cathepsin III-like) in brain homogenates, which in turn destroyed the most susceptible triosephosphate dehydrogenase enzyme.[129a]

An inhibition (17–82%) of anaerobic glycolysis was also found in embryonic chick tissue 2 to 4 days after infection with western equine encephalitis virus (WEE).[130] Specific immune antiviral serum could protect against this inhibition. Nickle and Kabat[131] compared the inhibition of poliomyelitis and WEE infected mouse brain and found differences from normal oxygen consumption and anaerobic glycolysis as well as differences in the types of inhibition by the two viruses.

b. Respiratory Studies on Other Virus-Host Systems

Bronfenbrenner and Reichert[132] found that emulsions of tissues infected with herpes and rabies viruses had no difference in respiration as compared with normal tissues.

Kempner[133] found demonstrable oxygen consumption and CO_2 evolution in plasma obtained from chickens suffering from fowl plague at the height of disease. Normal plasma could not respire. Irvine-Jones and Schoenthal[134], however, could not confirm this with plasma taken from hog-cholera pigs or measles patients. Neither these nor the normal plasmas from respective hosts were able to show aerobic or anaerobic respiration.

Bauer[135] studied the dehydrogenase activity of brains from mice infected with yellow fever, lymphocytic choriomeningitis, and lymphogranuloma

[129a] E. Racker and I. Krimsky, *J. Exptl. Med.* **85**, 715 (1947); *J. Biol. Chem.* **173**, 519 (1948); I. Krimsky and E. Racker, *ibid.* **179**, 903 (1949).
[130] J. Victor and C. H. Huang, *J. Exptl. Med.* **79**, 129 (1944).
[131] M. Nickle and H. Kabat, *J. Exptl. Med.* **80**, 247 (1944).
[132] J. J. Bronfenbrenner and P. Reichert, *Proc. Soc. Exptl. Biol. Med.* **24**, 176 (1926).
[133] W. Kempner, *Klin. Wochschr.* **6**(2), 2386 (1927).
[134] E. Irvine-Jones and L. Schoenthal. *Proc. Soc. Exptl. Biol. Med.* **27**, 163 (1929).
[135] D. J. Bauer, *Brit. J. Exptl. Path.* **28**, 440 (1947).

inguinale. These were found to be significantly higher than normal brain tissue in xanthine oxidase and pyruvic dehydrogenase. Activities in succinate, lactate, alpha-glycerophosphate and malate showed no difference. He later infected chick embryos with yellow fever and followed the development of xanthine oxidase in membrane and embryo.[136] The infected embryos were nearly always higher than normal with increase noticeable on the 13th day. From 13th to 18th days, activity fell back to normal due to lessening of active proliferation of the virus. The additional activity was suggested to be an extra synthesis of enzyme required for the virus metabolism.

c. Enzyme Activities of Tumors Resulting from Virus Action

Barron[137] found succinic dehydrogenase to be lacking or inhibited in tumor tissue of Rous chicken sarcoma and infectious myxoma of rabbits. Whereas normal tissue showed endogenous activity which was increased by the addition of succinate, the tumors produced by the viruses had a markedly reduced, or no, endogenous activity which was unaffected by succinate addition. Experiments with herpetic encephalitis (brain), rabbit virus III (testicle), neurovaccine Levaditi (testicle) and fowl pox (fowl eye and skin) were inconclusive because of high endogenous activity. A trend towards partial inhibition was, however, manifested. Rabbit myxoma also showed low respiration in glucose, and high aerobic and anerobic glycolysis with a U ($Q_{CO_2}^{N_2} - 2QO_2$) value (Pasteur reaction) of $+1.5$. Warburg[138] has characterized tumor growth with marked aerobic and anaerobic glycolytic activity and low respiration in glucose whereas the normal tissues (see Murphy and Hawkins[139] for exceptions) have high respiration, slight anaerobic, and no aerobic glycolysis (Pasteur effect). Thus, rabbit myxoma, an overgrowth produced by a virus, behaves in a similar manner. Crabtree[140] showed that all tissues characterized by active epithelial hyperplasia produced by viruses (fowl pox, vaccinia in chicks, and human warts) correspond to the characteristics of malignant tumors described by Warburg. Where there was no hyperplasia, such as vaccinia in rabbits and rabies in guinea-pig brain, the activity corresponded to that of the normal noninfected tissue. The Rous chicken sarcoma was found to be variable. Crabtree concluded that the characteristics of malignant tumor found by Warburg are not specific for malignant tissue but are a common feature of pathological overgrowths.

[136] D. J. Bauer, *Nature* **161**, 852 (1948).
[137] E. S. G. Barron, *J. Exptl. Med.* **55**, 829 (1932).
[138] O. Warburg, *J. Cancer Research* **9**, 148 (1925).
[139] J. B. Murphy and J. A. Hawkins, *J. Gen. Physiol.* **8**, 115 (1925–1928).
[140] H. G. Crabtree, *Biochem. J.* **22**, 1289 (1928).

d. The Action of Bacteriophage on the Enzymatic Activities of the Host Cells

The experiments carried out with animal viruses are in the main rendered unreliable by the many uncontrolled variables inherent in such analyses. In all cases we find a rather crude reproducibility of results, which are obviously reflections of uncontrollable biological flux and variations. At present, it is impossible to determine or control the proportion of cells which are infected. If only a small proportion of the total cells are affected by the presence of the virus, their metabolism would be lost in the overall activity of the noninfected portion. Furthermore, since inflammatory processes are excited by the presence of the virus, the high enzymatic activity of leucocytes and other inflammatory proliferations would render the results uninterpretable. Additional difficulties are presented with the introduction of impurities, e.g., proteolytic enzymes.

In the bacterial viruses, a system is available where most of these objections do not exist. The number of cells infected can be accurately controlled; inflammatory variables are absent; and the bacteria can be easily purified for enzymatic studies. Surprisingly, however, little has been done in analyzing normal and infected bacteria for differences in metabolism.

Cohen and Anderson[141] found that there is no difference between the normal *E. coli* and those infected with coliphage T_2 and T_4 as to rate of oxygen uptake and RQ. Cohen[142] discusses the enzymatic nature of the bacterial viruses in a recent review. In *E. coli* infected with phage T_2 there is increased production of desoxyribonucleic acid, which parallels the increase in the number of phage particles (Cohen[143]).

Monod and Wollman[144] demonstrated the inability of infected *E. coli* organisms to produce adaptive enzymes for the utilization of lactose. Both the normal and phage infected cells could utilize glucose but only the normal cells were able to utilize lactose. However, when the organisms were previously grown in lactose, utilization occurred regardless of the phage infection.

III. The Relation of Hormones to Enzymes

Hormones are chemical substances manufactured by specialized body cells in the endocrine glands. These glands, as chemical laboratories, are supplied by the blood stream with raw materials that are converted into hormones. The finished, highly potent products are returned to the blood stream and are distributed to every living cell in the body to perform specialized physiological functions. These glands are situated in various parts of the body.

[141] S. S. Cohen and T. F. Anderson, *J. Exptl. Med.* **84**, 511 (1946).
[142] S. S. Cohen, *Bact. Revs.* **13**, 1 (1949).
[143] S. S. Cohen, *J. Biol. Chem.* **174**, 281 (1948).
[144] J. Monod and E. Wollman, *Ann. inst. Pasteur* **73**, 937 (1947).

Generally speaking, hormones regulate the rate or end point of biochemical or physiological reactions. Small amounts of hormones reaching all parts of the body, influence the functions of organs in the form of growth and energy metabolism. As regulators of growth and energy metabolism, trace amounts of hormones are adequate for exercising their functions. Enzymes mediate specific chemical reactions. Hormones may act through biocatalysts and influence a sequence or chain of chemical reactions by acting on some key reactions in the system. However, within recent years, investigations on hormonal problems have shown a close interrelationship between the hormonal function and enzyme activities of certain organisms. Enzymes are proteins with or without a prosthetic or a coenzyme group. On the other hand, nonprotein substances such as epinephrine (adrenaline), derived from the adrenal medulla, thyroxine, derived from the thyroid gland, and steroids derived from the ovarium and corpus luteum, testicle, and adrenal cortex exercise specific hormonal functions. The answers to the question of whether or not these factors exercise their function as chemical individuals *per se* or in combination with specific proteins, does not appear as yet to be conclusive. However, at least, thyroxine has been shown to be a component of thyroglobulin. Even in this case, the function of thyroxine may involve the participation of supplementary factors other than the specific thyroid globulin. For example, Rossiter[145] found that, in the presence of glucose, pyruvate, or succinate, brain brei from thyroid and vitamin B_1 treated rats has a higher oxygen uptake than brei from controls that received vitamin B_1 only. *In vitro* addition of thyroglobulin causes an increase in oxygen uptake of brei of rat brain with both glucose and pyruvate. *In vitro* addition of thyroxine does not cause any increase under these conditions.

The field is young and the picture to be drawn from the hormone-enzyme relationship is as yet incomplete. Under the circumstances, one can only sketch briefly the possible effects of hormones on the various overall enzyme activities of organs.

1. Insulin

Insulin, isolated by Banting and Best in 1922, is dramatically helpful in diabetes, somehow aiding in carbohydrate oxidation as well as glycogen formation. The disappearance of ketosis may be secondary.

a. Amino Acid Metabolism

Bach and Holmes[146] reported that in *in vitro* experiments with sliced liver insulin inhibits the transformation of certain amino acids into carbohydrate and this inhibition is accompanied by a reduction in urea forma-

[145] R. J. Rossiter, *J. Endocrinol.* **2**, 165 (1940).
[146] S. J. Bach and E. G. Holmes, *Biochem. J.* **31**, 89 (1937).

tion. Stadie *et al.*[147] found that insulin inhibits the deamination of D-amino acids by liver slices, though the L-amino acids were not affected, indicating a relationship between insulin action and gluconeogenesis. The fact that liver slices from diabetic cats produce ketones at a higher rate than those from the normal animal, and that there is an inhibition of the formation of ketone bodies by insulin in the presence of fructose, fumarate, and lactate by diabetic liver slices has lent support to the view (Stadie *et al.*[148]) that insulin inhibits the formation of ketone bodies.

b. Hexokinase Activity

In *in vitro* studies, Price *et al.*,[149, 150] Cori[150a], and Colowick *et al.*[150b] reported that in muscle extracts from rats made diabetic with alloxan, the inhibition of the hexokinase reaction is abolished by insulin. A similar inhibition can be produced by addition of anterior pituitary extract to muscle extracts from normals. Here again insulin abolishes the inhibition. The view is expressed that the inhibition is due to the presence of an inhibitory substance which is counteracted by insulin. Certain cortical extracts which alone have no effect on hexokinase in normal extracts, greatly intensify the inhibitory effect of added or previously injected anterior pituitary extract. With muscle extracts from diabetic rats, cortical extract alone often produces a marked and prolonged inhibition. This inhibition likewise is abolished by insulin. Insulin had no effect on the hexokinase reaction. The conversion of glycogen to lactic acid in muscle extracts was not inhibited by the pituitary. Repeating the experiments of the above investigators, Broh-Kahn and Mirsky[151] obtained fluctuating action by pituitary extracts. They extracted a substance from spleen which inhibited hexokinase, and the reversal by insulin of the inhibition was most inconstant. They also found that the muscle extracts from rats rendered diabetic by alloxan displayed normal hexokinase activity which was not affected by insulin. On the basis of their results they concluded that the disorders of glucose metabolism are not necessarily due to an inhibition of hexokinase activity. Stadie and Haugaard[152] studied the extracts of muscle from normal rats and from rats 3 to 20 days after alloxanization for hexokinase activity. Adrenal cortical extract with or without insulin did not show a significant effect on muscle extracts from either normal or alloxanized rats. The diabetic state induced by alloxan was shown not to alter the rate of hexokinase

[147] W. C. Stadie, F. D. W. Lukens, and J. A. Zapp, *J. Biol. Chem.* **132**, 393 (1940).
[148] W. C. Stadie, J. A. Zapp, and F. D. W. Lukens, *J. Biol. Chem.* **132**, 423 (1940).
[149] W. H. Price, C. F. Cori, and S. P. Colowick, *J. Biol. Chem.* **160**, 633 (1945).
[150] W. H. Price, M. W. Slein, S. P. Colowick, and G. T. Cori, *Federation Proc.* **5**, 150 (1946).
[150a] C. F. Cori, *Harvey Lectures* **41**, 253 (1945–46).
[150b] S. P. Colowick, G. T. Cori, and M. W. Slein, *J. Biol. Chem.* **168**, 583 (1947).
[151] R. H. Broh-Kahn and I. A. Mirsky, *Science* **106**, 148 (1947).
[152] W. C. Stadie and N. Haugaard, *J. Biol. Chem.* **177**, 311 (1949).

reaction when compared to that of the control groups of rats. No initial slow phase of hexokinase reaction was observed in extracts of muscle from normal rats, alloxanized rats, or in extracts of kidney from normal or alloxanized rats.

In a later study, Stadie *et al.*[152a] reported the demonstration of highly significant increases in the synthesis of glycogen over the controls in a medium containing glucose and isolated rat hemidiaphragm which had been equilibrated in a phosphate-saline medium with 0.1 unit insulin per ml. for 1 minute (optimal period). After equilibration with insulin, washing in 25 ml. phosphate-saline for a few times and washing for 60 minutes did not appreciably eliminate the insulin effect. These findings indicated to them that insulin had firmly combined with the structural unit of muscle cells of the diaphragm, as a first step in the action of insulin on the mechanism of intact tissue.

It was reported[152b] that the metabolic state induced by injection of alloxan in the rat diminishes the ability of the diaphragm to combine with insulin. Adrenalectomy and hypophysectomy had no effect on this reaction. A crystalline growth hormone preparation (Armour and Co.) or crude anterior pituitary extract, which was injected 20 hours previously, completely prevented the combination of insulin and diaphragm. Crude pituitary extracts *in vitro* impaired the ability of the normal rat diaphragm to combine with insulin. An effect *in vitro* of highly purified growth hormone preparations could not be demonstrated; a rat diaphragm equilibrated for 5 minutes in a solution containing 0.5 mg. crystalline growth hormone per ml. failed to have an effect on the subsequent response of the diaphragm to insulin.

c. Diphosphothiamine

Foa *et al.*[153] reported that injection of insulin into dogs is followed by a temporary rise in the amount of diphosphothiamine in blood which was associated with a decrease in the amount of inorganic phosphate. There was a greater increase in diphosphothiamine when thiamine is injected after the administration of insulin. The administration of insulin is followed also by an increase in the adenosine-triphosphate of the liver, which serves as phosphate donor for thiamine. The injection of thiamine hydrochloride into depancreatized hyperglycemic dogs is not followed by significant changes in the blood concentration of diphosphothiamine, glucose, and inorganic phosphate.

[152a] W. C. Stadie, N. Haugaard, J. B. Marsh, and A. G. Hills, *Am. J. Med. Sci.* **218**, 265 (1949).

[152b] W. C. Stadie, N. Haugaard, A. G. Hills, and J. B. Marsh, *Am. J. Med. Sci.* **218**, 275 (1949).

[153] P. P. Foa, J. A. Smith, and H. R. Weinstein, *Arch. Biochem.* **13**, 449 (1947).

d. Formation of Fat from Glucose

Stetten and Klein[154] found that in rats and rabbits, the rate of fatty acid formation from the high carbohydrate diet is far below normal in the diabetic animal, and that the rate of hepatic lipogenesis was tremendously increased by administration of insulin. Fawcett[155] reported that tissues of rats injected with insulin contain more lipase in brown fat tissue. Heretofore lipase has not been reported in brown fat tissue.

e. Phosphatases

Cantor *et al.*[156] found that 4 days after alloxan administration, serum alkaline phosphatase activities were double the normal value. Drabkin and Marsh[157] also reported that the well-established alloxan-diabetic state in rats is associated with increased activities of both acid and alkaline liver phosphatase. Treated effectively with insulin, there was a return toward normal levels of liver phosphatase activity, suggesting that phosphatases have a significant role in the diabetic state. These observations show that the maximum increase in the activities of the serum alkaline phosphatases and liver acid and alkaline phosphatases comes about after the 4th day of alloxan administration. (For a review on the subject see Drabkin.[157a])

2. THYROXINE

a. Phosphatases

Ponz[158] reported that subcutaneous injection of thyroxine (0.5 mg. daily for 3 to 4 days) into rats causes an increase of alkaline phosphatase activity in the small intestine. According to Kochakian and Bartlett.[159] however, animals injected with DL-thyroxine showed a decrease in "alkaline" (pH 9.8) phosphatase. In all the experiments there was no change in the activities of arginase and "acid" (pH 5.4) phosphatase of the liver or any of the enzymes of the kidney. Williams and Watson[160] had previously reported that the administration of thyroxine or parathyroid extract causes a drop in the phosphatase content of bone. Wood and Ross[161] found that like other proteins, such as egg and serum albumin, parathyroid hormone accelerates the liberation of phosphate by kidney phosphatases. It has no peculiar effect, even in a concentration of 75 units/ml. of digestion solution, which can be attributed to its function as a hormone.

[154] D. Stetten, Jr. and B. V. Klein, *J. Biol. Chem.* **159**, 593 (1945); **162**, 377 (1946).
[155] D. W. Fawcett, *Science* **105**, 123 (1947).
[156] M. M. Cantor, J. Tuba and P. A. Capsey, *Science* **105**, 476 (1947).
[157] D. L. Drabkin and J. B. Marsh, *J. Biol. Chem.* **171**, 455 (1947).
[157a] D. L. Drabkin, *Proc. Am. Diabetes Assoc.* **8**, 3 (1948).
[158] F. Ponz, *Rev. Espan. Fisiol.* **1**, 173 (1945).
[159] C. D. Kochakian and Mary N. Bartlett, *J. Biol. Chem.* **176**, 243 (1948).
[160] H. L. Williams and E. M. Watson, *Endocrinology* **29**, 250, 258 (1941).
[161] T. R. Wood and W. F. Ross, *J. Am. Chem. Soc.* **64**, 2759 (1942).

b. *Cytochrome c*

Tissières[162] reported that cytochrome c content of the muscle of the hind legs of the rat is reduced after thyroidectomy or treatment with methylthiouracil. (Thiouracil interferes with the production of thyroxine.) Treatment of the thyroidectomized or normal rats with thyroxine raised the cytochrome c content of the muscle. Thyroxine treatment of normal rats lowered the weight of the thyroid and raised that of the adrenal; treatment with methylthiouracil caused the reverse effect of greater magnitude. Tissières[162a] later found that the prolonged administration of thyroxine caused an increase in oxidation reduction enzymes, particularly cytochrome c, in various tissues of the male rats. Oral ingestion of dinitrophenol or desoxycorticosterone acetate, or the subcutaneous injection of testosterone had no effect on the cytochrome c content of the tissues. At about the same time Drabkin[163] reported that after thyroidectomy or thiouracil administration there was a striking reduction in total body cytochrome c (in all tissues examined), although the changes were of greater magnitude in skeletal muscle than in other tissues. The administration of thyroxine caused increases in cytochrome c in all tissues. These observations suggest that thyroid hormone may act through its effect on cytochrome c content in tissues. Tipton[164] has reported that the activity of succinic oxidase and cytochrome oxidase increased significantly in the liver tissue of rats fed desiccated thyroid substance. After adrenalectomy these enzyme systems decreased in activity. The increase in enzyme activity after thyroid feeding is not so great if the hyperthyroid rats are adrenalectomized during the thyroid feeding.

That there appears to be a direct relationship between the activity of the rat or guinea pig thyroid and oxidase content of the tissue is demonstrated by Mainini[165] as measured by the oxidation of *p*-phenylenediamine. This activity was increased by administration of thyrotropin or implantation of hypophysis. Decreased activity followed hypophysectomy.

c. *Creatine and D-Amino Acid Metabolism*

Treatment with thyroxine subcutaneously causes a lowering of muscle creatine and phosphocreatine in rabbits (Wang[166]). Thyroidectomy causes no increase of creatine but increase of phosphocreatine occurs. Thyrotoxicosis causes temporary creatinuria. These data show that thyroid alone

[162] A. Tissières, *Arch. intern. Physiol.* **54**, 305 (1946).
[162a] A. Tissières, *Arch. intern. Physiol.* **55**, 252 (1948).
[163] D. L. Drabkin, *Federation Proc.* **7**, 483 (1948).
[164] S. R. Tipton, M. J. Leath, I. H. Tipton and W. L. Nixon *Am. J. Physiol.* **145**, 693 (1946).
[165] C. Gallini Mainini, *Rev. Soc. Argentina Biol.* **19**, 205 (1943).
[166] E. Wang, *Acta Med. Scand., Suppl.* **169**, 81 pp. (1946).

exerts a direct influence upon creatine metabolism, since adrenalectomy and hypophysectomy were without any effect.

Klein[167] reported a decrease in the content of D-amino acid oxidase in the tissues of thyroidectomized rats, and an increase in content in the tissues of animals maintained on an adequate diet supplemented with thyroid tissue. He did not find an association with a change in flavine-adenine-dinucleotide of the enzyme, but, probably, with a concentration of the protein moiety.

d. Vitamin A

The conversion of carotene to Vitamin A through the influence of thyroid hormone is reported by Drill and Truant.[168] Normal rats could be maintained with supplements of either Vitamin A or carotene. However, thyroidectomized rats could not be maintained with carotene, as exhibited by ocular changes and weight-loss, as well as increased mortality.

3. STEROID HORMONES

One of the principal functions of the hormones of the adrenal cortex is to influence the rate of gluconeogenesis from protein. On the other hand, many of the androgenic steroids stimulate protein anabolism. Studying the mobilization of protein, Berman *et al.*[169] reported that adrenalectomy inhibits the deposition of protein after partial hepatectomy and prevents the increase in fat and water content of the regenerating liver fragment. Friedgood *et al.*[170] studied the protein regeneration of rats adrenalectomized at the time of hepatectomy. Adrenalectomy reduced the amount of liver protein regeneration in rats both protein fed and protein depleted before operation. Rats, protein depleted for 2 weeks and subjected to the double operation, were given daily injections of desoxycorticosterone acetate, 11-dehydrocorticosterone acetate and 11-dehydro-17-hydroxycorticosterone (acetate) during the 2 week postoperative period. All stimulated hepatic protein metabolism and caused the same or greater regeneration of new liver protein than occurred in animals with intact adrenals. Desoxycorticosterone caused the greatest increment. Cowie and Folley[171] reported that there was a marked inhibition of milk production in the adrenalectomized rats. The lactation could, in part, be maintained with desoxycorticosterone acetate. According to Tipton,[172] untreated adrenalectomized rats showed decreased cytochrome oxidase activity in heart, kidney, and liver, and decreased cytochrome c concentration in kidney and liver, but not in heart

[167] J. R. Klein, *J. Biol. Chem.* **128**, 659; **131**, 139 (1939).
[168] V. A. Drill and A. P. Truant, *Endocrinology* **40**, 259 (1947).
[169] D. Berman, M. Sylvester, E. C. Hay, and H. Selye, *Endocrinology* **41**, 258 (1947).
[170] C. E. Friedgood, H. M. Vars, and J. W. Zerbe, *Federation Proc.* **8**, 200 (1949).
[171] A T. Cowie and S. J. Folley, *J. Endocrinol.* **5**, 14, 24, (1947).
[172] S. R. Tipton, *Endocrinology* **34**, 181 (1944).

tissue. Total liver and kidney cytochrome oxidase was also decreased. Losses were prevented by adrenal extract.

a. Phosphatases

Studies have shown a relationship between the action of the adrenal hormones and the enzyme activities of certain organs. Williams and Watson[160] reported that adrenal cortical extract and corticosterone cause a decrease in the phosphatase content of rats' femurs while desoxycorticosterone acetate produced an increase.

Kutschen and Wüst[173] had previously reported that phosphatase activity in the kidney and small intestine of adrenalectomized guinea pigs was about 50 and 30%, respectively, of that of the normal animal. Desoxycorticosterone restored the activity. Folley and Greenbaum[174] reported that there is a drop in alkaline phosphatase activity of the kidney of adrenalectomized rats, and desoxycorticosterone acetate restores it to normal level.

In a series of studies, working with extracts and crystalline hormones, Kochakian and his associates arrived at a definite conclusion that increase in the alkaline phosphatase of liver is a property of the C-11 steroid hormones of the adrenal cortex. The increase in enzyme activity did not parallel the degree of gluconeogenesis. Adrenalectomy slightly increased the alkaline phosphatase of the liver of young adult male rats. Adrenal cortical aqueous extract on the fifth postoperative day greatly increased this enzyme. Adrenalectomy resulted on the fifth postoperative day in a small decrease in the "alkaline" phosphatase of the kidney. Testosterone propionate produced marked increase in this enzyme in adrenalectomized animals. None of the above treatments produced a significant change in the "acid" phosphatase of either liver or the kidney. In a comparative study, Kochakian and Bartlett[159] found that aqueous (beef) adrenal cortical extract, lipoextract (hog adrenals), and 11-dehydrocorticosterone acetate produced very marked increases in the "alkaline" phosphatase of the liver of fasted rats when injected eight times at hourly intervals. The increase in enzyme activity did not parallel the degree of glycogenesis. Thyroxine produced a marked depletion of liver glycogen and a decrease in the "alkaline" phosphatase. Epinephrine produced a tremendous deposition of liver glycogen but did not affect the activity of the enzyme. Kochakian,[159, 175], Kochakian and Fox[175a], and Kochakian *et al.*[176]; Vail and Kochakian[177] found that

[173] W. Kutscher and H. Wüst, *Z. physiol. Chem.* **273**, 235 (1942).

[174] S. J. Folley and A. L. Greenbaum, *Biochem. J.* **40**, 46 (1946).

[175] C. D. Kochakian, *J. Biol. Chem.* **161**, 115 (1945); *Am. J. Physiol.* **145**, 118 (1945); *Recent Progress in Hormone Research* **1**, 177 (1947).

[175a] C. D. Kochakian and R. P. Fox, *J. Biol. Chem.* **153**, 669 (1944).

[176] C. D. Kochakian, Mary N. Bartlett, and J. Gongora, *Am. J. Physiol.* **153**, 210 (1948).

[177] Virginia N. Vail and C. D. Kochakian, *Am. J. Physiol.* **150**, 580 (1947).

certain androgens decrease the "alkaline" (pH 9.8) phosphatase both in total amount and per gram of tissue and increases the "acid" (pH 5.4) phosphatase of the kidneys of normal and castrated mice. All of the steroids that increase the size of the kidney decrease the "alkaline" phosphatase but increase the "acid" phosphatase. In both instances the changes were related to the change in kidney size. The enzymes of the liver and intestine were not significantly changed as a result of testosterone propionate treatment or castration.

According to Atkinson,[178] in the untreated castrated mouse, large amounts of alkaline phosphate are present in longitudinal muscles of uterus and small amounts in the circular muscles. Occasionally small amounts are found in uterine glands and epithelium. Injection of estrogen was followed by marked increase of phosphatase in uterine glands, epithelium, and circular muscles. Progesterone and testosterone do not produce this effect. Progesterone given concurrently with estrogen does not alter enzyme response to estrogen.

Verzar and Montigel[179] found that minced muscle from adrenalectomized animals shows lowered rate of phosphorylation of glycogen; desoxycorticosterone specifically restored it to normal activity. Determining the kinetics of phosphorolysis by hashed muscle of normal and adrenalectomized animals, Montigel[180] reported that desoxycorticosterone is an important component of the enzyme system that phosphorylates glycogen in muscle.

b. Arginase

According to Kochakian[175] the liver of the mouse contains the greatest amount of arginase. The other tissues contain relatively little or no enzyme. The arginase activity of the liver and intestines are not affected either by castration or steroid stimulation. The enzyme content of kidney, on the other hand, undergoes marked changes especially after stimulation by certain steroid hormones. The increase after castration is due to decrease in kidney mass. Testosterone, testosterone propionate, androstanol-17(a)-one-3, and androstanediol-3(a),7(a) decrease the arginase activity at the lower dose levels. When the dose is increased so that the kidney mass increases to greater than normal size, there is an increase in arginase activity, which continues to increase even though there is no further significant increase in kidney size. The presence of a 17-methyl group in the very active steroids, e.g., 17-methyltestosterone and 17-methylandrostanediol-3(a),17(a), causes an immediate increase in arginase activity. On the other hand, the less active steroids bring about a prolonged decrease in arginase activity.

Kochakian and Bartlett[159] report that their data provide evidence that

[178] W. B. Atkinson, and H. Elftman *Proc. Soc. Exptl. Biol. Med.* **62**, 148 (1946).
[179] F. Verzar and C. Montigel, *Helv. Chim. Acta* **25**, 22 (1942); *Nature*, **149**, 49 (1942).
[180] C. Montigel, *Helv. Chim. Acta* **26**, 883 (1943).

a change in liver arginase level is not essential for glyconeogenesis from protein under the stimulation of the C^{11} steroids. Furthermore, intense glycosuria and glyconeogenesis during alloxan diabetes are not accompanied by any change in arginase activity of the rat liver. The above observations represent a contradiction of the report by Fraenkel-Conrat *et al.*[181] that steroid hormones administered subcutaneously or orally to immature female rats after hypophysectomy or adrenalectomy markedly increase the arginase activity of the homogenized liver. According to Folley and Greenbaum,[182] liver, kidney, and mammary gland arginase levels of rats, adrenalectomized on the 4th day of lactation and autopsied on the 17th day, were significantly lower than those of pair-fed sham-operated lactating rats. Arginase per gram moist tissue was reduced most in the mammary gland (one seventh) and least in the kidney (to one half). Liver contains 9 times the amount in mammary tissue and 28 times the amount in kidney. These ratios are much the same in intact lactating rats on a reduced food intake, but in adrenalectomized rats the activity of the mammary gland relative to liver falls slightly below that of the kidney.

c. Cholinesterase

According to Mihailesco and Radulesco[183] estrone in concentrations greater than 25 units per ml. inhibits cholinesterase, thus allowing acetylcholine to be stored and improving capillary circulation. On the other hand, Berkhäuser[184] reported that a glycerol solution of estradiol dipropionate has no effect on cholinesterase activity of rat liver *in vitro*. *In vivo* it increases the cholinesterase content of liver secondary to increase of acetylcholine content. Sawyer and Everett[185] and Everett and Sawyer[186] found that serum cholinesterase in rats parallels the estrogen level. Castration in the female resulted in a decline while in the male a gradual rise ensued; estradiol produces an elevation while testosterone causes a decrease in activity. Progesterone showed no effect.

d. Estrinase

Bergstrom *et al.*[187] reported that potato phenol oxidase inactivates estradiol, which parallels exactly the capacity to oxidize *p*-cresol. Zondek and

[181] H. Fraenkel-Conrat, M. E. Simpson, and H. M. Evans, *Am. J. Physiol.* **138**, 439 (1943).

[182] S. J. Folley and A. L. Greenbaum, *Biochem. J.* **43**, 581 (1948).

[183] V. V. Mihailesco and M. Radulesco, *Bull. acad. méd. Roumanie* **18**, No. 1/3, 56 (1946).

[184] H. Berkhäuser, *Verhandl. Ver. schweiz. Physiol.* **18**, 19 (1941).

[185] C. H. Sawyer and J. W. Everett, *Endocrinology* **39**, 307 (1946).

[186] J. W. Everett and C. H. Sawyer, *Endocrinology* **39**, 323 (1945).

[187] S. Bergstrom, H. Theorell and A. Westman, *Arkiv. Kemi Mineral. Geol.* **19B**, 7 pp. (1944).

Sklow[188] reported that liver, hyacinth bulbs, potatoes, cauliflower, beet roots, and bran contain an enzyme, estrinase, which inactivates estrone. Estrinase resembles tyrosinase but is not identical with it since cauliflower contains estrinase but not tyrosinase and meal worm larvae contain tyrosinase but not estrinase.

According to Zondek and Finkelstein[189] excised livers of rats with vitamin B deficiency are devoid of the capacity to inactivate estrone *in vitro*. This ability still remains at a high level in the intact rat. It has been shown that monkey liver is relatively inefficient in the inactivation of estrogen *in vivo* (Hooker *et al.*[190]).

e. Succinoxidase

McShan *et al.*[191] reported that the phenolic group of various estrogens inhibited succinoxidase of rat tissues. This reaction left the biological activity of estrogens intact. Blocking of the phenolic group of androgens by converting them into sulfuric acid esters removed the inactivating action on succinoxidase.

Progressive increase in succinic dehydrogenase activity of the corpus luteum of pregnancy in rats to the eleventh day of pregnancy has been reported by Meyer *et al.*[192] The increase is believed to be associated with increasing production of progesterone and possibly other ketosteroids. According to McShan *et al.*[193] the malic dehydrogenase activity of rat corpora lutea of pregnancy increases through the first half of pregnancy, reaching a high level at 11 days, then decreases through the second half of pregnancy and through lactation. The activity of this enzyme increases during lactation, reaching a maximum level at the 20th day. At this time there is also a slight increase in activity of the ovarian residue. The cytochrome oxidase activity of corpora lutea of pregnancy maintains a high value at 7 and 15 days of pregnancy and decreases to a value of the 20th day of lactation. The cytochrome oxidase activity of corpora of lactation increases during lactation and reaches a high level on the 20th day.

Wight and Burk[194] reported that diethylstilbesterol inhibits the succinoxidase activity of mitochondrial elements obtained from tumor homogenates. This inhibition was markedly reduced or entirely eliminated by the simultaneous addition of progesterone or testosterone.

[188] B. Zondek and J. Sklow, *Proc. Soc. Exptl. Biol. Med.* **49**, 629 (1942).
[189] B. Zondek and M. Finkelstein, *Science* **105**, 259 (1947).
[190] C. W. Hooker, V. A. Drill and C. A. Pfeiffer, *Proc. Soc. Exptl. Biol. Med.* **65**, 192 (1947).
[191] W. H. McShan, R. K. Meyer, and W. F. Erway, *Arch. Biochem.* **15**, 99 (1947).
[192] R. K. Meyer, S. W. Soukup, W. H. McShan, and C. Biddulph, *Endocrinology* **41**, 35 (1947).
[193] W. H. McShan, W. F. Erway, and R. K. Meyer, *Arch. Biochem.* **16**, 379 (1948).
[194] K. Wight and D. Burk, *Federation Proc.* **8**, 265 (1949).

4. Growth Hormones

An important function of the growth hormone derived from the pituitary gland is to retain nitrogen, since true growth results from the accumulation of proteins. According to Li[195] the injections of the growth hormone induce a significant lowering of the urinary nitrogen within 24 hours. Bennett and Li[197] reported that alloxan diabetic rats (which still have some insulin) also retain nitrogen after growth hormone treatment. The growth hormone has been found to increase the alkaline phosphatase content in the plasma of hypophysectomized rats (Li *et al.*[198]). It causes a rise in the plasma inorganic phosphorus level of such animals (Li[195]).

According to Li,[195] adrenocorticotropic hormone activity is found in the nonprotein nitrogen of the peptic hydrolyzate of adrenocorticotropic hormone. It is dialyzable through collodion membranes. It is composed of about seven amino acid residues in peptide form. This hormone is reported to exhibit a retardation of the somatic growth of normal rats (Evans *et al.*[199]; Becks *et al.*[200]) and is considered a specific growth inhibiting substance. It increases urinary nitrogen excretion in normal rats (Gordon *et al.*[201]) and reduces the alkaline phosphatase content in the plasma of both hypophysectomized and normal rats (Li *et al.*[198]). The latter effect is neutralized by growth hormone injections. Ingle *et al.*[202] have shown that adrenocorticotropic hormone produces glycosuria in normal rats, and since similar results were obtained with 17-hydroxycorticosterone it has been concluded that adrenocorticotropic hormone increases the secretion of steroids. That this hormone enhances diabetes and opposes insulin has been shown by Bennett and Li.[197] On the other hand, Anderson and Long,[203] from experiments with the isolated pancreas, have concluded that growth hormone inhibits insulin secretion by a direct action on the islet cells. Neither adrenal cortical hormones nor thyroxine inhibit insulin secretion.

5. Epinephrine

a. Glycogen Metabolism

With resting frog muscle kept anaerobically in Ringer's solution and epinephrine, Hegenauer and Cori[204] observed marked increase of the break-

[195] C. H. Li, *Ann. Rev. Biochem.* **16**, 291 (1947).
[196] C. H. Li, C. Kalman, and H. M. Evans, *J. Biol. Chem.* **169**, 625 (1947).
[197] L. L. Bennett and C. H. Li, *Endocrinology* **39**, 63 (1946).
[198] C. H. Li, C. Kalman, H. M. Evans, and M. E. Simpson, *J. Biol. Chem.* **163**, 715 (1946).
[199] H. M. Evans, M. E. Simpson, and C. H. Li, *Endocrinology* **33**, 237 (1943).
[200] H. Becks, M. E. Simpson, C. H. Li, and H. M. Evans, *Endocrinology* **34**, 305 (1944).
[201] G. S. Gordon, C. H. Li, and L. L. Bennett, *Proc. Soc. Exptl. Biol. Med.* **62**, 103 (1946).
[202] D. J. Ingle, C. H. Li, and H. M. Evans, *Endocrinology* **39**, 32 (1946).
[203] E. Anderson and J. A. Long, *Endocrinology* **40**, 98 (1947).
[204] A. H. Hegenauer and G. T. Cori, *J. Biol. Chem.* **105**, 691 (1934).

down of glycogen. Hexosemonophosphate was found to accumulate in amounts equivalent to the inorganic phosphate which disappeared. Also Bendall and Lehmann[205] reported that *in vitro* rabbit and rat liver slices with epinephrine causes breakdown of glycogen. In intact cells, however, epinephrine accelerates phosphorylase activity and there is an increase in liver glycogen. According to Kochakian and Bartlett,[159] the injection of epinephrine (0.05 mg.) produced within 1 hour a tremendous deposition of glycogen in the liver of rats fasted for 18 hours (control 120 mg., epinephrine liver, 613 mg.).

b. Interaction between Epinephrine and Oxidases

According to Joyet-Lavergne[206] addition of epinephrine *in vitro* increased the intracellular oxidizing power of brain and muscle tissues of guinea fowl, tail, heart, muscle, and brain tissues of the salamander, pincer and cephalothorax muscle, heart, and branchi of the male crab, and the foot and intestinal tissue of the snail (*Gregorina polymorphia* and *Steinima ovalis*). It also increased the oxidation of leuco dyes more readily after treatment with epinephrine. Epinephrine has a direct action on the living cell, increasing the rate and intensity of intracellular oxidations. Vacca[207] reported that rabbits given three successive injections of epinephrine show, after an hour or so, marked increase in catalase and peroxidase titer of blood after the first injection, and diminished titer of these enzymes after each succeeding injection of epinephrine. Richter and Blaschko[208] reported that epinephrine, oxidized at pH 5 with catechol oxidase, takes up approximately one molecule of oxygen per mole. The oxidation product was suggested to be the unstable adrenochrome.

Epinephrine, as well as flavin and flavoprotein, in the presence of cozymase I, was reported to be capable of acting as a carrier in the oxidation of the malic or lactic acid dehydrogenase systems (Green and Richter[209]). Epinephrine and leucoadrenochrome are easily oxidized by the indophenol-cytochrome system. The dehydrogenation of succinic acid to fumaric acid and malic acid by epinephrine (Marquardt[210]) may indicate that an *o*-quinone is formed which acts as a hydrogen transport.

Govier[211] reported that α-tocopherol inhibits succinic dehydrogenase of homogenized rat muscle and liver. Epinephrine produces an increase in oxygen uptake by neutralizing the inhibitory effect of α-tocopherol. Govier believes that the increase of metabolic activity following addition of epinephrine to α-tocopherol inhibited succinic dehydrogenase can be used

[205] J. R. Bendall and H. Lehmann, *Nature* **148**, 538 (1941).
[206] P. Joyet-Lavergne, *Compt. rend.* **215**, 384 (1942) **216**, 818 (1943); **217**, 327 (1943).
[207] C. Vacca, *Boll. soc. ital. biol. sper.* **20**, 826 (1945).
[208] D. Richter and H. Blaschko, *J. Chem. Soc.* 601, (1937).
[209] D. E. Green and D. Richter, *Biochem. J.* **31**, 596 (1937).
[210] P. Marquardt, *Klin. Wochschr.* **17**, 14, 45 (1938); **18**, 252, 287 (1939).
[211] W. M. Govier, V. Bergmann, and K. H. Beyer, *J. Pharmacol.* **85**, 140, 143 (1945).

as a source of energy for synthesis of cocarboxylase from added thiamine and pyrophosphate.

Friedenwald and Herrimann[212] reported that the oxidation of epinephrine and certain other quinones by the cytochrome oxidase system or by polyphenoloxidase resulted in the production of unstable unidentified substances capable of inhibiting animal oxidase. The mechanism of inhibition appears to consist in the oxidation of a —SH group in the amine oxidase.

Mitalo[213] reported that injection of epinephrine in chickens, guinea pigs, rabbits, rats, and dogs causes a very marked diminution of indophenol oxidase activity of nervous system. Bhagvat and Richter[214] do not consider the inactivation of epinephrine by the phenolase systems likely because of the absence of catechol oxidase from mammalian tissues. However, Caddon and Dill[215] suggest that the organism may contain some highly specific phenolase. That this suggestion seems to have a reasonable basis is indicated by the following observation. Heirman[216] and Bacq[217] found that the oxidation of epinephrine in the tissue yielding adrenoxine, or a closely related substance, plays an essential role in the organism. Adrenoxine possesses greater mydriatic action than epinephrine (Heirman and Goffart[218]) and cardioinhibitory properties (Heirman[216]).

Barcia[219] observed that epinephrine incubated at 37°C. for 30 to 90 minutes with phenolase prepared from the press juice of *Agaricus campestus* is converted to adrenoxine. Helmer and Kohlstaedt[220] observed that the pressor action of epinephrine as measured by the pressor response of a pithed cat was rapidly destroyed by the horse radish-peroxidase and hydrogen peroxide system. Destruction was considerably increased by 0.0001 *N* iodine in potassium triodide.

According to Schapira,[221] the amine oxidase which oxidizes adrenaline oxidizes *phenethylamine* to *phenylacetaldehyde* which can be detected by its powerful hyacinth odor. By this test the amine oxidase was found in every animal tissue tested, including the adrenal medulla.

Martin *et al.*[222] observed that the oxidation of epinephrine by tyrosinase

[212] J. S. Friedenwald and H. Herrmann, *J. Biol. Chem.* **146**, 411 (1942).
[213] M. Mitalo, *Boll. ital. biol. sper.* **22**, 651 (1946).
[214] K. Bhagvat and D. Richter, *Biochem. J.* **32**, 1397 (1938).
[215] J. F. Caddon and L. V. Dill, *J. Biol. Chem.* **143**, 105 (1942).
[216] P. Heirman, *Compt. rend. soc. biol.* **126**, 1264, 1267 (1937); **127**, 343 (1938); *Arch. intern. physiol.* **46**, 404 (1938).
[217] Z. M. Bacq, *Arch. intern. physiol.* **50**, 141 (1940).
[218] P. Heirman and M. Goffart, *Compt. rend. soc. biol.* **132**, 84 (1939).
[219] R. C. Barcia, *Arch. soc. biol. Montevideo* **13**, 68 (1947).
[220] O. M. Helmer and K. G. Kohlstaedt, *Science* **102**, 422 (1945).
[221] G. Schapira, *Compt. rend. soc. biol.* **139**, 36 (1945).
[222] G. J. Martin, C. T. Ichniowski, W. A. Wisansky, and S. Ansbacher, *Am. J. Physiol.* **136**, 66 (1943).

is accelerated by aminophenol and hydroxybenzoic acid, and inhibited by aniline, benzoic acid and the three isomeric aminobenzoic acids.

Wallseh and Rakow[223] observed that certain oxidation products of epinephrine exercise a marked inhibitory action on choline esterase. This inhibition was also observed with physostigmine, indol and *N*-methylindol. Süllmann[224] found that oxygen uptake of linoleic acid and lecithin in the presence of lipoxidase (from soybeans) is inhibited in the following order by pyrocatechol, hydroquinone, α- and β-naphthol, *p*-aminophenol, 3,4-dihydroxyphenylalanine, epinephrine, and *dl*-α-tocopherol.

For an excellent treatment of the mechanism of the oxidation of phenolic compounds the reader is referred to Nelson and Dawson.[225]

IV. The Relation of Vitamins to Coenzymes

It is now common knowledge in enzymology that certain vitamins are incorporated into the coenzyme groups of oxidative enzymes. Their physiological functions are, therefore, intimately linked with the functions of these enzymes.

Nicotinamide is the structural component of cozymase I and II, or di- and triphosphopyridine nucleotide (DPN and TPN) respectively. In these combinations they function as the coenzymes of cozymase dehydrogenases.

Riboflavin is the structural component of the coenzyme groups of flavoproteins. Flavin-adenine dinucleotide is the coenzyme group of a large number of flavoproteins which mediate the transfer of hydrogen individually or in combination with cozymase dehydrogenase. Flavin mononucleotide is the coenzyme group, for example, of cytochrome reductase and L-amino acid oxidases.

Thiamine is the component of cocarboxylase (diphosphothiamine), the coenzyme group of carboxylase involved in the decarboxylation of pyruvate and certain other α-ketoacids and of pyruvic acid and aldehyde ketolase. For extensive literature, see Sumner and Somers.[226]

Pyridoxine in the form of pyridoxal phosphate has been found to function as coenzyme of the following systems:

(a) Decarboxylase involved in the decarboxylation of various amino acids (Taylor and Gale[227]; Gale[228]; Schales *et al.*[229]; Schales and Schales[230]).

[223] H. Waelsch and H. Rakow, *Science* **96**, 386 (1942).
[224] H. v. Süllmann, *Helv. Chim. Acta* **26**, 1114 (1943).
[225] J. M. Nelson and C. R. Dawson, *Advances in Enzymol.* **4**, 99 (1944).
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[228] E. F. Gale, *Advances in Enzymol.* **6**, 1 (1946).
[229] O. Schales, V. Mims, and S. S. Schales, *Arch. Biochem.* **10**, 455 (1946).
[230] O. Schales and S. S. Schales, *Arch. Biochem.* **11**, 155 (1946).

(b) Transaminase involved in the transamination reactions (Snell[231]; Lichstein *et al.*[232]; Gunsalus and Umbreit[233]; O'Kane and Gunsalus[234]).

(c) Tryptophanase involved in the breakdown of tryptophan into indole and alanine, or pyruvate and ammonia (Happold and Hoyle[235]; Wood *et al.*[236]; Dawes *et al.*[237]).

(d) Involved in the synthesis of tryptophan from anthranilic acid via indol and serine (Umbreit *et al.*[238]; Sweigert[239]).

Biotin. Various studies tend to show that biotin is a component of a coenzyme involved in the fixation of carbon dioxide. Biotin mediates the formation of oxalacetate from pyruvate and carbon dioxide. And, since oxalacetate is the precursor of aspartic acid, biotin appears to play a significant role in the tricarboxylic cycle and in the synthesis of aspartic acid and glutamic acid.

Koser[240] showed that aspartic acid exerted a biotin-sparing effect for *Torula cremoris*, and Burk and Winzler[241] indicated that biotin might mediate the utilization of carbon dioxide. In view of the fact that in the presence of excess biotin aspartic acid could be eliminated from the medium used for the growth of most lactic acid bacteria, Stokes *et al.*[242] suggested that biotin functions in the synthesis of aspartic acid. They could not show an altered rate of transamination in biotin deficiency, indicating that biotin was not involved in transamination reactions. Lardy *et al.*[243a,b] likewise found that, in the growth of *Lactobacillus arabinosus*, oxalacetate would promote growth on a biotin- and aspartic-acid deficient medium and that in a biotin-rich medium the growth could be stimulated by bicarbonate as source of carbon dioxide, which would indicate a role for biotin in the fixation of carbon dioxide. Shive and Rogers[244] reported that α-ketoglutarate relieved the biotin inhibition, which was interpreted to indicate that decreased biotin synthesis affected first the formation of α-ketoglutarate.

[231] E. E. Snell, *J. Am. Chem. Soc.* **66**, 2082 (1944).
[232] H. C. Lichstein, I. C. Gunsalus, and W. W. Umbreit, *J. Biol. Chem.* **161**, 311 (1945).
[233] I. C. Gunsalus and W. W. Umbreit, *J. Biol. Chem.* **170**, 415 (1947).
[234] D. E. O'Kane and I. C. Gunsalus, *J. Biol. Chem.* **170**, 425, 433 (1947).
[235] F. C. Happald and L. Hoyle, *Biochem. J.* **29**, 1918 (1935).
[236] W. A. Wood, I. C. Gunsalus, and W. W. Umbreit, *J. Biol. Chem.* **170**, 313 (1947).
[237] E. A. Dawes, J. Dawson, and F. C. Happold *Biochem. J.* **41**, 426 (1947).
[238] W. W. Umbreit, W. A. Wood, and I. C. Gunsalus, *J. Biol. Chem.* **165**, 731 (1946).
[239] B. S. Schweigert, *J. Biol. Chem.* **168**, 283 (1947).
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[243] H. A. Lardy, R. L. Potter, and C. A. Elvehjem, *J. Biol. Chem.* **169**, 451 (1947).
[243a] H. A. Lardy, R. L. Potter, and R. H. Burris, *J. Biol. Chem.*, **179**, 721 (1949).
[243b] P. R. MacLeod and H. A. Lardy, *J. Biol. Chem.* **179**, 733 (1949).
[244] W. Shive and L. L. Rogers, *J. Biol. Chem.* **169**, 453 (1947).

Lichstein and Umbreit[245] found that aging of the cell suspensions of *E. coli* in acid buffer rendered the cells deficient with respect to the ability to release carbon dioxide from systems containing fumaric, malic, and aspartic acids. The addition of biotin reactivated the enzyme systems. Ochoa *et al.*[246] suggested that in the liver biotin catalyzes the reversible decarboxylation of oxalacetate to pyruvate and CO_2.

Pantothenic Acid. According to Lipmann *et al.*[247] and Novelli and Lipmann[248] pantothenic acid is a component of a coenzyme group which was named Coenzyme A. Coenzyme A functions as the general coenzyme for acetylation (Kaplan and Lipmann[249]). In the liver, acetate is claimed to react with ATP to form a compound with the properties of acetyl phosphate. Since acetate is known to occupy an important place in the formation and degradation of fatty acids, and in oxidative pathways for carbohydrates, this enzyme assumes even greater importance. Lipmann[250] has employed a test system for acetylation of aromatic amines by liver preparations to assay for the presence and activity of coenzyme.

The first observation about the role of pantothenate in various enzyme systems was reported by Dorfman *et al.*[251] They found that pantothenate accelerates the rate of oxidation of pyruvate by *Proteus morgani*. Hill[252] found that pantothenate increases the aerobic and anaerobic metabolism of pyruvate. Increased oxygen uptake was likewise found with the oxidation of C_4- and C_5-dicarboxylic acids, and lactate in the presence of pantothenate. Four fold increase in oxygen uptake with malate, but not succinate, was observed. Sevag and Green[253] found that in the presence of glucose only pantothenate enables a strain of tryptophan-requiring *Staph. aureus* to dispense with the requirements for tryptophan. Apparently, pantothenate mediated certain reactions in glucose metabolism in an amino acid environment which led to the synthesis of tryptophan. Kersey and Porter[254] reported that pantothenate accelerated the oxidative deamination of DL-glutamic acid, DL-aspartic acid and DL-serine by *Proteus morgani*. They found also that pantothenate accelerates the oxidation of α-ketoglutarate, succinate, fumarate, oxalacetate, and pyruvate. In all these

[245] H. C. Lichstein and W. W. Umbreit, *J. Biol. Chem.* **170**, 329 (1947).

[246] S. Ochoa, A. Mehler, M.Blanchard, T. H. Jukes, C. E. Hoffmann, and M. Regan, *J. Biol. Chem.* **170**, 413 (1947).

[247] F. Lipmann, N. O. Kaplan, G. D. Novelli, L. C. Tuttle, and B. M. Guirard, *J. Biol. Chem.* **167**, 869 (1947).

[248] G. D. Novelli and F. Lipmann, *J. Biol. Chem.* **171**, 833 (1947).

[249] N. O. Kaplan and F. Lipmann, *Federation Proc.* **6**, 266 (1947).

[250] F. Lipmann, *J. Biol. Chem.* **160**, 173 (1945).

[251] A. Dorfman, S. Berkman, and S. A. Koser, *J. Biol. Chem.* **144**, 393 (1942).

[252] G. M. Hills, *Biochem. J.* **37**, 418 (1943).

[253] M. G. Sevag and M. N. Green, *J. Biol. Chem.* **154**, 719 (1944); *J. Bact.* **48**, 631 (1946).

[254] R. C. Kersey and J. R. Porter, *Proc. Soc. Exptl. Biol. Med.* **69**, 379 (1948).

instances, pyruvate was found as one of the reaction products. Pyruvate yields reaction products upon which pantothenate acts. According to McElroy and Dorfman[255] acetyl-methylcarbinol accumulates during the oxidation of pyruvate by *Proteus morgani* in the absence of added pantothenate, and they suggested that a pantothenic acid containing coenzyme is concerned with the utilization of acetylmethyl carbinol or some closely related substance. Carboxylation of acetylmethylcarbinol to α-acetolactic acid by *Staph. aureus* is suggested by Watt and Krampitz[256].

Ravel and Shive[257] and Shive *et al.*[258] reported that cysteic acid interfered with the synthesis of pantothenic acid which was inferred from the competitive inhibition between aspartic acid metabolism and cysteic acid. Glutamic acid, β-alanine, citric acid, *cis*-aconitic acid, and α-ketoglutaric acid reverse the cysteic acid inhibition of the growth of *E. coli*. A combination of oxalacetate and pyruvate, or acetate showed a lesser activity. These results were interpreted to indicate an acetylation function for pantothenic acid in the tricarboxylic acid cycle. In the growth of *Lactobacillus arabinosus*, oleic acid, in the form of 'tween 80 , relieved the inhibition by cysteic acid, which suggested to them a function of pantothenic acid in acetate condensation to form fatty acids.

Shive and Macow[259] reported that aspartic acid, via β-alanine is a precursor of pantothenic acid. Billen and Lichstein[260] found that washed cells of *Rhizobium trifolii* produces β-alanine from aspartic acid. Virtanen and Laine[260a] had previously claimed that *Rhizobium leguminosarum* decarboxylates aspartic acid with the formation of β-alanine.

As a component of a coenzyme, pantothenate is involved also in the acetylation of choline in the presence of acetate, adenosine triphosphate, potassium ion, cysteine, and choline acetylase (Nachmansohn and Weiss[261]).

p-Aminobenzoic Acid and Folic Acid. *p*-Aminobenzoic acid in combination with glutamic acid and a pterine residue has been shown to be a component of pteroylglutamic acid, *N*-(4-{[(2-amino-4-hydroxy-6-pteridyl) methyl]-amino}benzoyl)glutamic acid (Hutchings *et al.*[262]). It has been maintained that the latter is involved in the synthesis of purines, but there is as yet no conclusive evidence to support this.

Vitamin K. A relationship between a vitamin K-like naphthoquinone

[255] O. E. McElroy and A. Dorfman, *J. Biol. Chem.* **173**, 805 (1948).
[256] D. Watt and L. O. Krampitz, *Federation Proc.* **6**, 301 (1947).
[257] J. M. Ravel and W. Shive, *J. Biol. Chem.* **166**, 407 (1946).
[258] W. Shive, W. W. Ackermann, J. M. Ravel, and J. E. Sutherland, *J. Am. Chem. Soc.* **69**, 2567 (1947).
[259] W. Shive and J. Macow, *J. Biol. Chem.* **162**, 451 (1946).
[260] D. Billen and H. C. Lichstein, *J. Bact.* **57**, 267 (1949).
[260a] A. I. Virtanen and T. Laine, *Enzymologia* **3**, 266 (1937).
[261] D. Nachmansohn and M. S. Weiss, *J. Biol. Chem.* **172**, 677 (1948).
[262] B. L. Hutchings, E. L. R. Stokstad, J. H. Boothe, J. H. Mowat, C, W. Waller, R. B. Angier, J. Semb, Y. Subba Row, and A. de Grunigen, *J. Biol. Chem.* **168**, 705 (1947).

and luciferin, coenzyme group of luciferase, has been suggested. Kluyver *et al.*[263] suggested that dihydroluciferin is either identical with, or closely related to 2-(hydroxyacetyl)-1,4-naphthohydroquinone, which would make it related to vitamin K derivatives whose synthesis by bacteria has been demonstrated by Dam *et al.*[264]

Lyons[265] believes that a vitamin K-like substance, 2-methyl-1,4-napthoquinone, is a component of thrombin. The production of fibrin involves the release of sulfhydryl groups in fibrinogen which are oxidized to —S—S— bridges as a result of oxidation by the above mentioned naphthoquinone or by a vitamin K-like component of thrombin. In this manner, many molecules of fibrinogen form —S—S— bridges among themselves and thus form a network in the form of fibrin. Transformation of fibrinogen —SH into a fibrin gel could also be brought about by the addition of minute amounts of 2-methyl-1,4-naphthoquinone. The presence of such a compound in thrombin was indicated by a colorimetric reaction.

V. Action of Chemotherapeutic Agents on Enzymes

1. Inhibition of Bacterial Oxidative Enzymes in Relation to the Antibacterial Action of Sulfonamides

Despite the similarity in the basic pattern of the reactions which are mediated by the same functional type of enzymes present in different cells, the enzyme proteins of each species differ in their pattern and behavior toward agents that are not native to a given cell. *Penicillin*, which is a normal and apparently nontoxic metabolite of certain strains of Penicillium, is a highly toxic substance and antibiotic for a number of bacteria, and exercises little or no apparent effect on other species of bacteria. Sulfonamides which are effective drugs against *Streptococcus pyogenes* are of no value as antibacterial chemotherapeutic agents, for example, against *E. typhosa*. These antibacterial agents which are specifically effective against the above-mentioned bacteria are relatively nontoxic against the human or animal hosts despite the fact that they form complexes with the host proteins.

An effective antibacterial agent must interfere with the enzyme processes of a bacterium, and be detrimental for its normal physiological activities. This process is intimately related to a specific affinity for certain key enzyme proteins of a given drug-sensitive bacterium; not all the proteins in a given bacterium possess this affinity for one or more of the

[263] A. J. Kluyver, G. J. M. Van der Kirk, and A. Van der Burg, *Proc. Koninkl. Nederland Akad. Wetensch.* **65**, 886 (1942).

[264] H. Dam, J. G. Glavind, S. Orla-Jensen, and A. D. Orla-Jensen, *Naturwissenschaften* **29**, 287 (1941).

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TABLE I

Drugs	Inhibition of	Source of enzyme	Reversed by	References
Sulfonamides	Respiration	Bacteria		See the text for numerous studies
Sulfathiazole	Pyruvate dismutation	*E. coli*, Baker's yeast, Brewer's yeast, *Staph. aureus*	*p*-Aminobenzoic acid, cocarboxylase	Sevag *et al.*[266, 266a, 267]
Sulfathiazole	Growth in glucose	*Staph. aureus*	Riboflavin + pantothenate	Sevag and Green[268]
Sulfathiazole	Oxidation of glutamate, succinate, lactate	*P. avicida*, *Streptococcus hemolyticus*		Frei[269]; Frei and Jesierski [270]
Sulfapyridine	Dehydrogenases; glucose, lactate, glycerol	Pneumococcus		MacLeod[271]
Sulfathiazole	Oxygen uptake, dehydrogenase	Pnemococcus	Methylene blue, riboflavin	Sevag and Gots;[80] See text also
Sulfanilamide	lactic dehydrogenase	Gonococcus		Bucca[272]
Sulfonamides	Glucose-6-dehydrogenase	Top ale yeast	TPN	Altman[273]
Sulfonamides	Lactate oxidation, pyruvate disimutation	*E. coli*		Fox[274]
Sulfonamides	Luciferase	Cypridina and bacteria		Johnson and Chase;[275] Johnson[276]

[266] M. G. Sevag and Myrtle Shelburne, *J. Bact.* **43**, 421, 447 (1942).
[266a] M. G. Sevag, Mrytle Shelburne, and S. Mudd, *J. Bact.* **49**, 65 (1945).
[267] M. G. Sevag, Jane Henry, and Ruth A. Richardson, *J. Bact.* **49**, 139 (1945).
[268] M. G. Sevag and M. N. Green, *J. Bact.* **48**, 623 (1944).
[269] W. Frei, *Schweiz. med. Wochschr.* **72**, 763 (1942).
[270] W. Frei and A. Jezierski, *Schweiz. Z. Path. u. Bakt.* IX, 277 (1946).
[271] C. M. MacLeod, *Proc. Soc. Exptl. Biol. Med.* **41**, 215 (1939); *J. Am. Med. Assoc.* **113**, 1405 (1939).
[271a] C. M. MacLeod and G. Daddi, *Proc. Soc. Exptl. Biol. Med.* **41**, 69 (1939).
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[273] K. I. Altman, *J. Biol. Chem.* **166**, 149 (1946).
[274] C. L. Fox, Jr., *J. Bact.* **43**, 68 (1942).
[275] F. H. Johnson and *A.* M. Chase, *J. Cell. Comp. Physiol.* **19**, 151 (1942).
[276] F. H. Johnson, *Advances in Enzymol.* **7**, 215 (1947).
[276a] F. H. Johnson, D. Rexford, and E. N. Harvey. *J. Cellular Comp. Physiol.* **33**, 133 (1949).

TABLE 1—*Continued*

Drugs	Inhibition of	Source of enzyme	Reversed by	References
Sulfonamides	Coagulase	*Staph. aureus*		Spink and Vivino[277]
Sulfanilamide	Cytochrome oxidase	Gonococcus		Bucca[278]
Sulfonamides	Cytochrome reductase	Top ale yeast		Altman[273]
Sulfanilamide	Sucrose	Yeast		Ercoli and Ravazzoni[278]
Sulfanilamide	Amylase	*Aspergillus oryzae*		Ercoli and Ravazzoni[278]
Sulfanilamide	Rapidase, amylase	Bacterial		Ercoli and Ravazzoni[278]
Sulfonamides	Succinic and all dehydrogenases	Pig and rat liver, heart and kidneys		Pražak[279]
Sulfathiazole (succinyl-)	Carboxylase, fumaric oxidase	Rat liver	Liver extract	Pilgrim and Elvehjem[280]
Sulfonamides	Cytochrome oxidase	Rat thyroid		Paschkis *et al.*[281]
Sulfanilamide	Cytochrome C	Horse heart		Altman[273]
Sulfanilamide (*p*-hydroxyamino-)	Catalase	Liver	Serum, serum albumin, hemin	Sevag *et al.*[281a]
Sulfanilamide (*p*-hydroxymino-)	Cytochrome oxidase,	Beef heart		Collier[282]
	Cytochrome C, catalase	Beef heart, guinea pig liver		
Sulfonamides	Catalase	Liver, bacteria		Schuler and Meier[283]
Sulfonamides	Peroxidase	Horse radish		Lipmann[284]
Sulfanilamide (and PABA)	Catalase, peroxidase			Carrara and Chiancone[285]

[277] W. W. Spink and J. J. Vivino, *Proc. Soc. Expl. Biol. Med.* **50**, 37 (1942).

[278] A. Ercoli and C. Ravazzoni, *Rend ist. lombardo sci., Classe sci. mat. nat.* **73**, 573 (1939–40); *Chem. Abstracts* **37**, 3110.

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[284] F. Lipmann, *J. Biol. Chem.* **139**, 977 (1941).

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TABLE 1—*Continued*

Drugs	Inhibition of	Source of enzyme	Reversed by	References
Sulfonamides	Tyrosinase	Potato	$CuSO_4$, ICH_2COOH	Paschkis *et al.*[286]
Sulfonamides (and PABA)	Tyrosinase	Potato		Baur and Ruf[287]
Sulfanilamide (and PABA)	Tyrosinase	*Psalliota compestris*		Wisansky *et al.*[288]
Sulfanilamide	Carbonic anhydrase	Erythrocytes, Gastric mu-		Mann and Keilin[289]
Sulfanilamide	Carbonic anhydrase	Rabbit	Partially by PABA	Van Goor[290]
Sulfanilamide (Thiaphene-2)	Carbonic anhydrase	Erythrocytes		Davenport[291]
Acetylsulfonamide, *p*-Methylphenyl-sulfonamide Caproyl sulfonamide	Carbonic anhydrase	Erythrocytes		Locke *et al.*[292]
Sulfanilamide	Alkaline phosphatase	Hen		Genest and Bernard[293]
Soluseptazine	Phosphatase	Dog serum		de Elio and Sanchez[294]
Sulfonamides	Phosphatase			
Sulfonamides	Alkaline and acid Phosphatases	Serum		Paget and Vittu[295]
Sulfanilamide	Phosphatase	Rat femur bone		Silver and Golding[296]
Sulfonamides	Phosphomonoesterase	Human erythrocytes	PABA, SA/PABA = 100	Paget and Vettu[295]

[286] K. E. Paschkis, A. Cantarow, W. M. Hart, and A. E. Rakoff, *Proc. Soc. Exptl. Biol. Med.* **57**, 37 (1944).

[287] E. Baur and H. Rüf, *Helv. Chim. Acta* **25**, 523 (1942).

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[291] H. W. Davenport, *J. Biol. Chem.* **158**, 567 (1945).

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[293] P. Genest and R. Bernard, *Rev. Can. Biol.* **5**, 586 (1946).

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TABLE I—*Concluded*

Drugs	Inhibition of	Source of enzyme	Reversed by	References
Sulfonamides	Pseudocholin-esterase	Human serum		Paget and Dhellemmes [297]
Sulfonamides (and PABA)	Cholinesterase	Blood serum		Zeller[298]
Sulfanilamide	Cholinesterase	Human brain and serum Guinea pig serum		Zeller[298]

known antibacterial agents. The enzyme proteins of certain bacterial species appear to lack absolutely a measurable affinity for the known drugs even in a simple *in vitro* environment.

An affinity between an enzyme protein and a drug must result in a chemical reaction resulting in an irreversible inactivation of the key enzyme, or form a reversible complex resulting in bacteriostasis. In either case, the bacteria would be incapable of multiplication.

In view of the complexity of the biochemical processes employed by microorganisms for growth purposes, and lack of a satisfactorily clear definition of the intimate relationships of the step-by-step progressing reactions involved in the growth mechanism, it is at present impossible to establish a direct relationship between the chemotherapeutic action of a drug and its inhibition of one or more enzymes mediating certain reactions or steps in a synthetic process.

Despite the above considered complexities and difficulties, students of cell physiology recognize the well-established fact that there are certain basic processes which are critical in the biology of a given bacterium. An antibacterial drug to be effective must be capable of irreversibly blocking such processes. The blockage of such processes by a drug must, therefore, be closely analyzed to gain an insight into the mechanism of antibacterial drug action.

In any growth environment, a bacterial cell must first metabolize foodstuffs to derive energy and materials necessary for the synthesis of essential components, such as vitamins, purines, pyrimidines, nucleic acids, polysaccharides, lipides, new amino acids, proteins, enzymes, etc. A bacterial cell cannot make use of available food molecules if it lacks ability or is inhibited from tapping the energy stored in the constituents of a complete

[297] M. Paget and G. Dhellemmes, *Compt. rend.* **224**, 503 (1947).
[298] E. A. Zeller, *Verhandl. Ver. schweiz. Physiol.* **21**, 43 (1942).

amino acid medium. It is often necessary that glucose or its intermediary products be present as a ready source of energy for the utilization of the preformed amino acids. Since energy metabolism is mediated by oxidative enzymes, they constitute the mediators of the basic biochemical processes common to all cells. An interference by drugs with the function of these enzymes can therefore effectively function as antibacterial chemotherapeutic action. It does not necessarily mean that the action of antibacterial agents is limited only to these sites to be effective.

a. Inhibition of Bacterial Respiration by Sulfonamides

In view of the above considerations, various investigators who undertook the study of the problem of the mode of action of sulfonamides began their study with the determination of the interference by these drugs with the oxidative mechanism of pathogenic bacteria. The subject has already been reviewed by Henry[299] and Sevag.[300] For later discussion, certain pertinent data will be discussed here. Barron and Jacobs,[301] Chu and Hastings,[302] Ely,[303] Dorfman *et al.*,[304, 305, 306] Kohn and Harris[307] (see Henry[299]), and Clifton and Loewinger[308] reported inhibitions of bacterial respiration by sulfonamides ranging from negligible to 85%. Frei,[269] Frei and Jisierski[270] reported that from 0.25 to 0.75 mg. of sulfathiazole/ml. inhibits from 40 to 85% of the oxidation of glucose, glutamic acid, succinic acid, and lactate by *Pasteurella avicida* and *Streptococcus pyogenes* in the presence and absence of blood or serum.

Since the weight of cells was not determined where growth occurred, it was not possible, on the basis of the above cited observations, to correlate the inhibition of respiration with the inhibition of the growth of bacteria by sulfonamides. Sevag and Shelburne[266] reported the result of a series of studies representing a direct correlation between the degree of inhibition of oxidation enzymes and that of growth in a given system using *Streptococcus pyogenes* and pneumococci as test organisms.

Measuring simultaneously at various time intervals, in the presence of yeast extract, serum, glucose, and sulfanilamide, the increase in the number of streptococci and the milligrams of nitrogen produced and the respiration,

[299] R. J. Henry, *Bact. Revs.* **7**, 175 (1943).
[300] M. G. Sevag, *Advances in Enzymol.* **6**, 33 (1946).
[301] E. S. G. Barron and H. R. Jacobs, *Proc. Soc. Exptl. Biol. Med.* **37**, 10 (1937).
[302] H. I. Chu and A. B. Hastings, *J. Pharmacol.* **63**, 407 (1938).
[303] J. O. Ely, *J. Bact.* **38**, 391 (1939).
[304] A. Dorfman, L. Rice, S. A. Koser, and F. Saunders, *Proc. Soc. Exptl. Biol. Med.* **45**, 750 (1940).
[305] A. Dorfman, L. Rice, and S. A. Koser, *J. Biol. Chem.* **140**, XXXIII (1941); *J. Bact.* **43**, 69 (1942).
[306] A. Dorfman and S. A. Koser, *J. Infectious Diseases* **71**, 241 (1942).
[307] H. I. Kohn and J. S. Harris, *J. Pharmacol.* **73**, 343 (1941).
[308] C. E. Clifton and I. E. Loewinger, *Proc. Soc. Exptl. Biol. Med.* **52**, 225 (1943).

the inhibition of both aerobic and anaerobic oxidation was demonstrated to result in proportional inhibition of growth. In view of these relationships they[266] formulated the "Inhibition of Respiration" theory as the mode of action of sulfonamides. A recalculation of our data by Henry[299] on a per cell or mg. N basis showed that in actively growing cultures, approximately 65% inhibition by sulfanilamide of aerobic respiration or approximately 45% inhibition of anaerobic growth resulted or accompanied complete bacteriostasis. It was likewise shown (Sevag and Shelburne.[266] Sevag *et al.*[267] and Sevag and Gots[80]) that the respiration of pneumococcus, type I, in the presence of yeast extract and glucose was 38–42% inhibited by sulfanilamide, which was accompanied by a 45% inhibition of growth; 0.0038 *M* sulfathiazole caused 65% inhibition of the oxygen uptake by pneumococcus, type I, in the presence of glucose.

In a careful study, Fisher and Armstrong[309] compared the effects of sulfathiazole and *n*-propyl carbamate on the rates of growth and of oxygen consumption by cells of *E. coli*. The effects exercised by both compounds were found to be very similar. Concentrations of sulfathiazole or of *n*-propyl carbamate which were just sufficient to stop growth completely, lowered the rate of oxygen consumption per unit of bacterial protoplasm to a value approximately 50% of that seen in the absence of the inhibitor. Since both of these two compounds produce appreciable inhibition of the rate of oxygen consumption while they are inhibiting growth, the authors stated that "the possibility that the effect on oxygen consumption is the immediate cause of the effect on growth must be entertained." Armstrong and Fisher[310] found that the inhibition of oxygen consumption that is associated with ammonia fixation, by both sulfathiazole and *n*-propyl carbamate, closely parallels the inhibition of growth by these compounds. These inhibitors exercised no effect on the rate of oxygen consumption by the cells after the rate of growth had fallen and the cells were in the resting state. It was, therefore, pointed out that these observations would be adequately accounted for if growth depended on a discrete fraction of the total oxygen consumption of the growing cell.

b. Inhibition of Bacterial Dehydrogenases by Sulfonamides

MacLeod,[271] and MacLeod and Dadi[271a] reported that dehydrogenase activity of pneumococci in the presence of glycerol, lactate, and pyruvate, measured by methylene blue reduction, is inhibited by sulfapyridine; Gots and Sevag[311] could not confirm these observations. MacLeod reported that the drug did not inhibit the glucose dehydrogenase activity of pneumococci. Clifton and Loewinger[306] though, demonstrated the inhibition by

[309] K. C. Fisher and F. H. Armstrong, *J. Gen. Physiol.* **30**, 263 (1947).
[310] F. H. Armstrong and K. C. Fisher, *J. Gen. Physiol.* **30**, 279 (1947).
[311] J. S. Gots and M. G. Sevag, *J. Bact.* **56**, 709 (1948).

sulfanilamide of the aerobic and anaerobic respiration of *E. coli* but could not demonstrate an inhibition of the reduction of methylene blue. Brazda and Rice[312] likewise failed to show the inhibition of the D-alanine dehydrogenase. Bucca[272] working with gonococci demonstrated the inhibition of lactic dehydrogenase by sulfanilamide but not that of glyceric acid dehydrogenase. Fox[274] determined manometrically the effects of sulfonamides on certain enzymes of *E. coli*. He reported that, aerobically, the oxidation of lactate, and anaerobically, the dismutation of pyruvate is depressed. In contrast, he did not find an inhibition of lactic dehydrogenase. This sulfonamide inhibition of the aerobic lactate oxidation and that of pyruvate dismutation was found to be approximately proportional to the bacteriostatic action in growing cultures with equivalent sulfonamide concentrations.

In connection with the above reported failures of an inhibition of dehydrogenase activities by sulfonamides, Sevag and Gots[80] found that methylene blue antagonizes the inhibitory action of sulfonamides on the oxygen uptake by pneumococci in the presence of glucose. In the Thunberg technique, when the order of the addition of various factors was varied, only the system in which methylene blue was added last from the side arm of the Thunberg tube showed 57% inhibition of the reduction of the dye by sulfathiazole. This inhibition indicates that sulfonamides and methylene blue may compete for the same enzyme site, probably flavoprotein.

c. Sulfonamide Inhibition of Hydrogen Transfer in Bioluminescence

Johnson and Moore[313] reported that sulfanilamide (SA) readily inhibits bacterial luminescence. This inhibition appeared at a slightly lower concentration of SA than the amount required for the inhibition of growth. It is believed (Johnson *et al.*[314, 315]) that sulfonamides fall in a group of narcotics together with barbiturate, chloral hydrate, and *p*-aminobenzoic acid, which decrease the light intensity of luminous bacteria. The inhibition by *p*-aminobenzoic acid was observed with a concentration higher than that of SA. The results showed that the action of these inhibitors is on the enzyme *luciferase* (Johnson[276]).

On the basis of available spectroscopic data and the energy changes involved, it has been suggested that the luciferin-luciferase system may be related to a flavoprotein with luciferin as the carrier. The light-emitting organ of lampyrids contains excessively large concentrations of flavin

[312] F. G. Brazda and J. C. Rice, *Proc. Soc. Exptl. Biol. Med.* **49**, 5 (1942).

[313] F. H. Johnson and K. Moore, *Proc. Soc. Exptl. Biol. Med.* **48**, 323 (1941).

[314] F. H. Johnson, D. E. S. Brown and D. A. Marsland, *J. Cellular Comp. Physiol.* **20**, 269 (1942).

[315] F. H. Johnson, H. B. Eyring, and R. W. Williams, *J. Cellular Comp. Physiol.* **20**, 247 (1942).

(Johnson[276]). Flavin has recently been isolated from luminous bacteria and appears to be no different from the flavin or other sources. Luminescence involves hydrogen transfer. The inhibition of luminescence by various inhibitors may also involve an interference with the function of flavoprotein, though proof is as yet lacking (Johnson *et al.*[276a]).

The inhibition of luminescence by 1,4-naphthohydroquinone, and by redox indicators, *methylene blue*, *pyocyanine*, *phthiocol*, is due to an interference with the hydrogen transfer required for luminescence. The inhibitors are hydrogenated by acting as hydrogen carriers, shifting the bacterial luciferin into a more oxidized state. Unlike the mechanism of the inhibition of bacterial luminescence by redox indicators, the inhibition of luminescence by sulfonamides, *p*-aminobenzoic acid and the narcotics is an interference with the hydrogen transfer to luciferin by way of the light emitting system.

d. Antagonism between Sulfathiazole, Methylene Blue and Riboflavin

In a study with pneumococcus, type I, the antagonisms among sulfathiazole, methylene blue and riboflavin were determined by Gots and Sevag.[316]

In these experiments sulfathiazole exercised from 30 to 68% inhibition of oxygen uptake in the presence of glucose. Both methylene blue and riboflavin individually antagonized sulfathiazole inhibition. Methylene blue and riboflavin present together in the system completely abolished sulfathiazole inhibition. This abolition of sulfathiazole inhibition is more effective if pneumococci are allowed to stand with riboflavin for one or two hours before sulfathiazole is added to the system. However, notwithstanding, this effective antagonism to sulfathiazole by riboflavin alone or together with methylene blue, there is a gradual increase in the inhibition by sulfathiazole zole by displacing both riboflavin and methylene blue from the site of the reaction. This displacement mechanism indicated by a rise from 0 to 35% inhibition is due to the greater affinity exercised by sulfathiazole for the susceptible site of the enzyme than by methylene blue and riboflavin. In view of the operation of this displacement mechanism, in growth experiments neither methylene blue nor riboflavin would be capable of counteracting the growth inhibitory action of sulfathiazole. A consideration of these relationships is a necessary condition in the understanding of the antibacterial action of a given drug. A drug that is readily counteracted by the factors present in a nutritional environment could not serve as an effective chemotherapeutic agent. The success of sulfonamides as antibacterial agents against certain pathogenic organisms must therefore be governed by the fact that none of the antagonists so far studied are effective in displacing sulfonamides from the site of reaction.

[316] J. S. Gots and M. G. Sevag, *J. Bact.* **58**, 585 (1949).

The above observations likewise show that pneumococcal *flavoprotein* is most likely the site of the action of sulfathiazole, and riboflavin and methylene blue as well (see further, Gots and Sevag,[311] Sevag and Gots[80]).

e. Inhibition of Pyruvate Metabolism by Sulfonamides

Having gained the information that the sulfonamides interfere with the energy metabolism of bacterial cells resulting in or accompanied by the inhibition of growth, the question of which key oxidation enzymes sulfonamides are able to block naturally had to be considered. The effect of sulfonamides on the metabolism of pyruvate formed as the terminal intermediary product of glucose metabolism was investigated in this laboratory.

Due to the presence of the thiazole ring in the coenzyme cocarboxylase involved in the metabolism of pyruvate and in sulfathiazole, the idea was entertained (Sevag *et al.*[266a]) that sulfathiazole would exercise a more specific inhibitory action on the pyruvate metabolism than the other sulfonamides.

It was found (Sevag *et al.*[266a]) that in comparison with the other sulfonamides, sulfathiazole is the most powerful inhibitor of the enzyme system involved in the metabolism of pyruvate. In agreement with our observation according to Rammelkamp and Jewell[317] and Strauss *et al.*[318] sulfathiazole is about four times as effective as sulfadiazine, eight times as effective as sulfanilamide, and five or six times as effective as sulfapyridine on the growth of *Staph. aureus*. Similar results were obtained with *E. coli*. The greater inhibition by sulfathiazole of the pyruvate metabolism and of the growth of *Staph. aureus* and *E. coli* suggests a causal relationship and makes the relation of the blockage of pyruvate by sulfathiazole to the latter's antibacterial action more real than apparent.

Another set of data related to the critical role of pyruvate metabolism was obtained in a comparative study of the susceptibility to sulfonamides of the growth of *Staphylococcus aureus* in the presence of glucose, and of pyruvic acid (see Table I, Sevag and Green,[268] Sevag *et al.*,[319]).

It is evident from the above data that growth of staphylococci is susceptible to sulfonamide in the presence of either glucose or pyruvate, and it takes a sixteen-fold greater amount of sulfanilamide to produce an inhibitory effect equal to that of sulfathiazole.

f. Antagonism between Cocarboxylase and Sulfathiazole

The inhibition by sulfathiazole of pyruvate metabolism by yeast and *Staphylococcus aureus* is antagonized by cocarboxylase (Sevag *et al.*[266a]). With yeast, one molecule of cocarboxylase antagonized more than 8000

[317] C. H. Rammelkamp and M. L. Jewell, *Proc. Exptl. Biol. Med.* **48**, 27 (1941).
[318] E. Strauss, J. H. Dingle, and M. Finland, *J. Immunol.* **42**, 313, 331 (1941).
[319] M. G. Sevag, E. Steers, and M. Forbes, *Arch. Biochem.* **25**, 85 (1950).

molecules of sulfathiazole. The experiments were carried out with intact yeast and yeast washed with alkaline phosphate.

Similarly, the inhibition by sulfathiazole of the pyruvate metabolizing enzymes in bacteria measured manometrically was antagonized by cocarboxylase; one molecule of cocarboxylase counteracted the inhibitory effect of 215 molecules of sulfathiazole.

g. Antagonism of p-Aminobenzoic Acid to the Inhibitory Action of Sulfathiazole on Pyruvate Metabolism

Sevag and Shelburne[266] working with *Streptococcus pyogenes*, and Clifton and Loewinger[308] working with *E. coli* showed that the inhibition of respiration by sulfanilamide is antagonized by *p*-aminobenzoic acid.

Sevag *et al.*[267] likewise found that *p*-aminobenzoic acid antagonizes the inhibition by sulfathiazole of pyruvate metabolism. The molar ratios of sulfathiazole to *p*-aminobenzoic acid in these experiments were comparable in magnitude to those observed in growth experiments.

The inhibition of the carboxylase activity of yeast by sulfathiazole shows that sulfathiazole exercises a strong affinity for the enzyme carboxylase, which contains cocarboxylase as the coenzyme group that only in part is structurally related to the substituent groups in the sulfathiazole molecule. In bacteria, the metabolism of pyruvate, according to Lipmann,[320] is mediated by an enzyme system involving the participation of cocarboxylase (thiamine pyrophosphate) and flavin-adenine-dinucleotide respectively, as coenzymes. The inhibition of the metabolism of pyruvate by sulfathiazole would therefore appear to involve an action on both the flavoprotein and the thiamine enzyme. This is in agreement with the observations already discussed above that sulfonamides interfere with the dehydrogenase activities of various bacteria, the flavoproteins of pneumococcus and the carboxylase activity of yeast. Sulfonamides inhibit, therefore, in addition to the thiamine enzyme, enzyme systems, such as flavoproteins which contain flavin-adenine-dinucleotide or riboflavin monophosphate as coenzyme, with no structural similarity to sulfonamides or to *p*-aminobenzoic acid, which, as discussed above, counteracts these inhibitions by sulfathiazole and sulfanilamide. The neutralization of sulfathiazole inhibition by methylene blue and riboflavin shows that the inhibitions and anti-inhibitions by these substances are independent of structural similarity. These substances combine with the protein moiety of the enzymes not because it contains a structural analogue, but because of an inherent affinity between the protein molecule and the nonprotein substances discussed here, and the energy yielding substrates as well. An unbiased grasp of these relationships should serve as basis for an understanding of the mechanism of the action of antibacterial drugs.

[320] F. Lipmann, *Cold Spring Harbor Symposia Quant. Biol.* 7, 248 (1939).

h. The Inhibition of Amino Acid Synthesis by Sulfonamides

It is now well established that in the chain of reactions leading to the synthesis of amino acids and protein, the first product of critical importance in carbohydrate metabolism is phosphopyruvic acid. Phosphopyruvate is the source of "acetylphosphate," which plays an important role in the acetylation reactions, yielding condensation and other products. What is more important, pyruvate stands at the threshold of the tricarboxylic acid cycle that provides cells growing actively in the presence of glucose or pyruvate with other α-keto acids, oxalacetic, oxalsuccinic acid, and α-ketoglutaric acid. These α-keto acids are active components of transamination reactions for the synthesis of the corresponding amino acids and certain other amino acids as well, and thereby the synthesis of proteins. Growth of the drug-sensitive staphylococci in a medium containing pyruvate in place of glucose is also sensitive and is comparable to the sensitivity of growth in glucose-containing medium (Sevag and Green,[268] Sevag *et al.*[319]). It is reasonable to conclude that the blockage of pyruvate metabolism can stop any of the reactions associated or dependent on pyruvate metabolism and may be considered as a critical site of sulfonamide action.

In the absence of glucose, the synthesis of amino acids by bacteria from other amino acids is dependent on oxidative deamination reactions to provide the cells with the liberated energy and with α-keto acids necessary for the transaminations and the syntheses of other α-keto acids corresponding to the amino acids to be synthesized. The enzymes that catalyze the oxidative deamination of amino acids are known as D- and L-amino acid dehydrogenase (Stumpf and Green,[321] Green *et al.*,[322] Blanchard *et al.*[323]). The enzymes are flavoproteins with flavin monophosphate as the coenzyme group of L-amino acid dehydrogenase and flavin-adenine-dinucleotide as the coenzyme group of D-amino acid dehydrogenase.

Lichstein and Cohen[324] have shown that various bacteria contain highly active transamination enzyme systems. By these systems the bacteria make use of metabolic pathways that involve pyruvate-alanine, oxalacetate-aspartate, glutamate-glutarate, etc. metabolism for the synthesis of other amino acids paving the way for the synthesis of proteins.

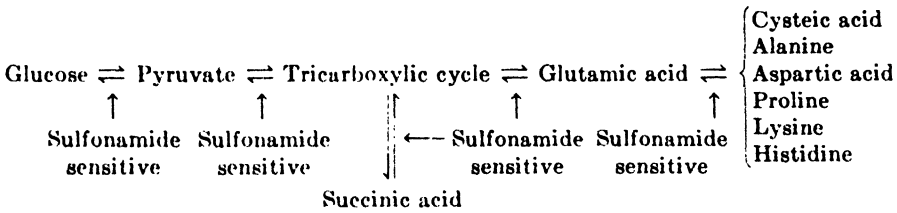
i. Blockage of Tricarboxylic Acid Cycle by Sulfonamides

The inhibition of pyruvate metabolism and the synthesis of glutamic as well as other amino acids from each other by sulfonamides was discussed

[321] P. K. Stumpf and D. E. Green, *J. Biol. Chem.* **153**, 387 (1944).
[322] D. E. Green, D. H. Moore, V. Nocito, and S. Ratner, *J. Biol. Chem.* **156**, 383 (1944).
[323] M. Blanchard, D. E. Green, V. Nocito, S. Ratner (and D. H. Moore). *J. Biol. Chem.* **161**, 583 (1945).
[324] H. C. Lichstein and P. P. Cohen, *J. Biol. Chem.* **157**, 85 (1945).

above. This, integrated with the findings (Frei[269]) that sulfathiazole inhibits the oxidation of glutamic acid and succinic acid by *Pasteurella avicida*, shows that the pathways leading from pyruvate, succinate and amino acids to the tricarboxylic acid cycle, the most important metabolic pool involved in the growth mechanism, is blocked by sulfonamides. The fact that sulfonamides block the metabolism of amino acids in these reactions would indicate that one of the enzymes susceptible to sulfonamides may be flavoprotein.

Steers and Sevag[325] and Sevag and Steers[326] found that of these amino acids, the synthesis of glutamic acid by *Staphylococcus aureus* is one of the most sensitive sites. Five micrograms of sulfathiazole per milliliter completely inhibited this synthesis and inhibited those of methionine and lysine 50% or less. Winkler and DeHaan,[326a] in a study with *E. coli* and *Salmonella typhimurium* growing in glucose-salt medium, reported that successively increasing concentrations of sulfanilamide inhibited the enzyme systems involved in the synthesis of methionine, xanthine, and serine (in this order). The synthesis of methionine was most sensitive to this drug. In the study by Steers and Sevag[325] the synthesis of glutamic acid was many times as sensitive as those of methionine and lysine. The above considerations are schematized below:



j. Interference by Sulfathiazole with the Functions of Riboflavin, Pantothenate, and Tryptophan

The consideration of the various effects of sulfonamides up to this point include: (a) Inhibition of the respiration of bacteria associated or accompanied with a parallel inhibition of growth; (b) inhibition of the various bacterial flavoproteins and thiamine protein enzymes involved in the metabolism of pyruvate by resting and by growing cells; (c) inhibition of succinic acid and glutamic acid oxidases; (d) inhibition of the synthesis of amino acids, among which the synthesis of glutamic acid, methionine, lysine (*Staph. aureus*), methionine, and serine (*E. coli*), in the order given, were most sensitive to sulfonamides. Integration of these effects shows that the tricarboxylic acid cycle, which constitutes one of the most important metabolic pools in the growth mechanism, is blocked by sulfonamides.

[325] E. Steers and M. G. Sevag, *Arch. Biochem.* **24**, 129 (1949).
[326] M. G. Sevag and E. Steers, *Arch. Biochem.* **24**, 144 (1949).
[326a] K. C. Winkler and P. G. DeHaan, *Arch. Biochem.* **18**, 97 (1948).

In addition to the above inhibitions, sulfonamides interfere with the functions of pantothenate which appears to play, as discussed elsewhere in this article, a critical role in the reactions involved in the tricarboxylic acid cycle and in other systems.

It has been observed (Sevag and Green[268]) that the inhibition of growth observed in the absence of tryptophan but presence of glucose not only is abolished but there is a 38% acceleration of the growth in the combined presence of tryptophan, riboflavin, pantothenate and glucose. Since this effect related to pantothenate is not produced in the absence of glucose, the role of pantothenate in producing this acceleration is intimately related to an effort of pantothenate on the metabolic end products of glucose, such as pyruvate and acetylphosphate discussed elsewhere in this article.

Riboflavin abolished the inhibition by sulfathiazole of the growth dependent on amino acid metabolism by virtue of the fact that flavoproteins are involved in the oxidative deamination occurring during the amino acid metabolism. The metabolism of the oxidative deamination products, α-keto acids, which likewise are obtained from glucose metabolism are accelerated by pantothenate (Kersey and Porter[254]). Thus, flavoprotein, or its coenzyme group, and pantothenate in the form of coenzyme A function hand in hand to produce the above discussed sulfonamide-antagonizing effects.

That pantothenate is a critical vitamin in the sulfonamide picture is indicated by the results obtained by Miller *et al.*[327] Comparing the synthesis of pteroylglutamic acid, pantothenate, biotin, and nicotinic acid by sulfonamide-susceptible and sulfonamide-resistant pairs of related *E. coli* strains, they found that nicotinic acid synthesis by both the susceptible and the resistant strain was not inhibited by sulfathiazole. The synthesis of the other three vitamins by the susceptible strain was sensitive to sulfathiazole; of these three vitamins, the synthesis of folic acid was the least sensitive. When the sulfathiazole-resistant strain was grown in a drug-containing medium, its synthesis of pantothenic acid was increased, nicotinic acid was not changed, biotin was depressed slightly, and folic acid was moderately inhibited. Thus sulfathiazole has been shown to have greater effect on pantothenic acid synthesis than on folic acid synthesis; in other words, the interference by sulfathiazole with the synthesis and function of pantothenate is more critical than the synthesis of folic acid. Shive and Macow[259] made the interesting observation that aspartic acid is a precursor in the biosynthesis by *E. coli* of the β-alanine portion of pantothenic acid. If one accepts this conclusion, the inhibition of the oxidative deamination of glutamic acid involving the mediation by flavoprotein, of succinic acid, and of pyruvate metabolism by sulfathiazole would result in the inhibition of the synthesis of aspartic acid and thereby of pantothenate. The interference by sulfonamide with the function of flavoproteins

[327] A. Katherine Miller, P. Bruno, and R. M. Berglund, *J. Bact.* **53**, 9 (1947).

would seem to play a critical role in these reactions. At this point it might be pointed out that the synthesis of riboflavin by bakers yeast is not blocked by sulfonamides (Eppright and Williams[328]). Sulfonamide, therefore, interferes with the function and not with the synthesis of riboflavin.

In view of the above considerations, it becomes clear now why the combined presence of riboflavin and pantothenate counteracts the inhibitory action of sulfathiazole on the growth of *Staphylococcus aureus* (Sevag and Green[268]).

k. Failure of Folic Acid to Function as Growth Factor for L. Arabinosus 17–5 and as an Antagonist to Sulfonamides

Lampen and Jones[329] stated that the inhibition by sulfadiazine of the growth of certain enterococci, i.e., *Streptococcus faecalis*, Ralston, is noncompetitively antagonized by pteroylglutamic acid, pteroyltriglutamic acid, and thymine. *L. arabinosus* requires *p*-aminobenzoic acid for growth. This requirement can be met by the presence of the preformed pteroylglutamic acid, and there is no inhibition of growth in its presence. On this basis, it was concluded that sulfonamides block the step involving the synthesis of folic acid via *p*-aminobenzoic acid. Pteroylglutamic acid did not relieve the inhibition by sulfonamides of the growth of *Staphylococcus aureus*, pneumococci and *E. coli*. It is clear, therefore, that sulfonamide action in these and many other organisms is not related to the synthesis of pteroylglutamic acid, or that this synthesis is not a critical factor in the metabolism and growth of these organisms related to the action of sulfonamides. As discussed below, the postulates of Lampen and Jones have been found to lack experimental basis. It has been found by Koft and Sevag[330], Koft *et al.*[330a], and Sevag *et al.*[330b] that it is a decomposition product and not PGA *per se* which is a growth stimulant for *L. arabinosus* 17-5. Growth stimulation by PGA is directly related to the degree of decomposition. Aged PGA (80% decomposed, calculated as *p*-aminobenzoylglutamic acid (PABG)) is almost 100-fold more active than intact PGA, as are also *p*-aminobenzoic acid (PABA) and PABG. In systems containing 3% phosphate (pH 6.8) to maintain neutrality, unlike PABA, PABG and aged PGA, intact PGA fails to stimulate growth earlier than after 72 hours. These data show that PGA *per se* is not active, but that it is the breakdown products of PGA which stimulate the growth of this organism.

The antagonism of PGA *per se* to sulfonamides reported by Lampen and Jones[329] is apparent rather than real, and is controlled by the following: (1) an incubation period which is associated with the decomposition of

[328] M. A. Eppright and R. J. Williams, *J. Gen. Physiol.* **30**, 61 (1946).
[329] J. O. Lampen and M. J. Jones, *J. Biol. Chem.* **166**, 534 (1946); **170**, 133 (1947).
[330] B. W. Koft, and M. G. Sevag, *J. Am. Chem. Soc.* **71**, 3245 (1949).
[330a] B. W. Koft, M. G. Sevag, and E. Steers, *J. Biol. Chem.*, in press.
[330b] M. G. Sevag, B. W. Koft, and E. Steers, *J. Biol. Chem.*, in press.

TABLE II

Drugs	Inhibition of	Enzyme source	Inhibition reversed by	References
Proflavine	Respiration	*T. equiperdum*		Issekutz[331]
Proflavine	Respiration	*T. rhodesiense*		Fulton and Christophers[332]
Proflavine	Respiration	*P. knowlesi*		Fulton and Christophers[332]
Proflavine	Respiration (and growth)	*Staph. aureus*	AMP, ATP, DPN, NA	Martin and Fisher[333]
Proflavine	Hydrogen transport (and growth)	*E. coli*, *Streptococcus pyogenes*	Riboflavin, phenazine, methylene blue	McIlwain[334]
Proflavine	Growth	*L. casei*	Riboflavin	Madinaveitia[335]
Proflavine	Respiration	Baker's yeast	AMP, ATP, NA (partial)	Massart, *et al.*[336, 336a]
Proflavine	Dehydrogenases of glucose, HDP, ethyl alcohol (and growth)	Pneumococcus	Riboflavin	Gots and Sevag[311]; Sevag and Gots[60]
Atabrine	Respiration	*T. rhodesiense*, *P. knowlesi*		Fulton and Christophers[332]
Atabrine	Growth	*E. coli*	Spermidine, Spermine, trimethylene-tetramine, thiamine, riboflavin, glutathione, pantothenic acid, nicotinic acid	Silverman and Evans[337]; Miller and Peters[338]

[331] B. von Issekutz, *Arch. exptl. Path. u. Pharm.* **173**, 479 (1933).
[332] J. D. Fulton and S. R. Christophers, *Ann. Trop. Med. Parasitol.* **32**, 77 (1938).
[333] G. J. Martin and C. V. Fisher, *J. Lab. Clin. Med.* **29**, 383 (1944).
[334] H. McIlwain, *Biochem. J.* **35**, 1311 (1941).
[335] J. Madinaveitia, *Biochem. J.* **40**, 373 (1946).
[336] L. Massart, G. Peeters, J. de Ley, and R. Vercauteren, *Experientia* **3**, 119 (1947).
[336a] L. Massart, G. Peeters, A. van Houcke and A. Lagrain, *Arch. intern. pharmacodynamie* LXXV, 141 (1947).
[337] M. Silverman and E. A. Evans, Jr., *J. Biol. Chem.* **154**, 521 (1944).
[338] A. Katherine Miller and L. Peters, *Arch. Biochem.* **6**, 281 (1945).

TABLE II—*Continued*

Drugs	Inhibition of	Enzyme Source	Inhibition reversed by	References
Atabrine	Growth	*L. casei*	Riboflavin	Madinaveitia[338]
Atabrine	Growth	*L. arabinosus*	Glutathione, thioglycollic acid	Fraser *et al.*[339]
Atabrine	Growth	Pneumococcus	Riboflavin	Gots and Sevag[311]; Sevag and Gots[80]
Atabrine	Respiration	*P. gallinaceum*		Silverman *et al.*[340]; Oldham and Kelsey[341]
Atabrine	Oxidation of glucose, pyruvate, succinate	*P. lophurae*	AMP, ATP[a]	Bovarnick *et al.*[342]
	Hexokinase	*P. lophurae*		Bovarnick *et al.*[342]
Atabrine	D-Amino oxidase	Lamb kidney	FAD[b]	Hellerman et al.[343]
Atabrine	D-Amino oxidase, oxygen consumption by glucose, lactate, pyruvate, malate, fumarate, citrate	Rat liver, brain and kidney slices	FAD	Wright and Sabine[344]
Atabrine	Flavoprotein	Yeast	Flavin mononucleotide	Haas[345]
Atabrine	Glucose-6-dehydrogenase	Yeast	Glucose-6-phosphate	Haas[345]

[a] Adenylic acid reversed also the inhibitions by plasmoquine, sulfathiazole, auramine. SN6911 (3-methyl-4-(4-dimethylamino)-7-chloroquinolinediphosphate) and SN10,447 (4-(4-diethylamino-1-methylbutylamino)-2,3-dimethylquinolinediphosphate).

[b] D-Amino acid oxidase was inhibited also by plasmoquine, sulfanilamide, benzoic acid, *p*-aminobenzoic acid. FAD reversed these inhibitions with the exception of those of *p*-aminobenzoic and benzoic acids.

[339] H. F. Fraser, F. Irreverre, and M. M. Grenan, *Proc. Soc. Exptl. Biol. Med.* **61**, 72 (1946).

[340] M. Silverman, J. Certhaml, L. G. Taliaferro, and E. A. Evans, *J. Infectious Diseases* **75**, 212 (1944).

[341] F. K. Oldham and F. E. Kelsey, *J. Pharmacol.* **83**, 288 (1945).

[342] M. R. Bovarnick, A. Lindsay, and L. Hellerman, *J. Biol. Chem.* **163**, 523 (1946).

[343] L. Hellerman, A. Lindsay, and M. R. Bovarnick, *J. Biol. Chem.* **163**, 553 (1946).

[344] C. J. Wright and J. C. Sabine, *J. Biol. Chem.* **155**, 315 (1944).

[345] E. Haas, *J. Biol. Chem.* **155**, 321 (1944).

TABLE II—*Continued*

Drugs	Inhibition of	Enzyme source	Inhibition reversed by	References
Atabrine	Hexokinase, Phosphoglyceraldehyde and pyruvate-lactate dehydrogenases	Yeast, *P. gallinaceum*		Speck and Evans[346]; Marshall[347]
Atabrine	Dehydrogenases of glucose, HDP and ethyl alcohol	Pneumococcus	Riboflavin[c]	Sevag and Gots[80]
Atabrine	Dephosphorylation of cocarboxylase (Phosphatase)	Yeast		Silverman[348]
Atabrine	Synthesis of thiamine	*Torula utilis*		Silverman[348]
Atabrine	Choline esterase	Tissue		Wright and Sabine[349]
Atabrine (and pamaquine)	Quinine oxidase	Rat liver		Chen[350]
Quinine	Respiration	*T. rhodesiense*, *P. knowlesi*.		Fulton and Christophers[322]
Quinine	Respiration	*P. gallinaceum*		Silverman *et al.*[340]; Oldham and Kelsey[341]; Ceithaml and Evans[351]
Quinine	Oxidation of glucose	*P. lophurae*	AMP	Bovarnick *et al.*[342]
Quinine	D-Amino acid oxidase	Lamb kidney	FAD	Hellerman *et al.*[343]
Quinine	Hexokinase, pyruvate-lactate dehydrogenase	*P. gallinaceum*		Marshall[347]

[c] Riboflavin also antagonized the inhibitions by propamidine.

[346] J. F. Speck and E. A. Evans, *J. Biol. Chem.* **159**, 71, 83 (1945).
[347] P. B. Marshall, *Brit. J. Pharmacol.* **3**, 1, 8 (1948).
[348] M. Silverman, *J. Biol. Chem.* **178**, 423 (1949).
[349] C. J. Wright and J. C. Sabine, *J. Pharmacol.* **93**, 230 (1948).
[350] G. Chen, *Proc. Soc. Exptl. Biol. Med.* **66**, 313 (1947).
[351] J. Ceithaml and E. A. Evans, Jr., *Arch. Biochem.* **10**, 397 (1946).

TABLE II—*Continued*

Drugs	Inhibition of	Enzyme source	Inhibition reversed by	References
Quinine	Growth	*L. casei*	Riboflavin[d]	Madinaveitia[335]
Quinine	Dehydrogenase (and growth)	*E. coli.*	Spermine, spermidine,[e] riboflavin, cozymase	Miller and Peters[333] Johnson and Lewin[352]
3,5-Aminoacridine	Oxidation of glucose, pyruvic, lactic, and oleic acids, asparagine	*E. coli*		Ferguson and Thorne[353]
Propamidine	Oxidation of alanine asparagine	*E. coli*		Bernheim[354]
	Glucose, pyruvic acid	Rat brain		
Propamidine	Oxidation of glucose, lactic acid	*E. coli*		Wien *et al.*[355]
Dibromopropamidine Hexamidine Iodohexamidine	Choline	Rat liver		Wien, *et al.*[355]
Diamidine (undecane) Stilbamidine	Diphosphoglyceraldehyde dehydrogenase, and probably pyruvate decarboxylation	*T. evansi*		Marshall[347]
Propamidine	Dehydrogenases	Pneumococcus	Riboflavin	Gots and Sevag[311]; Sevag and Gots[80]
Diamidines	Amine Oxidase	Rabbit liver		Blaschko and Duthie[355a]

[d] Riboflavin also reversed the inhibition of the growth by the following substances: Quinine, atabrine, propamidine, and methylene blue.

[e] Spermine and spermidine also antagonized the inhibition by propamidine.

[352] F. H. Johnson and I. Lewin, *J. Cellular Comp. Physiol.* **28**, 1, 23, 47, 77 (1946).

[353] T. B. Ferguson and S. O. Thorne, Jr., *J. Pharmacol.* **86**, 258 (1946).

[354] F. Bernheim, *Science* **98**, 223 (1943).

[355] R. Wien, J. Harrison and W. A. Freeman, *Brit. J. Pharmacol.* **3**, 211 (1948).

[355a] H. Blaschko and R. Duthie, *Biochem. J.* **39**, 347 (1945).

TABLE II—*Continued*

Drugs	Inhibition	Enzyme source	Inhibition reversed by	References
Stilbamidine, pentamidine	Growth	*E. Coli*, *Staph. aureus*, *L. donovani*, *Tr. vaginalis*.	Sod. nucleate	Bichowsky[356]
Phenylarsine oxide (As^{+++})	Hexokinase	*T. evansi*		Marshall[347]
Penicillin	Adenylpyrophosphatase	*Cl. sporogenes*		Gros and Macheboeuf[357]
Penicillin	Coagulase	Staphylococci		Agnew *et al.*[358]
Penicillin	Urease			Vargas and Escubos[359]
Penicillin	Bone phosphatase			Natarajan and De[360]
Penicillin	Cholinesterase	Guinea pig serum		Frommel *et al.*[361]
Penicillin	Pancreatic enzymes			Vargas and Escubos[359]
Penicillin	Catalase	*Staph. aureus*		Cimino and Cimino[362]
Penicillin	Oxidation of sodium ribonucleate	*Staph. aureus*		Krampitz and Werkman[363]
Penicillin	Inhibition of the assimilation of certain amino acids requiring energy	*Staph. aureus*		Gale[364]
Penicillin	Coupled oxidative and reductive deamination	*Cl. sporogenes* *Cl. saccharobutyricum*		Gros *et al.*[365]
Streptomycin	Coagulase	Staphylococci		Agnew *et al.*[358] Walker *et al.*[356a]

[356] L. Bichowsky, *Proc. Soc. Exptl. Biol. Med.* **57**, 163 (1944).
[357] F. Gros and M. Macheboeuf, *Compt. rend.* **224**, 1736 (1947).
[358] S. Agnew, M. Kaplan, and W. W. Spink, *Proc. Soc. Exptl. Biol. Med.* **65**, 38 (1947).
[356a] B. S. Walker, M. A. Derow, N. K. Schaffer, *J. Bact.* **56**, 191 (1948).
[359] J. J. Vargas and J. J. Escubos, *Rev. expán. fisiol.* **1**, 50, (1945).
[360] S. Natarjan and N. N. De, *Current Sci. India* **15**, 289 (1946).
[361] E. Frommel, A. Goldfieder and J. Piquet, *Acta Pharmacol. Toxicol.* **2**, 207 (1946).
[362] S. Cimino and G. Cimino, *Boll. soc. ital. biol. sper.* **23**, 233 (1947).
[363] L. O. Krampitz and C. H. Werkman, *Arch. Biochem.* **12**, 57 (1947).
[364] E. F. Gale, *Nature* **160**, 407 (1947).
[365] F. Gros, M. Macheboeuf and U. Rambech. *Ann. inst. Pasteur* **75**, 446 (1948).

TABLE II—*Concluded*

Drugs	Inhibition of	Enzyme source	Inhibition reversed by	References
Streptomycin	Coupled oxidative and reductive deamination	*Cl. sporogenes*		
	Dephosphorylation of purine nucleotides resulting in failure of the formation of adenylic acid, required for Stickland reaction	*Cl. saccharobutyricum*		Gros *et al.*[366]
Tyrothricin (gramicidin)	Transphosphorylation, Glucose metabolism, synthesis of ATP and acetyl phosphate	*Cl. sporogenes*		Gros *et al.*[367]
Tyrothricin (gramicidin)	Deamination of δ-NH_2 in arginine	*Cl. sporogenes* *Cl. saccharobutryicum*		Gros *et al.*[366]
Tyrothricin tyrocidine	Alanine dehydrogenase	*Cl. sporogenes* *Cl. saccharobutyricum*		Gros *et al.*[366]
Tyrothricin	Stickland reaction (Coupled oxidative and reductive deamination)	*Cl. sporogenes* *Cl. saccharobutyricum*		Gros *et al.*[366]

PGA and is necessary for any antagonism to sulfonamides, (2) the concentration of PABG and/or PABA which brings about a competitive type of antagonism; PABG content of 0.01 γ of PGA, though adequate for optimal growth, is inadequate to antagonize 1 γ of sulfonamide, and (3) the rapid increment of acidity in the medium which has practically no buffering capacity (0.1 g.% phosphate buffer). The increased acidity suppresses the

[366] F. Gros, M. Macheboeuf and S. Jenlin, *Ann. inst. Pasteur* **75**, 242 (1948).
[367] F. Gros, M. Macheboeuf and C. Latterade, *Ann. inst. Pasteur* **75**, 311 (1948).

inhibitory capacity of sulfonamide and augments the antisulfonamide capacity of the liberated PABG. In a well-buffered medium containing 3.0 g.% phosphate (pH 6.8) the complete inhibition of growth by sulfonamides persists in the presence of PGA without a detectable reversal. These data do not support the postulate of Lampen and Jones[329] that sulfonamides interfere with the synthesis of PGA via PABA or PABG.

2. Acridines and Diamidines

A survey of the inhibitions caused by the acridines, quinine, arsenicals, diamidines, etc. shows that these drugs act upon various respiratory and other types of enzymes. It is clearly shown that a given drug acts or combines with the protein moieties of different enzmyes listed in the table. These affinities between a drug and various enzyme proteins are, therefore, independent of structural similarity between the drugs and the coenzyme groups of the enzymes. For example, atabrine, which is structurally related to flavin, a derivative of the coenzyme group of flavoproteins, inhibits not only the flavoproteins but also hexokinase, phosphatase, cholinesterase, etc. These inhibitions show that various proteins possess similar affinities for a given drug. The neutralization by riboflavin of the inhibition of atabrine (or methylene blue), however, could be related to specific action of atabrine on flavoproteins (Wright and Sabine,[344] Haas,[345] Sevag[300]), unless it can be shown that riboflavin is capable of also combining nonspecifically with the protein moieties of other enzymes, such as hexokinase, phosphatase, etc. We have as yet no direct evidence concerning the latter possibility.

In the experiments of Wright and Sabine, the atabrine treated D-amino acid oxidase did not inhibit the oxidation of succinic acid and *p*-phenylenediamine, showing that atabrine is unable to inhibit the cytochrome-cytochrome oxidase system. Likewise in the experiments of Haas, atabrine failed to interfere with the cytochrome-cytochrome oxidase system. The interference with cytochrome reductase was specifically reversed by flavin mononucleotide, which is the coenzyme group of cytochrome reductase. In the experiments of Sevag and Gots[80] the atabrine inhibition of pneumococcal dehydrogenases was antagonized by riboflavin but not by nicotinic acid and thiamine, indicating a specific relationship between the site of inhibition and riboflavin antagonism.

Conversely, the inhibition of D-amino acid oxidase, a flavoprotein, by structurally different inhibitors, such as atabrine, quinine, plasmopuine, sulfonamides, benzoic acid and *p*-aminobenzoic acid, (Hellerman *et al.*[343]), and the reversal of certain of these inhibitions by riboflavin shows that inhibitors of different structural make up exercise affinities for the protein moiety of a given flavoprotein. Reversal of these inhibitions by flavin

adenine dinucleotide shows that the coenzyme exercises a greater affinity than these inhibitors. Failure of FAD to reverse the inhibitions by benzoic acid and *p*-aminobenzoic acid may indicate that the latter two substances exercise a greater affinity for the protein than the former. Or it may be that these agents combine with groups of flavoprotein other than those involved in the combination with flavin-adenine-dinucleotide.

Reversal by adenylic acid, adenosine triphosphate, and partially by nucleic acid (Martin and Fisher,[333] Massart *et al.*[336a] Bovarnick *et al.*[342] Hellerman *et al.*[343]) of the inhibition by acridines has been stated to be due to chemical combination between the acridines (methylene blue as well) and the nucleotides and nucleic acids (Massart *et al.*[336a]). In this manner, the concentration of the acridines are reduced partially or completely in the reaction system, and the inhibition is thereby abolished. In these systems, apparently, the inhibitors exercise a stronger affinity for the nucleotides than for the proteins of the oxidative enzymes. Reversal by alkaline earth metals of the inhibitions by acridines (Massart *et al.*[368]) may be attributed to a reaction between the metals and the susceptible enzyme groups thus preventing inhibitions by acridines.

The data presented in the table show that atabrine and acridines inhibit the respiration of yeast, oxidation of glucose, pyruvate, succinate, hexosediphosphate, glucose-6-phosphate, phosphoglyceraldehyde and amino acids; they inhibit the activities of hexokinase, flavoprotein, cytochrome reductase various dehydrogenases, synthesis of thiamine, etc. of the various microorganisms. Therapeutically active diamidines act in a similar manner. In these respects these drugs resemble sulfonamides as discussed here. In view of the above types of inhibitions it could be concluded that these chemotherapeutic agents paralyze the parasites by blocking their critical enzymes, such as those involved in oxidative mechanisms.

Another effect would result from a combination between acridines and nucleoproteins and nucleic acids. Wagner-Jauregg[369] demonstrated the existence of stoichiometric compounds between adenylic acid or adenosine triphosphate on the one side, and acridines on the other. McIlwain[334] prepared such complexes using yeast nucleic acid and proflavine. Massart and his associates isolated a compound between rivanol (lactate of 2-ethoxy-5,7-diaminoacridine) and yeast nucleic acid containing 4 molecules of rivanol to 1 molecule of nucleic acid.

A combination between an acridine and adenylic acid and adenosine triphosphate would deprive any cell of its most important wheel of energy metabolism and therefore paralyze it completely. If we agree with the

[368] L. Massart, G. Peeters, and J. Wuyts-Robiette, *Arch. intern. pharmacodynamie* **75**, 162 (1947).

[369] Th. Wagner-Jauregg, *Z. physiol. chem.* **239**, 188 (1936).

hypothesis that the genes are nucleoprotein in nature, a combination between an acridine and nucleic acid (or nucleoprotein in a cell) could cause mutational changes evolving a resistance mechanism.

3. Streptomycin Action

Cohen[370] found that streptomycin combines with nucleic acids to form polymeric compounds. The size of the compounds depended on the combining ratios of the bivalent base to multivalent nucleates. He also reported that streptomycin precipitates *E. coli* phage. Bracco and von Euler[371] reported that streptomycin inhibits the formation of chlorophyll by seeds, e.g., barley. Their chloroplasts were free from chlorophyll. Streptomycin was likewise found to precipitate chloroplastin. Provasoli *et al.*[372] reported that streptomycin abolished the formation of chlorophyll by several strains of *Euglena gracilis* under both *nonproliferating*, and growth conditions. Loss of this ability persisted for generations in streptomycin-free media. Massart *et al.*[373,373a] found that streptomycin prevented the staining of yeast by trypaflavine.

Schuler[374] observed that streptomycin acts on the respiration of *E. coli* and staphylococci. The inhibition of the respiration of staphylococci by streptomycin is reported also by Hirsch and Dosdogru.[375] In both instances a period of latency preceded the active inhibition.

Fitzgerald *et al.*[376] reported that streptomycin inhibits the oxidation of benzoic acid by washed suspensions of *Mycobacterium tuberculosis* and *M. lacticola*. However, when the cells had been put through the induction period in the presence of 5 to 50 μg. benzoic acid, streptomycin did not exercise an inhibitory effect on the oxygen uptake of the post-induction period. They concluded that the induction period corresponded to the formation of an "adaptive enzyme" and that streptomycin had no inhibitory effect on the oxidation once this "adaptive enzyme" is formed.

Nelson and Dawson[377] have reviewed the literature and discussed the mechanism of the oxidation of the benzene nucleus in a critical manner. These authors, and Evans[378] who subjected to a careful chemical analysis

[370] S. S. Cohen, *J. Biol. Chem.* **166**, 393 (1946).
[371] M. Bracco and H. von Euler, *Kemiska Arbeten Nyföljd* II. **10** (1947).
[372] L. Provasoli, S. H. Hutner, and A. Schatz, *Proc. Soc. Exptl. Biol. Med.* **69**, 279 (1948).
[373] L. Massart, G. Peeters and A. Van Houcke, *Experientia* III, 289 (1947).
[373a] L. Massart, G. Peeters, J. de Ley, R. Vercauteren, and A. Van Houcke, *Experientia* **3**, 288 (1947).
[374] W. Schuler, *Experientia* **3**, 110 (1947).
[375] J. Hirsch and S. Dosdogru, *Arch. Biochem.* **14**, 213 (1947).
[376] R. J. Fitzgerald, F. Bernheim and D. B. Fitzgerald, *J. Biol. Chem.* **175**, 195 (1948).
[377] J. M. Nelson and C. R. Dawson, *Advances in Enzymol.* **4**, 99 (1944).
[378] W. C. Evans, *Biochem J.* **41**, 373 (1947).

the oxidation of phenols and benzoic acid by various bacteria, have shown that the induction period corresponds to the formation of mono- and dihydroxybenzene derivatives. These catalyze the accelerated oxidation of the benzoic acid. Addition of a few molecules of the preformed dihydroxybenzene derivatives to such systems eliminates the induction period. It is therefore possible that streptomycin inhibited the formation of mono- and dihydroxy derivatives from benzoic acid and not the production of an "adaptive enzyme."

Henry *et al.*[379] studied the aerobic oxidation of glucose, pyruvate, lactate and glycerol by *S. aureus* and *B. cereus* and found that acetate was the only substance tested for that was found to be present consistently in greater amounts in the presence of streptomycin than in the absence of streptomycin. Acetate also was found to accumulate in increased amounts during the endogenous respiration of the susceptible strain of *B. cereus* in the presence of inhibitory concentrations of streptomycin. The oxidation and utilization of acetate by the susceptible strain of *B. cereus* was completely blocked. With this organism, but not with *Staph. aureus*, the total hydrolyzable reducing substances of the cell were found in decreased amounts when growth occurred in the presence of streptomycin, which was interpreted to indicate a blockage of the oxidative assimilation of carbohydrate.

Under anaerobic conditions, streptomycin (1 μg. streptomycin/ml.) inhibited the growth, and the metabolism of glucose and pyruvate by *B. cereus*, and the metabolism of pyruvate by *S. sonnei*. With *Staph. aureus* the anaerobic growth was inhibited, but the anaerobic metabolism of pyruvate and glucose was not inhibited by streptomycin.

The accumulation of acetate resulting from the inhibition by streptomycin of the oxidation of glucose, pyruvate, lactate, and glycerol might indicate that the utilization of acetate for condensation reactions, and the initiation of the tricarboxylic acid cycle is inhibited. These results and those observed by Geiger and Umbreit seem to corroborate each other.

A real beginning was made by Geiger[380] in the study of the mode of action of streptomycin on bacteria, and was extended by Umbreit.[381]

Geiger showed that when the washed cells of *E. coli* are shaken for 3 hours with fumarate, or succinate, malate, oxalacetate, glucose, lactate, glycerol (but not pyruvate), the oxidation of serine, alanine, leucine, and glutamate were accelerated. The accelerated oxidations were inhibited by streptomycin. The inhibition of the oxidation by streptomycin of aspartate did not require the above pretreatment. Geiger interpreted these

[379] J. Henry, R. J. Henry, R. D. Housewright and S. Berkman, *J. Bact.* **56**, 527 (1948).
[380] W. D. Geiger, *Arch. Biochem.* **15**, 227 (1947).
[381] W. W. Umbreit, *J. Biol. Chem.* **177**, 703 (1949).

results to indicate that unidentified intermediates formed from the oxidation of fumarate were necessary for amino acid metabolism. The role of these hypothetical intermediates in the oxidation of amino acids was inhibited by streptomycin. The transamination reactions were not affected by streptomycin.

According to Umbreit, streptomycin prevents the formation of the hypothetical intermediate from fumarate, etc., rather than by interfering with its action once it is formed. The oxidation of both pyruvate and fumarate under suitable conditions is inhibited by streptomycin. Streptomycin is believed to prevent the oxidation of pyruvate by way of condensation with oxalacetate, which is supposed to be an intermediate of fumaric oxidation. The previous oxidation of fumarate stimulates the oxidation of threonine. This stimulation is likewise inhibited by streptomycin.

The following findings are of interest in connection with the above observations. Green *et al.*[382] reported that the addition of from 1 to 3% pyruvate or fumarate to nutrient broth supported growth of *E. coli* in the presence of from 10 to 150 μg./ml. of streptomycin. Salts of succinic, formic, malic, and maleic acids also exerted some antagonistic effect on streptomycin, lactose, glycerol, glycerophosphate, and lactic, acetic, and propionic acids had no effect on the growth-inhibiting action of streptomycin on *E. coli*. Pyruvate and fumarate also protected *P. vulgaris* but not *A. aerogenes* and *S. aureus*.

4. Action of Quinones on Enzymes in Relation to Antibacterial Action

Certain antibiotics, such as javanicin, fumigatin (3-hydroxy-4-methoxy-2,5-toluquinone), citrinin, etc. are quinone type of compounds. Fischer *et al.*[383] believe that the antibacterial activity of certain tetramethyldiaminodiphenylmethane dyes is due to the presence of quinoid structures.

The inhibition of various enzymes by quinones has been known for some time. *p*-Benzoquinone has been shown to inhibit muscle succinic dehydrogenase (Wieland and Frage,[384] Wieland and Lawson[385]), milk aldehyde dehydrogenase (Wieland and Mitchell[386]) alcohol dehydrogenase of *Acetobacter suboxidans* and yeast (Wieland and Pistor,[387] Wieland and Claren[388]), urease (Quastel[389]), papain (Bersin and Logemann,[390] Hoffmann-Osten-

[382] S. R. Green, W. P. Iverson, and S. A. Waksman, *Proc. Soc. Exptl. Biol. Med.* **67**, 285 (1948).
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[384] H. Wieland and K. Frage, *Ann.* **477**, 1 (1929).
[385] H. Wieland and A. Lawson, *Ann.* **485**, 193 (1931).
[386] H. Wieland and W. Mitchell, *Ann.* **492**, 156 (1932).
[387] H. Wieland and H. J. Pistor, *Ann.* **535**, 205 (1938).
[388] H. Wieland, O. B. Claren, and P. Couceiro, *Ann.* **509**, 182 (1934).
[389] J. H. Quastel, *Biochem. J.* **27**, 1116 (1933).
[390] T. Bersin and W. Logemann, *Z. physiol. Chem.* **220**, 209 (1933).

hof and Biach[391]), carboxylase (Kuhn and Bernert[392]), bacterial pyruvic oxidase (Wallenfels[393]) and plant lipoxidase (Süllmann[394]). Karrer and Viscontini[395] reported that the yeast carboxylase activity is reduced by high concentrations of various quinones, ketones, and phenols. Gemmill[396] reported that naphthoquinones inhibit glycolysis in frog muscle enzyme systems which could be reversed by cysteine.

Wendell[397] reported that 2-hydroxy-3-alkylnaphthoquinone and many of the naphthoquinones which are active against infections with *P. lophurae* and *P. knowlesi*, strongly inhibited the respiration of these parasites at concentrations as low as 10^{-6} *M*. Glucose is not affected, but lactate oxidation is inhibited by effective naphthoquinones. According to Ball *et al.*[398] 2-hydroxy-3-alkylnaphthoquinone probably inhibits malaria parasite and yeast respiration via cytochromes *b* and *c*. Bueding *et al.*[399] reported that methylnaphthoquinone inhibits aerobic glycolysis more than respiration in schistosomes. Michaelis and Thatcher[400] reported that 50 μg./ml. of citrinin inhibited the lactate dehydrogenase system and the oxygen uptake in glucose and lactate by *Staph. aureus*, and showed no effect on citrinin insensitive *E. coli* in these respects.

Wieland[401] demonstrated that quinones serve as hydrogen acceptors, and hydroquinones as hydrogen donators in biological oxidation systems. Luminescence involves hydrogen transfer (Johnson[402]). McElroy and Kipnis[403] reported that methylnaphthoquinone inhibits luciferin oxidation. As discussed earlier, Spruit and Schuiling[404] reported that 1,4-naphthohydroquinone causes 50% inhibition of luminescence, Redox indicators, methylene blue, pyocyanine, phthiocol, etc. similarly inhibited luminescence. These acted as hydrogen acceptors, shifting bacterial luciferin into a more oxidized state and thereby causing inhibition of luminescence. Suggestion has been made by Spruit (see Johnson[402]) that luciferin consists of 1,4-naphthohydroquinone containing a side chain with a ketohydroxy group substituted at position 2. The inhibition of luminescence by 1,4-naphtho-

[391] O. Hoffmann-Ostenhof and E. Biach, *Experientia* **2**, 405 (1946).
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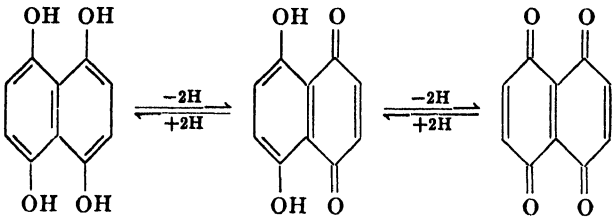
[393] K. Wallenfels, *Chemie. Die* **58**, 1 (1945).
[394] H. v. Süllmann, *Helv. Chim. Acta* **26**, 2253 (1943).
[395] P. Karrer and M. Viscontini, *Helv. Chim. Acta* **30**, 268 (1947).
[396] C. L. Gemmill, *J. Pharmacol.* **95**, 116 (1949).
[397] W. B. Wendel, *Federation Proc.* **5**, 406 (1946).
[398] E. G. Ball, C. B. Anfinsen, and O. Cooper, *J. Biol. Chem.* **168**, 257 (1947).
[399] E. Bueding, L. Peters, and J. F. Waite, *Proc. Soc. Exptl. Biol. Med.* **64**, 111 (1947).
[400] M. Michaelis and F. S. Thatcher, *Arch. Biochem.* **8**, 177 (1945).
[401] H. Wieland, Über den Verlauf der Oxydations vorgange. Ferdinand Enke Verlag, Stuttgart, 1933; Silliman Lectures, Yale Univ. New Haven, Conn., 1931.
[402] F. H. Johnson, *Advances in Enzymol.* **7**, 215 (1947).
[403] W. D. McElroy and D. M. Kipnis, *J. Cellular Comp. Physiol.* **30**, 359 (1947).
[404] C. J. P. Spruit and A. L. Schinling, *Rec. trav. chim.* **64**, 219 (1945).

hydroquinone appears to be an interference with the hydrogen transfer involved in luminescence. Not finding a parallelism between the antibacterial action of quinones and the inhibition exercised on urease, catalase and papain-like enzymes of bacteria, it was pointed out by Hoffmann-Ostenhof[405]) that the most active antibacterial quinone, 2-methylnaphthazarine (a derivative of 5,8-dioxynaphthaquinone), possibly undergoes a reversible oxidation reduction.



Dihydro-naphthazarine Naphthazarine Diquinone

Of the naphthazarines, javanicin (Arnstein *et al.*[406]) has been reported to exercise the strongest antitubercular activity. Javanicin contains acetonyl substitution giving perhaps to this substance lipophilic properties to account for its activity against acid fast bacteria. Wallenfels[394] advanced the hypothesis that the antibacterial action of quinones might be due to an interference with carboxylase, pyruvic, and other dehydrogenases.

Potter and DuBois[407] have discussed the possibility that quinones react with sulfhydryl groups in succinoxidase. Quinones are believed to inactivate the sulfhydryl containing enzymes such as urease, succinic oxidase, etc. It has been proposed by several investigators (Cavallito and Haskell,[408] Geiger and Conn,[409] Callwell and McCall[410]) that quinone type of antibiotics function by inactivating the sulfhydryl groups of bacterial enzymes, since these inhibitions are reversed by cysteine, thioglycollate, etc. Cavallito *et al.*[411] reported that the inhibitory action of gliotoxin, which possibly has a dithio structure, is reversibly inactivated by cysteine. They assume that —S—S— group of gliotoxin can oxidize the —SH group of enzymes. However, Stanley and Mills[412] report that hydrogen peroxide and potassium permanganate inactivate gliotoxin, an effect which does not appear to point to an —S—S— $\rightleftharpoons$ 2—SH relationship between gliotoxin and the

[405] O. Hoffman-Ostenhof, *Experientia* III 137, 176 (1947).
[406] H. R. V. Arnstein, A. H. Cook, and M. S. Lacey, *Nature* **157**, 333 (1946).
[407] V. R. Potter and K. P. J. DuBois, *J. Gen. Physiol.* **26**, 391 (1943).
[408] C. J. Cavallito and T. H. Haskell, *J. Am. Chem. Soc.* **67**, 1991 (1945).
[409] W. B. Geiger and J. E. Conn, *J. Am. Chem. Soc.* **67**, 112 (1945).
[410] C. A. Callwell and M. McCall, *J. Bact.* **51**, 659 (1946).
[411] C. J. Cavallito, J. H. Bailey, and W. F. Warner, *J. Am. Chem. Soc.* **68**, 715 (1946).
[412] N. F. Stanley and J. M. Mills, *Australian J. Exptl. Biol. and Med. Sci.* **24**, 133 (1946).

sulfhydryl groups of enzymes. According to Ball *et al.*[398] the inhibition by 2-hydroxy-3-alkylnaphthoquinones of the respiration of malaria parasite and yeast did not seem to be due to effects upon sulfhydryl systems and was not reversed by cysteine. Hoffmann-Ostenhof as discussed above could not find a parallelism between the antisulfhydryl and antibacterial activities of quinones.

5. Penicillin Action

Despite many attempts to elucidate the mechanism of the antibacterial action of penicillin our knowledge remains limited. Chain *et al.*[413] reported that, during the resting phase, even large concentrations of penicillin had no effect on the rate of oxygen uptake by staphylococci. During the early lag and logarithmic phases of multiplication, penicillin exerted a strong inhibitory effect on, and eventually completely stopped, the oxygen uptake of the bacteria. Hirsch and Dosdogru[375] reported that penicillin halts respiration of staphylococci after a latency period. Similarly, Schuler[374] found that penicillin acts on the respiration of *E. coli* and staphylococci after a latent period. Krampitz and Werkman[363] reported that penicillin G interferes with the dissimilation of ribonucleic acid and consequently with its assimilation during growth. Gale[364] proposed that penicillin interferes with the assimilation of amino acids by Gram-positive organisms. Of the amino acids he tested, the diffusion of glutamic acid, glutamine, aspartic acid, and histidine into the interior of the cells was assumed to require an energy source, such as glucose. It would seem difficult, however, to know whether the blockage of the passage of amino acids into the cells is a result of the bactericidal action of penicillin or of a direct interference. The findings of Hunter and Baker[413a] would appear to contradict the postulate of Gale that penicillin interferes with the assimilation of preformed amino acids rather than their synthesis. They showed that the growth of a strain of *B. subtilis* (non-producer of penicillinase) which utilized ammonium salts as source of nitrogen and grew in a salt glucose synthetic medium devoid of amino acids, was just as sensitive to penicillin as it was in a "complete" medium (tryptose phosphate buffer) regardless of wider changes in the age of the culture, the size of the inoculum, and the time of reading. Gros and Macheboeuf[357] reported that penicillin inhibits the hydrolysis of adenosine triphosphate by washed suspensions of *Clostridium sporogenes*.

Cavallito and Bailey[414] and Osborn[415] have reported that cysteine antagonizes penicillin. Cavallito found that cysteine inactivates penicillin,

[413] E. Chain, E. S. Duthie, and D. Callow, *Lancet* **1**, 652 (1945).
[413a] T. H. Hunter and K. T. Baker, *Science*, **110**, 423 (1949).
[414] C. J. Cavallito and J. H. Bailey, *Science* **100**, 390 (1944).
[415] E. M. Osborn, *Brit. J. Exptl. Path.* **24**, 227 (1943).

citrinin, gliotoxin, clavacin, pyocyanine, etc. Cavallito *et al*[416] believe that a large class of antibacterial agents act by interfering with the sulfhydryl groups of enzymes. Cavallito[417] considers it not necessary that penicillin bind protein —SH in direct proportion to the number of —SH groups in proteins, since some types of —SH groups react only sluggishly or not at all with penicillin. In an intensive study, Cavallito[418] reported that the ideal thiol type of inactivation for penicillin should possess a basic amino group (primary through tertiary) on the carbon adjacent to the carbon carrying the sulfhydryl group, with substitutents on the nitrogen smaller than ethyl (methyl or hydrogen). The cysteine unit in proteins would, therefore, show greatest reactivity toward penicillin when its amino group is not acetylated. Whether such groups or cysteine units exist in intact enzyme proteins is not known.

According to Simon,[419] the molecular ratio of cysteine to penicillin necessary for inactivation decreases as the concentration of penicillin decreases. At high concentrations of penicillin a ratio representing fewer molecules of cysteine than of penicillin is sufficient for inactivation. Iodine solution fails to reverse the inactivation of penicillin by cysteine. On this basis, a reaction between cysteine and penicillin resulting in the disintegration of penicillin is surmised. This reaction is assumed to liberate a substituted cysteine compound of penicillamine type, which can then start a chain reaction by combining with more penicillin.

The inactivation of penicillin by cysteine is different from those of streptomycin, and arsphenamines (Eagle[420]). In the latter case an oxidation reduction with the As=As group of arsphenamines, using four —SH groups, occur. In streptomycin, a combination with at least two molecules of cysteine is considered to take place for complete inactivation. Iodine reverses this combination, setting the active streptomycin free.

In connection with the inactivation of the enzyme protein —SH by antibiotics as the mode of their action, Diczfalusy and von Euler[421] pointed out that if the —SH groups in the enzyme proteins are the common site of action of penicillin, streptomycin, pyocyanine, gliotoxin, etc. resistance to penicillin developed by staphylococci should increase their resistance to the other antibiotics. But, in no way was the sensitivity to any of the other antibiotics affected.

[416] C. J. Cavallito, J. H. Bailey, T. H. Haskell, J. R. McCormick, and W. F. Warner, *J. Bact.* **50**, 61 (1945).
[417] C. J. Cavallito, *Science* **105**, 235 (1947).
[418] C. J. Cavallito, *J. Biol. Chem.* **164**, 29 (1946).
[419] R. D. Simon, *Brit. J. Exptl. Path.* **29**, 202 (1948).
[420] H. Eagle, *J. Pharmacol.* **66**, 436 (1939).
[421] E. Diczfalusy and H. v. Euler, *Arkiv. Kemi Mineral. Geol.* **25A**, 1 (1947).

Chapter 5

Cytochemical Foundations of Enzyme Chemistry

By A. L. Dounce

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I. Introduction

In recent years, attention has been increasingly directed to a study of discrete units of the cell from the standpoint of general chemical constitution and enzymatic function. It seems apparent that a mixture in a single aqueous phase of all of the enzymes known to occur in a cell could hardly carry out the manifold and orderly enzymatic activity known to go on in cells, and therefore it becomes necessary to attempt to analyze the distribution of enzymes and their substrates among various components of the cell. Some of the techniques that have been applied for this purpose will be discussed in this chapter. However, neither time nor space will permit an exhaustive survey of the literature pertaining to all phases of cytochemistry. It is to be hoped that authors whose work has not been included will be charitable enough to realize that some selection of material has been inevitable, and that in addition the writer no doubt is unaware of many papers that have been published in the field of cytochemistry. In general, work will be covered that seems to have bearing on the enzymatic constitution of the various cellular components and on the relationships between enzyme distribution and cell physiology. The localization of substrates in various parts of the cell will be considered to some extent as well as the intracellular distribution of the enzymes, since both substrates and enzymes must be taken into account if cellular metabolism is to be understood.

The various cellular components or cellular regions which will be discussed are the cytoplasm as a whole as well as the various cytoplasmic granules, including the mitochondria, secretory granules, and microsomes; the Golgi apparatus; and the nucleus as a whole, as well as its constituents, the chromosomes and the nucleolus. Emphasis will be placed upon physicochemical techniques for separating the various cell components in relatively large quantities, but histochemical techniques and microdissection also will be touched upon in a later section.

II. Enzyme Chemistry of Cytoplasm as Deduced from Physicochemical Studies

If we consider an actively metabolizing cell such as the liver cell, whose nucleus comprises only 6 to 10% of the total cellular volume,[1] it seems almost certain that the cytoplasm must account for a greater amount of the metabolism than the nucleus, assuming that the enzymes and substrates involved are about equal in concentrations in the cytoplasm and the nucleus. This argument is strengthened by the knowledge that thus far only a few enzymes seem to be higher in concentration in the cell nucleus than in the cytoplasm, while certain enzymes, such as succinic dehydrogenase, appear to be lacking in the nucleus.[2, 3] Since the cytoplasm thus appears to be of great importance in cellular metabolism, and since cellular metabolism, especially of a cell such as the liver cell, is of great complexity, it is logical to attempt to analyze the structure of cytoplasm by all available methods in order to render comprehensible the manifold and complex reactions taking place there.

The above statements should, however, not be construed in such a way as to minimize the importance of metabolism that may go on in the cell nucleus. This metabolism must be of primary importance in dividing cells; in addition indirect evidence is available which indicates that the cell nucleus may be vital even to resting cells, and hence be an actively functioning unit here rather than an inert body that becomes active only during cell division. This point will be discussed later on.

In this section on cytoplasmic components, the following topics will be discussed: mitochondria, secretory granules, microsomes, particulate glycogen, miscellaneous particles of animal cell cytoplasm, and chloroplasts of plant cells. The evidence presented will be chiefly that obtained by a study of material separated by physicochemical methods. Evidence obtained by other methods will be briefly considered later on.

1. Large Granules

a. Mitochondria

The mitochondria are discrete bodies visible in the cytoplasm of many types of cells following the use of proper fixation and staining.[4, 5] The stain Janus Green B is stated to be quite specific for mitochondria.[7, 8, 9] Mito-

[1] A. Marshak, *J. Gen. Physiol.* **25**, 275 (1941).
[2] W. C. Schneider, *J. Biol. Chem.* **165**, 585 (1946).
[3] A. L. Dounce and G. T. Beyer, *J. Biol. Chem.* **174**, 585 (1948).
[4] E. V. Cowdy, editor, General Cytology. Univ. of Chicago Press, Chicago, 1924.
[5] E. B. Wilson, The Cell, Macmillan, New York, 1925.
[6] G. Bourne, editor, Cytology and Cell Physiology. Clarendon Press, London, 1942.
[7] L. Michaelis, *Arch. mikroskop. Anat. Entwicklungsmech.* **55**, 558 (1900).

chondria are roughly about 1 μ in diameter, but they tend to be elongated and of non-uniform shape. The reader is referred to references 4, 5, and 6 for details of the cytology of mitochondria. Many of the conclusions reached by cytological techniques alone should perhaps be taken with a large grain of salt, as will be seen by comparing them with conclusions derived from direct chemical experiments. An excellent electron microscopic photograph of guinea pig liver cells showing the mitochondria has been published by Claude and Fullham.[10] A photograph of mitochondria of liver cells, isolated by an improved method,[21] is shown in Fig. 1.

Bensley and Hoerr have been accredited by Lazarow[11] as the first to isolate mitochondria from differential centrifugation of disrupted cell preparations.[12] Guinea pig liver was the source of material. The mitochondria were isolated by differential centrifugations of mortar-ground liver suspended in cold 0.82% NaCl of a pH of about 6.0. The authors stated that during the procedure the mitochondria swelled and became cone-shaped or round; a small amount of material also seemed to be lost from them. The total lipide content was about 43 to 44%, but lecithin and cephalin were said to be low or absent. The preparation was examined microscopically at various stages after suitable staining to determine the state of purity.

It seems possible that Bensley and Hoerr had preparations of mitochondria in 1934 which could have been used for extensive enzyme studies. Their failure to find phospholipid is however undoubtedly an error.

Extensive work has been carried out by A. Claude on the fractionation of cell granules by differential centrifugation of saline extracts of various types of cells. In 1940, Claude[13] reported the chemical composition of particles isolated from chick embryo cells, mouse embryo cells, chicken tumor I, a spontaneous mouse tumor, and a transplantable mouse sarcoma originally produced by benzpyrene. The chemical composition of these particles isolated from all of these tissues was quite similar. The particles were stated to be of sizes ranging from 50 to 150 mμ in diameter, and were thought to represent isolated mitochondria, chiefly because of the chemical composition and because of a statement by Cowdry[8] that in certain instances the breadth of mitochondria could range from 50 to 200 mμ. Chemical analysis of the particles showed that they consisted of lipide, protein, and ribonucleic acid, while histochemical studies[8, 14, 15] of mitochondria had indicated that these bodies were composed of protein and phospholipides in large part.

[8] E. V. Cowdry, *Carnegie Inst. Washington Pub. No. 271. Contrib. Embryol.* **8**, 39 (1918).
[9] R. Noel, *Arch. Anat. micros.* **19**, 1 (1923).
[10] A. Claude and E. F. Fullham. *J. Exptl. Med.* **83**, 499 (1946).
[11] A. Lazarow, *Biol. Symposia* **10**, 9 (1943).
[12] R. R. Bensley and N. L. Hoerr, *Anat. Record* **60**, 449 (1934).
[13] A. Claude, *Science* **91**, 77 (1940).
[14] C. Regaud, *Compt. rend. soc. biol.* **65**, 718 (1908).
[15] A. Guilliermond, Les Constituants Morphologiques du Cytoplasm. Hermann et Cie, Paris, 1934.

In 1941, Claude[16] reiterated his belief that the fine granules isolated from certain cells were mitochondria. To illustrate his point, he presented dark field photographs of cells of a transmittable rat leukemia showing granules, presumably mitochondria, within the cells, and some of his isolated particles were photographed in the same field. The sizes and shapes of the intracel-

FIG. 1. Photograph of isolated liver cell mitochondria. Fixation, 1% O_3O_4; staining 1% safranine; magnification, 2400 ×. (Hogeboom, G. H., Schneider, W. C., and Pallade, G. E., *J. Biol. Chem.* **172**, 619, 1948.)

lular particles and isolated particles were thought to compare favorably. In the same paper, a study of the granules of guinea pig liver cells led him to the conclusion that the large granules, thought by Bensley and Hoerr to be mitochondria[12] were rather granules of a different nature called secretory

[16] A. Claude, *Cold Spring Harbor Symposia Quant. Biol.* **IX**, 263 (1941).

granules, and it is implied that here too the mitochondria were the fine particles.

In 1943, Claude[17] abandoned the idea that the small particles, of sizes ranging from 50 to 150 mμ were mitochondria and proposed the term *microsome* to describe them. The large granule fraction was still referred to as the secretory granule fraction however.

In 1944,[18] the suggestion was made by Claude that the large granule fraction of liver cells, consisting mainly of particles from 0.5 to 3 μ in diameter were mixtures containing unknown proportions of mitochondria and secretory granules, and this view has been commonly held until recently. In later papers, Hogeboom *et al.*[19] and Claude[20] were of the opinion that the large granule fraction which could be isolated from disrupted liver cells by differential centrifugation consisted chiefly of mitochondria. One line of evidence which these authors offered in favor of this hypothesis is that the secretory granules of the liver cell can be caused to diminish greatly in number by feeding the animal, while the mitochondria remain as before feeding. Therefore by avoiding the use of fasted animals, the only large granules of abundance in the cytoplasm are the mitochondria.

One of the chief obstacles in identifying the large granule fraction prepared from cell homogenates was the swelling of the mitochondria in isotonic salt or buffer solutions. This swelling, which apparently could not be entirely avoided even by careful control of the pH and use of low temperature throughout the isolation procedure, caused the mitochondria to assume rounded or spherical shapes so that they were no longer identifiable by their microscopic appearance. If distilled water instead of saline solutions was used for washing, the swelling was worse and eventually complete disruption and disappearance of the mitochondria occurred. This obstacle has recently been overcome by the use of hypertonic sucrose solutions as a medium instead of the saline solutions formerly employed. A detailed account of the new procedure is given by Hogeboom *et al.*[21] (See photograph of isolated mitochondria in Fig. 1 of this chapter, which is taken from this paper.) The large granule fraction thus isolated was stated to consist almost entirely of mitochondria, which stained with Janus Green B after treatment with aniline acid fuchsin. The secretory granules which were originally present in the homogenate were stated to migrate centripetally with the fat globules and thus to be removed from the mitochondria. Moreover, a proportion of the secretory granules were thought to disintegrate in

[17] A. Claude, *Science* **97**, 451 (1943).

[18] A. Claude, A.A.A.S. Research Conference on Cancer. American Association for the Advancement of Science, Washington, D. C., 1945, p. 223.

[19] G. H. Hogeboom, A. Claude, and R. D. Hotchkiss, *J. Biol. Chem.* **165**, 615 (1946).

[20] A. Claude, *J. Exptl. Med.* **84**, 61 (1946).

[21] G. H. Hogeboom, W. C. Schneider, and G. E. Pallade, *J. Biol. Chem.* **172**, 619 (1948).

the sucrose solutions, thus being lost. In this laboratory, it has been found that the method of preparing mitochondria of Hogeboom, Schneider, and Pallade (21) using hypertonic sucrose solutions is relatively easy to carry out and that it gives preparations of excellent microscopic appearance (unpublished).

The work of Hogeboom, Schneider, and Pallade undoubtedly represents a major advance in the isolation of mitochondria from cell homogenates. The question of whether the mitochondria thus prepared are contaminated with an appreciable proportion of secretory granules will gradually be settled as new investigations repeat and extend this work. At the present time the claims of the authors to have isolated highly purified mitochondria appear to be well founded.

The enzymatic composition of mitochondria is at the present time under investigation and only a relatively meager amount of authentic information is available. Much of the information that is at hand derives from studies of large granule fractions which were obtained before the use of hypertonic sucrose solutions was introduced.

Warburg[22] has been accredited by Claude[23] as the first to make a systematic attempt to separate cytoplasmic granules by differential centrifugation. Warburg's cell particles no doubt were impure; but enough information was obtained to make it appear likely that most or all of the oxygen consumption of the cell-free extracts of guinea pig liver was attributable to the large granules contained therein. Lazarow[11] and Barron[24] reported that large granules obtained from guinea pig liver consumed oxygen and possessed succinic dehydrogenase activity. However, this was also true of fractions composed of smaller particles. Stern[25, 26] stated that cytochrome oxidase and succinic dehydrogenase were both associated with particles 50 to 200 mμ in diameter obtained from heart muscle. But Hogeboom *et al.*[19] found that the cytochrome oxidase and succinic dehydrogenase activity of particulate components of the cytoplasm of rat liver was carried largely or exclusively by the large granules, now thought to be the mitochondria. Moreover, the activity was entirely associated with particulate components and was not present in the supernatant solutions. The same findings were independently reported by Schneider[2], and were confirmed by Schneider *et al.*[27] Finally, repetition of this work by Hogeboom *et al.*[21] showed that the cytochrome oxidase and succinic dehydrogenase activity of highly purified mitochondria was associated exclusively with the large granule fraction

[22] O. Warburg, *Arch. ges. Physiol.* (*Pflüger's*) **154**, 599 (1913).
[23] A. Claude, *J. Exptl. Med.* **84**, 51 (1946).
[24] E. S. G. Barron, *Biol. Symposia* **10**, 27 (1943).
[25] K. G. Stern, *Cold Spring Harbor Symposia Quant. Biol.* **7**, 312 (1939).
[26] K. G. Stern, *Biol. Symposia* **10**, 291 (1943).
[27] W. C. Schneider, A. Claude, and G. H. Hogeboom, *J. Biol. Chem.* **172**, 451 (1948)

of liver cell homogenates prepared by the use of hypertonic sucrose, which, as has been stated, probably consists almost entirely of mitochondria.

Recent work by Lehninger[28] and by Schneider[29] has also demonstrated that the fatty acid oxidase of liver cells is carried by the mitochondria. The latter were prepared by sucrose method.

Schneider[2] made a detailed investigation of the distribution of the enzymes cytochrome oxidase, succinic dehydrogenase, and adenosine triphosphatase in rat liver cells. Mitochondria, an unfractionated supernatant, and a nuclear fraction were separated, using saline solutions as dispersing media. The nuclear fraction contained some mitochondria and whole cells. The cytochrome oxidase and succinic dehydrogenase were found to be in greatest concentration in the mitochondria, where their concentration was about four times that of the whole tissue. Since the concentrations of these enzymes were very low in the supernatant from isolation of the mitochondria (unfractionated residue) and since the concentrations in the nuclear fraction were only about half those in whole tissue, it is not surprising that 70% or more of the total amounts of these enzymes were found in the mitochondria. The ratio of cytochrome oxidase to succinic dehydrogenase was the same in the three fractions, and moreover Schneider emphasizes the fact that all of the enzymatic activity of the homogenate could be accounted for by adding the activities present in the various fractions. In contrast to cytochrome oxidase and succinic dehydrogenase, adenosine triphosphatase was found to be more generally distributed among the three fractions.

Schneider also compared primary hepatomas of rats with normal rat liver in respect to distribution of enzymes along the cell fractions listed above.[30] Numerous chemical analyses also were made. There seemed to be less large granular (mitochondrial) material in the hepatoma cells than in the normal liver cells. Succinic dehydrogenase had disappeared from the hepatoma tissue, while adenosine triphosphatase had migrated from the mitochondria to the unfractionated residue. Cytochrome oxidase was lower in the tumor than in the normal tissue. As with normal tissue, cytochrome oxidase was associated chiefly with mitochondria in the case of the hepatoma.

The adenosine triphosphatase concentration was about equal in the nuclear and in the large granule fraction in the case of normal rat liver, but in the hepatoma its concentration in the large granule fraction was considerably higher than in the nuclei.

In fairness to the author, it should be stated that in the last two papers mentioned, he referred to a "large granule fraction" rather than to mitochondria. However, the more recent work of Hogeboom *et al.*,[21] already mentioned, makes it seem probable that the particles consisted principally of mitochondria.

The study of the distribution of enzymes in malignant tissue as derived from Schneider's work with hepatomas may or may not be generally valid, since he appar-

[28] A. L. Lehninger, *J. Biol. Chem.* **172**, 847 (1948).
[29] W. C. Schneider, *J. Biol. Chem.* **176**, 259 (1948).
[30] W. C. Schneider, *Cancer Research* **6**, 685 (1946).

ently used primary hepatomas. These primary hepatomas contain unknown numbers of nonmalignant cells and also presumably still contain the poisonous products derived from dimethylaminoazobenzene and therefore can be expected to differ from transplanted tumors.

From the standpoint of enzyme chemistry, the result of greatest certainty established by Schneider was the occurrence of high concentrations of the three enzymes studied in the mitochondria, and the fact that most of the cytochrome oxidase and succinic dehydrogenase of the cell are to be found in the mitochondria. The enzyme studies on the nuclear fraction may be less valid because of the uncertainty about the state of purity of the latter fraction.

The distribution of cytochrome C in the cytoplasm of rat liver cells has also been studied by Schneider *et al.*[27] The distribution and activity of cytochrome C was markedly affected by the medium used during the isolation procedures. Thus when water was used as the suspending medium, cytochrome C was found both in the large granule (mitochondria) fraction and the microsome fraction. The cytochrome C in the large granule fraction was in this case only slightly active in the succinic oxidase system, and it was thought by the authors that a secondary adsorption of dissolved cytochrome C by the large granules and microsomes had occurred. This idea was strengthened by the observation that the cytochrome C could be largely removed both from the large granules and the microsomes (previously obtained from water suspensions) by washing with isotonic saline.

When the large granules (mitochondria) were isolated by differential centrifugation, using isotonic saline instead of water as the suspending medium, most of the cytochrome C was in the large granules, and furthermore this cytochrome C was active in the succinic oxidase system. The microsomes in this case were found not to contain cytochrome C. This work demonstrated the importance of the type of medium employed in preparing cell constituents, and showed that redistribution among cell particulates of at least some enzymatic components can occur if the proper medium is not used. The swelling of the mitochondria in water probably initiated the redistribution of cytochrome C when water was used as suspending medium. That the distribution of all enzymes is not easily affected by the type of suspending medium used is shown by the fact that cytochrome oxidase and succinic dehydrogenase distribution was the same whether water or saline was employed. In both cases these two enzymes always occurred in the mitochondria and not in the microsomes or in the supernatant solution.

The effect of the medium, especially of saline, on the composition of cellular constituents recalls to mind the fact that a large amount of protein can be removed from liver cells simply by washing with saline.[31] The question arises as to the source of this protein. Does it come from mitochondria, from

[31] A. E. Mirsky and A. W. Pollister, *Proc. Natl. Acad. Sci.* **28**, 344 (1942).

microsomes, or from the continuous internal aqueous phase now believed to exist within the cell?[32] If even part of this protein comes from the mitochondria, it follows that a saline solution has limitations as a medium for the isolation of mitochondria, and the same argument might be applied to the preparation of mitochondria using hypertonic sucrose solutions. Thus, it can be seen that considerable study of the effect of conditions must be made when the distribution of new enzymes within the cytoplasm is studied by methods similar to those outlined above.

The dispersion medium can have an important effect on the activity of enzymes of large granules of the liver even after isolation. Thus Lehninger[28, 33] found that the fatty acid oxidase of liver cells was not active in the absence of neutral salt ions such as NaCl or KCl. This is most probably an effect of the neutral salt ions on the physiochemical integrity of the granules rather than a direct activating effect on the enzyme. Potter has made similar observations.[34, 35] He found that aqueous suspensions of cell granules prepared according to Lehninger would not oxidize octanoate, whereas suspensions prepared using saline would catalyze the oxidation. Potter at first was inclined to ascribe these results to the presence of unbroken cells in the saline-prepared material, but later on[36] stated that the results were related to the integrity of some subcellular unit which we now realize must have been the mitochondria.

Claude in 1944[18] summarized findings on the distribution of some enzymes in the cells of mammalian liver, before the use of a strong sucrose solution as the homogenizing medium had been introduced. Cytochrome oxidase, succinic dehydrogenase, α-glycerophosphate dehydrogenase, D-amino acid oxidase, adenylpyrophosphatase, phosphatase, and ribonuclease were found to be associated principally with the large granule fraction, and hence according to present concepts, with the mitochondria. The coenzyme for D-amino oxidase had to be added to the washed granules to restore full activity of this enzyme, since the coenzyme was apparently lost as the large granules were washed. Catalase was present in the purified large granules, but most of this enzyme was found in the supernatant after centrifugation of the large granules and microsomes. Transaminase was present but low in concentration in the large granules, being mostly in the supernatant. Malic dehydrogenase and a phosphate transferring enzyme acting upon adenosine triphosphate also were in the supernatant. The microsomes (to be discussed later on) were not found to contain any of the above-mentioned enzymes in significant amounts.

[32] A. Claude and R. Chambers, *Biol. Symposia* **10**, 91; 111 (1943).
[33] A. L. Lehninger and E. P. Kennedy, *J. Biol. Chem.* **173**, 753 (1948).
[34] V. R. Potter, *J. Biol. Chem.* **163**, 437 (1946).
[35] V. R. Potter and H. L. Klug, *Arch. Biochem.* **12**, 241 (1947).
[36] V. R. Potter, *J. Biol. Chem.* **169**, 17 (1947).

Quite recently, Schneider and Potter[37] have found that oxaloacetic oxidase activity is present in liver cell mitochondria to the extent of about 45% of the activity of whole homogenate, and in kidney mitochondria to the extent of about 32% of the activity of whole homogenate. However, the addition of nuclei, which carry only 8% of the activity of kidney homogenate, to the isolated mitochondria increases the activity of the latter up to the value of about 62% of that of whole homogenate. An explanation for this latter finding is not available at present. In this work, the mitochondria were prepared using hypertonic sucrose solution.

Bensley has recently published a paper[38] indirectly concerned with the enzyme composition of mitochondria. He concluded that the principal pigment of isolated mitochondria is oxidized phospholipide. He assumes that oxidation of phospholipide occurs in the mitochondria in the intact cell, and that the mitochondria may simply be temporary aggregates of complex composition, "consisting of a number of major components and many trace substances including enzymes and vitamins." He believes that . . . "this idea is further supported by the fact that in prolonged inanition the mitochondria rapidly disappear from the pancreatic cell and are rapidly reconstituted after feeding. These structures are in no sense permanent organelles of the protoplasm but, in case of need, may be utilized for the nourishment of the cell." The latter conclusion apparently was reached by Bensley earlier.[39]

This concept is certainly not in accord with the idea of a definite morphology of the mitochondria including possibly an external membrane and an internal structure.[40, 41] It is moreover not in accord with the most recent concepts of the enzymatic composition of mitochondria.

Before leaving the topic of mitochondria, it might be well to include a summary of their general composition. If the large granule fraction of mammalian liver, obtained by Claude, is considered to consist chiefly of mitochondria, rather than secretory granules, the chemical composition of mitochondria of guinea pig liver is approximately as shown in Table I.[20]

A more recent paper by Barnum and Huseby[42] also gives analyses of the large granule fraction from mouse liver. Their work is summarized in Table II.

In summarizing this section on mitochondria it can be stated that these bodies apparently have been isolated from cells, at least from mammalian liver cells, in relatively pure state, and that they contain most of the cytochrome oxidase and succinic dehydrogenase of the cell. They also contain other enzymes, and probably at least one coenzyme, namely, the coenzyme for D-amino oxidase (flavine-adenine-dinucleotide). They appear to possess an internal structure and possibly a limiting membrane. In gross chemical

[37] W. C. Schneider, and V. R. Potter, *J. Biol. Chem.* **177**, 893 (1949).
[38] R. R. Bensley, *Anat. Record* **98**, 609 (1947).
[39] R. R. Bensley, *Anat. Record* **69**, 341 (1937).
[40] A. Claude and E. R. Fullam, *J. Exptl. Med.* **81**, 51 (1945).
[41] K. R. Porter, A. Claude, and E. F. Fullam, *J. Exptl. Med.* **81**, 233 (1945).
[42] C. P. Barnum and R. A. Huseby, *Arch. Biochem.* **19**, 17 (1948).

composition, they appear to consist of protein, ribonucleic acid, and lipide, including a large percentage of phospholipide. The ash is appreciable (in the neighborhood of 4%).

b. Secretory Granules

Less information about secretory granules is available from physicochemical studies than about the mitochondria. The secretory granules are relatively large granules (1 to 5 μ in diameter), which are more nearly

TABLE I

COMPOSITION OF MITOCHONDRIA OF GUINEA PIG LIVER

Material	Percentage
—SH—	Present
Lipide	About 25
Inositol	About 0.5
Phospholipide	About 17
Acetalphospholipide	Present
Nitrogen	About 11
Phosphorous	About 1
Sulfur	About 1
Copper	About 0.03
Iron	About 0.03
Ash	About 4

TABLE II

COMPOSITION OF LARGE GRANULE FRACTION OF GUINEA PIG LIVER

Material	Percentage
N	12.1
P	1.11
Ribonucleic acid	3.7
Lipide	27.4
Phospholipide as per cent of total lipide	56.6

round than the mitochondria, and which form in cells known to secrete enzymes, mucins, etc. In some cases they can be seen in a different part of the cell from the area occupied by the mitochondria. They tend to increase in numbers shortly before the cells secrete. The salivary glands and the pancreas are good examples of organs that secrete enzymes, and whose secreting cells produce secretory granules, or zymogen granules as they are more apt to be called when composed chiefly of enzymes. Liver cells also contain secretory granules; in this case the granules accumulate on fasting and tend to disappear after feeding.[20] In the case of the liver, it is possible that the

secretory granules are responsible for the formation of bile; but it should also be recalled that the liver produces plasma proteins such as albumin, thrombin, and fibrinogen. Whether these proteins are formed in the cell as secretory granules cannot be stated. Mast cells are another example of cells that form secretory granules; heparin occurs in these cells as secretory granules[43, 44]. (Earlier references are given in these papers.)

If cells are studied that contain considerable numbers of secretory granules in addition to the mitochondria, it seems very probable that the isolation of pure mitochondria or pure secretory granules could not be accomplished by the usual differential centrifugation technique, even with the use of strong sucrose solution as suspending medium. Instead, one would expect to obtain a large granule fraction consisting of secretory granules and mitochondria in roughly the proportions found in the intact cell. A possible method of effecting a separation of such a mixture into its two components would be specific gravity technique, such as that used by Behrens in obtaining nuclei (see below under nuclei), if it should happen that the specific gravity of secretory granules and mitochondria were of sufficient difference to permit application of this procedure.

Claude in his earlier work on the large granules of mammalian liver believed that this fraction was composed of unknown proportions of mitochrondria and secretory granules. It has been explained already that the proportion of secretory granules in the liver cell can be considerably lowered by feeding the animal, and that in any case the secretory granules of the liver cell do not appear to be very stable and also tend to migrate to the centripetal pole of the centrifuge tube in strong sucrose solutions[21]. Since Claude found little difference in composition of his large granules according to whether he used fasted or fed animals, it seems likely, especially in view of the two findings just mentioned, that his large granule fraction consisted mainly of somewhat swollen mitochondria, and his results with the large granule fraction have therefore been considered in the section on mitochrondria. It must be admitted however that some secretory granules may have been present, and that even in the preparation obtained through the use of sucrose, some secretory granules may be present together with the mitochondria.

It is thought by some authors that the mitochondria are in some way responsible for the formation of the secretory granules,[45] but this view is certainly not proved.

It now seems fairly certain that in the cases of cells which secrete enzymes, these enzymes are formed within the cell, often as zymogens, in

[43] H. Holmgren and O. Wilander, *Z. mikroskop. anat. Forsch.* **42**, 242 (1937).

[44] E. Jorpes, H. Holmgren, and O. Wilander, *Z. mikroskop. anat. Forsch.* **42**, 279 (1937).

[45] E. S. Duthie, *Proc. Roy. Soc. London* **B114**, 20 (1933–1934).

the form of secretory granules. These granules then seem to be extruded through the cell wall in some manner which leaves the cell intact and ready to produce more granules. It is possible to observe this process microscopically as has been done in the case of the pancreas by Covell, using anesthetized white mice as subjects and stimulating an enhanced secretion of granules by the intraperitoneal injection of secretin or pilocarpine[46].

In the case of the pancreas, secretory or zymogen granules have been found in the ducts. The reader is referred to a detailed article by Gage on the zymogen granules of the pancreas of various cold blooded and warm blooded forms. Many references are included[47].

Claude has mentioned the isolation of secretory granules from the pancreas[17, 48]. Isotonic saline had to be used, since distilled water caused activation of the pancreatic lipase which destroyed the granules. The granules finally isolated by centrifugation were in the form of a loose opaque sediment, yellow or yellow-green in color. The percentage of nitrogen was about 12%; phosphorus about 2%; sulfur about 0.5%; and lipide, which was mostly phospholipide, about 20%. These so-called secretory granules thus were very similar in gross chemical composition to the particles isolated by Claude from liver.

Since acinar cells of the pancreas contain considerable numbers of secretory granules, especially before the animal has eaten, it might be thought that they would serve as an ideal source for obtaining these granules in purified form. However, the lipase and proteases of the pancreas complicate the situation, since they can, no doubt, destroy the granules if activated. Whether Claude's large granules from the pancreas were mainly secretory granules or mainly mitochondria, or a mixture of both in roughly equal amounts, remains a question. It will be necessary to await further work with improved techniques before accurate information about secretory granules is available.

The problem of isolation of the secretory granules in pure form is one which should be of considerable interest to the enzyme chemist. Secretory granules composed of mucin must be quite different from those composed of enzymes. If we knew whether one or several enzymes can exist in a single secretory granule, we might have information of some value in elucidating the mechanism of the synthesis of proteins.

It has been tacitly assumed that cells of a given type in an organ such as the pancreas which secretes a variety of enzymes are equivalent. But as a matter of fact, is it even certain that there is not more than one kind of enzyme-secreting cell? Problems of this sort must be answered if we are to gain a complete understanding of cell physiology and biochemistry.

[46] W. P. Corell, *Anat. Record* **40**, 213 (1928).
[47] H. S. Gage, *Trans. Am. Microscop. Soc.* **64**, 151 (1945).

2. Small Granules

a. Microsomes

The microsomes are small particles in the size range of 50 to 150 mμ which occur in the cytoplasm of at least most kinds of animal cells. In general they are not resolvable or at the most barely resolvable with the light microscope, and must be observed in the intact cell by means of dark field illumination. The term microsome was proposed by Claude[17] when he realized that these small particles were not mitochondria.

The microsome fraction of most cells not of such a highly specialized nature as muscle cells for instance, can be isolated by first homogenizing the tissue in physiological saline or hypertonic sucrose solutions near 0°C. by means of a ground glass homogenizer or by grinding with sand and then by resorting to differential centrifugation in the cold. The microsomes are sedimented by prolonged centrifugation at centrifugal forces close to 18,000 or 20,000 times gravity, and form nearly transparent pellets on the bottoms of the centrifuge tubes. A number of references to the preparation of microsomes from various tissues is given by Claude.[48] A more recent article which includes work on microsomes and a submicrosome fraction from mouse liver cell cytoplasm has been written by Barnum and Huseby.[42] Schneider *et al.* have also included results of enzyme studies on the microsome fraction of liver and tumor tissues.[2, 27, 49]

Until recently, enzymes were not known to occur in the microsome or submicrosome fraction. Although cytochrome C was for a time thought to occur in the microsomes,[20] this finding was apparently an artifact, as has been explained already.[27]

It is true that cytochrome oxidase and succinic dehydrogenase have been found by Stern to occur in a particulate fraction from ground muscle which corresponds in size to the microsome fraction of ordinary cells.[25, 26] However, it is necessary to subject muscle to considerable grinding before obtaining such material, and it is not clear whether the particles exist as such in the muscle cytoplasm, or whether they are produced during the grinding by being broken off from larger particles.

The reported finding of succinic dehydrogenase in the microsome fraction by Lazarow[50] has not been confirmed by other workers.

Recently evidence has appeared indicating that certain enzymes may be associated with the microsome fraction of cells and indeed may occur there in concentrations several times higher than concentrations found elsewhere in the cell. An example is the finding of esterase in the microsome fraction of

[48] A. Claude, *Biol. Symposia* **10**, 111 (1943).
[49] G. A. LePage and W. C. Schneider, *J. Biol. Chem.* **176**, 1021 (1948).
[50] A. Lazarow, *J. Biol. Chem.* **140**, Proceedings Page LXXV (1941).

liver cells by Omachi *et al.*[51] The microsome fraction contained an average of 47% of the total amount of the enzyme in the entire cytoplasm. The concentration of esterase in the microsomes was 4.2 times that of its concentration in the whole tissue. However, esterase was also found in the cell nuclei, the microsomes, and the supernatant from the microsome fraction. Still more recently, Hogeboom,[52] using the hypertonic sucrose method for fractionation, has found that 58% of rat liver cell coenzyme I—cytochrome C reductase is present in the microsome fractions, only 32% being in the mitochondria and 5% in the supernatant solution.

In evaluating work of this sort, one must keep in mind results such as those obtained in the case of cytochrome C, already discussed. The question remains open as to whether the esterase of Omachi, Barnum, and Glick[51] was originally present in the mitochondria and later became transferred to the microsomes during the process of preparing them. This criticism may or may not apply to the work on D.P.N.-cytochrome C. reductase.

In another recent paper by Jeener,[53] it has been found that the thrombokinase activity of liver cells is concentrated in the microsome fraction rather than in the mitochondria. This paper is of interest in that it supports earlier work of Chantrenne[54] which purported to show a considerable range of particle sizes of cytoplasmic granules rather than a grouping into two or three sizes as claimed by most of the American workers. Moreover, by extracting with Edsal's solution, it was possible to obtain a ribonucleoprotein complex which contained about 20% ribonucleic acid, starting from granules of any desired size. The smaller the size of the particle used as a starting material, the higher was the yield of ribonucleoprotein obtained therefrom. It is suggested that the cytoplasmic granules originate as aggregates of very fine nucleoprotein particles which gradually grow and become transformed into an interior part consisting of various bound enzymes such as we know occur in mitochondria, and an exterior part consisting of material similar to the original nucleoprotein particles. Extracting with Edsal's solution is presumed to remove this exterior layer of undissolved fine nucleoprotein granules, leaving the interior material (consisting of enzymes in part). This idea is supported by the reportedly high concentration of fine granules rich in ribonucleic acid in the cytoplasm of embryonic cells, and by comparing the results of studies of cytoplasmic granules with the results of certain studies of virus molecules.

The gross chemical composition of microsomes produced from cells such as liver cells is somewhat similar to that of the large granule fraction or

[51] A. Omachi, C. P. Barnum, and D. Glick, *Proc. Soc. Exptl. Biol. Med.* **67**, 133 (1948).
[52] G. H. Hogeboom, *J. Biol. Chem.* **177**, 847 (1949).
[53] R. Jeener, *Biochimica et Biophysica Acta* **2**, 633 (1948).
[54] H. Chantrenne, *Biochimica et Biophysica Acta* **1**, 437 (1947).

mitochondria. The chief difference is that the lipide and the ribonucleic acid are present in considerably higher amounts than in the large granules. The composition of microsomes of guinea pig livers is shown in Table III constructed from the work of Claude[20] and Barnum and Huseby.[42] This table shows the presence of a submicrosome fraction in which the ribose nucleic acid is even higher than in the microsome fraction (16% on the average). The work of Chantrenne[54] also deals with very small particles rich in ribose nucleic acid.

If Chantrenne's picture of the structure of isolated cytoplasmic granules is considered, one is tempted to inquire whether such a structure might possibly be an artifact produced by adsorption of fine microsome granules on larger granules composed chiefly of protein. It should be recalled that

TABLE III

COMPOSITION OF MICROSOMES AND SUBMICROSOMES OF MAMMALIAN LIVER

	Guinea pig microsomes %	Rat liver microsomes %	Mouse liver microsomes[a] %	Submicrosomes[a] %
Nitrogen	9.2	9.0	10.3	13.2
Phosphorous	1.5	1.7	1.87	1.9
Lipide	40 to 45		35.1	16.8
Phospholipide	29		35.1[b]	
R.N.A.			9.1	
Inositol	12			
N.A.				16.0
Sulfur	0.8			
Copper	0.02			
Iron	0.03			

[a] Barnum and Huseby.
[b] Per cent total lipide.

Claude[17] observed an apparent disintegration of large cytoplasmic granules under certain conditions, yielding small particles similar to microsomes.

The function of microsomes and their origin in the cell remains to be discovered. It is not known whether the microsomes originate in the nucleus or are manufactured in the cytoplasm. Their high content of ribose nucleic acid might lead one to suspect that they play a role in protein synthesis in some manner as yet unexplained.

b. Particulate Glycogen

This substance was obtained in 1942 from liver cells by Lazarow.[55, 56] It sedimented in a period of half an hour at a centrifugal force of about 12,000 times

[55] A. Lazarow, *Science* **95**, 49 (1942).
[56] A. Lazarow, *Anat. Record* **84**, 31 (1942).

gravity along with some of the microsome fraction after removal of the large granule fraction, forming a single tightly packed white layer below the more loosely packed microsomes. The white layer of glycogen can be purified by resedimenting in saline suspensions, after removal of the microsomes.[11]

Particulate glycogen thus obtained was found to consist of 92 to 93.5% glycogen, and approximately 1% protein on a dry weight basis. The remaining 6% of material unaccounted for was thought to be principally water, which is very difficult to remove completely. In the fresh state, the glycogen particles contain about 75% water. Lipide is not present.

The particulate glycogen may be dissociated into smaller units or dispersed into solution by the usual reagents employed in the chemical preparation of glycogen, e.g., trichloracetic acid, strong alkali, and prolonged heating. This would make it appear that the particles are dependent upon the small amount of protein contained within them for integrity.

Particulate glycogen is mentioned because it demonstrates one method employed by the cell in disposing of excess substrate material and yet keeping it available for immediate use when needed. The glycogen particles, although small, are in a way analogous to secretory granules, the point of major difference being that they are not secreted as such but first are broken down into glucose. If we are to understand the cellular biochemistry of glycogen and glucose completely, these glycogen particles must be taken into account.

Other work on particulate glycogen will be found in one of the papers of Claude[20] and in a paper by Mitchell and Wislocki.[57]

3. Miscellaneous Cytoplasmic Granules

a. Ferritin

Stern and Wyckoff[25] found that the iron-containing protein ferritin sedimented from liver extracts at a rate indicating a molecular weight of the order of several million. Thus ferritin might be thought of as belonging to the class of cytoplasmic granules.

b. Melanin

Melanin granules stated to contain melanin or a similar substance were first isolated by Claude from the livers of *Amphiuma* (an amphibian) and in smaller yield from guinea pig liver cells.[58]

A more recent paper in which Tswett column adsorption is used to separate cytoplasmic granules[59] refers to the separation of melanin-containing granules from Harding-Passey mouse melanoma. Since these particles possessed enzyme activity, they were probably inhomogenous.

4. Chloroplasts

It is not the purpose of this chapter in general to cover enzymatic work on plant cells. However, it may be worthwhile to mention the isolation of chloroplasts, since this work may eventually prove to be an important step in the eludication of the mechanism of photosynthesis.

[57] A. J. Mitchell and G. B. Wislocki, *Anat. Record* **40**, 261 (1944).
[58] A. Claude, *Trans. N. Y. Acad. Sci.*, (Ser. II) **4**, 79 (1942).
[59] V. T. Riles, M. L. Hesselbach, S. Fiala, and M. W. Woods, *Science* **104**, 361 (1949).

A number of references are found in Glick's book on *Techniques of Histo- and Cytochemistry* to the earlier isolation of chloroplastic material. Granick[60] and Neish[61] seem to have been the first or among the first to obtain intact chloroplasts. The technique employed was to grind the leaves with sand in a mortar with 0.5 molar glucose solution and subsequently to isolate the chloroplasts by differential centrifugation, preferably in the cold.

A considerable amount of work has been done in recent years on the enzyme systems of isolated chloroplasts. Although the chloroplasts will not carry out photosynthesis, they will carry out a photochemical reaction, presumably dependent on chlorophyll, which is stated to result in oxygen evolution, if certain quinones are present for catalysts or substrates (see last reference on chloroplasts). A different type of reaction catalyzed by chloroplasts has been claimed to liberate hydrogen.[62] Evidently the complete process of photosynthesis is dependent upon enzyme systems and substrates that reside in parts of the cell other than the chloroplasts, or upon enzymes and substrates which have been washed out of the chloroplasts during their isolation. It might be of some interest to attempt to prepare chloroplasts by a modification of the Behrens technique for preparing isolated cell nuclei (see under cell nuclei) in order to be more certain of retaining substrates, coenzymes, etc.

Krossing in 1940[63] reported that chloroplasts of spinach cells contain catalase, chlorophyllase, amylase, and saccharase, but lack the enzyme peroxidase.

Polyphenol oxidase has been stated to be present in the chloroplasts of tea leaves but absent from the chloroplasts of spinach leaves.[64] The reduction of *o*-dinitrobenzene in the light by chloroplasts has been reported by Gurevich.[65]

For an excellent discussion of chloroplasts, the reader is referred to a chapter by Granick in *Photosynthesis in Plants*.[66] Another chapter by Holt and French deals with the photochemical liberation of oxygen by isolated chloroplasts (p. 277).

5. Discussion of Section II

It is possible that the above presentation may have implied a greater simplicity in the arrangement of the cell than actually exists. For instance,

[60] S. Granick, *Am. J. Botany* **25**, 558 (1938).
[61] A. C. Neish, *Biochem. J.* **33**, 293 (1939).
[62] E. A. Boichenko, *Compt. rend. acad. sci. U.R.S.S.* **52**, 521 (1946) (In English). Taken from *Chem. Abstracts* **41**, 3177 (1947).
[63] G. Krossing, *Biochem. Z.* **305**, 359 (1940).
[64] L. P. Li and J. Bonner, *Biochem. J.* **41**, 105 (1947)
[65] A. A. Gurevich, *Compt. rend. acad. sci. U.R.S.S.* **55**, 263 (1947) (In English). Taken from *Chem. Abstracts* **41**, 5921 (1947).
[66] S. Granick, in Photosynthesis in Plants. Edited by J. Frank and W. E. Lommis, Iowa State College Press, Ames, Iowa, 1949, p. 113.

the cytoplasmic granules were rather arbitrarily divided into a few classes, chiefly the large granules (mitochondria and secretory granules) and the small granules (microsomes, particulate glycogen, etc.). The work of Chantrenne in particular, that of Jeener, and to a certain extent that of Barnum and Huseby, who isolated two submicrosome fractions, has shown that under certain conditions it is possible to segregate cytoplasmic granules into almost any number of size classes desired, depending upon the manner of centrifugation. However, Chantrenne did not have access to the new method for isolating mitochondria by differential centrifugation in sucrose solutions, and it is possible that some of his particles were artifacts derived from breakdown of the mitochondria and secretory granules. It would be well to keep his work in mind, however, when repeating the isolation of mitochondria with sucrose solutions, and when studying the so-called microsome fraction.

The low enzyme content of the microsome fraction, coupled with its high nucleic acid content, is at present not readily explainable. It may be that more enzymes need to be studied before it is possible to state that the microsomes are really low in enzyme content in general. Perhaps the microsomes, submicrosomes, etc. are centers of synthesis for cytoplasmic proteins. They may possibly originate, at least in part, in the nucleus (cf. work of Caspersson, to be discussed subsequently), and on the other hand they may represent at least in part factors that are synthesized in the cytoplasm (cf. the work of Spiegelman, Sonneborn, etc. to be discussed subsequently).

The mitochondria and secretory granules on the other hand would seem definitely to arise in the cytoplasm. There is indirect evidence from microscopic observations that the secretory granules arise at or in the immediate vicinity of mitochondria, and later migrate to positions in the cytoplasm near their position of exit from the cell.[45] How the mitochondria themselves are produced or duplicated does not seem to be known at the present time. The role of the lipide and phospholipide in the mitochondria (and also the microsome and submicrosome fractions) is at the present time still a mystery.

Nothing has been said thus far about the continuous internal phase of cytoplasm, which persumably exists and which presumably contains the water-soluble constituents of the cell in solution. In a recent paper, LePage and Schneider[67] state that some of the enzymes of glycolysis are in the supernatant solution after removal by centrifugation of the cytoplasmic particles. This is no certain indication that these enzymes were present originally in the continuous internal phase, since they might have been dissolved out of the mitochondria, for example. Schneider[30] has reported a curious difference in distribution of adenosinetriphosphatase between large granules and an unfractionated residue (consisting of a small quantity of large granules to-

[67] G. A. LePage and W. C. Schneider, *J. Biol. Chem.* **176**, 1021 (1948).

gether with the microsomes and soluble cytoplasmic material) in comparing normal liver with primary hepatomas produced by feeding dimethylaminoazobenzene to rats. The concentration of this enzyme was more than six times higher in the large granules than in the unfractionated residue in the case of normal rat liver; but in the case of the primary hepatomas, the enzyme was only about 1.3 times more concentrated in the large granule fraction than in the unfractionated residue. This change in ratio was caused by a lowering of the concentration of enzyme in the large granule fraction in tumor relative to normal liver, as well as to a very marked enhancement in concentration of the enzyme in the unfractionated residue in the case of tumor relative to normal liver. The concentration of "A.T.P.—ase" in the original homogenate was higher in the case of the hepatomas than in the case of normal liver, in spite of the fact that the tumor cells are smaller than normal liver cells and contain considerably less of the large granule fraction than liver.

III. Studies of Cell Nuclei and Chromosomes Isolated by Physicochemical Procedures

1. Whole Nuclei

a. Miescher's Work on Pus Cell Nuclei and Nuclei of Fish Spermatozoa

Many years ago, Miescher[68] isolated nuclei from pus cells by first digesting away the cytoplasm with pepsin-HCl and then separating the nuclei by centrifugation. His studies on these nuclei culminated in the recognition of nucleic acid as a material heretofore unknown in tissue. Although the procedure employed by Miescher resulted in microscopically recognizable nuclei, it is apparent that it was so drastic as to render the nuclei unfit for studies of enzymes, and most proteins. Many ordinary proteins which escaped digestion by the pepsin-HCl presumably could have been extracted by the acid and thus lost. It is a question as to whether even the histone would remain in nuclei prepared in this way. Perhaps the only other major constituent besides the nucleic acid which could be profitably studied in such nuclei would be the lipides. The method of pepsin-HCl digestion has been incorporated in a more recent procedure for preparing muscle cell nuclei by Stoneburg.[69]

Miescher also isolated nuclei from fish spermatozoa, by washing with distilled water to remove the nonnuclear material. Nuclei were then isolated by differential centrifugation. Pollister and Mirksy[70] state however that his procedure does not yield nuclei that are completely free of nonnuclear pieces. Such nuclei, although they may not be completely free from cyto-

[68] F. Miescher *in* Hoppe-Seyler, F., Medizinisch-chemische Untersuchungen. A. Hirschwald, Berlin, 1871, Vol. 4, p. 441.

[69] C. A. Stoneburg, *J. Biol. Chem.* **129**, 189 (1939).

plasm, are presumably isolated by a sufficiently mild procedure to permit subsequent study of almost any constitutents, including proteins and enzymes, which were not lost by extraction from the nuclei during their preparation. However, the state of enzyme chemistry was not sufficiently advanced at the time Miescher first obtained nucle from fish spermatozoa to make possible an enzyme study of these nuclei, and apparently little or no enzyme work has been carried out with such nuclei since then.

The chief work thus far carried out on nuclei of fish spermatozoa comprises studies of nucleic acid and protamine.[70-76] These two constituents account for a very high proportion of the solid material of the defatted nuclei. The reader is referred to the work of Pollister and Mirsky for recent advances along these lines.

b. Mammalian Spermatozoa Nuclei

Mirsky and Pollister[70] found that the nucleoprotein of bull spermatozoa, unlike that of trout spermatozoa nuclei, was not readily soluble in 1 *M* NaCl. No explanation for this finding could be offered. The cytochrome oxidase content of the nuclei of bull spermatozoa has been investigated by Zittle and Zitin[77]. The nuclei were obtained following treatment with sonic vibrations which caused the tails and midpieces to become separated. The tails, midpieces, and nuclei were then each isolated separately by differential centrifugation. The cytochrome oxidase concentration of the tails was about 24 times that of the nuclei, while the cytochrome oxidase content of the midpieces was about 12 times that of the nuclei. Further work on studies of nucleic acid, lipide, and protein nuclei of bull spermatozoa nuclei were made by Zittle and O'Dell.[78]

c. Work on Bird and Snake Erythrocyte Nuclei

Early work on bird erythrocyte nuclei yielded gelatinous material useful chiefly for studies of nucleic acid and histone.[79, 80] Other early experiments on bird and snake erythrocyte nuclei are to be found in references 81 and 82.

[70] A. W. Pollister and A. E. Mirsky, *J. Gen. Physiol.* **30**, 101 (1946).
[71] V. T. Miescher, Die Histochemischen und Physiologischen Arbeiten. F. C. W. Vogel, Leipsig, 1897, Vol. 2, p. 55.
[72] E. Masing, *Z. physiol. Chem.* **66**, 262 (1910).
[73] A. Kossel, The Protamines and Histones. Longmans, Green, and Co., New York, 1928.
[74] A. Kossel, *Münch. med. Wochschr.* **58**, 65 (1911).
[75] H. Steudel, *Z. physiol. Chem.* **83**, 12 (1913).
[76] H. Steudel, *Z. physiol. Chem.* **90**, 291 (1941).
[77] C. A. Zittle and B. Zitin, *J. Biol. Chem.* **144**, 99 (1942).
[78] C. A. Zittle and R. A. O'Dell, *J. Biol. Chem.* **140**, 899 (1941).
[79] T. L. Brunton, *J. Anat. Physiol.* **4**, 91 (November 1869).
[80] D. Ackermann, *Z. physiol. Chem.* **43**, 299 (1904-05).
[81] P. Plosz, *Med. Chem. Untersuch.* **4**, 461 (1871).
[82] C. Hörhammer, *Biochem. Z.*, **39**, 270 (1912).

Warburg obtained bird erythrocyte nuclei in somewhat damaged condition by the use of freezing and thawing to break the cells.[83] Miyake also used this technique.[84] Negelein studied the respiration of goose erythrocyte nuclei.[85] A low respiratory rate was observed.

More modern work on bird erythrocyte nuclei has been done by Yakushiji[86, 87]. Yakushiji used slightly hypertonic saline solutions of saponin, digitonin, sodium oleate, and sodium cholate for liberating the nuclei from the cells and isolated the liberated nuclei by differential centrifugation. He made an elaborate study of the effect of various conditions on the isolation of nuclei and undoubtedly succeeded in preparing nuclei of a high quality according to the criterion of good microscopic appearance. He states that a mixture of alcohol and ether removes the residual stroma which remains attached to the nuclei. His work did not however include enzyme studies.

Laskowski[88] later prepared nuclei from chicken erythrocytes by laking with lysolecithin in approximately isotonic saline solution. Laskowski ob-

TABLE IV

RESPIRATION OF CHICKEN ERYTHROCYTE NUCLEI

pH	Q_{O_2}
6.0	0.094
6.5	0.13
7.0	0.21
7.5	0.19

tained excellent nuclei in this manner and studied their respiration, which was very low. Nuclei prepared by lysolecithin maintain a very tenuous stroma that may be overlooked unless a very careful microscopic scrutiny is made of the preparation. Some of the results of Laskowski's studies of the chicken erythrocyte nuclei are given in Table IV.

In the presence of glucose the Q_{O_2} of the nuclei rose to 0.3 at pH 7.3. With the nuclei in saturated NaCl, the Q_{O_2} at first rose, and then respiration ceased. Agglutination did not lower the Q_{O_2} appreciably.

In a later paper Laskowski and Ryerson[89] made a study of the solubility of nucleoprotein of the bird erythrocyte nuclei under various conditions. Nucleoprotein could be solubilized by treating the nuclei with water or

[83] A. Warburg, *Z. physiol. Chem.* **70**, 413 (1910).
[84] M. Miyake, *Keijo J. Med.* **4**, 247 (1933).
[85] E. Negelein, *Biochem. Z.* **158**, 121 (1925).
[86] N. Yakushiji, *Keijo J. Med.* **7**, 521 (1936).
[87] N. Yakushiji, *Keijo J. Med.* **7**, 276 (1936).
[88] M. Laskowski, *Proc. Soc. Exptl. Biol. Med.*, **49**, 354 (1942).
[89] M. Laskowski and D. L. Ryerson, *Arch. Biochem.* **3**, 227 (1943).

with 5% NaCl solution. The nucleoprotein was least soluble in physiological saline, in agreement with the work of Mirsky and Pollister,[70] but this is no indication that other material is not removed from the nuclei in physiological saline solution. Gels were observed when the nuclei were suspended in strong saline solution, which rendered difficult the isolation of soluble nucleoprotein fractions. Soluble nucleoprotein could be recovered however by suction filtration through cellite of the gel produced by treatment of the nuclei with 5% NaCl solution. It now seems likely that the soluble nucleoprotein thus collected was obtained as the result of a slight autolysis occurring during the filtration. Reasons in support of such an idea will be given later on.

Lan and Dounce[90] prepared chicken erythrocyte nuclei in 1943 by breaking the cells with saponine in 0.9% NaCl. The previous work of Yakashiji (previously cited) unfortunately had been overlooked and was not referred to in their paper. The nuclei thus prepared showed a Q_{O2} of 0.4 at 37.5°C. in 0.9% NaCl buffered to pH 7.4 with phosphate, and a Q_{O2} of 0.26 in 0.11 molar (isotonic) phosphate of pH 6.8 at 25°C. The total lipide was about 14%, and by direct Dische determinations the desoxyribonucleic acid was stated to be about 45%. The latter figure was based on an impure standard and is erroneous; it should have been about 38% as reported by Dounce in a later article[91]. Even this value is no doubt too high since the direct Dische method is now known to give erroneously high results. A yellow pigment shown to be xanthophyll was found in the nuclei.

The acid phosphatase content of chicken erythrocyte nuclei was determined by Dounce and Seibel[92] and it was found that the activity of the isolated nuclei was about the same as that of whole washed chicken erythrocytes on a dry weight basis.

The writer is not aware of any further work on enzyme systems of bird erythrocyte nuclei. As a matter of fact, the bird erythrocyte is a cell of such limited metabolic function that enzyme studies on the whole cell or its components may be of dubious value as far as drawing general conclusions is concerned.

Recent work by Melampy[93, 94] on bird erythrocyte nuclei has been concerned with the amino acid content of the hydrolyzed material. Among other results the arginine content of the nuclei was found to be high, relative to that of the whole cell, and the tryptophan and histidine contents of the nuclei were low compared to those of cytoplasm. These results presumably reflect in part the high histone content of the nuclei, since histone is low in tryptophan and high in arginine. This is particularly true since it is likely that protein other than histone is lost from the nuclei during their prepara-

[90] A. L. Dounce and T. H. Lan, *Science* **97**, 584 (1943).
[91] A. L. Dounce, *J. Biol. Chem.* **151**, 235 (1943).
[92] A. L. Dounce and D. Seibel, *Proc. Soc. Exptl. Biol. Med.* **54**, 22 (1943).
[93] R. M. Melampy, *Proc. Soc. Exptl. Biol. Med.* **65**, 213 (1947).
[94] R. M. Melampy, *J. Biol. Chem.* **175**, 2 (1948).

tion, because of solubility in physiological saline solution. The amino acid content of the whole cells can, on the other hand, be thought of as reflecting to a considerable extent the composition of chicken hemoglobin.

Work on snake erythrocyte nuclei has been carried out by Villela.[95] This work was not concerned with enzymes, but the lipide content and the desoxyribonucleic acid content of the nuclei were measured. The former was about 12.7% for two species of snakes; the latter ranged from 56 to 64% in six species of snakes. Since 5% citric acid was used to wash the nuclei, after washing with isotonic saline, these values for desoxyribonucleic acid are probably very much higher than they should be, chiefly because of loss of material other than nucleic acid during the washing procedure.

Using nuclei washed only with saline, thiamine was found to be present in a concentration of about 80 μg./g., and nicotinamide in a concentration of about 122 μg./g. These values probably are not of great value either, owing to probable loss of low molecular weight water-soluble material from the nuclei during the washing procedure.

d. Studies of Arbacia Egg Nuclei Obtained by Centrifugation Techniques

A technique has been worked out by the Harveys[96–103] for separating Arbacia egg cells into a heavy, nonnucleated half and a lighter, nucleated half by centrifugation at 7000 r.p.m. of a sea water suspension of the eggs which overlays a solution of 0.95 molar sugar solution. The constituents of the unbroken eggs, which take a position at the interface between the saline and sugar solutions, first become stratified into a top oily layer, a clear layer just below this, a third thin granular layer, a yellowish yolk layer still lower, and a red pigmented layer at the bottom. Such stratification can be observed microscopically with the centrifuge running.

Breakage of the cells finally takes place, usually at the yolk layer. The lighter nucleated halves go to the top of the saline solution, the red pigmented halves go to the bottom of the tube, and unbroken eggs remain at the interface.

The white nucleated halves at the top of the saline layer can be collected and recentrifuged in the same manner as before to remove contaminating nonnuclear material. After this second centrifugation, the top layer is said to consist mainly of nuclei with a small amount of oily material as a contaminant. Using this method, sufficient material can be collected for studies using the Warburg or Fenn apparatus.

Shapiro[104] using unfertilized eggs of the sea urchin, *Arbacia punctulata*,

[95] G. G. Villela, *Proc. Soc. Exptl. Biol. Med.*, **66**, 398 (1947).
[96] E. N. Harvey, *Biol. Bull.* **61**, 273 (1931).
[97] E. B. Harvey, *Biol. Bull.* **62**, 155 (1932).
[98] E. B. Harvey, *Biol. Bull.* **62**, 155 (1932).
[99] E. B. Harvey, *Biol. Bull.* **66**, 228 (1934).
[100] E. B. Harvey, *Biol. Bull.* **71**, 101 (1936).
[101] E. B. Harvey, *Biol. Bull.* **75**, 170 (1938).
[102] E. B. Harvey, *Biol. Bull.* **78**, 202 (1940).
[103] E. B. Harvey, *Biol. Bull.* **78**, 412 (1940).
[104] H. Shapiro, *J. Cellular Comp. Physiol.* **6**, 101 (1935).

found that the light half of the egg obtained by the Harvey centrifugation technique showed an oxygen uptake nearly equal to that of the whole cell, while the pigmented half without a nucleus, which possessed most of the yolk and the pigment echinochrome, showed an oxygen consumption of about 88% that of the whole cell. The oxygen uptake calculated for the sum of the two half eggs was about 29% greater than that of the unbroken egg; hence disruption of the egg caused changes in the activity of the oxidative systems.

On fertilization, the light halves showed an increase in oxygen uptake of about 2.7-fold, as did the whole cells, but the pigmented halves did not show such an increase. This work is difficult to interpret in terms of the respiratory activity of the cell nucleus, since the light halves of the eggs contained much material besides nuclei.

Boell *et al.*[105] studied the cytochrome oxidase of the half cells of *Arbacia punctulata*, also obtained by the method of the Harveys. Here the lighter nucleus-containing halves showed a somewhat greater oxygen consumption upon addition of *p*-phenylenediamine than the heavier pigmented halves. Whether this result can be interpreted to mean that the concentration of cytochrome oxidase is higher in the nucleus of the arbacia egg than in the cytoplasm seems doubtful in view of the considerations mentioned just previously.

e. Mammalian Somatic Cell Nuclei Isolated from Aqueous Tissue Homogenates by Differential Centrifugation

Miescher isolated the nuclei of pus cells in a drastic procedure which involved the use of pepsin-HCl to remove cytoplasm.[68] These nuclei were collected by centrifugation and as a result of studies of the material thus isolated Miescher was lead to the discovery of nucleic acid.

This work of Miescher is mentioned, because it is the first recorded attempt of which this writer is aware to prepare nuclei on an ordinary chemical scale from cells, and because it is the basis of some more modern work on isolating cell nuclei. It might be well to mention at this point, that merely because a cell or one of its components has been isolated in a state of good microscopic appearance, it does not necessarily follow that the cell or its constituent has not lost considerable material that originally was present. This point will be emphasized later on. It is also obviously true that in spite of a normal microscopic appearance of a cell or a cell component, enzymes contained therein may have been completely inactivated. As far as the author knows, there is no method, except possibly that of microdissection, which can be used to isolate a cell nucleus in such a condition as to have within it in an undamaged state all components originally present.

[105] E. J. Boell, R. Chambers, E. A. Glancy, and K. G. Stern, *Biol. Bull.* **79**, 352 (1940).

Therefore, one must choose a method of isolation suitable to a study of the particular constituent in mind. This statement may well apply to other cell components such as mitochondria, but we do not yet have evidence on the point. Although the method of Miescher probably denatured enzymes completely and removed much material from the nuclei, it was nevertheless of great value since it left the nucleic acid in a more or less undamaged state within the nuclei and thus permitted a separation of this material from a mass of cytoplasm.

Abderhalden and Kashiwado[106] in 1912 studied nuclei isolated from thymus (by means of pepsin-HCl). Anaphylaxis as produced by thymus nuclear material and nuclear material from bird erythrocyte nuclei indicated that the protein components of these two types of nuclei were not identical, as might have been surmised. No enzyme studies were carried out.

Stoneburg in 1939 capitalized upon the histological observation of Crossmon[107] that strong solutions of citric acid cause a disintegration of cytoplasm and at the same time liberate nuclei in an apparently undamaged condition (microscopically). By treatment of tissue with 5% citric acid followed by differential centrifugation, Stoneburg was able to isolate cell nuclei in a fair degree of purity. He then reverted to the pepsin-HCl treatment of Miescher to remove the last traces of cytoplasm and isolated the nuclei, after washing, again by the use of differential centrifugation. These nuclei were used primarily for a study of lipides, and of course would have been useless for enzyme studies. The method represented an advance over the technique of Miescher however, and nuclei prepared in this way could be used for a study of desoxyribonucleic acid as well as lipides. It is possible that some ribonucleic acid might be lost, however.

Later on Marshak[108, 109] applied the initial steps of the method of Stoneburg to various tissues and was able to obtain very pure nuclei, judging from microscopic appearance, by the use of 5% citric acid and differential centrifugation without recourse to the pepsin-HCl treatment. In particular Marshak obtained very good nuclei from liver, whereas Stoneburg had been unable to obtain any results with his technique from this tissue. Marshak studied phosphorous turnover in the nucleic acid and phospholipide fractions of his nuclei, using radioactive phosphate. Haven and Levy[110] later used the method of Marshak to obtain liver and tumor nuclei, with the modification of using slightly less citric acid. The nuclei were used for phospholipide studies. However, even these nuclei had been prepared at too low a pH to permit enzyme studies. It is of interest in con-

[106] E. Abderhalden and T. Kshiwado, *Z. physiol. Chem.* **81**, 285 (1912).
[107] G. Crossmon, *Science* **85**, 250 (1937).
[108] A. Marshak, *Science* **92**, 461 (1940).
[109] A. Marshak, *J. Gen. Physiol.* **25**, 275 (1941).
[110] F. L. Haven and S. R. Levy, *Cancer Research* **2**, 797 (1942).

nection with the work on lipides that sphingomyelin was not found in the nuclei, although sphingomyelin was present in the whole tissue. Lecithin and cephalin were present in the nuclear lipide.

The writer of this chapter in 1943 found that it is possible to obtain nuclei from certain tissues by using very little citric acid, if the pH is rigorously controlled.[92, 111] For example, it is possible to prepare nuclei from normal rat or rabbit liver at pH 4.0 by the use of a Waring blendor for mincing the tissue in very dilute ice-cold citric acid. If the pH employed was between 4 and 6, it became very hard to isolate nuclei owing to agglutination of cytoplasmic material to such an extent that the nuclei became mixed with a large mass of well-packed sediment upon centrifugation. At

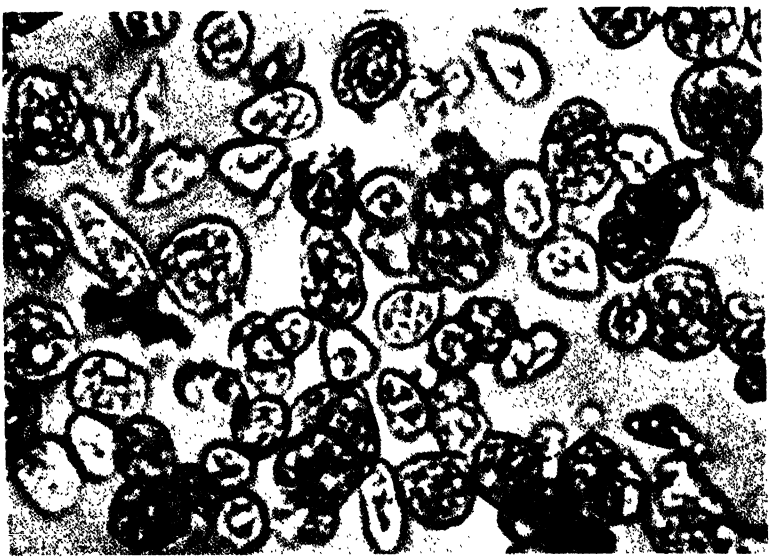

FIG. 2. Nuclei isolated from rat liver at pH 6.0. (Dounce, A. L., *J. Biol. Chem* **174,** 859 1948.)

pH 6.0 however, it was possible again to obtain nuclei provided that the pH was held within very closely specified limits. A photograph of nuclei of normal rat liver, prepared in this way, is shown in Fig. 2. At pH 5.7 sufficient agglutination of cytoplasm occurred to become very troublesome, whereas above 6.2 few or no nuclei could be isolated at all even though no agglutination of cytoplasm occurred. In the latter case the nuclei became dissolved or disintegrated during the mixing in the Waring blendor and the subsequent centrifugations, presumably as the result of an autolytic action of some sort, acting together with the shearing action of the blendor. Since nuclei once isolated do not dissolve at pH values of 6.2 to 7.5, it may

[111] A. L. Dounce, *J. Biol. Chem.* **147,** 685 (1943).

be that the cytoplasm is responsible for this autolysis. More recently, nuclei have been obtained at pH 6.0 from liver, kidney, and pancreas by an improvement of our original method.[3]

It was immediately possible to study enzymes in nuclei prepared at pH 6.0, and even in a few cases in nuclei isolated at pH 4.0. A summary of the early results will be found in a paper by the author.[111] The early failure to find catalase in the nuclei has not been confirmed in later work, and this result probably is to be considered as erroneous, possibly attributable to our lack of a refrigerated centrifuge when this work was carried out.

Studies of glycogen, desoxyribonucleic acid, and total lipide were also made using nuclei prepared at pH 4.0 and 6.0. Glycogen was found to be absent from the nuclei. The desoxyribonucleic acid content of nuclei prepared at pH 6.0 varied from about 13 to about 20% as determined by the direct Dische determination. Since the direct Dische determination is now known to give somewhat high results, these values are too high and should have been approximately 10.4 to 16%, according to a recent comparison by us of the direct Dische determination with the Schneider determination for desoxyribonucleic acid which we now use. The desoxyribonucleic acid content of nuclei prepared at pH 3.8 to 4.0 varied from about 20 to 23%, and these values should have been corrected to about 16 to 19%.

The total lipide content of nuclei prepared at pH 6.0 from normal rat livers as determined by continuous extraction and dry weight measurements, varied from 7.5 to 10.8%. In the case of nuclei prepared from normal liver at pH 3.8 to 4.0, a strain difference was suspected, since nuclei from Osborne-Mendel rats prepared at this pH range showed a total lipide content of 3.2%, whereas nuclei prepared from Wistar strain rats showed a total lipide content of 6.0 to 7.2%. However, too few results were available to be certain that this difference was significant.

Our values for total lipide of isolated rat liver cell nuclei are considerably lower than those of Williams *et. al.*[112] We believe the difference in these results is to be ascribed to the fact that our determinations of total lipide were by direct dry weight determinations, whereas those of Williams *et al.* were obtained by adding the results of various isolated lipide fractions. In the latter case, an overlapping of fractions, would cause some lipide to be counted twice or more.

It is not the purpose of the paper to enter into a detailed discussion of the nucleic acid or lipide contents of isolated nuclei. For this work, the papers of Haven and Levy[110] and of Williams and collaborators[112] are to be recommended. But the variation in the concentration on a dry weight basis of total lipide as well as desoxyribonucleic acid of nuclei isolated by various procedures has a bearing on the apparent concentrations of enzymes in the nuclei, and hence it is mentioned at this point. It is just now becoming clear that even during the mildest preparation of nuclei in aqueous media, loss in dry weight occurs.

All of our earlier work with cell nuclei prepared at pH 6.0 or at pH 4.0 and lower using citric acid involved adding small pieces of liver to ice cold citric acid and crushed ice in a Waring blendor, followed by homogenization for a fixed length of time. It was objected by Dr. Claude in a private communication that this procedure must subject the first portion of tissue

[112] H. H. Williams, M. Kaucher, A. J. Richards, and E. Z. Moyer, *J. Biol. Chem.* **160**, 227 (1945).

added to a pH lower than the pH finally obtained at the end of the homogenizing procedure. It was found by us that the pH rose so rapidly after the addition of a small amount of liver that this objection did not appear to be very serious, but nevertheless the fact remained that some nuclei must have been subjected to lower pH values that others. To overcome this difficulty, a modified procedure was devised whereby the frozen tissue was homogenized in ice water for a minute or less, and then tenth molar citric acid was added dropwise with the blendor still running until the pH finally attained was 6.0 or 4.0 as desired.

The improved method is somewhat more exacting and difficult to carry out than our earlier method. For instance, the pH must be kept within 0.1 pH unit of 6.0 during the original blending and during the first two or three washings. Sometimes, a few drops of very dilute citric acid must be added to the suspended material in the first washing to avoid an increase in pH above 6.1 which will lower the yield or ruin the preparation (unpublished).

Using this procedure it was possible to isolate nuclei from kidney and pancreas as well as liver[3] at pH 6.0. In working with kidney and pancreas, special steps had to be used to remove fiber.

It was found that a number of glycolytic enzymes occurred in liver cell nuclei (aldolase[113], enolase, 3-phosphorglyceraldehyde dehydrogenase, lactic dehydrogenase (this enzyme was measured using diphorase and methylene blue in a Thunberg tube), and phosphorylase (unpublished)). In most cases where the enzyme activity per dry weight was measured (aldolase, enolase, lactic dehydrogenase) the concentration in the nucleus was in the neighborhood of half that of whole tissue. Phosphorylase was present (unpublished) in a somewhat lower relative concentration and it was necessary to grind the nuclei so as to produce considerable fragmentation before phosphorylase could be demonstrated in appreciable amount. The enzyme arginase was studied in considerable detail.[3] It was found that the addition of $MnSO_4$ to nuclei at 25°C. produced no activation of arginase, whereas a very appreciable activation is obtained using whole liver homogenate. In the case of arginase, the activity per dry weight of isolated nuclei was higher than that of whole liver. This result may simply mean that no arginase is lost in preparing the nuclei, while other water-soluble material, especially low-molecular weight constituents are lost.

Catalase was found to be present in nuclei isolated at pH 6.0 by the improved method in concentrations comparable to those existing in the whole tissue (unpublished). A similar result was found for kidney. Aldolase was also found in kidney cell nuclei isolated by the improved procedure. Pancreas cell nuclei on the other hand seemed to lack aldolase and catalase. However, lipase and esterase were found in these nuclei in concentrations

[113] A. L. Dounce and G. T. Beyer, *J. Biol. Chem.* **174**, 159 (1948).

TABLE V

ENZYMES OF ISOLATED CELL NUCLEI

Enzyme	Activity per dry weight of nuclei expressed as per cent of activity per dry weight of fresh whole tissue homogenated[a]
RAT LIVER CELL NUCLEI	
Aldolase[a]	40
D-Amino oxidase[b]	100
Arginase[a]	113
Catalase[a]	50–80
Choline oxidase[b]	0
Cytochrome oxidase[a,c]	50–60
Cytochrome C	Low
Enolase[a]	50
Esterase	50
Alkaline phosphatase	192
Acid phosphatase	25–30 (minimal)
Phosphorylase[a]	30 (after grinding)
Lactic dehydrogenate[a]	40
Succinic dehydrogenase[a]	0
Uricase[b]	100
RAT KIDNEY CELL NUCLEI	
Aldolase[a]	40
Arginase[a]	0
Catalase[d]	50–80
BEEF PANCREAS NUCLEI	
Esterase[a]	50
Lipase[a]	50
CHICKEN ERYTHROCYTE NUCLEI (pH 7.0)	
Acid phosphatase	100
CHICKEN LIVER NUCLEI	
Arginase[a]	Zero in nuclei and whole cell
CHICKEN KIDNEY NUCLEI	
Arginase[a]	0

[a] Fresh whole tissue was homogenized in distilled water in a ground glass homogenizer.
[b] Work of T. H. Lan.[114]
[c] Based upon Waring Blendor homogenate at pH 6.0.
[d] Nuclei obtained at pH 6.0 by our improved method. Other nuclei (except those of chicken erythrocytes) were prepared at pH 6.0 by our original method.

about half those of whole pancreas (unpublished). The results of our enzyme determinations on isolated nuclei are summarized in Table V.

[114] T. H. Lan, *J. Biol. Chem.* **151**, 171 (1943).

As far as we are able to judge, the isolation of nuclei at pH 6.0 by our improved method constitutes a reliable procedure for obtaining nuclei using aqueous extractants, which are in general suitable for enzyme studies. It must be kept in mind, however, that appreciable losses of many water soluble constituents, including enzymes and proteins, probably occur. The method can be adapted with some difficulty to small scale procedure using a ground glass homogenizer[3] although best results are obtained when using about 50 g. of material with the Waring blendor for homogenizer.

Schneider was able to isolate a fraction from cell homogenates used in preparing mitochondria, in which apparently all of the nuclei accumulated.[2, 30] This work has been extended to the use of sucrose homogenates.[21, 29, 115, 116] It is unfortunately difficult to ascertain the state of purity of the nuclei in these fractions from reading the literature on the subject. In our experience, considerable numbers of whole cells have been present in these fractions, and the clumping of the nuclei makes an estimation of extraneous particulate material somewhat difficult. It seems likely that losses in water-soluble material (including enzymes and proteins) also would occur from nuclei prepared in hypertonic sucrose solution, but this point has not yet been established.*

We have never been successful in obtaining mammalian liver or kidney cell nuclei using physiological saline for aqueous suspending medium and using the Waring blendor as a homogenizer. This has generally been due in large part to inability to break the cells in the blendor. Nor have we yet succeeded in obtaining nuclei using hypertonic sucrose solution with the Waring blendor. Either we have not found the proper conditions or this suspending medium is not adapted to our general type of homogenizing with the Waring blendor.

It has, however, proved to be possible to prepare pancreas nuclei using distilled water alone without the addition of any citric acid. It may be that pancreas nuclei could also be prepared using physiological saline. Why pancreas nuclei should not become dissolved or disintegrated above pH 6.0 is not known.

Schneider[2, 30] working with nuclear concentrates from normal liver and primary hepatomas of rat liver induced by feeding dimethylaminoazobenzene, found cytochrome oxidase and succinic dehydrogenase in the nuclear fraction from normal liver but concluded that the presence of the enzymes was probably due to contamination with whole cells and large

[115] G. H. Hogeboom, *J. Biol. Chem.* **177**, 847 (1949).

[116] W. C. Schneider and van R. Potter, *J. Biol. Chem.* **177**, 893 (1949).

* Arnesen *et al* (K. Arnesen, Y. Goldsmith, and A. D. Dulaney, *Cancer Research*, **9**, 669 (1949)) find that desoxyribonucleic acid is extracted from nuclei obtained by differential centrifugation of homogenates prepared with 8.5 or 30% sucrose solutions.

granules. Adenosine triphosphatase on the other hand seemed to be in higher concentration in the nuclear fraction from normal liver than in the whole tissue homogenate, and was about equal in concentration to its concentration in the large granule fraction. Hence one might conclude that adenosine triphosphatase is a constituent of the cell nucleus in the case of normal rat liver.

In nuclear concentrates from hepatomas, the observed cytochrome oxidase and succinic dehydrogenase again were presumed to be present as the result of contamination with cytoplasmic material. As has been mentioned already, the distribution of adenosine triphosphatase was different in the case of normal liver and primary hepatoma. The activity of this enzyme per dry weight of material was less in the case of hepatoma nuclei than in the case of normal liver cell nuclei, but there was still sufficient activity so that this enzyme may well have been present in the nuclei.

Schneider emphasizes the necessity of recovering the total enzyme activity of the homogenate by adding the activities of the various fractions, in order to be certain that some activation or inactivation of enzyme does not occur during the fractionation procedure. This is no doubt a highly desirable accomplishment in many instances, but it is not helpful in deciding whether a cellular fraction which constitutes a small percentage of the total cell volume (such as the nucleus of the liver cell) possesses enzyme intrinsically, or whether the enzyme is present merely as the result of contamination with a fraction especially rich in that enzyme. It appears to us that one must work with fractions of considerable purity to decide the latter point.

A reinvestigation of the cytochrome oxidase and succinic dehydrogenase activities of normal rat liver cell nuclei isolated at pH 6.0 has been made by Dr. F. G. Smith and the writer (unpublished). It was found that the cytochrome oxidase activity of the nuclei per dry weight was about 60% that of whole liver homogenate taken from the Waring blendor after adjustment to pH 6.0 with citric acid. This result is the same as previously reported by one of us.[111] However, it is true that the adjustment to pH 6.0 with citric acid weakens the cytochrome oxidase activity considerably, so that one cannot fairly compare the activity of the nuclei with that of fresh homogenate, unless one assumes without apparent reason that the cytochrome oxidase of the isolated nuclei is less affected than the cytochrome oxidase of the whole homogenate. If the latter apparently unjustified assumption is made, the cytochrome oxidase activity of the nuclei per dry weight is about 10 to 15% that of fresh liver.

These new results have convinced us that highly purified nuclei from normal rat liver do contain cytochrome oxidase, although it is still barely possible that adsorption of mitochondria by the nuclei could account for

this. It seems to us however that for the time being at least the burden of proof remains on anyone who wishes to maintain that cytochrome oxidase is absent from the nucleus of the normal rat liver cell.

We have still been unable to detect succinic dehydrogenase in purified nuclei of normal rat liver, and hence conclude in agreement with Schneider that it is not present there. Hogeboom *et al.*[21] state that all of the succinic dehydrogenase of the liver cell is present in mitochondria obtained by the improved technique using hypertonic sucrose, so that their result in this case is in agreement with ours.

Esterase was reported to be present in a nuclear concentrate of normal liver cells in about 50% of its concentration in whole liver homogenate by Omachi *et al.*[51] This result is in essential agreement with the result reported earlier by the writer[111] although exact comparison of the two pieces of work is not possible in view of the crude method for determining activity used by the writer, and in view of the fact that only nuclear concentrates were used by Omachi, Barnum, and Glick.

Von Euler and collaborators[117, 118] isolated nuclei from thymus and liver by a modification of the writer's earlier technique, but used these nuclei mainly for a study of nucleic acids. The tissue was homogenized at pH 4.0 (using dilute citric acid) and the nuclei were isolated by centrifugation at pH values from 5.0 to 6.0. Succinic dehydrogenase and lactic dehydrogenase activities were extremely low in concentration in the nuclei. However, lactic dehydrogenase activity was measurable and was enchanced by the addition of coenzyme I. The catalase activity of nuclei of Jensen sarcoma was negligible compared with that of the cytoplasm.

Several difficulties make these results unreliable. For example, the pH of the homogenization was too low for successful work with most enzymes; and no diaphorase was added when lactic dehydrogenase was estimated. We have repeated work with lactic dehydrogenase in the presence of excess diaphorase and coenzyme I, using nuclei prepared by our improved method at pH 6.0, and have found the activity of lactic dehydrogenase per unit dry weight of nuclei to be about 40% that per dry weight of whole fresh liver homogenate (unpublished). We do not yet know whether freezing lowered the enzymatic activity of the homogenate, so that this value of 40% represents a minimal value.

Von Euler and collaborators[117] also found that an initial high viscosity of nuclei rapidly fell at 20°C. when the nuclei were brought to pH 7.0–7.5 with alkali. This change was thought to be the result of enzyme action of some sort.

[117] H. von Euler, I. Fischer, H. Hasselquist, and M. Jaarma, *Arkiv Kemi, Mineral. Geol.* **21A**, No. 12 Page 1 (1945).

[118] H. von Euler, L. Hahn, H. Hasselquist, M. Jaarma, and M. Lundin, *Sartryck ur Svensk Kem. Tid.* **57**, 217 (1945).

Some further statement about the desoxyribonucleic acid content of isolated cell nuclei seem necessary at this point in order to gain insight as to the magnitude of the losses in nuclear material thát occur when nuclei are isolated by aqueous extraction procedures. In our earlier work, we assumed that an inappreciable amount of desoxyribonucleic acid would be lost at pH 4.0, and in view of later work this assumption seems still tenable. Nuclei prepared at pH 6.0 were assumed to have lost some desoxyribonucleic acid. This assumption seemed logical in view of the variability of the desoxyribonucleic acid content of these nuclei, and indeed may have been correct. It is also possible that the lack of a refrigerated centrifuge may have contributed to variability in analyses of nuclei prepared at pH 6.0. However, recent work in this laboratory has indicated that nuclei prepared by the improved method at pH 6.0 using a refrigerated centrifuge contain about 14% desoxyribonucleic acid as measured by the Schneider technique. This is essentially the same amount of desoxyribonucleic acid as occurs in nuclei obtained at pH 4.0.

It was demonstrated that when nuclei are prepared using citric acid at pH values of 4.0 or lower, the desoxyribonucleic acid content of the nuclei, based on dry weight, rapidly rises as the pH of the preparation falls. This finding undoubtedly caused by a removal of more and more histone and possibly other protein from the nuclei as the pH falls.

Extraction with saline also causes loss of dry weight from isolated nuclei. Indeed, saline may cause loss of much more protein than dilute citric acid at pH 6.0. This point will be mentioned again in the discussion of nuclei prepared by Behrens' procedure. The values reported by us for desoxyribonucleic acid in chicken erythrocyte nuclei[119] are so high (in the neighborhood of 35 to 40% by the direct Dische determination) that it seems certain that much material other than nucleohistone must have been extracted from this nuclei. The same can be said for the snake erythrocyte nuclei prepared by Villela[95] which showed an even higher content of desoxyribonucleic acid calculated on a dry weight basis. Here the nuclei were originally suspended in isotonic saline and then washed with strong citric acid. The combination of these two factors no doubt removed most of the material other than histone, desoxyribonucleic acid, and possibly some insoluble structural protein, and lipide. Hepatic cell nuclei of rat liver which were prepared by Villela and Ubatuba[120] from saline perfused livers by means of ice-cold 5% citric acid also must have lost a great deal of material other than desoxyribonucleic acid, since the concentration of the latter material on a dry weight basis was about 30%.

The above statements are borne out by microscopic observations. We ourselves had reported that nuclei prepared at pH 6.0 by our early method lost protein and appeared to shrink when extracted with NaCl. It has been observed by others that chicken erythrocyte nuclei obtained by the lysolecithin or saponin method of laking in 0.85 per cent NaCl are smaller and appear more condensed than they do in the unlaked erythrocyte.

Finally, in a recent paper, Pollister and Leuchtenberger have demonstrated more directly the loss of protein from nuclei as a result of extraction with physiological saline solution.[121] Thus, the finding of Mirsky and Pollister[31, 122] that nucleohistone (i.e., histone and nucleic acid in salt combination) is very insoluble in physiological saline, apparently has led many workers to the false assumption that all nuclear con-

[119] A. L. Dounce, *J. Biol. Chem.* **151**, 221 (1943).
[120] G. G. Villela and F. Ubatuba, *Rev. brasil biol.* **8**, 35 (1948).
[121] A. W. Pollister and C. Leuchtenberger, *Proc. Natl. Acad. Sci.* **35**, (No. 1) 66 (1949).
[122] A. E. Mirsky, *Advances in Enzymol.* **3**, 1 (1943).

stituents, at least those of a high molecular weight, have maximal insolubility in physiological saline.

All of the foregoing discussion indicates that in determining the amount of an enzyme per dry weight of isolated nuclei, the results can probably be considered at best as semiquantitative, and in some cases only of a qualitative nature, if aqueous solvents were used in isolating the nuclei. This does not mean that enzyme studies of isolated nuclei are not of value, but it does mean that caution must be exercised in interpreting results and that the problem must eventually be approached from as many angles as possible, including studies of nuclei obtained by microdissection, studies of nuclei produced by the Behrens technique, studies of nuclei by means of histochemical techniques, and so forth.

It may be of some interest at this point to outline a problem concerning the state of nucleic acid in the nucleus which at the present time is just beginning to be clearly defined, and which no doubt involves enzyme chemistry. We demonstrated originally that nuclei prepared at pH 4.0 yielded a stiff gel which persisted in very dilute solutions after the addition of sufficient ammonia to raise the pH to 8.0 or higher. Laskowski observed gel formation when chicken erythrocyte nuclei were treated with strong NaCl solution. We confirmed this finding and also observed gel formation upon the addition of ammonia. Miescher had observed gel formation when he treated fish spermatozoa with strong NaCl solutions[123] and Mirsky and Pollister confirmed this.[70] Bensley and Hoerr had observed a gel formation when liver tissue previously extracted exhaustively with physiological saline was treated with dilute ammonia.[124] The latter authors ascribed their gel to the presence of a supposedly new cytoplasmic constituent, "plasmosin," but we believe, as apparently Mirsky does, that this view was erroneous, and that the gel was chiefly due to the desoxyribonucleic acid remaining in the cell nuclei.

Recently we have observed gel formation upon adding 5 or 10 % NaCl or ammonia or dilute NaOH to nuclei of normal rat liver cells which were obtained by the Behrens technique (see below). In this case, it is necessary to raise the pH above 9.0 to get good gel formation upon the addition of ammonia or NaOH. It is also possible to obtain gel formation by adding NaOH to rat liver nuclei prepared at pH 4.0. Nuclei prepared below pH 4.0 will also produce gels with alkali.

An explanation of the chemistry of this gel formation is due in part to Dr. Elmer Stotz of this laboratory. The theory is as follows:

Gel formation is due to the formation of an alkali metal or ammonium salt of desoxyribonucleic acid. In the case of the Behrens nuclei, where no

[123] F. Miescher, Die Histochemischen und Physiologischen Arbeiten. F. C. W. Vogel, Leipzig, 1879, Vol. 2 p. 63.

[124] R. R. Bensley and N. L. Hoerr, *Anat. Record* **60**, 251 (1934).

aqueous solvents were employed in the preparation, no lowering of the pH occurred, and the desoxyribonucleic acid presumably is present as a salt with histone. The addition of NaCl causes a metathetical reaction as follows:

$$NaCl + Hs\text{-}D.N.A. \longrightarrow Na\text{-}D.N.A. + HsCl^*$$

The addition of alkali produces the same result as follows:

$$NaOH + Hs\text{-}D.N.A. \longrightarrow Na\text{-}D.N.A. + HsOH^*$$

* D.N.A. = desoxyribonucleic acid
Hs = histone
HsCl = histone chloride
HsOH = free histone base

In the latter case the pH must be raised to the vicinity of the isoelectric point of the histone, at least, if all of the latter is to be displaced from the nucleic acid. The same reactions hold for nuclei of chicken erythrocytes, prepared at pH 7.0 in physiological saline. Nuclei prepared at pH 4.0 on the other hand presumably contain much free desoxyribonucleic acid and histone citrate, if the histone has not been extracted. Reactions accounting for gel formation would then be as follows:

$$D.N.A. + NaOH \longrightarrow Na\text{-}D.N.A. + H_2O.$$

Here the pH need be raised only high enough to neutralize the free D.N.A. (pH 8.0), since the histone is already neutralized with citrate. The addition of NaCl to nuclei prepared at pH 4.0 produces no gel, since there is no reaction between a strong free acid and a highly ionized salt:

$$D.N.A. + NaCl \longrightarrow \text{No reaction}$$

No place has been made in the above mentioned scheme for rat liver nuclei prepared at pH 6.0 by the dilute citric acid method. These nuclei apparently should produce a gel upon the addition of either NaCl or alkali, but in fact they do not. Instead, an alkali metal salt of a viscous highly polymerized desoxyribonucleic acid is produced from which residual nuclei can be removed by high speed centrifugation. This sodium desoxyribonucleate, most conveniently produced by treating the pH 6.0 nuclei with 5% NaCl[111] appears to be similar or identical to material prepared by the Hammarsten procedure[125] or by the technique of Mirsky and Pollister.[126] The question naturally arises as to why no gel is produced. Although an exact answer cannot be given, it can be stated that in all probability some sort of autolytic action has solubilized the nucleic acid without causing extensive depolymerization, and has thereby rendered it incapable of gel formation. This statement is borne out by the fact that nuclei prepared at pH 4.0 by dilute citric acid, as well as Behrens type nuclei, gradually lose

[125] E. Hammarsten, *Biochem. Z.* **144**, 383 (1924).
[126] A. E. Mirsky and A. W. Pollister, *J. Gen. Physiol.* **30**, 117 (1946).

their ability to form a gel if allowed to stand for a few hours at room temperature at pH 7.0. Evidently the enzymes necessary for this autolysis reside within the nucleus itself.

It was previously noticed by us in studying nuclei prepared at pH 4.0 or lower using the citric acid procedure that very little of the desoxyribonucleic acid was extractable from the nuclei at pH 7.0 to 8.0 with 5% NaCl solution. Lately it has been possible apparently to extract some sodium desoxyribonucleate from Behrens type nuclei (see below) by means of 10% NaCl, but it is a question as to whether a solution of sodium desoxyribonucleate was really formed. The extracted material formed the same type of gel as the original material and did not form the viscous but non-gelled solutions typical of the Hammarsten or Mirsky-Pollister sodium desoxyribonucleate. The interpretation of this finding depends to a considerable extent upon the nature of the gel in question. These gels which are formed by nuclei under appropriate conditions do not have a truly homogeneous appearance. Upon extending the gel in a slanted test tube with a stirring rod, the material, at least while it is reasonably concentrated, can be seen to possess a faintly visible inhomogeneity which however is not easily visible as long as the gel is quiescent. The gel also possesses the curious phenomena of rubberband-like elasticity. This can be demonstrated by stirring a fairly dilute gel rapidly in one direction, for example in a clockwise direction looking down upon the test tube or beaker. When stirring is quickly stopped, the gel can be seen, on account of small air bubbles trapped therein, to rotate in a counter clockwise direction, thus acting as if it had become twisted up during the original stirring or rotation, and then spontaneously had become untwisted when the twisting force was removed. Such behavior is indicative of a rather gross macroscopic structure rather than a fine molecular structure consisting of even very large molecules. Moreover, if a nuclear gel produced by adding 10% NaCl solution to Behrens type nuclei is coagulated by being poured into enough distilled water to reduce the NaCl concentration to about 1%, very fine fibers that have a beaded appearance after staining with aqueous toluidine blue can be seen microscopically under oil immersion.

All of these qualitative observations have lead us to the tentative conclusion that nuclear gels are really produced as the result of a hydration and swelling of the chromosomes in the nuclei, resulting finally in a considerable disruption of nuclear structure and eventually of an uncoiling of the chromatin threads. The latter phenomenon accounts for the persistence of the gel after great dilution. Incidentally, on diluting these gels, considerable work is required to bring the gel into complete dispersion in the new volume of diluting medium. At first the gel remains segregated in the added diluent.

We are then lead to the conclusion that in all probability gel formation

means the formation of a highly hydrated and swollen, but nevertheless insoluble alkali metal or ammonium salt of desoxyribonucleic acid, and that extractable desoxyribonucleic acid means the formation of an alkali metal or ammonium salt of desoxyribonucleic acid that has become solubilized by some sort of intranuclear autolysis and thereby been set free. It was thought possible that the nature of the autolysis in question might be the initial action of desoxyribonucleodepolymerase on the desoxyribonucleic acid in the nucleus, and hence an attempt was made to obtain nuclei at pH 6.0 in isotonic citrate solution, with the hope that the citrate might act as an effective inhibitor of the desoxyribonucleodepolymerase.[127] It was possible to obtain nuclei of a somewhat peculiar microscopic appearance by this procedure, but no gel could be formed from them. Hence it was concluded that the autolysis probably does not consist of the action of desoxyribonucleopolymerase, although this point is by no means proved. It appears to us that a clearing up of the nature of the autolysis discussed above would constitute another step in our understanding of the enzymatic chemistry of the cell nucleus.

It has been claimed by the Stedmans[128–130] that a new acid-protein termed *chromosomin* (not to be confused with the chromosin of Mirsky) makes up the bulk of the protein of the cell nucleus and that desoxyribonucleic acid, if present at all, must be there in relatively unimportant amounts, chiefly in the "nuclear sap." This claim, which was originally put forth with no detailed supporting evidence, and which was in fact supported by circular arguments, appears to the present writer to conflict with all well-established evidence about the chemical constitution of the cell nucleus. This idea of the Stedmans has been criticized in a veiled and open manner by several writers, and it appears unnecessary to consider it here in detail.

Before leaving the topic of the nucleic acid of cell nuclei, a further interesting and perhaps very important finding should be mentioned. Marshak[109] and particularly Brues *et al.*[131] found that the turnover of the phosphate of desoxyribonucleic acid as measured by radioactive phosphate uptake is very low in normal liver cells but is much higher in regenerating liver cells or in tumor cells. This finding was established by isolating whole nuclei from the tissues in question, using strong citric acid. In contrast, the turnover of the phosphate of ribonucleic acid was equally high in resting and dividing cells. The fact that the phosphorous of the desoxyribonucleic acid does not have a high turnover rate in the resting cell should not necessarily be taken to indicate that desoxyribonculeic acid has no *function* in the nucleus of the resting cell. It may well indicate however that the *synthesis* of desoxyribonucleic acid is very slow in the resting cell.

[127] M. McCarty, *J. Gen. Physiol.* **29**, 123 (1946).
[128] E. Stedman and E. Stedman, *Nature* **152**, 267 (1943).
[129] E. Stedman and E. Stedman, *Nature* **152**, 504 (1943).
[130] E. Stedman and E. Stedman, *Nature* **152**, 556 (1943).
[131] A. M. Brues, M. M. Tracy, and W. E. Cohn, *J. Biol. Chem.* **155**, 619 (1944).

Recently, Marshak[132] purports to show by studies involving radioactive phosphate that cell nuclei of normal and regenerating rat liver initially incorporate phosphate into some new type of nucleic acid hithertofore not described, and that the phosphate thus taken up is later transferred to ribose nucleic acid of the cytoplasm in normal liver and to desoxyribonucleic acid of the nuclei in regenerating liver. These conclusions are reached largely from measuring soluble nucleotides formed by incubating the isolated nuclei alone or with ribonuclease and desoxyribonuclease in saline solution or in bicarbonate buffer, and also from measuring the specific activity of these fractions with respect to P^{32}. The statement is made that this new "precursor type" of nucleic acid is different from the ribonucleic acid of cytoplasm since its specific activity with respect to P^{32} is much higher.

Marshak apparently has demonstrated that in both regenerating and normal liver, P^{32} is not incorporated into the desoxyribonucleic acid fraction of the nucleus to an appreciable extent in the first 3 hours after administration. The best indication of this result is the finding of no appreciable radioactivity in the desoxyribonucleic acid fraction extracted by the method of Schmidt and Thannhauser[133] (erroneously called by Marshak the method of Schneider who made use of it[134]). The work with the added enzymes is somewhat ambiguous however, since it was not demonstrated that the desoxyribonuclease was free from ribonuclease and vice-versa. Moreover, the insolubility in 1 *M* NaCl[91] of desoxyribonucleic acid in nuclei prepared by means of strong citric acid was apparently not appreciated by Marshak, nor was the possibility that some ribonucleic acid might be extracted by the strong citric acid which he employed. The writer can see little reason at the present time for using 5% citric acid in preparing liver cell nuclei.

Marshak found that autolysis of the nuclei in respect to nucleic acid occurred at 37° in saline, and to a greater extent in bicarbonate buffer. The latter result is not surprising since it is doubtful whether the pH in the interior of the saline-washed nuclei could be nearly as high as seven. The findings of Marshak which appear to have demonstrated the presence in the cell nucleus of enzymes which can depolymerize nucleic acid, support similar studies of von Euler and collaborators, already mentioned. However, more work remains to be done to prove this point. Apparently the enzymes of the nuclei which can depolymerize nucleic acid must be extraordinarily resistant to acid.

This writer is not convinced that Marshak has demonstrated the presence of a new "precursor type" of nucleic acid. The fact that the specific activity of the cytoplasmic ribonucleic acid is lower than that of the "pre-

[132] A. Marshak, *J. Cellular Comp. Physiol.* **32**, 381 (1938).
[133] G. Schmidt and S. J. Thannhauser, *J. Biol. Chem.* **161**, 84 (1941).
[134] W. C. Schneider, *J. Biol. Chem.* **164**, 747 (1946).

cursor nucleic acid" in no way proves them to be different materials. It is more probable that the alleged "precursor type" nucleic acid of Marshak is simply ribonucleic acid. The latter statement is strongly supported by work recently reported by Barnum and Huseby[135] who show that the ribonucleic acid of cell nuclei takes up radioactive phosphorous from inorganic phosphate far more rapidly than the ribonucleic acid of cytoplasm. The ribonucleic acid of the submicrosome fraction, although lagging behind the ribonucleic acid of the nuclei in speed of uptake of radioactive phosphate, is nevertheless ahead of the ribonucleic acid of mitochondria and microsomes. The latter fractions both take up the radioactive phosphate very slowly. This work lends some support to the idea of a flow of ribonucleic acid from the nucleus to the cytoplasm.

f. Studies of Nuclei Isolated by the Technique of Behrens

In 1932 Behrens[136] was able to isolate cell nuclei from heart muscle, brain, liver, pancreas and thymus by a method which did not involve the use of any aqueous solvents. This work was for the time being more or less unnoticed, but it was of the greatest importance in that it provided a means of checking analytically the amount of material lost from nuclei prepared by using aqueous extractants. This type of work was not however carried out to any great extent by Behrens. Briefly, the method of Behrens consists in lyophilizing the tissue, grinding in a ball mill to break the cells, and sifting to remove large particles and fiber, followed by isolation of the nuclei using a specific gravity flotation method with mixtures of benzene and carbon tetrachloride as solvents. The latter procedure depends upon the fact that the nuclei have a greater specific gravity than the whole cell or most of the components of cytoplasm. Of course lipide studies cannot be made upon nuclei obtained by the method of Behrens.

In 1939, Behrens[137] reported that arginase was present in nuclei prepared by his method from liver in about the same concentration as in the cytoplasm, but that lipase was very low in concentration in these nuclei as compared with its concentration in cytoplasm. This result may be compared with unpublished work of our own in which we have found both lipase and esterase in nuclei obtained at pH 6.0, using aqueous solvents, from pancreas, and esterase in nuclei obtained from liver, all in concentrations of about one-fourth to one-half that of the corresponding whole tissue. We also confirmed Behrens' results with arginase using nuclei obtained by a modification of his method.[138] A photograph of nuclei of normal

[135] C. P. Barnum and R. A. Huseby, *Federation Proc.* **8**, 182 (1949).
[136] M. Behrens, *Z. physiol. Chem.* **209**, 59 (1932).
[137] M. Behrens, *Z. physiol. Chem.* **258**, 27 (1939).
[138] A. L. Dounce, G. H. Tishkoff, S. R. Barnett, and R. M. Freer, *Federation Proc.* **8**, 194 (1949).

rat liver obtained by us using our modification of Behrens' procedure is shown in Fig. 3.

Behrens also reported the preparation of cell nuclei from thyroid.[139] Finally, the new method of cell fractionation was applied to plant tissue[140] and in subsequent work, Behrens and collaborators proved that the nuclei of cereal germ contain desoxyribonucleic acid[141] and that the cytoplasm of cereal germ contains ribonucleic acid.[142] Thus the older idea that desoxyribonucleic acid occurred only in animal cells was definitely disposed of, and the true picture, i.e., the localization of the desoxyribonucleic acid in cell nuclei whether in the plant or animal kingdom, was established.

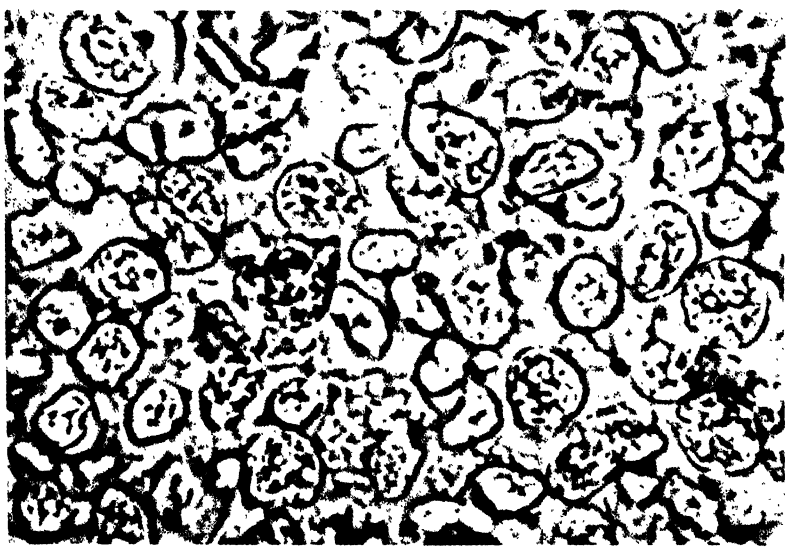

FIG. 3. Nuclei isolated from rat liver by the Behrens' technique. (Dounce, A. L. and collaborators.)

Subsequent workers have utilized various modifications of Behrens' procedure for obtaining nuclei from animal cells. For instance Williams and collaborators[143] studied the distribution of vitamins between nucleus and cytoplasm of heart muscle and mouse cancers, using a modified Behrens' procedure to obtain the nuclei.

Microbiological studies indicated that nicotinic acid, riboflavin, pantothenic acid, thiamine, and folic acid are two to four times as concentrated

[139] M. Behrens, *Z. physiol. Chem.* **232**, 263 (1935).
[140] M. Behrens, *Z. physiol. Chem.* **220**, 97 (1933).
[141] R. Feulgen, M. Behrens, and S. Mahdihassan, *Z. physiol. Chem.* **246**, 203 (1937).
[142] M. Behrens, *Z. physiol. Chem.* **253**, 185 (1938).
[143] E. R. Isbell, H. K. Mitchell, A. Taylor, and R. J. Williams, *Univ. Texas Pub. No. 4237*, pp. 81–3 (1942).

in the nuclei as in the whole cell in the case of heart muscle. Inositol and biotin appeared to be more concentrated in the cytoplasm than in the nuclei, while pyridoxine was equally concentrated in the cytoplasm and nucleus. In the cancer tissues studied, on the other hand, the vitamins in general seemed to be less concentrated in the nuclei than in the whole tissue.

A study of the proteins of nuclei isolated from calf thymus by the Behrens' technique was made by Mayer and Gulick.[144] These authors studied the protein make-up of the isolated nuclei and estimated the nucleic acid content by analyzing for total phosphorous (a procedure which is quite erroneous). A sulfur-containing protein fraction was isolated from the nuclei which was precipitable isolectrically at pH 5.8 to 6.2 and which was soluble in 5% NaCl. This fraction no doubt must have contained many individual proteins.

A study of some metals of isolated cell nuclei of bovine thymus, human tonsil; and bovine supramammary lymph gland was made by Williamson and Gulick[145] A modification of the Behrens' technique was used in which acetone dehydration was used instead of lyophilization. The isolated nuclei contained on the average of 1.35% calcium and about 0.07% magnesium, as against 0.73% calcium in whole thymus cells and 0.02% magnesium in the same cells. Thus calcium and magnesium seem to be more concentrated in the nuclei than in the cytoplasm. The percentage of calcium and magnesium did not seem to vary from one type of nucleus to another.

Lately we have undertaken a study of Behrens type nuclei in this laboratory, using normal rat liver and rat carcinoma 256.[138] The original method of Behrens, which is cumbersome and defective in the lyophilization step, was modified to some extent. Our lyophilization was carried out in a period of 48 hours using a vacuum pump with dry ice-acetone traps to freeze out the evaporated water. Grinding was carried out in the cold using a ball mill, with the frozen, powdered liver suspended in petroleum ether. Fiber was removed by straining through cheese cloth and specific gravity flotation and differential centrifugation was carried out in the cold, using benzene-carbon tetrachloride mixtures. Details of this work will appear elsewhere.

The chief findings of interest were first that the desoxyribonucleic acid of Behrens type nuclei is only about 6% as measured by Schneider's method. This probably indicates either that much material other than desoxyribonucleic acid has been lost from nuclei prepared at pH 4.0 or 6.0 in aqueous medium, or that the Behrens nuclei are only 40 to 50% pure. From the method of preparation and microscopic observation, the latter possibility is very improbable. A second finding was that the desoxyribonucleic acid content of Walker tumor nuclei is higher than that of normal

[144] D. T. Mayer and A. Gulick, *J. Biol. Chem.* **146**, 433 (1942).
[145] M. B. Williamson and A. Gulick, *J. Cellular Comp. Physiol.* **23**, 77 (1944).

rat liver nuclei, contrary to results previously obtained by us.[91] This indicates that less material is lost from tumor nuclei than from liver nuclei when both are prepared in aqueous media, although much material also is lost from the tumor nuclei. A third finding was that the nucleic acid of Behrens type nuclei is not easily extractable in 5% sodium chloride solution and forms a gel as previously discussed, upon the addition of NaCl solution or alkali. A fourth general result was that some enzymes can be measured in Behrens type nuclei, while others are destroyed or nearly destroyed by the method of preparation. Arginase appears to be undamaged; catalase is 50% inactivated, and aldolase is almost completely inactivated.

Finally, free amino acids were measured by paper chromatography in the isolated nuclei and whole cells. No appreciable differences in amino acid patterns were found between nuclei and whole cells, either in the case of normal liver or tumor.

The work outlined above has made it quite clear that great losses of material, undoubtedly including protein, occur when nuclei are prepared in aqueous media at pH 6 or 4 in the case of liver. Nucleic acid analyses make it most probable that the same statement holds for bird erythrocyte nuclei prepared at pH 7.0. Whether the statement applies to nuclei obtained by the hypertonic sucrose technique is not yet clear, but there seems no reason to suppose that the latter nuclei should retain any more of their dry weight than nuclei prepared by other procedures using aqueous media. It is also possible that chromosomes and mitochondria prepared in aqueous media lose some protein, but this is not yet known to be true.

2. Studies of Isolated Chromosomes

Claude and Potter[146] published a method for the isolation of chromatin threads from leukemia cells in 1943. The threads were extended, generally doubled, and stained in a banded manner with the Feulgen stain. From the photomicrographs in the article, these chromatin threads appeared to be in an excellent state of purity. About 3% lipide was present in the isolated chromatin threads which may or may not have been an impurity according to Claude. No further work on chromatin threads of which the writer is aware has been published by Claude. A photograph of the chromatin threads of Claude and Potter is shown in Fig. 4.

Slightly earlier than the date of appearance of Claude's paper, Mirsky and Pollister[147] announced the preparation of chromatin threads from liver and other tissue which were thought possibly to be isolated chromosomes, but details of the method were not published until 1947.[148, 149] By this time

[146] A. Claude and J. S. Potter, *J. Exptl. Med.* **77**, 345 (1943).
[147] A. E. Mirsky and A. W. Pollister, *Biol. Symposia* **10**, 247 (1943).
[148] A. E. Mirsky and H. Ris, *J. Gen. Physiol.* **31**, 1 (1947).
[149] A. E. Mirsky, *Cold Spring Harbor Symposia Quant. Biol.* **12**, 143 (1947).

it was certain that the threads were indeed chromosomes. Mirsky's chromosomes appear to be coiled for the most part and possess sufficient morphology so that a given kind of chromosome can be found repeatedly in a smear. A photograph of these coiled chromosomes is shown in Fig. 5. It is of interest that both Claude and Mirsky used whole tissue rather than isolated nuclei as starting material for obtaining isolated chromosomes. Indeed Mirsky states that he has been unable to obtain isolated chromosomes starting from isolated nuclei. It seems apparent that a slight autolysis, presumably caused by enzymes of the cytoplasm, must occur in order to

FIG. 4. Chromatin threads isolated from mouse leukemia cells according to Claude. Left hand figure at 500× magnification, right hand figure at 1600× magnification. Photograph kindly furnished by Dr. Claude. (Claude, A. and Potter, J. S., *J. Exptl. Med.* **77**, 345, 1943.)

"loosen" the chromosomes and allow them to become dispersed. Since the desoxyribonucleic acid of the isolated chromosomes is extractable with molar NaCl, and since the chromosomes were prepared at high pH in aqueous medium, one may probably assume with safety that enough autolysis has occurred to prevent gelability of the chromosomes, although this statement is not made by Mirsky. The marked instability of isolated chromosomes in physiological saline has been noted by Mirsky and Ris.[150] It is recognized that autolytic changes must occur even during preparation of the chromosomes. It is shown also that not all of the histone can be split

[150] A. E. Mirsky and H. Ris, *J. Gen. Physiol.* **31**, 7 (1947).

off from the nucleic acid of isolated chromosomes with HCl or H_2SO_4. Whether this latter finding can have anything to do with gel formation is a matter of uncertainty. The last two papers mentioned are especially recommended to the reader.

The only knowledge of the enzymatic composition of the isolated chromosomes of which the writer is aware is the statement by Mirsky that the

FIG. 5. Suspension of isolated thymus chromosomes according to Mirsky (*Cold Spring Harbor Symposia Quant. Biol.* **12**, (1947)). Aceto-orcein; magnification = 1200×.

isolated chromosomes are rich in alkaline phosphatase and also in the autolytic enzymes of unspecified nature which render them very unstable on standing.

Since Mirsky used 0.9% NaCl in preparing isolated chromosomes it is not surprising, in view of discussion already concluded, that desoxyribonucleohistone should comprise a very high percentage of the total mass of the chromosomes. It would be of interest if chromosomes could be isolated by Behrens' technique, to compare the desoxyribonucleohistone content of such chromosomes with those obtained by the method of Mirsky. It has

in fact been determined by Pollister and Leuchtenberger[121] by microabsorption technique that the concentration of desoxyribonuculeoprotein in chromosomes as they exist in the cell is lower than in the isolated chromosomes of Mirsky. It might also be of interest to compare the desoxyribonucleohistone content of chromsomes isolated with water as the suspending medium with that of chromosomes isolated using physiological saline as the suspending medium.

Chromosomes have been isolated more recently by Gopal-Ayengar and Cowdry[151] from mouse epidermis made hyperplastic by metholcholanthrene, from normal mouse epidermis, and transplantable mouse epidermal carcinoma produced by methylcholanthrene. Enzymes were not studied, but the desoxyribonucleic acid content of the chromosomes of hyperplastic epidermis was found to be below that of the normal epidermis, while the desoxyribonucleic acid of the tumor chromosomes was considerably above normal. This work is important and of considerable interest to those working in cancer research, but in view of difficulties already mentioned in connection with isolated whole nuclei, complete evaluation of interpretation of the results may not be possible at the present time.

3. Discussion of Section III

It now seems clear that many enzymes, for example alkaline phosphatase and important enzymes of the glycolytic system including the dehydrogenases, are normally present in at least two kinds of cell nuclei (liver and kidney), but that oxidative systems must be deficient there, since succinic dehydrogenase, for instance, is almost certainly lacking. It is also clear that the enzymatic make-up of nuclei can be variable, since liver cell nuclei, for example, are rich in arginase, but apparently this enzyme is absent from the nuclei of kidney cells.

Esterase and catalase appear to be present in both liver and kidney cells. Cytochrome oxidase appears to be present in the nuclei of liver cells at least. Pancreas nuclei seem to be very deficient in aldolase but contain esterase, lipase and some catalase. This deficiency in aldolase may reflect the extremely low values of aldolase in whole pancreas or it may be that aldolase is more easily lost from pancreas nuclei than from liver and kidney nuclei.[152]

Autolytic enzymes that destroy the gelability of desoxyribonucleic acid appear to be present in the nuclei of all tissues thus far studied, and in fact may be present in isolated chromosomes.

It is still too early to state just what type of metabolism goes on in the cell nucleus. Recent studies in this laboratory on the substrates of Behrens type nuclei have shown no difference between the pattern of free amino

[151] A. R. Gopal-Ayengar and E. V. Cowdry, *Cancer Research* **7**, 1 (1947).
[152] J. W. Sibley and A. L. Lehninger, *J. Biol. Chem.* **177**, 859 (1949).

acids in the nucleus and cytoplasm of normal rat liver cells from rats fed *ad libitum* on a standard fox chow diet. Glutathione also was present in the nucleus and cytoplasm in approximately equal concentrations.

The composition of isolated nuclei has been shown to depend upon the method of preparation, since on the one hand considerable water-soluble material is undoubtedly extracted in using aqueous methods, and on the other hand lipide is extracted in using the Behrens' technique. It is therefore desirable to use a method of preparation that can be shown, if possible, not to damage or extract a component being studied, and also, if possible, to determine the amount of the component in question per nucleus rather than per dry weight of nuclei. The latter procedure involves counting nuclei, however, and it must be admitted that this is not easy or accurate in many instances, because of the great tendency for the nuclei to clump together, if prepared at any pH except 6.0. Strong citric acid (or other acid) can be used in diluting the nuclei before counting to minimize clumping, and staining should be avoided if possible, since this favors clumping.

IV. Studies of the Chemistry of Cell Components as Deduced from Special Physiochemical Techniques.

1. Special Centrifugation and Microdissection Techniques, Singly or Combined

In work of this sort, a very small number of cells is used as a rule, and the estimation of enzymes in the cell fragments therefore must be carried out by special micromethods, most of which have been developed in the laboratory of Linderstrøm-Lang. The micromethods will not be discussed here, since they are covered in Glick's new book on technique.[153] A discussion of the methods will also be found in a chapter by Linderstrøm-Lang and Holter in Bammann and Myrbäck, Die Methoden der Fermentforschung.[154] The procedures have been applied, for example, to echinoderm eggs and to amoebas.

Holter and Linderstrøm-Lang[155] found that in the eggs of the echinoderm *Dendraster excentricus*, significant amounts of the enzymes catalase and peptidase were not present in the cell nucleus. This was also true of *Amoeba proteus*. In general, using amoebae or echinoderm eggs, the enzymes peptidase and catalase were well correlated in activity with the amount of "hyaline cytoplasmic matrix" estimated to be present in the cell components studied, and hence these enzymes were thought to be present in this

[153] D. Glick, Techniques of Histo- and Cytochemistry. Interscience, New York, 1949.

[154] Die Methoden der Fermentforschung. Edited by Bammann and Myrbäck, Thieme, Leipsig, 1940, p. 1132.

[155] H. Holter and K. Linderstrøm, *Sitzber. Akad. Wiss. Wien Mathem.-naturw. Klasse Abt. II-b* **145**, 898 (1936); or *Monatsh.* **69**, 292 (1936).

hyaline cytoplasmic matrix rather than in the mitochrondria, nucleus, or other large formed elements of the cell. The technique of the Harveys (references previously cited) was used in centrifuging the cells, and microdissection was used only when necessary to separate previously unseparated cell halves.

In 1936, Holter[156] reported, in agreement with the above mentioned work, that peptidase occurred in the "hyaline cytoplasmic matrix" in three species of marine ova (*Arbacia punctulata, Echinarachnius parma,* and *Chaetopterus pergamentaceus*).

This work with peptidase was confirmed, in general, by Holter[157] using amoebas, although the authors state that in this case the results were at best semi-quantitative.

On the other hand, Holter and Doyle[158] reported that the amylase of amoeba proteus appeared to be localized in the mitochondria.*

Some interesting observations have been made concerning the function of the nucleus in amoebas. For instance, Lynch in 1919[159] reported a set of observations on nucleate and nonnucleate halves of amoebas which had been separated by microdissection. It was found that the enucleated half could live almost as long as a normal amoeba deprived of food, whereas the nucleated half seemed to behave like a more or less normal amoeba. The nucleus appeared to enable the amoeba to utilize glucose plus urea in its nutrition or metabolism, for without the nucleus, the addition of glucose plus urea was harmful although glucose alone was beneficial. The motion of the amoeba was not seriously affected by lack of the nucleus; indirect (and somewhat inconclusive) evidence was offered to show that the nucleus did not take an important part in cellular oxidations. Very logical arguments were offered on this last point however. It was also reported that the nonnucleate halves were somewhat more sensitive to high or low temperature and to cyanide than the nucleate halves.

The question as to whether an enucleated amoeba can digest food has been a matter of some controversy. Verworn[160] stated that denucleated amoebas could ingest food but could not digest it. On the other hand, Hofer[161] stated that enucleated amoebas could continue the processes of respiration, digestion, and excretion for several days.

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[156] H. Holter, *J. Cellular Comp. Physiol.* **8,** 179 (1936).

[157] H. Holter and M. J. Kopac, *J. Cellular Comp. Physiol.* **10,** 423 (1937).

[158] H. Holter and C. Doyle, *Compt. rend. trav. lab. Carlsberg. Ser. chim.* **22,** 219 (1938).

* Brachet (J. Brachet, *Arch. exptl. Zellforsch. Gewebezüch.* **22,** 541 (1938–39)) in a study of nuclei of frog eggs obtained by microdissection, found that oxidation and CO_2 production by the nuclei were very low compared with the corresponding effects in cytoplasm. On the other hand, peptidase and esterase were nearly as high in concentration in the nucleus as in the cytoplasm.

[159] V. Lynch, *Am. J. Physiol.* **48,** 258 (1919).

[160] M. Verworn, Psycho-physiologische Protisten-Studien, Experimentelle Untersuchungen. Jena, 1889. Cited by M. J. Kopac and H. Holter, *J. Cellular Comp. Physiol.* **10,** 434 (1937).

[161] B. Hofer, *Jena. Z. Naturw.* **24,** 105 (1890). Cited by M. J. Kopac, and H. Holter, *J. Cellular Comp. Physiol.* **10,** 434 (1937).

As early as 1912, Gruber[162] analyzed the previously existing biological literature on the function of the nucleus and cytoplasm in the amoeba and made a rather exhaustive study of the mechanism of movement of the amoeba and the role of the nucleus in maintainance of normal movement and a number of other chemical and physiological functions.

Micro-operative technique was used to obtain large and small portions of enucleated cytoplasm and nucleate cytoplasm. It was found that the maintainance of normal movement is dependent upon the nucleus in some indirect way. Loss of the nucleus did not prevent ingestion of food, but it was definitely shown to hinder digestion of ingested food. It was surmised that digestive enzymes are synthesized within the nucleus and subsequently pass out into the cytoplasm; consequently an enucleate amoeba can digest small particles of food until its content of cytoplasmic enzyme is exhausted, and then digestion fails. At this point partially digested food particles are ejected.

The presence of a nucleus was found to be required for the maintainance of normal rhythm of the contractile vacuole, but contractile vacuoles could be formed and would even contract at a somewhat lowered rate for a time without the presence of a nucleus.

Nucleate and enucleate halves of an amoeba were stated to behave similarly towards alternation in tempeature (contrary to the work of Lynch previously cited). A considerable study was made of the effect of temperature changes on the amoeba. Large pieces of enucleate amoebic cytoplasm behaved more nearly like normal nucleate amoebas than small ones.

If small nucleated amoebas were produced by removing most of the cytoplasm by microdissection, these forms with difficulty could be induced to ingest food. At first the nuclei shrank in size, but later both the cytoplasm and nucleus grew if food ingestion could be maintained, until finally an amoeba of normal cytoplasmic and nuclear size was regenerated. At this point, but not before this, the operated amoeba could divide in a normal manner.

Enucleate amoebas even if large always lost function gradually and eventually died. One author is quoted as stating that he could keep enucleated halves alive as long as 30 days, following operation. Needless to say the enucleate halves did not divide or grow.

It can be seen that this work of Gruber is in agreement with the later work of Lynch, already discussed, except for one or two minor points.

More recently work of a similar nature has been carried out by Clark[163]. Clark found that large organisms (rotifers) could not be digested by protoplasm of the amoeba in the absence of the nucleus, and suggested that digestive enzymes are secreted as zymogens by the nucleus and later are activated by factors in the cytoplasm. The loss of digestive enzymes appeared to be gradual after removal of the nucleus, as reported by Gruber and Lynch, so that a recently enucleated amoeba could ingest and kill small organisms presumably by digestion, whereas after 4 or 5 days food was ingested infrequently and once ingested lived for exceptionally long periods in the food vacuole.

In multinucleate amoebas, according to Clark, only one nucleus is necessary for digestive function. If in a bi- or trinucleate amoeba one or two nuclei respectively are damaged by pricking with a dissecting needle, these damaged nuclei are digested and the amoeba is normal as long as one nucleus remains, but if a mononucleate amoeba is deprived of the function of its single nucleus in this way, the organism be-

[162] K. Gruber, *Arch. Protistenk.* **25**, 316 (1912).
[163] A. M. Clark, *Australian J. Exptl. Biol. Med. Sci.* **21**, 215 (1943).

haves like an enucleate amoeba and does not digest the nuclear remnants. Amoebas with destroyed nuclei "lived" only for 2 to 3 days, whereas Clark was able to keep enucleate amoebas alive for periods as long as a week.

The work of Clark thus seems to confirm quite definitely the idea that the normal digestive function of the amoeba is dependent upon the presence of an intact nucleus. Clark states that all previous workers have recognized the importance of the nucleus in amoebas for synthetic metabolic processes, and this statement is supported by the articles of Gruber and Lynch.

Clark also studied the permeability of the nuclear membrane of amoebas by noticing how fast certain dyes could be taken up by the nucleus. Microinjection was used to introduce dye substance directly into the cytoplasm. The membrane was found to be quite permeable to all the dyes studied, whereas the cytoplasmic surface or plasmalemma was impermeable to some of the dyes. Thus nuclear membrane seems to have a higher degree of permeability than the cell membrane, at least in the case of amoebas.

Clark found that a peroxidase occurs normally in the nucleus and the cytoplasm of the amoeba. This enzyme disappears from the cytoplasm of starved amoebas but reappears when feeding is permitted, in the normal amoeba. However in the enucleate amoeba the enzyme does not reappear even when food has been ingested. This indicates that the peroxidase is probably formed within the nucleus as such or as a zymogen, and later passes out into the cytoplasm.

Permeability of the nuclear membrane of nuclei isolated from frog eggs by microdissection[164] has been studied by Duryee.[165] In this connection it is of interest that Duryee[166] has shown that particles called nucleoli migrate to the surface of the nucleus of the frogs egg and then rupture through the nuclear membrane in some way into the cytoplasm, leaving some small residues inside the nuclear membrane. The nucleoli appear to be formed from outgrowths of the chromosomes ("lampbrush chromosomes"). Nucleolar migration also can be observed in insects.[167,168]

In a more direct approach to the problem of enzyme distribution within the cell, Bundling in 1941[169] studied catalase distribution in cells of the salivary glands of chironomus larvae using the technique of microdissection to separate nuclei from cytoplasm. It was found that cytoplasm produced copious decomposition of H_2O_2 as observed qualitatively by noting the evolution of gas bubbles, whereas nuclei which were broken open after isolation and treated with H_2O_2 did not cause appreciable gas bubble formation. It was concluded therefore that catalase was present in the cytoplasm of this cell but was low in concentration or absent in the nucleus. As has been mentioned, our own observations with nuclei isolated at pH 6.0 from liver, kidney, and pancreas cells by our improved method show the presence of a considerable amount of catalase, although we had previously found no appreciable catalase in nuclei prepared by our original procedure, possibly because of lack of a refrigerated centrifuge.

[164] W. R. Duryee, *Arch. exptl. Zellforsch. Gewebezücht.* **19**, 171 (1937).
[165] W. R. Duryee, *Proc. Am. Sci. Congr. 8th Congr.* **3**, 45 (1940).
[166] W. R. Duryee, Cytology, Genetics, and Evolution. Univ. of Pennsylvania Press, Philadelphia, Pa. 1941, pp. 129–141.
[167] R. A. R. Gresson, *Proc. Roy. Soc. Edinburgh* **13**, 32 (1932).
[168] R. A. R. Gresson, *Quart. J. Microscop. Sci.* **73**, 177 (1929).
[169] I. M. Bundling, *J. Cellular and Comp. Physiol.* **17**, 133 (1941).

Before leaving the subject of special centrifugation techniques and microdissection, mention should be made of the extensive work of Kite and Chambers in this field.[170-173] Space does not permit a complete review of this important and fundamental work. Cell membranes and nuclear membranes were found to be present in most cells studied (but polymorphonuclear leucocytes were said to have no definite cell membrane); the cytoplasm of many cells (but not all) appeared to be a highly viscous material (and in the case of polymorphonuclear leucocytes an actual jelly); and the nucleus was usually but not invariably in the form of a gel (the starfish nucleus was found to be an exception). The nucleolus and chromosomes also were gel-like. The chromosomes were dense structures. The nuclei were very susceptible to injury, and pricking them with a dissecting needle would cause death of the cell. In binucleate macrophages, contrary to the results of Clark with amoebas (previously cited[163]) it was found that pricking of one nucleus caused a swelling of both nuclei and then death of the cell. The swollen nuclei then became granular.

The technique of microdissection will not be discussed. References will be found in the above mentioned articles.

2. Microspectrographic Studies

Much of the important work in this field has been done by Caspersson and collaborators[174-181] in studies of the nucleic acid and protein content of various cellular components, including the nucleus, nucleolus, and the cytoplasm. This work does not give direct information about localization of enzymes, but it permits certain tentative conclusions to be drawn about the type of enzyme activity going on in various parts of the cell. Space permits only very meager mention of the work here. The reader is referred to Glick's book on histo- and cytochemistry[153] for details of the methods employed and references to the work of other authors.

The basis of Caspersson's work is the spectrographic analysis of portions of the microscopic image of a cell, especially in the ultraviolet range. A microscope using quartz lenses or a reflecting microscope must be employed. Since the absorption spectrum of nucleic acid (both ribo- and desoxyribo-) shows a well defined maximum at approximately 260 mμ with a very high absorption coefficient, it is possible with little difficulty to localize regions containing nucleic acid, although by absorption spectroscopy alone it is not possible to distinguish ribo- from desoxyribonucleic acid. It is also true that mono- and dinucleotides will interfere, but interference by these

[170] R. Chambers, *in* General Cytology. Edited by E. V. Cowdry, Univ. of Chicago Press, Chicago, 1924, p. 237.
[171] G. L. Kite, *Am. J. Physiol.* **32**, 146 (1913).
[172] G. L. Kite, *J. Infectious Diseases* **15**, 319 (1914).
[173] R. Chambers, *Proc. Roy. Soc. London* **B109**, 380 (1931).
[174] T. Caspersson, *J. Roy. Microskop. Soc.* **60**, 8 (1940).
[175] T. Caspersson, *Naturwissenschaften* **3**, 33 (1941).
[176] T. Caspersson and L. Santesson, *Acta. Radiol. Suppl.* **46**, 1 (1942).
[177] Same as reference [176].
[178] T. Caspersson, H. Landstron-Hyden, and L. Aquilonius, *Chromosoma* **2**, 11 (1941).
[179] T. Caspersson, *Arch. exptl. Zellforsch. Gewebezücht.* **22**, 650 (1939-b).
[180] T. Caspersson, *Chromosoma* **5**, 562 (1940).
[181] T. Caspersson and B. Thorell, *Chromosoma* **2**, 132 (1941).

substances can be avoided by washing them out from the fixed tissues. In any case the concentration of the nucleic acid is high enough so that the latter type of interference usually will be negligible.

Protein also can be localized, since proteins absorb in general from 270 to 290 mμ, owing to the tryosine and tryptophan contained therein.

Caspersson claims to be able to distinguish ordinary albumin-globulin type protein from histone, by proper analysis of his absorption curves, but this claim seems to have been erroneous.[182,183] However it seems certain that regions of the cell which are high in protein can easily be localized.

The absorption coefficient of nucleic acid is much higher than that of proteins, so that if a cell is photographed in ultraviolet light at or near 260 mμ, the approximate distribution of nucleic acid within the cell, particularly within the nucleus can be seen in the photograph at a glance, as is well known. The banded structure of giant chromosomes of the salivary gland of diptera larvae is an example of this phenomenon.

Since it is known from chemical studies that desoxyribonucleic acid is absent or at least present only in thus far undetectably low concentration in cytoplasm, any region of high nucleic acid concentration observed microspectrographically in cytoplasm (such as around the nucleus in some types of cells) can be taken as meaning a high concentration of ribonucleic acid. The situation is not so clear in nuclei however, since it is known from chemical studies that both desoxyribo- and ribonucleic acid occur there. Even in isolated chromosomes both desoxyribo- and ribonucleic acids are found according to Mirsky. Hence in studying nuclei with microspectrography alone, the results must in general be reported as total nucleic acid.

However, it is possible by treating the cell with crystalline ribonuclease to remove ribonucleic acid, and then to get the distribution of the remaining desoxyribonucleic acid. A recent article on the action of ribonuclease on fixed tissues has been published by Stowell and Zorzoli.[184] In this way Brachet[185] and Caspersson and Schultz[186] have demonstrated that in some cases the *nucleolus* contains a high concentration of ribonucleic acid but no desoxyribonucleic acid; but Davidson and Waymouth[187] were unable to prevent staining of the nucleoli of liver cells with toludine blue by previous treatment with ribonuclease, although staining of cytoplasm was prevented by this procedure. This finding might be taken to indicate the presence of desoxyribonucleic acid in the nucleoli, but correct interpretation of the work seems difficult.

It is possible in certain cases to estimate fairly well the total amount of a constituent such as nucleic acid in a cell component, using microspectrography. For instance Caspersson[188] found 11.2×10^{-6} μg. nucleic acid in the leptotene spermatocyte nucleus of the large grasshopper by the ultraviolet method.

The principal conclusions of Caspersson and collaborators of interest to us are that the nucleolus is rich in ribose nucleotides at the same time that these substances are found to be high in concentration in cytoplasm. Furthermore nucleotides are said to be concentrated around the nuclear membrane in certain types of cells. From these findings it might be concluded

[182] A. W. Pollister and H. Ris, *Cold Spring Harbor Symposia Quant. Biol.* **12**, 147 (1947).
[183] A. E. Mirsky and A. W. Pollister, *Trans. N. Y. Acad. Sci.*, Ser. II **5**, 187 (1943).
[184] R. E. Stowell and A. Zorzoli, *Stain Technol.* **22**, 51 (1947).
[185] J. Brachet, *Compt. rend. Soc. biol.* **133**, 88 (1940).
[186] T. Caspersson and J. Schultz, *Proc. Natl. Acad. Sci.* **26**, 507 (1940).
[187] J. N. Davidson and C. Waymouth, *Proc. Roy. Soc. Edinburgh* **62**, 96 (1944).
[188] T. Caspersson, *Chromosoma*, **1**, 147 (1939).

that ribonucleic acid from the nucleus, and perhaps from the nucleolus, actually diffused out into the cytoplasm, although other interpretations are possible. In this connection one should consider the work of Duryee and others (previously cited) on nucleolar migration. Caspersson believes, as the result of work done by himself and his collaborators, that the cell nucleus is a very important site of protein synthesis, but it must be stated that he has in no wise proved this idea although he has made it seem plausible enough. Other evidence in favor of the idea that nucleic acid participates in protein synthesis will be found in an article by Greenstein on nucleoproteins.[189]

Another way of demonstrating localization of nucleic acid within the cell is to use a staining method that is more or less specific for nucleic acid, and then to use microphotometric methods to measure the stain. Stowell has, for example, adapted the Feulgen staining reaction for desoxyribonucleic acid to this type of procedure. References to his work will be found in Glick's book on histo- and cytochemistry.[153] Pollister and Ris[182] describe methods of this sort for the determination of nucleic acid and proteins within cells. This is not microspectrography in the strict sense of the word, but it is microphotometry, which is a very closely related technique. Such work can be of immense importance in investigating possible losses of material occurring when a cellular constituent is isolated by physicochemical procedures. For instance Pollister and Leuchtenberger[121] demonstrated quite conclusively that the amount of protein relative to nucleic acid is much higher in the nucleus as it exists in the cell than it is in nuclei or chromosomes isolated by aqueous procedures. This finding agrees with work carried out in the writer's laboratory on nuclei obtained by a modified Behrens' technique, where protein extraction cannot occur.

V. Histochemical Procedures

1. Enzyme Substrates and Minerals

a. Staining Procedures

It is possible in certain instances to localize high-molecular weight substances that are not easily diffusable by treating fixed microscopic sections of a tissue with some specific or relatively specific color-producing reagent. A number of references to specific methods will be found in Glick's book[153] and in the review article by Dempsey and Wislocki.[190]

One of the best illustrations of this type of procedure is the staining of desoxyribonucleic acid by the Feulgen reagent, which is simply Schiff's aldehyde reagent made up in a standard manner suitable for tissue work. The desoxyribonucleic acid must be subjected to a partial hydrolysis (usually with 1*N* HCl) before the reagent will func-

[189] J. R. Greenstein, *Recent Advances in Protein Chem.* **1**, 209 (1944).
[190] E. W. Dempsey and G. W. Wislocki, *Physiol. Revs.* **26**, 1 (1946).

tion for desoxyribonucleic acid. For the details of the method, as well as other methods for staining nondiffusable substrates in cells, the reader is referred to the book of Glick, already cited.

There has been some disagreement as to the specificity of the Feulgen reagent for desoxyribonucleic acid, but apparently if the acetyl phospholipides are removed by extraction with fat solvents, the reagent does localize desoxyribonucleic acid quite well. In agreement with chemical studies on isolated cell components, the Feulgen staining technique shows that desoxyribonucleic acid is present in appreciable amounts only in the cell nucleus. Furthermore, if the stain is used on isolated chromosomes, a banded staining is obtained[146] indicating a banded concentration distribution of desoxyribonucleic acid, as has been inferred from microspectrographic studies.

The Feulgen staining reaction is probably optimal when the purine bases have been split off from the nucleic acid molecule, leaving two free pentose aldehyde groups per each two pyrimidine residues left unhydrolized, according to Di Stefano.[191] Under optimal conditions, measurement of the amount of fuchsin dye in a cell nucleus by microcolorimetry can lead to a computation of the amount of desoxyribonucleic acid contained therein. For cartilage cell nuclei, according to Di Stefano, the value thus computed comes out to be approximately 2.44×10^{-6} μg. per nucleus. By macro methods and counting, as applied to isolated cell nuclei, Vendrely and Vendrely[192] found about 5×10^{-6} to 6×10^{-6} μg. of desoxyribonucleic acid per cell nucleus in the case of liver, as already stated. Pollister and Ris report[182] from microphotometric studies with staining procedures that calf thymus lymphocytes contain 1.0×10^{-6} μg. of nucleic acid per nucleus. Caspersson[188] reported that he found 11.2×10^{-6} micrograms of nucleic acid per nucleus of large grasshopper spermatocyte nuclei, using ultraviolet microabsorption spctroscopy.

In general, it can be stated that the results of nucleic distribution and the amounts present in cells and cell nuclei acid as determined by microphotometric methods are in fairly good agreement with the results of chemical work on a macro scale coupled with counting, and with microspectrographic methods.

Schiff's reagent also has been used to localize glycogen within the cell, after treating the sections with chromic acid. In this case salivary amylase can be used to remove the glycogen so that a control section can be made which does not possess glycogen.

The distribution of glycogen within the cell is of some interest. Methods for localizing this substance will be found in Glick's book. It was found by Chipps and Duff[193] using Best's carmine stain, that glycogen appears in the nuclei of liver cells in uncontrolled diabetes, and in some other pathological conditions, whereas it is normally absent from the nucleus. We also have found glycogen to be absent in nuclei isolated from cells of normal rat liver, although glycogen was always present in our homogenates. It seems possible that the presence of glycogen in the nucleus in diabetes may be a reflection of the abnormally high concentration of glucose which is presumably present in the liver cell. We have found some phosphorylase (Cori enzyme) in nuclei isolated from normal rat liver cells and hence it seems

[191] H. S. Di Stefano, *Proc. Natl. Acad. Sci.* **34**, 75 (1948).
[192] R. Vendrely and C. Vendrely, *Experientia* **4**, 436 (1948).
[193] M. D. Chipps and G. L. Duff, *Am. J. Path.* **18**, 645 (1942).

reasonable to assume that nuclei could form glycogen if sufficient substrate for the synthesis (Cori ester) could be accumulated there.

Histochemical procedures for localizing other cellular constituents will be found in Glick's book, but most of these seem to be of somewhat doubtful significance. Lipide can be localized by the use of Sudan dyes.

b. Microincineration

Microincineration is a technique which makes it possible to burn off the organic matter of a tissue section without causing appreciable change in localization of the nonvolatile elements, and then to analyze chemically or spectroscopically for various elements remaining in the ash. Also the variation in the total amount of ash present in various parts of the section can be studied by means of dark-field illumination. The method of microincineration coupled with dark-field illumination, or microspectroscopy, appears to be better adapted to a determination of the distribution of certain elements among whole groups of cells rather than among the various components of a single cell. The reader is referred to Glick's book on histo- and cytochemistry for a brief description of the method and references to the literature on this subject.

A different procedure for locating certain metals, namely calcium plus magnesium, in different parts of a tissue or even of a single cell is the method of emmission electron microscopy. Frozen sections of the tissue in question are dehydrated at −63°C. and are then imbedded in paraffin and placed on a polished, chemically treated nickel cathode that is part of a low power electron microscope. The pressure is reduced to a very low value and the cathode is heated slowly until it finally attains a temperature in the neighborhood of 700–800°C. This results in an incineration of the section. An accelerating voltage is then applied to draw off electrons from the cathode. Electrons are emitted as the result of some sort of catalytic action wherever the concentration of calcium plus magnesium is high in the incinerated section. These electrons are focused to form an image on a fluorescent screen or on a photographic plate. The image thus corresponds to the distribution of calcium plus magnesium in the cell. The reader is referred to Glick's book and to an article by G. H. Scott[194] for details and references.

A number of photographs are published in the article referred to by Scott. These pictures show that the amount of calcium plus magnesium is generally high in the cell nucleus, the cell membrane, the anisotropic disks of muscle, the zymogen granules of pancreas, and in certain definite locations in some epithelial cells. Microincineration studies made in conjunction with emission electron microscope studies show that potassium salts and perhaps other salts must be high in concentration in parts of the cell where the concentration of calcium plus magnesium is low. In young cells (embryonic) or tumor cells, the cytoplasmic calcium plus magnesium seems to be lower than in fully differentiated cells. Other results are given in Scott's article.

c. Radioautography

This procedure consists in placing a section of tissue containing some radioactive material on a fine-grained photographic film or plate in the dark, and then, after a suitable time interval, developing the film and enlarging the image produced by the action of radioactive material. References to uses of the method will be found in

[194] G. H. Scott, *Biol. Symposia* **10**, 277 (1948).

Glick's book. It is possible to localize a radioactive material in a group of cells very nicely, but it is difficult to obtain images corresponding to various components of a single cell, since the radioactive particles tend to affect the photographic plate at a distance from the emission point. This can be seen at a glance from photographs published by Boyd and collaborators.[195] Glycine was the radioactive material and C^{14} the radioactive element contained therein. Radioautographs were taken of erythrocytes, polymorphonuclear cells, and lymphocytes. Grains of silver caused by exposure to the radiation from C^{14} are seen around the periphery of the cells. It is thus impossible to locate accurately the source of the radioactive particle *within* the cell.

Radioautography, or autoradiography as it is now often called, is a useful means of determining what cells accumulate a metal in cases of exposure of an animal to the given metal by some route. This procedure was used considerably during the war to locate the deposition sites of uranium, plutonium, etc. Frozen sections can be used in many instances. Some caution must be observed in using the method, since nonradioactive chemicals in the sections may also reduce the silver bromide to metallic silver if the section is applied to the emulsion directly (Boyd, G. A., private communication).

A very interesting recent example of the application of radioautography to investigation of a problem in metabolism is furnished by the work of LeBlond and coworkers.[196] These investigators wished to study the uptake of radioactive phosphorous from inorganic phosphate by the desoxyribonucleic acid of various tissues. Sections were prepared in the usual manner by fixing, dehydrating, imbedding in paraffin, and cutting. The paraffin was dissolved out after the sections were placed on the slides. This procedure was calculated to remove practically all of the phosphate-containing compounds except desoxyribonucleic acid and ribonucleic acid. It should be noted however that phosphoprotein, if such material exists in cells in appreciable quantities, would not be removed.

In order to remove ribonucleic acid, the sections were treated with ribonuclease until the cytoplasm no longer was basophilic to the dye pyronine. Residual phosphate was assumed to be present chiefly or exclusively in desoxyribonucleic acid.

Autographs were made by coating the sections with photographic emulsion (a new process for which references are given by the authors). Development of the emulsions after exposure for 24 hours showed radioactivity in many tissues, and it was shown by the authors that all of these tissues possessed dividing cells. Positive results were obtained in some cases after exposures of only 2 hours. It was stated that an autograph reaction was observed overlying the positions of the nuclei of the epithelial cells in the lower part of the villi of the small intestine. In contrast, parenchymatous organs such as pancreas, thyroid, etc. showed no radioactivity and hence presumably had not incorporated P^{32} into their desoxyribonucleic acid.

If treatment of the sections with ribonuclease was omitted, the autographs were assumed to show the location of ribonucleic acid as well as desocyribonucleic acid. In this way large amounts of newly formed ribonucleic acid were located in the liver, kidney, adrenal cortex, and many epithelia. Strangely, however, none was thus localized in the pancreas, salivary glands, and thyroid.

This work is in good agreement with the previously discussed findings of Brues, Tracy, and Cohn, and Marshak, who made use of isolated nuclei in their studies. It seems likely, therefore, to be reliable on the whole and to be worth extending in the future.

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[195] G. A. Boyd, G. W. Casarett, K. I. Altman, T. R. Noonan, and K. Solomon, *Science* **108**, 529 (1948).

[196] C. P. LeBlond, C. E. Stevens, and R. Bogoroch, *Science* **108**, 531 (1948).

2. Methods of Enzymatic Histochemistry

A number of enzymes have been localized in cells by histochemical staining procedures. Again, details of the methods used will be found in Glick's new book on histo- and cytochemistry.

a. Alkaline Phosphatase

One of the best known and most reliable of these methods is the method of Gomori for localizing alkaline and acid phosphatase. Briefly, the procedure consists of making a microscopic section of tissue by fixing in acetone, replacing the acetone by xylene, imbedding in paraffin at a temperature not over 56°C., and cutting into sections 4 to 8 μ in thickness. The sections are placed on slides and the paraffin is removed with xylene. Then the sections are passed through alcohol of graded concentrations to water and are incubated for 1 to 24 hours as needed with substrate. The latter consists of 2% sodium glyceryl phosphate in an appropriate buffer. Calcium chloride or lead nitrate also is present to precipitate phosphate *in situ* as fast as it is formed. Usually sodium barbital is used as buffer for alkaline phosphatase (pH 9.4) and acetate buffer for ordinary acid phosphatase (pH 4.7).

In the case of alkaline phosphatase, the calcium chloride is used to precipitate the liberated phosphate. The calcium phosphate thus formed is changed to cobaltous phosphate, after rinsing out the buffer substrate with water, by adding cobaltous acetate or some other cobaltous salt. The excess soluble cobaltous salt is washed out with water and the cobaltous phosphate is changed to black cobaltous sulfide by treatment of the section with ammonium sulfide. This black precipitate is well localized and permits one to determine microscopically where phosphate has been liberated from the substrate within a cell, or which type of cells in a tissue have liberated the greatest amount of inorganic phosphate.

The writer has had no personal experience with the method, but has examined a number of slides and photographs of intestinal epithelium, kidney, and nervous tissue prepared by Drs. Emmel, Smith, and Kochakian of the University of Rochester, showing beautiful localizations of the liberated phosphate. A photograph showing the distribution of alkaline phosphatase in cells of intestinal epithelium is given in Fig. 6.

In general, the alkaline phosphatase as determined by Gomori's method is found to be highly concentrated in the brush border of the tubular epithelium of the proximal convoluted tubules of mammallian kidney. It is present also in high concentration in the border of intestinal epithelial cells and in the region of the Golgi apparatus of these cells.[197, 198] Alkaline phosphatase is also present in the cytoplasm and nuclei of liver

[197] V. Emmel, *Anat. Record* **91**, 39 (1945).
[198] V. Emmel, *Anat. Record* **95**, 159 (1946).

cells, neurons, and a number of other types of cells. It can even be localized in chromosomes and nucleoli. In addition, it sometimes is visualized as a heavy perinuclear ring.

The cell nucleus is apt to show a more intense staining than the cytoplasm. This is true for instance of liver cells of the rat, and often of neurons of the central nervous system. The results of Gomori's method agree with our own work on alkaline phosphatase concentration in the nuclei of rat liver cells. Alkaline phosphatase seems to be present in the mitochondria of kidney cells. Emmel has histochemical evidence to indicate that the alkaline phosphatase of kidney may differ from that of intestine.[198, 199] This work has been disputed by Gomori,[200] but nevertheless seems sound to this writer. If the work turns out to be correct, as seems probable, it

FIG. 6. Alkaline phosphatase in duodenal epithelium of the hamster, showing concentration of the enzyme in the cuticular border, region of the Golgi apparatus, and nuclei. The section was prepared by fixation, paraffin embedding, and Gomori phosphatase staining, without counter-staining. (Courtesy of Dr. V. Emmel, University of Rochester.)

means that a histochemical comparison of the amounts of alkaline phosphatase in different cells of the same animal could be misleading, owing to possible differences in sensitivities of the different enzymes to the fixation. Hence supporting chemical work would be desirable. The possibility also exists that the so-called "alkaline phosphatase," which may consist of more than one phosphomonoesterase, may differ in sensitivity to fixation from species to species.

Kochakian has made a study of the effect of hormone treatment on the amount of certain enzymes in kidney and liver. In two of his papers, the Gomori technique for alkaline phosphatase is used in investigating this problem.[201, 202]

[199] V. Emmel, *Anat. Record*, **96**, 423 (1946).
[200] G. Gomori, *Proc. Soc. Exptl. Biol. Med.* **70**, 7 (1949).
[201] V. N. Vail and C. D. Kochakian, *Am. J. Physiol* **150** (No. 4), 580 (1947).
[202] C. D. Kochakian, *Am. J. Physiol.* **152**, (No. 2) 257 (1948).

In connection with the Gomori histochemical method for phosphatase, an interesting discussion of lack of rigor in cytochemical work is given by Danielli.[203] Most or all of the criticisms raised by Dr. Danielli are logically very sound, but often the specific objections which are raised against a procedure to demonstrate lack of rigorous proof of its validity are improbable, and in one or two instances are now known to be untrue. This article is answered by Baker[204] and a reply is given by Danielli in an article immediately following. It is unfortunately true that most work in cytochemistry consists only in the gradual accumulation of evidence, often quite fragmentary, which finally becomes strong enough so that certain conclusions can be safely drawn. It is also true that biologists often do not feel qualified to support their histochemical procedures with careful kinetic studies using homogenates, and on the other hand that chemists are apt to proceed in ignorance of well established cytological information. Nevertheless so many biologists and chemists are now interested in this field that the mistakes of one worker or group of workers are likely to be discovered and pointed out by someone in reasonably short time. Moreover, the whole preliminary survey in cytochemistry work would quickly come to a halt if each method at the outset had to be set on completely logical ground. Very little work in biochemistry seems to be rigorously proved; usually evidence gradually accumulates which finally establishes a discovery quite thoroughly or which shows it in all probability to be false.

Danielli[205] later made a critical study of the alkaline phosphatase method of Gomori with the intent of answering critical questions raised by him relative to the validity of the method. He was able to show how much destruction of enzyme occurred during preparation of the sections, and that the destruction was fairly uniform in different cytological sites. By using phenolphthalein phosphate as a substrate and also by forming diazonium salts *in situ* of the phenol liberated from disodium phenol phosphate, he showed that the cytological sites of enzyme activity were the same in both cases as those obtained using the Gomori technique. There is no doubt that his work greatly strengthens the validity of the results obtained with the Gomori method, In spite of this, Danielli has only adduced additional evidence and has not proved his point in the sense of proving a mathematical proposition.

b. Acid Phosphatase

The histochemical procedure is less satisfactory for this enzyme than for alkaline phosphatase, but fair results have been obtained. The enzyme appears to be generally present in both cytoplasm and nuclei, and in some

[203] J. F. Danielli, *Nature* **147**, (1946).
[204] J. R. Baker and F. K. Sanders, *Nature* **158**, 129 (1946).
[205] J. F. Danielli, *J. Exptl. Biol.* **22**, 110 (1946).

instances can be found in higher concentration in the nuclei than in the cytoplasm.[206] Acid phosphatase as well as alkaline phosphatase seem to be partly located in large cell granules in many instances, according to Moog and other authors.[207]

It should be remembered that enzyme stability may differ among animal species, so that the fixation process might damage acid phosphatase in one species more than in another. Moreover, if not all acid phosphatases are identical, as seems likely, they may differ in sensitivity in various cell types within a given species or within a given animal for that matter. Thus a comparison of acid phosphates among different cell types and among different animals by the histochemical method could be somewhat misleading without a considerable amount of supporting chemical evidence.

References to an interesting new application of the histochemical method for acid phosphatase can be found in an article by Smith.[208] It is apparently possible to localize acid phosphatase in nervous tissue even after formalin fixation, and the method constitutes an excellent way to visualize nerve cells and fibers. More important, however, is the change in acid phosphatase within a neuron after sectioning of the axon. The acid phosphatase gradually increases in amount, concomitant with the resulting chromatolysis, to a point where cells whose axons have been sectioned at a distance can easily be located among cells with undamaged axons. This constitutes a new and powerful means to trace fiber pathways and localize neurons corresponding to given fibers. A photograph showing this phenomenon is given in Fig. 7.

It still seems to be a matter of some dispute as to whether specific phosphatases occur in cells other than the alkaline phosphatase already described and two or three acid phosphatases. Gomori[200] finds no essential differences in distribution of phosphatase activity among a number of substrates at pH 5, 7, and 9 in testing a number of human and guinea pig tissues, and hence concludes that a number of specific phosphatases do not exist. This work is contrary to the results of Dempsey[209] and to that of Kabat and coworkers.[210] Apparently it will be necessary to carry out careful chemical investigations using homogenates and purified enzymes before this question can be settled with certainty. The inability to study kinetics by the histochemical procedure as well as the generally damaging effects of fixation render the histochemical technique unsuitable by itself for determining with certainty whether a very few or a relatively large number of specific phosphatases exist, in the opinion of this writer.

It is utterly impossible in this limited chapter to cover the relatively enormous literature on histochemical determinations of phosphatases within

[206] G. Gomori, *Arch. Path.* **32**, 189 (1941).
[207] F. Moog and H. B. Steinbach, *J. Cellular Comp. Physiol.* **28**, 209 (1946).
[208] W. K. Smith, *Anat. Record* **102**, No. 4, 523 (1948).
[209] E. W. Dempsey, *Am. J. Anat.* **80**, 1 (1947).
[210] W. Newman, I. Feigin, A. Wolf, and E. A. Kabat, *Federation Proc.* **8**, 232 (1949).

cells and tissues. It is therefore to be hoped that the mentioning of a few papers on the subject will not cause great annoyance to those whose papers have not been included.

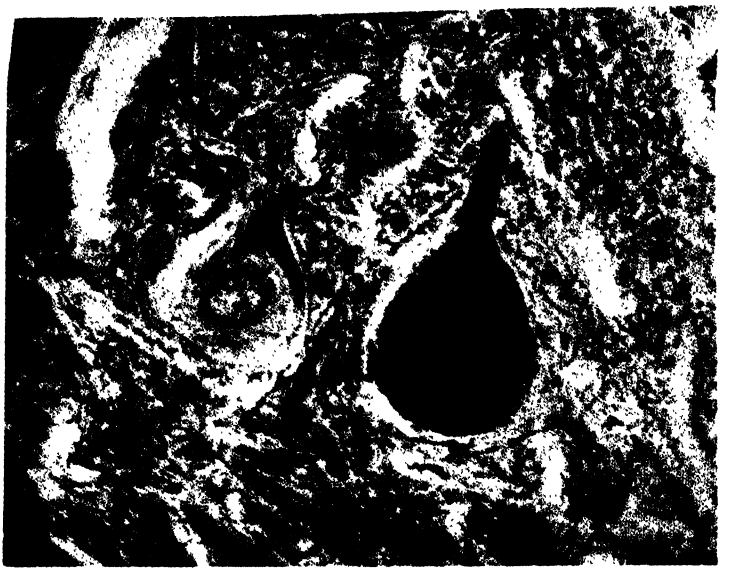
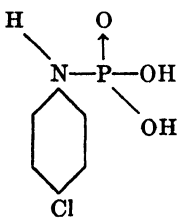


FIG. 7. Acid phosphatase in neurons, showing localization in perinuclear region and region of origin of axon in normal cell, and intense reaction in entire cell whose axon has been cut. (Courtesy of Dr. W. K. Smith, *Anat. Record*, **102**, 523, 1948.)

c. Phosphoamidase

This enzyme was demonstrated histochemically by Gomori.[211] The substrate was *p*-chloranilidophosphoric acid,



which was used in acetate buffer at pH 5.4 to 5.8 with $Pb(NO_3)_2$ added to fix the liberated phosphate, and with $MnCl_2$ added to activate the enzyme.

The results were somewhat erratic but in good sections the enzyme was found to be present in liver, kidney tubules, and small intestine, as well as in other tissues. Usually the enzyme was mainly present in cytoplasm but in some cells also it was also present in the cell nuclei.

[211] G. Gomori, *Proc. Soc. Exptl. Biol. Med.* **69**, 407 (1948).

d. Aldolase

Aldolase was determined histochemically by Allen and Bourne[212] who utilized the Gomori technique for localizing phosphate liberated from triose phosphate by treatment with alkali. The phosphate was first precipitated *in situ* with magnesia mixture. Fluoride was added to block alkaline phosphatase and iodoacetate was added to prevent further enzymatic breakdown of the triose phosphates. The pH of the determination was not stated, but calculations based on the ammonium chloride buffer used indicate that it may have been in the neighborhood of 9.5. It was stated that fluoride was finally found not to be needed, since alkaline phosphatase activity was not encountered under the conditions of the experiment. This latter finding seems somewhat strange.

The method was stated not to be entirely satisfactory since a dark precipitate formed on the outside of some of the sections, for unknown reasons.

Using this technique, aldolase was found in heart and striated muscle and was also found in liver and kidney. Lung however gave very little reaction. No marked intracellular segregation of the enzyme could be discovered; instead, it appeared to be diffusely distributed and to be present in both nuclei and cytoplasm.

It is difficult to evaluate this work until more papers on the subject have appeared. The purity of the substrate might have some effect on the results.

e. Choline Esterase

Gomori has devised a method for the histochemical demonstration of choline esterase.[213] A choline ester of a higher fatty acid such as myristoyl choline was used as a substrate, in the presence of a mixed buffer system consisting of maleic acid and tris(hydroxymethyl)aminomethane buffer. Cobaltous acetate was used to precipitate the liberated fatty acid, and the cobalt in the precipitate was then converted to sulfide by the addition of ammonium sulfide as in the Gomori technique for alkaline phosphatase. A small amount of $CaCl_2$, $MgCl_2$, and $MnCl_2$ was added since the reaction was thereby improved for some reason.

The interpretation of results seems unclear, since a highly purified enzyme from the electric eel failed to hydrolyze the higher choline esters useful in the histochemical procedure. Nevertheless the hydrolysis of the higher esters by crude tissues was inhibited by prostigmine, a specific inhibitor of choline esterase.

As determined by the histochemical procedure, choline esterase was present in the sympathetic nervous system of most species. It was often localized in cell nuclei and sometimes in fibers. Satellite cells of the sympathetic ganglia showed intense staining of nuclei using lauroyl choline as substrate,

[212] R. J. L. Allen and G. J. Bourne, *J. Exptl. Biol.* **20**, 61 (1943).
[213] G. Gomori, *Proc. Soc. Exptl. Biol. Med.* **68**, 254 (1948).

whereas palmitoyl choline caused no staining of the satellite cells but did cause intense staining of nerve fibers. Rat and guinea pig tissues were too low in enzyme activity to give clear-cut results, whereas dog and mouse tissues gave much more intense and clear-cut pictures. This may well be the result of differences in sensitivity of the enzymes of various species to the fixation and the dehydration procedure, which involves the use of acetone, alcohol, collodion, paraffin, chloroform, and xylene.

It is of interest to the writer that this method shows the presence of enzyme in the cell nuclei. It might be asked whether enzymes in the nucleus are better protected against the action of the fixative etc. than enzymes in the cytoplasm. It seems to be too early to evaluate this work fully.

f. Lipase

Gomori[214, 215] also has devised a histochemical technique for the determination of lipase, in which "tween 40" or "tween 60" is used as substrate. These are palmityl and stearyl esters respectively of hexitols in which most of the hydroxyl groups are esterified, leaving the substrates still water-soluble however. Maleate buffer of pH 7.4 was used and $CaCl_2$ was added to precipitate calcium salts of the liberated fatty acids. The calcium salts were later converted to lead salts with $Pb(NO_3)_2$ and the lead was converted to sulfide with ammonium sulfide as usual. Acetone fixation and dehydration was employed followed by treating of the tissue with benzene, and imbedding in paraffin prior to sectioning. The paraffin was subsequently removed with xylene, and graded concentrations of alcohol were then used to return the section gradually to water solution.

Lipase could be found in dog and rat liver, pancreas, lung, kidney, and other tissues. In the pancreas the site of the enzyme was the zymogen granules; the islets of Langerhans were negative. The enzyme was present in the coarse granules of rabbit liver cells. Considerable work was done on the distribution of lipase in the intestinal tract, where it was found in the zymogenic cells of the fundus of human stomach, and in the atrial and pyloric regions as well. The enzyme was present in the deep layers of the esophagus, and the salivary glands as well as in the duodenum and the rest of the small intestine.

Apparently this histochemical method for lipase is not well adapted at its present stage of development to an exactly study of the fine distribution of enzyme within a single cell, or at least is not as reliable as the alkaline phosphatase method. It was not stated in the papers of Gomori and was not clear from the photographs in most cases exactly how the enzyme was distributed intracellularly. For instance, it was apparently not determined whether it was present or absent from the nucleus.

[214] G. Gomori, *Proc. Soc. Exptl. Biol. Med.* **58**, 362 (1945).
[215] G. Gomori, *Arch. Path.* **41**, 212 (1946).

In using a high molecular substrate such as the one employed, the question of its penetration to all parts of the cell undoubtedly should be given careful consideration.

A slightly improved technique for the histochemical determination of lipase is given by Gomori.[216]

g. Dopa Oxidase

This enzyme apparently can be demonstrated histochemically, by observing the formation of a dark deposit upon incubating the tissue with l-dihydroxyphenylalanine at pH 7.4. References will be found in Glick's book. However, the formation of melanin apparently can be induced without the addition of the substrate in cells known to contain small amounts of melanin, and attacks have been made on the specificity of the histochemical oxidase reaction on the grounds that insufficient controls have been carried out.[217] Apparently this is another example of an attempt to elucidate a confusing situation by the use of a histochemical procedure not supplemented with careful kinetic studies using homogenates.

It has been stated by proponents of the dopa oxidase reaction that the enzyme is absolutely specific for L-3,4-dihydroxyphenylalanine.

In photographs of cells stained by the dopa oxidase reaction, nuclei as well as cytoplasm appear to be stained, and a somewhat granular appearance can be noted if the staining is not too heavy.[218, 219]

In addition to melanoblasts, myeloidal leucocytes are stated to give an intense histochemical reaction owing to the presence of the nonspecific polyphenol oxidase.

General references to dopa oxidase can be found in Greenstein's book on the *Biochemistry of Cancer*, and in *Chemistry and Methods of Enzymes*, by Sumner and Somers. A recent paper denies the existence of a specific dopa oxidase; the alledged dopa oxidase is stated to be in reality L-tyrosinase.[220]

h. Peroxidase

Peroxidase, which occurs in high concentration in white cells of the myeloidal variety, can be localized by histochemical procedures, but according to Glick, diffusibility of products may interfere with proper localization of the enzyme within the cell, and hence this enzyme will not be considered further.

[216] G. Gomori, *Am. J. Clin. Path.* **16**, 347 (1946).

[217] H. Sharlit, W. Sachs, C. F. Sims, and B. H. Salzer, *Arch. Dermatol. Syphilol.* **45**, 103 (1942).

[218] G. R. Laidlaw and S. N. Balkberg, *Am. J. Path.* **8**, 477 (1932).

[219] G. R. Laidlaw, *Anat. Record* **53**, 399 (1932).

[220] A. R. Lerner, T. B. Fitzpatrick, E. Calkins, and W. H. Summerson, *J. Biol. Chem.* **178**, 185 (1949).

i. Cytochrome Oxidase and Succinic Dehydrogenase

The same statements just made about peroxidase apply to histochemical tests for cytochrome oxidase and succinic dehydrogenase. The reader is referred to Glick's book[183] for references to histochemical work with these enzymes.

j. Glycuronidase

Glycuronidase has recently been studied by Friedenwald and Becker[221] using an histochemical procedure. Two substrates were employed. One of these was prepared by diazotizing *o*-aminophenyl glucuronide and coupling the resulting diazonium salt with β-naphthol. The glucuronide of the red dye thus produced was sufficiently water soluble to serve as a substrate for glucuronidase, in acetate buffer at pH 5.0. The action of the enzyme caused liberation of the red dye which is insoluble in water and immediately precipitates at the site of the enzyme reaction.

The other substrate was the glucuronide of 8-hydroxyquinoline in acetate buffer at pH 5.0. The liberated 8-hydroxyquinoline is precipitated as the black ferric salt as rapidly as it is formed, as the result of incorporating $FeCl_3$ in the substrate mixture. The excess Fe^{+++} was removed with oxalate and the remaining ferric salt of 8-hydroxyquinoline was decomposed with $K_4Fe(CN)_6$, resulting in the formation of Prussian blue which is insoluble in alcohol and is not washed out during dehydration of the sections. Unfixed frozen sections were used with both substrates.

Glucuronidase activity was found to be the same with both substrates. The enzyme is present in kidney tubular cells, while the glomeruli are practically inactive. Hepatic parenchymal cells contain the enzyme. It is also present in the spleen and lymphatic nodules; it is scattered in lung parenchyma, and concentrated in bronchial mucosa. It is found in Brunner's glands of the duodenum and in the mucosa of the colon and ileum; it is also present in uterine endometrium and in seminal vesicles and epididymis.

The enzyme appeared to be present in the cell cytoplasm rather than in the nucleus. In this connection one might wonder whether the substrate was able to penetrate the nucleus.

This work on glycuronidase is very interesting and furnishes an excellent illustration of how a cytochemical method can be employed to ascertain the distribution of an enzyme among various cell types. The method may not be so well suited to studying the distribution of the enzyme within a cell, however, since unfixed frozen sections are used which make it difficult to obtain fine details of cell structure, and since the penetration of the substrates to all parts of the cell might possibly be in question.

[221] J. Friedenwald and B. Becker, *J. Cellular Comp. Physiol.*, **31**, 303–9 (1948).

k. Linderstrøm-Lang Technique of Histochemistry

An elegant type of procedure for determining the distribution of an enzyme among various cell types within a tissue or organ has been developed by Linderstrøm-Lang and collaborators. A summary of this sort of work written by Linderstrøm-Lang will be found in the Harvey Lectures for 1939.[222] Also, a recent review of Gersh[223] should be consulted, as well as the chapter by Blaschko and Jacobson in *Cytology and Cell Physiology*, edited by Bourne[6]. A discussion of the microchemical procedures which are involved is also given in Bamann and Myrbäck, *Die Methoden der Fermentforschung*, in the chapter by K. Linderstrøm-Lang and H. Holter,[154] as already has been stated.

Details of the method and results will not be given here. The procedure is to plot the amount of enzyme in serial histological sections against the number of cells of a given type in the same or in alternate or adjacent sections, in passing from one side of the tissue block to the other. In order to measure the enzyme in the section, special micromethods must be used owing to the smallness of the amount of material available. The localization of pepsin in the chief cells of the gastric mucosa is a classical example of the application of the technique.

Another interesting example of Linderstrøm-Lang histochemical procedure is the purported localization by Weil and Ely[224] of arginase in the cells of the proximal convoluted tubules of the kidney. The microtitrimetric method for determining the enzyme was originally developed by Linderstrøm-Lang and coworkers.[225]

In this work, the section of saline-perfused kidney partly frozen at −5°C. was bored out with a small cork borer, and the plug of tissue thus obtained was then sectioned, using a freezing microtome. Enzyme determinations were carried out on the sections.

A cylinder of tissue immediately surrounding the hole left by the first boring was then bored out using a slightly larger cork borer and boring concentrically to the hole already present. This cylinder is fixed, dehydrated, imbedded, and stained in the usual manner preparatory to histological observation. The assumption is then made that counting the cells of a given type in sections of the cylinder will yield numbers which are proportional to the numbers of cells of the same type in adjacent sections in the plug. In this way, numbers of cells of a given type can be plotted against distance through the plug, and hence can be correlated with enzyme ac-

[222] K. Linderstrøm-Lang, *Harvey Lectures* **34**, 214 (1938–1939).
[223] I. Gersh, *Physiol. Revs.* **21**, 242 (1941).
[224] L. Weil and J. O. Ely, *J. Biol. Chem.* **112**, 565 (1936).
[225] K. Linderstrøm-Lang and H. Holter, *Comptes rend. lab. Carlsberg, Ser. chim.* **21** (No. 2), 1 (1935).

tivity as distance through the plug is increased. Various corrections have to be made however, owing to the shrinkage of the tissue cylinder during fixation.

Weil and Ely claim to have localized arginase in this manner in the cells of the proximal convoluted tubules of the kidney of the rabbit. However, their correlation curves do not appear to this writer to establish their claim with absolute certainty, since for example the cells of distal convoluted tubules seem to show a reasonably good correlation with arginase activity except at the very beginning of the curve. It would seem that a more precise histological method would be required to be certain that arginase is not also present in the distal convoluted tubules. This impression is strengthened by the results of work being carried out in our laboratory by Mrs. Helen Conrad on cytochrome oxidase distribution in the kidney (unpublished).

It should be noted that in cases where alternate sections of tissue can be studied, owing to the ability of the tissue to withstand use of the freezing microtome histologically as well as chemically, much more accurate results probably can be obtained. This sort of work can be done with gastric mucosa, for instance.

Weil and Jennings[226] have also investigated cathepsin, aminopolypeptidase, dipeptidase, esterase, and amylase in rabbit kidney, and have showed that catheptic, aminopolypeptidase, and esterase activities can be demonstrated in all structural elements of the kidney. However, the cells of the proximal tubules appear to be more active than the cells of Henle's loop and the latter are more active than the cells of the collecting tubules, in respect to these three enzymes.

The distribution of dipeptidase was found to be the same except that this enzyme was absent in cells of the collecting tubules. Amylase was said to be associated with the cells of the proximal and distal convoluted tubules and with cells of the collecting tubules, but was absent from cells of the loop of Henle.

Still another method for investigating the distribution of enzymes among various types of cells within a tissue is to separate the cell types by methods somewhat similar to those used for isolating cell nuclei. An example of such a procedure is the separation of epithelial from stromal cells of the choroid plexus.[227] The tissue cells can be separated by agitating the tissue in saturated sodium sulfate solution at 38°C. and decanting the suspended epithelial cells. This process must be repeated a number of times to effect a good separation.

If media other than saturated sodium sulfate solution, such as physiological saline for instance, are used in separating the different cell types, some

[226] L. Weil and R. K. Jennings, *J. Biol. Chem.* **139**, 421 (1941).

[227] J. S. Friedenwald, H. Herrmann, and R. Buka, *Bull. Johns Hopkins Hosp.* **70** (No. 1), 14 (1942).

enzymes are rapidly extracted from the cells and are thus lost by passing into the solution, so that subsequent enzyme analyses may be invalidated.

Using saturated sodium sulfate for separation of the two cell types, it was found that the cytochrome oxidase-succinic dehydrogenase-fumarase system is located almost entirely in the epithelial cells of the choroid plexus, whereas the activities of lactic and malic dehydrogenases is greater per cell in the stroma than in the epithelium.

It is claimed that this work is in agreement with predications of the redox potentials of the two types of cells made by using oxidation-reduction dye indicators. It is considered possible that the difference in redox potential between the epithelium and stroma may constitute a source of energy for secretion of the cerebrospinal fluid by the choroid plexuses.

The distribution of choline esterase in the choroid plexus and also in the ciliary body of the eye have been studied by Herrmann and Friedenwald.[228] Epithelial cells were removed from stroma as described above, but the separation was more difficult and less clean-cut in the case of the ciliary body.

Choroid plexus and ciliary body (beef and pig) showed moderate and similar activities, the esterase activity of the stroma being high compared with that of the epithelium.

VI. Chemistry of Cell Surfaces

It has been suspected for some time that certain enzyme systems may be concentrated in cell surfaces and function there in the processes of absorption and excretion of various substances from the cell. In the case of alkaline phosphatase, high enzyme concentration in the surfaces of intestinal epithelial cells and cells of the proximal convoluted tubules of the kidney can be demonstrated with the Gomori technique.

The action of certain drugs has been thought to be concerned with the cell surface rather than the interior cell cytoplasm. The latter view has been supported by A. J. Clark,[229] who gives a number of references to work indicating that cell surfaces may be more sensitive to drugs than cell interiors.

Brinley[230] found that immersion of *Amoeba proteus* in water solutions of H_2S resulted in destruction of the cells, whereas microinjection of such solutions, even when the volume of the solution injected was nearly half the original volume of the cell, produced only transitory effects followed by rapid recovery of the amoebas. The same findings also were reported for cyanide solutions varying in concentration from $M/10$ to $M/3000$.[231, 232]

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[228] H. Herrmann and J. S. Friedenwald, *Bull. Johns Hopkins Hosp.* **70** (No. 1), 14 (1942).

[229] A. J. Clark, *in* Handbuch der Experimentellen Pharmakologie. Founded by H. Heffter, Springer, Berlin, 1937, Vol. IV; reprinted by J. W. Edwards, Ann Arbor, Michigan, 1944.

[230] F. J. Brinley, *Am. J. Physiol.* **85**, 355 (proceedings) (1928).

[231] F. J. Brinley, *Proc. Soc. Exptl. Biol. Med.* **25**, 305 (1928).

[232] F. J. Brinley, *J. Gen. Physiol.* **12**, 201 (1928).

Hiller[233] found that ethyl alcohol, chloretone, ether, and chloroform were not fatally damaging to *Amoeba dubia* when injected into the cytoplasm, but that they could readily kill the cells when the latter were immersed in aqueous solutions of the drugs.

Marsland[234] found that the effects of hydrocarbons on the surface and interior cytoplasm of *Amoeba dubia* were different.

The specific choline esterase of red blood cells is in the surface of the erythrocytes according to a letter to the editor by Paleus.[235] Here the "membrane" is taken to be identical with the stroma. The enzyme could not be eluted from the stroma. Whether one is justified in considering red cell stroma as identical with the cell membrane is left to the judgment of the reader.

By studying the pH activity curve of purified invertase and invertase in whole yeast cells, Wilkes and Palmer[236] adduced evidence indicating that this enzyme is located in or near the cell surface. Myrbäck and Vasseur[237] have evidence of a similar nature indicating that the enzymes lactase and trehalase are located in the cell surfaces of certain yeasts.

During the war, a study of the mechanism of action of uranium on the animal body lead the present writer to suspect that this material might be acting on the surfaces of cells of the kidney tubules. Recently papers has been published by Rothstein and Meier showing that hexavalent uranium in dilute solutions acts upon the surface of yeast cells in a remarkably specific manner. The latter cells were used because of their relative insensitivity to low pH and their suitability for experimental work, in contrast to the unavailability and unsuitability of kidney tubules.

Rothstein and Meier[238] found that glucose metabolism by yeast is blocked by hexavalent uranium, and that the site of action of the uranium is at the cell surface. In another paper, Rothstein, *et al.*[239] made a more detailed study of the inhibition of glucose metabolism at the yeast cell surface and concluded that the uranyl group forms a complex with some active group participating in glucose metabolism in the cell surface. Some different group, probably carboxyl radicals of proteins, complexes uranium at the cell surface at higher uranium concentrations.

In an earlier paper, Rothstein and Meier[240] showed that A.T.P.-ase is located at the surface of the yeast cell, and in addition have studied other phosphatases in yeast cell surface.

[233] S. Hiller, *Proc. Soc. Exptl. Biol. Med.* **24**, 427; same article repeated on page 938 (1927).

[234] D. Marsland, *J. Cellular Comp. Physiol.* **4**, 9 (1934).

[235] S. Paleus, *Arch. Biochem.* **12**, 153 (1947).

[236] B. G. Wilkes and E. T. Palmer, *J. Gen. Physiol.* **16**, 233 (1932).

[237] K. Myrbäck and R. Vasseur, *Z. physiol. Chem.* **227**, 171 (1943).

[238] A. Rothstein and R. Meier, *J. Cellular Comp. Physiol.* **32** (No. 3), 247 (1948).

[239] A. Rothstein, A. Frenkel, and C. Larrabee, *J. Cellular Comp. Physiol.* **32** (No. 3), 261 (1948).

[240] A. Rothstein and R. Meier, *J. Cellular Comp. Physiol.* **32** (No. 1), 77 (1948).

Lack of space makes it impossible to present the somewhat complicated evidence in the statements in these three papers, but as far as this writer can determine, the work is completely sound. The work in the first two papers mentioned constitutes a very ingenious and new application of uranyl uranium and its complexes to an elucidation of cell surface chemistry. The work in the last paper is based on the failure of radioactive phosphate in A.T.P. to come into equilibrium with the inorganic phosphate of the cell during and after hydrolysis of A.T.P. at the cell surface.

Barron and collaborators[241] have also presented work indicating that uranium blocks enzyme groups at the surface of yeast cells. Their evidence does not appear to be as conclusive as that of Rothstein, however.

Boell and Nachmansohn[242] found that choline esterase is concentrated in the sheath of the giant fiber of the squid, although this sheath is largely composed of connective tissue fibers. Practically none of the enzyme was found in the axoplasm. This means that choline esterase is highly concentrated at or close to the surface of the nerve fiber.

Nachmansohn and Steinbach[243] on the other hand found that succinic dehydrogenase was more concentrated in the axoplasm than the sheath of the giant fiber of the squid, and that it is more concentrated in the head ganglia than in the fiber. Choline esterase also was found to be more concentrated in the head ganglia than in the fiber. Diphosphothiamine was higher in concentration in the sheath than in the axoplasm.

The work on cell surface enzymes and chemistry is of considerable importance and ultimately no doubt will explain the mechanism of absorption and excretion at cell surfaces. The phosphatases in the surface seem to be necessary to hydrolyze organic phosphates so that the organic fragment subsequently can be absorbed and metabolized. However, in the subsequent absorption process, at least some of the organic molecules in question are again phosphorylated (glucose for instance, by hexokinase action in yeast). Thus a cell cannot metabolize phosphorylated sugars added to the medium, but nevertheless must phosphorylate sugars in the cell surface in the first step of absorbing and metabolizing them. In the case of kidney tubule cells and intestinal epithelial cells, if glucose is phosphorylated during the absorption process, it must subsequently be dephosphorylated, perhaps by phosphatases deeper within the epithelial cells.

It is at the present time impossible to define accurately the cell surface in which the above mentioned enzymes are located in exact terms or to state its thickness, on the basis of the available chemical evidence. All that can be stated is that there is some kind of surface of limited thickness that separates the internal cytoplasm from the surrounding medium and that

[241] E. S. G. Barron, J. A. Muntz, and B. Gazvoda, *J. Gen. Physiol.* **32**, 163 (1948).
[242] E. J. Boell and D. Nachmansohn, *Science* **92**, 513 (1940).
[243] D. Nachmansohn and H. B. Steinbach, *Science* **95**, 513 (1940).

contains certain enzymes in high concentrations. The reader is referred to a chapter by Danielli in *Cytology and Cell Physiology*[6] (page 68) for further information of a general nature on cell surfaces.

Nuclear Membrane

According to Baud and Fulleringer[244] alkaline phosphatase occurs in high concentration in the nuclear membrane of certain cells. Whether it has any role in the transfer of materials from the nucleus to cytoplasm or vice versa is entirely a matter of speculation. Stern[245] in a review article has dealt with the passage of various substances through the nuclear membrane. The study by Clark of the passage of dyes through the nuclear membranes of amoebas has been mentioned previously in this chapter. A recent electron microscope study of the nuclear membrane of amphibian oocytes has been made by Callan *et al.*[246]

VII. Enzymes and Genes

In closing this chapter, brief reference will be made to the relationship between enzymes and genes. The reader is referred to a very excellent review by Gulick on the "Chemical Formulation of Gene Structure and Gene Action"[247] for general background material. In addition, a number of review articles bearing on the subject have been written by Beadle.[248–251] Articles on the same subject have been written by Wright[252] and by Sturtevant;[253] and finally a recent article entitled "Beyond the Gene" by Sonneborn[254] is recommended. It is now quite well established from the work of Beadle and others that there is a one to one correspondence between at least some genes and cell enzymes. This means that the loss of a given enzyme can be inherited in a Mendelian manner (Lysenko notwithstanding), if the loss of enzyme has not been fatal to the cell. Much of this work is based on experiments with isolated spores of the fungus Neurospora, which have been subjected to the mutation-producing action of x-rays. In this work, loss of an enzyme was compensated for by including in the growth medium whatever substrate could no longer be synthesized by the fungus. In general, different enzymes were found to be destroyed or potentially destroyed in

[244] G. A. Baud and A. Fulleringer, *Compt. rend.* **227**, 645 (1948).
[245] C. Stern, *Am. Naturalist* **72**, 350 (1938).
[246] H. G. Callan, J. T. Randall, and S. G. Tomlin, *Nature* **163**, 280, (1940).
[247] A. Gulick, *Recent Advances in Enzymol.* **4**, 1 (1944).
[248] G. W. Beadle, *Am. Scientist* **34**, 31 (1946).
[249] G. W. Beadle, *Am. Scientist* **36**, 69 (1948).
[250] G. W. Beadle, *Physiol. Revs.* **25**, 643 (1948).
[251] G. W. Beadle, *Ann. Rev. Biochem.* **XVII**, 727 (1948).
[252] S. Wright, *Physiol. Revs.* **21**, 487 (1941).
[253] A. H. Sturtevant, *Am. Scientist* **36**, 225 (1948).
[254] T. M. Sonneborn, *Am. Scientist* **37**, 33 (1949).

different spores. The latter interesting finding has bearing on the problem of radiation damage in general, and means that in a given population of cells which are exposed to radiation, not all cells that are destroyed are necessarily destroyed by the same mechanism. Thus percentage loss of a given enzyme in a whole cell population may be immeasurably small, and an attempt to use enzyme tests to detect early changes caused by low doses of radiation may be fruitless.

It is also claimed by Sonneborn, Spiegelman, and others, that certain cytoplasmic factors can be inherited independently of gene mechanisms. What relationship these factors have to enzymes is not yet certain. However, a plausible theory bearing on this point is presented by Spiegelman and Kamen.[255]

It was shown by these authors that radioactive P^{32} is taken up by nucleoprotein from inorganic phosphate during growth of yeast cells. Subsequently, a flow of the P^{32} apparently takes place from the nucleic acid to some other fraction if the yeast cells are placed in a nitrogen-containing medium and allowed either to grow or to produce an "adaptive" enzyme such as mellibiase.* Such an outflow of P^{32} from the nucleic acid fraction does not occur if sodium azide or dinitrophenol are added to block growth or adaptive enzyme formation. Moreover, such an outflow of P^{32} from the nucleic acid fraction does not occur if the cells are allowed to ferment carbohydrate anaerobically in the absence of a nitrogen source so that growth or enzyme formation cannot occur.

The authors next review some of the work on adaptive enzymes and show that an adaptive enzyme can be maintained in the absence of the gene responsible for initiating its synthesis, simply by keeping the substrate for the enzyme in question constantly present in the culture medium. However, in the absence of any gene corresponding to the enzyme in question, the yeast cell finally loses its capacity to synthesize the "adaptive" enzyme if the substrate for this enzyme is withheld from the culture medium.

This sort of evidence taken together with the evidence of a similar nature such as the work on the paramecium killer factor of Sonneborn[254, 256, 257] leads to the hypothesis of the so-called plasma genes said to be first postulated by Wright.[252, 258]

The relationship between genes and plasma genes as conceived by Spiegelman and Kamen[257] might be diagrammed as shown in Fig. 8. The gene in the nucleus is assumed to send out a replica of itself (P_0) to the cytoplasm.

[255] K. Spiegelman and M. Kamen, *Science* **104**, 581 (1946).

* For the literature on adaptive enzymes the reader will find references in the article being discussed and in another chapter in this book by Spiegelman.

[256] T. M. Sonneborn, *Proc. Natl. Acad. Sci.* **29**, 329 (1943).

[257] C. D. Darlington, *Nature* **154**, 164 (1944).

[258] S. Wright, *Am. Naturalist* **79**, 289 (1945).

This replica in the cytoplasm then in turn allows the formation of replicas P_1, P_2, P_3, P_4, . . . P_n of itself in the cytoplasm. P_0, P_1, P_2, P_3, P_4, . . . P_n are all identical plasma genes.

If the plasma genes divide faster than they are supplied from the gene in the nucleus, according to the same authors, Mendelian inheritance will be obscured to a greater or lesser extent by "cytoplasmic inheritance." If on the other hand the limiting factor for production of the plasma gene is the gene itself in the nucleus, Mendelian inheritance will be observed.

Spiegelman and Kamen propose the idea that the self-duplicating unit is nucleoprotein, a thought fairly commonly entertained. The flow of P^{32} from the nucleic acid fraction is taken to be the result of the hypothetical role of the nucleic acid in furnishing energy for protein synthesis through

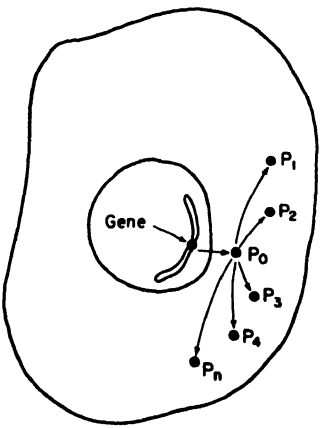

FIG. 8. Diagram of gene-plasma-gene relationship.

its phosphate bonds. Such a process would thus seem to be analogous to the loss of phosphate from Cori ester in the synthesis of glycogen.

The only substance thus far known to occur in cells in a number of varieties of the same order of magnitude as the number of genes in the cell is protein. It is therefore not surprising to find a correspondence between genes and enzymes, and indeed the genes may themselves represent a great variety of specific proteins. The only other substance that conceivably may occur in as rich a spectrum of individual components is nucleic acid. A tremendous number of possible different nucleic acids can be calculated if one assumes a molecular weight of only around 100,000 and retains complete freedom of choice in arranging the four nucleotides of the nucleic acid. Considering a nucleic acid molecule to be composed of 150 purines of two varieties and 150 pyrimidines of two varieties (which would lead to a molecular weight of about 90,000), Dr. D. Charles of the Atomic Energy Project of our University has calculated the possible number of isomeres, assuming

complete freedom of arrangement, as about 4×10^{87} (unpublished). The work of Avery and collaborators[259] on the mutation-like change of an unencapsulated pneumococcus to an encapsulated variety induced by desoxyribonucleic acid is indicative of specificity and hence suggests that variety of components of desocyribonucleic acid may exist in a given species.

The great tendency of nucleic acid molecules to associate in aqueous solutions unfortunately makes it very difficult to see how to effect satisfactory fractionation of this material, unless a way can be found to remove the high polarity of the phosphate groups by a sufficiently mild chemical reaction. For the present then the question of specificity of nucleic acid remains an unsolved problem.

Questions such as those just outlined are obviously concerned with the role of the nucleus in the resting cell and the function of the cytoplasm of the resting cell in synthesizing protein. If, as has been surmised, a flow of ribonucleic acid from the nucleus to cytoplasm really does occur in cells that are synthesizing protein, the question of function of this ribose nucleic acid when it gets to the cytoplasm presents an intriguing problem. Does it act to furnish templates for the synthesis of specific proteins? Or, are proteins being synthesized by the action of the cytoplasm without any participation by the nucleus? The synthesis of viruses by infected cells and the production of "plasma genes" make the latter possiblity seem probable in certain cases. On the other hand, it may be that the bulk of protein synthesis in the cell is dependent upon a functioning of the nucleus, even in the resting cell. The reader is referred to the paper by Stern,[245] who has collected evidence to show that genes exert their influence during the resting stage of the cell. If the latter statement is true, a knowledge of the chemistry and metabolism of the cell nucleus may turn out to be as important or even more important than a detailed cytochemical knowledge of the chemistry and metabolism of the cytoplasm, even though the cytoplasm in most cells obviously carried out almost quantitatively the aerobic oxidations of the cell and great bulk of the commonly studied metabolic reactions, especially those contributing to the organism as a whole.

VIII. Summary and Conclusions

Evidence has been presented which decisively demonstrates the occurrence of many enzymes in small cell particles, such as nucleoli, chromosomes, nuclei and mitochondria, and on cell surfaces. In some cases (cytochrome oxidase and succinic dehydrogenase) it seems clear that none of the enzyme occurs dissolved in the cytoplasmic matrix, and in the case of succinic dehydrogenase at least, all of the enzyme seems to be located within the mitochondria. The bulk of the cytochrome oxidase and cytochrome system is also located in the mitochondria, and in addition, it now

[259] O. T. Avery, C. M. McLeod, and M. J. McCarty, *J. Exptl. Med.* **79**, 137 (1944).

seems probable that mitochondria carry the entire system of enzymes participating in the Krebs cycle for the oxidation of carbohydrate. These particles also carry enzymes necessary for the oxidation of fatty acids.

The occurrence of enzymes in particles is no doubt of great significance in regulation of the chemistry of the cell. There must be areas of different pH and redox potential within a single cell, perhaps in part resulting from the localization of enzymes within the cell.

It would be of great advantage to know whether all mitochondria, or all secretory granules for instance, are alike in enzyme composition. If they are not, it might be possible to effect a separation of different classes of granules electrophoretically, after segregation according to particle size with centrifugation techniques, if difficulties of determining boundaries and keeping the material suspended could be overcome. It might even be possible to effect further purification of enzymes in this manner, and perhaps to draw conclusions about the mode of formation of the particulate enzymes if such electrophoretic experiments were successful. Another point which might be more easily answered is the question of how much increase in efficiency can be expected from a group of properly oriented enzymes working in a single particle (the cytochrome oxidase-succinic dehydrogenase system or the system of glycolytic enzymes, for instances) over and above the efficiency of the same enzymes operating in solution or on different particles.

It is still unsettled as to whether enzymes exist dissolved in the cytoplasmic matrix. Evidence indicates that they may thus exist, but the point is not indisputably settled in the opinion of this writer. It is probable that enzymes bound to particles are less likely to break through the cell membrane and become lost than enzymes that exist dissolved in solution. In view of the evidence presented by Whipple and his associates[260] that the liver cell, at least, must be quite permeable to certain plasma proteins, this point may be of more significance than one might imagine at first sight. Since some enzymes occur in plasma, apparently as the result of leakage from cells, it may be that small amounts of enzyme generally do occur dissolved in cytoplasmic matrix.

It has been demonstrated that in the case of tissue homogenates, enzymes can occur bound to microsomes and submicrosomes; but the significance of these results is not so clear as the finding of enzymes in the mitochondria. The question arises as to whether a transfer of enzyme could have taken place from the mitochondria or cytoplasmic matrix to the microsomes during the isolation of the latter particles. Considering the possibility of adsorption of enzymes, the enormous absorbing area of the microsomes certainly must be taken into account. Until it can be demonstrated that no escape of enzymes occurs from the mitochondria or secretory granules, and

[260] G. H. Whipple, *Am. J. Med. Sci.* **203**, 11 (1942).

that no great amount of the enzyme in question exists free in the aqueous phase, the possibility of absorption by microsomes stands as a menace against correct interpretation of the results of studies of the enzyme activity of isolated microsomes and submicrosomes. This menace is less in the case of mitochondria and nuclei, in the opinion of the writer, but still must be taken into account in these cases.

The question of function of microsomes and submicrosomes remains an enigma. The high ribose nucleic acid content of these particles is undoubtedly of significance, and it is possible that the particles act as sites of enzyme and protein synthesis in the cytoplasm. In certain types of cells, as has been pointed out, enzymes to be used extracellularly are known to accumulate in larger particles called secretory granules, and it is possible that the microsomes play a part in synthesis of this type of enzyme. It is possible that the microsomes may arise in the cell nucleus and later pass out into the cytoplasm.

It seems highly probable that a body as large as the cell nucleus must carry on a metabolism of its own, and hence it is not surprising to find enzymes in cell nuclei. It also seems clear that some enzymes known to occur in cytoplasm do not occur as such in nuclei, although of course they might be present there as zymogens. It is now known that any method for obtaining nuclei that depends upon the use of aqueous homogenates is likely to result in a loss of protein, and hence undoubtedly of enzymes, from the nuclei. Therefore, the results of quantitative studies of the enzyme content of nuclei are not accurate. The more soluble enzymes will appear to be low while the less soluble enzymes will appear to be high in concentration. Why part of the enzyme should remain in the nuclei while part is extracted is now known. Possibly the unextracted part is bound to nucleic acid, or to insoluble protein. Nucleic acid is known to be capable of complexing proteins if the pH is favorable relative to the isoelectric point of the protein; and nucleic acid has at least in some cases a protective action against denaturation.

Why an enzyme such as arginase should be present in high concentrations in the nucleus of the liver cell but not in the nucleus of the kidney cell is not clear. Arginase would not seem to be necessary for the metabolism of the cell nucleus, for if it were, it should be present in all cell nuclei in appreciable amounts.

It is as yet unknown whether any protein is lost from mitochondria during the process of isolation using hypertonic sucrose solutions. It would be very useful to investigate this point.

The question of possible adsorption of microsomes and submicrosomes by mitochondria or by nuclei during the processes of isolation of the latter cell fragments can be raised, but any such adsorption would not seem to be a greatly disturbing factor in enzyme studies in most cases, since

the microsomes thus far seem to contain fewer enzymes than mitochondria or nuclei. The ribose nucleic acid content of mitochondria is so low that it might be an adsorbed impurity.

The ultimate goal of cytochemical studies would seem to be the establishment of a sound cell physiology in which the location of enzymes and the flow of substrates within the cell would be well understood. The various processes of absorption and excretion, especially those requiring energy from the cell, probably will become comprehensible in the future as the result of cytochemical investigations of one sort or another.

A hypothesis concerning the possible effect of substrate concentration on nuclear function has been under consideration for some time by the writer as the result of an attempt to find some common denominator among the numerous known causes of cancer. Briefly, this hypothesis is that the ring of cytoplasm surrounding the nuclei of all animal cells capable of cell division may act as a screen which keeps the concentration of certain substrates low in the cell nucleus. For instance, if oxygen is considered as a substrate, it seems likely that enzymatic utilization of the oxygen by the mitochondria will lower the oxygen tension rapidly so that the average concentration of oxygen in the nucleus will be much lower than the average concentration in the outer zones of cytoplasm. The simplest type of cell to consider in this connection would be relatively nonpolarized cells such as liver parenchymal cells, but polarized cells such as those of the intestinal epithelium do not seem to offer any particular difficulties if the principal zone where oxygen enters the cell is at the basal portion, as seems probable.

Other substrates which might be considered similarly to oxygen are amino acids, glucose in the case of certain cells, and in fact any substrate which must enter a cell from the outside. Even substrates that are synthesized within the cytoplasm but not within the nucleus might be considered similarly.

If cell division should require that the concentration of certain key substrates be above a certain level in the nucleus, it is easy to see how a cytoplasmic screening, such as outlined above, could prevent a cell from dividing. Such screening by the cytoplasm might, however, be overcome in one of two ways: either the concentrations of substrates in the cytoplasm could be raised to such high values that the screening could no longer keep the substrate concentrations in the nuclei below the critical levels, or the screening ability of the cytoplasm might be weakened by a general lowering of cytoplasmic enzyme activity. In the case of regenerating liver of rats subjected to partial hepatectomy, the first situation presumably would be at hand, since the total portal supply would have to pass through the remaining liver and this should cause an increased rate of delivery of substrates, e.g., amino acids and glucose, to the cell.

In the case of cancer cells, the second situation presumably would be at hand, because of the lowered oxidative capacity and general lowering of enzyme activity of the cytoplasm which seems to occur. The great reserve of enzymes in the cytoplasm of cells such as those of the liver, in contrast to the lowered enzyme reserve in cancer cells, seems to favor this idea also. In cancer cells the enzymes seem more likely to be operating at full capacity than in noncancer cells.

If this hypothesis should contain a germ of truth, the degree of cytoplasmic screening ought to govern the frequency of cell division or at least to be one important factor governing this frequency. Cells with a very thin shell of cytoplasm, such as precursors of lymphocytes of the thymus and lymph nodes, should tend to have a

higher frequency of division than cells of the liver for instance. The hypothesis must, however, be applied only to cells known to be capable of division. There would be little meaning in trying to apply the idea to such highly differentiated cells as striated muscle cells and neurons for example, since these cells are incapable of dividing, no doubt because of structural peculiarities.

It should be noted that the hypothesis of cytoplasmic screening does not preclude the metabolic functioning of the cell nucleus in the resting cell. It merely states that if the metabolism of the cell nucleus is to be thrown into a sufficiently active state to cause cell division, the concentrations of certain substrates in the nucleus or at least the rates of delivery of these substrates to the nucleus must be above some threshold values.

Since cells with highly specialized metabolic functions, such as liver cells, must divert more of their basic substrates into specialized metabolic reactions necessary for the functioning of the body as a whole, one might expect a cytoplasmic screening effect to be greater in liver cells than, for example, in cells such as those of epidermis.

The hypothesis of cytoplasmic screening, which implies that increased blood supply or limited damage to cytoplasm or both would tend to favor cell proliferation, might be applicable to wound healing as well as to liver regeneration and to cancer. In the case of virus-induced cancer, the hypothesis still could hold, since the virus might act as an agent lowering the general enzyme activity of cytoplasm. As a matter of fact, cancer has been considered by some investigators as a disease of the cytoplasm.[261, 262]

It should be noted that the hypothesis of cytoplasmic screening is not easily refuted, but on the other hand it is not easily supported by direct experimental work. If the rate of delivery of substrates to the nucleus rather than their final concentrations in the nucleus is the more important factor, it may take years to find experimental techniques for approaching the problem. However, if the concentrations of certain substrates should be appreciably higher in the cytoplasm than in the nucleus, it may be possible to investigate the situation by applying the technique of Behrens to the preparation of nuclei and cytoplasm and then measuring the concentrations of the substrate in the isolated fractions. A possible difficulty with this apparently simple procedure is that diffusion of substrates between nucleus and cytoplasm may occur so rapidly even in the frozen state that concentration differences between nucleus and cytoplasm will be wiped out. The writer and coworkers have found that the absolute and relative concentrations of free amino acids in the cytoplasm and nuclei of liver cells of rats fed *ad libitum* on a fox chow diet are about the same, as judged by paper chromatography, which it must be admitted is not especially quantitative (unpublished). The crucial experiment of feeding the rats high amounts of protein digest and then repeating the work remains to be carried out.

The cytoplasmic screening hypothesis is indirectly supported by the work of Chipps and Duff,[193] who found that glycogen appears in the nuclei of liver cells in uncontrolled diabetes and in some other pathological conditions, whereas it is normally absent from the nucleus (see Section III, e, under nuclei). Since phosphorylase can be detected in ground liver cell nuclei, it seems logical to suppose that the excessive and persistant gluconeogenesis of diabetes may cause a flooding of the nucleus with glucose and a resulting formation of glycogen, provided that the glucose can be phosphorylated.

A piece of evidence that, on the other hand, does not seem at first sight to be easily reconcilable with the cytoplasmic screening hypothesis is furnished by the work on

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[261] H. G. DuBrey, *Science* **102**, 591 (1945).
[262] M. W. Woods and H. G. DuBrey, *Science* **104**, 469 (1946).

microdissection of amoebas already referred to. If too much of the cytoplasm is removed by microdissection, the amoeba will not divide until it has grown back to nearly normal size. Here, however, the trouble may be that the rate of digestion of food by the cell is lowered to such an extent that metabolizable substrates cannot accumulate anywhere in the cell fast enough to allow more than a very slow rebuilding of cytoplasm. The amoeba cannot be considered equivalent to a cell which receives predigested metabolites.

It should be remembered that a primitive cell tends to divide from time to time as long as sufficient substrate or nutrient is available and as long as the accumulation of waste products does not cause trouble. Cells which exist for the benefit of a whole organism must relinquish much of the tendency to divide, and an important problem is to find out what factors bring this about. In the primitive cell the whole economy is directed towards a synthesis of protoplasm, whereas in the specialized cell of a whole organism, the synthesis of protoplasm in many cases has become a secondary process.

This hypothesis of cytoplasmic screening has been presented because it appears to be plausible and quite generally applicable, and it illustrates that some sort of general theory of cell physiology may eventually be constructed from the result of cytochemical investigations. Of course it is possible that this particular hypothesis is quite erroneous. If it should be repugnant to some, it is to be hoped that they will be stimulated to carry out experiments to try to disprove it; if it should prove interesting to others, it is to be hoped that they will try to devise experiments to support it.

The chemistry of an organ or tissue can eventually be completely understood only if the distribution of enzyme systems among the various types of cells present in the organ or tissue is known. Some illustrations of enzymatic histochemistry were therefore included in this chapter.

ACKNOWLEDGEMENT

The writer wishes to acknowledge the support of the Donner Foundation and the U. S. Public Health Service during the time when this chapter was written.

CHAPTER 6

Modern Aspects of Enzymatic Adaptation

BY S. SPIEGELMAN

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I. Introduction

It is the purpose of the present article to attempt a critical analysis of the present status of the problem of enzymatic adaptation. Recent years have witnessed a considerable increase in both the interest and the amount of work done on this problem. It is being recognized by an increasing number of biological workers that the existence of enzymatic adaptation possesses implications for some of the most fundamental problems of modern biology. Perhaps most important is the recognition that this phenomenon may possibly represent a tool of no little power for the experimental analysis of the mechanism of enzyme formation. Data so obtained could, in principle, illuminate such problems as gene-enzyme relationships and cellular differentiation.

Except for references where pertinent, such general implications will not be discussed since they have been dealt with in great detail in several recent reviews.[1,2,3] Neither will an attempt be made at an exhaustive historical survey of the subject. An adequate account may be obtained by consulting several of the reviews[1,2,3,4,5] which have appeared in the last few years. We shall here concentrate our attention primarily on the methodology developed in recent years to investigate enzymatic variation in microorganisms and the results obtained.

A major portion of the advances of recent origin can be attributed to two innovations. One is the introduction of genetic principles into the experimental analysis of microbial variation. The other is the application of more rigid enzymological criteria to instances of enzymatic adaptation.

II. Some Examples of Enzymatic Adaptation

The capacity of microorganisms to vary their physiological and biochemical properties in response to cultivation in media of diverse composition has been known as long as microbiology has existed as a science. This variability constituted a source of endless annoyance to a majority of the early workers attempting to bring some order into the chaos which characterized the early stages in the development of the science. Indeed, one school, the monomorphists, sought to abolish this source of irritation by a sort of *executive fiat.*

One major aspect of the problem of microbial variation and its significance was opened for serious study with the introduction of the enzymological point of view into bacteriology. Wortmann[6] made a contribution of signal significance by recognizing that much could be learned through comparisons of organisms grown on two media which differed in the presence or absence of only one component. He showed that certain bacterial species could produce amylase only when grown in the presence of starch. Attention was thus focused on the possibility of experimentally analyzing the process under conditions which allowed the experimentalist to exert a measure of control over the factors involved in the phenomenon he was observing. Since Wortmann's observations, many other workers have shown that the presence or absence of a particular substance in the growth medium of a culture can influence, in a specific manner, the enzyme pattern exhibited by the culture.

Many of the earlier studies of enzymatic adaptation were done on molds.

[1] J. Monod, *Growth* **11**, 223 (1947).
[2] S. Spiegelman, *Symp. Soc. Exptl. Biol.* **2**, 286 (1948).
[3] S. Spiegelman, *Cold Spring Harbor Symposia Quant. Biol.* **11**, 256 (1946).
[4] E. F. Gale, *Bact. Revs.* **7**, 139 (1943).
[5] E. F. Gale, *Advances in Enzymol.* **6**, 1 (1946).
[6] J. Wortmann, *Z. physiol. Chem.* **6**, 287 (1882).

Duclaux,[7] in his *Traité de Microbiologie*, cites some of his own observations on enzyme production by the Aspergilli. He notes that saccharase is produced only when saccharose is present, and that proteases become detectable only if milk is included as a component of the medium. Went[8] studied similar phenomena in Neurospora. A series of papers by Kertesz[9,10] dealt with the effect of environmental factors on the production of saccharase by Penicillium. Besides the presence of substrate, other factors found to influence the output of saccharase were pH and potassium content of the medium. The pH optimum of this enzyme produced by the same organism under diverse conditions remained constant.

Phaff[11] has recently published some exceedingly interesting observations on the production of pectinase by *Penicillium chrysogenum*. Pectinase is a complex of a pectinesterase which hydrolyzes methanol from the esterified carboxyl groups of galacturonic acid units, and a polygalacturonase which hydrolyzes the D-glucosidic linkages between galacturonic acid units. Both enzymes are secreted into the medium under the proper conditions. Examination of 52 different carbon compounds for their capacity to stimulate production of this enzyme complex revealed that the only substances possessing significant activity were

1) the substrate itself, pectin

2) D-galacturonic acid, the product of hydrolysis, and substances which by decomposition can give rise to this compound

3) compounds possessing the same configuration on the last five carbons as D-galacturonic acid.

It is important to note that the examples of enzymatic adaptation mentioned thus far deal with instances of the production of extracellular enzymes. This fact has an important bearing on the nature of the process being observed and we shall return to it when these questions are discussed. In view of some of the obvious advantages possessed by extracellular enzyme systems, the paucity of systematic investigations into enzymatic adaptations in the molds is both surprising and regrettable. It seems highly probable that active investigation of such phenomena in these forms cannot fail to provide information fundamental to our understanding of the factors controlling enzyme formation.

The bacteria and the yeasts have constituted by far the most common objects of study to date. The number of enzyme systems examined is by now quite large and we need mention here only a few to indicate the extent and variety. Gale and Epps[12,5] examined the conditions required for the

[7] E. Duclaux, Traité de Microbiologie. 3 Vols., Masson et Cie., Paris, 1899.
[8] F. C. Went, *Jahrb. wiss. Botan.* **36**, 611 (1901).
[9] Z. I. Kertesz, *Fermentforsch.* **10**, 36 (1928).
[10] Z. I. Kertesz, *Plant Physiol.* **6**, 249 (1931).
[11] J. Phaff, *Arch. Biochem.* **13**, 67 (1947).
[12] E. F. Gale and H. Epps, *Biochem. J.* **38**, 232 (1944).

production of amino acid decarboxylases. Six distinct enzymes have been identified, each concerned with decarboxylation of one of the following amino acids: lysine, ornithine, arginine, tyrosine, histidine, and glutamic acid. Acid pH and, except for glutamic acid, the presence of the specific substrate during the growth of the culture are required for the production of the corresponding decarboxylase.

Dubos[13,14,15,16] made striking contributions to the problem in his series of studies on enzymes attacking the type-specific polysaccharides of Pneumococcus. Using an enrichment culture technique in which the polysaccharide constituted the sole source of carbon, an organism was isolated from soil which elaborated an enzyme capable of splitting type III polysaccharide. The enzyme was produced only when the organism was grown in the presence of the polysaccharide, or aldobionic acid, a product of its enzymatic hydrolysis. The degree of specificity exhibited by this adaptive hydrolytic enzyme was striking indeed, since it had no effect on any of the other type-specific polysaccharides, nor were these capable of inducing the formation of this enzyme under similar conditions. This situation obtained even in the case of type VIII polysaccharide which is quite similar to type III, both containing aldobionic acid and exhibiting serological cross reactions.

Another investigation which provides data pertinent to the problem of specificity is that of Mirick.[17] The reaction studied was the oxidation of *p*-aminobenzoic acid (PABA) to CO_2, H_2O, and NH_3 by organisms isolated from soil. Cultures grown in an ordinary casein hydrolyzate medium exhibit a small amount of activity. This activity could be considerably increased (up to 25-fold) by subculturing for a 12-hour period in the presence of 1% PABA, and was lost in a few hours after removal of the PABA. Only substances upon which the enzyme system could act were capable of inducing its appearance. An interesting illustrative example is provided by *o*-aminobenzoic acid (anthranilic acid). Cells grown in the presence of this isomer possess no increased capacity to oxidize PABA although they are adapted to the oxidation of anthranilic acid. Similarly, cells adapted to PABA show no evidence of being adapted to the ortho-isomer.

As illustrated by the outstanding investigations of Monod,[18] adaptive enzyme systems involving the metabolism of carbohydrates have played an increasingly important role in recent years in attempts to analyze the

[13] R. Dubos and O. T. Avery, *J. Exptl. Med.* **54**, 51 (1931).
[14] R. Dubos, *J. Exptl. Med.* **55**, 279 (1932).
[15] R. Dubos, *J. Exptl. Med.* **62**, 259 (1935).
[16] R. Dubos, *Ergeb. Enzymforsch.* **8**, 135 (1937).
[17] G. S. Mirick, *J. Exptl. Med.* **78**, 255 (1943).
[18] J. Monod, Recherches sur la croissance des cultures bacteriénnes. Hermann and Cie., Paris, 1942, 210 pp.

nature and significance of enzymatic adaptation. These will be considered in detail in later sections.

III. The Operational Description of Enzymatic Adaptation in Microorganisms and its Biological Significance

1. Adaptation during Growth

The basic elements of the phenomenon, as illustrated by the examples given in the previous section, can be adequately described in terms of the following operational concepts: a culture of organisms, either low or completely deficient in a defined enzymatic activity against a particular substrate is placed in contact with a medium containing the substrate. After the lapse of a period of time, the culture acquires the ability to metabolize the substrate in the manner specified. Subsequent contact with a medium lacking the adapting substrate, under the usual conditions, leads to the loss of the newly acquired enzymatic activity.

To be fruitful of precise and useful information, an analysis of this phenomenon must begin with a consideration of some more or less purely biological problems. These arise from the fact that large numbers of individuals are always involved in such adaptation experiments and the change observed is a modification in the biochemical properties of a population. The mechanisms available to an *individual cell* for adapting itself to an environmental change are limited by its genetic constitution and the physiological flexibility permitted by its particular degree of specialization. When, however, the adaptation of a *population of cells* is being considered, there must be added to the physiological pliability of its members the genetic plasticity of the group in terms of the numbers and kinds of variants it is capable of producing.

Because of this composite nature of populational adaptability, it is evident that any particular case of enzymatic adaptation in microorganisms could be attained by any one or more of the following mechanisms:

1) the natural selection of existent variants possessing the necessary enzyme patterns from a genotypically heterogeneous population,

2) the induction of enzyme by the substrate in all the members of a genetically homogeneous population,

3) a combination of the above two mechanisms in which the enzymes are induced by the substrate in certain variants and these are subsequently selected.

Much of the older and even some of the more recent[19] literature on enzymatic variation in microorganisms is difficult to interpret, because the

[19] C. N. Hinshelwood, The Chemical Kinetics of the Bacterial Cell. Oxford, Clarendon Press, 1946.

design of the experiments either completely ignored the necessity for deciding which of the above mechanisms was involved in the instance being studied, or was inadequate to do so. It is quite often extremely difficult to provide a definitive answer to such questions. The rarity of mutations in general is of relatively little value in arriving at a decision when microbial populations are being studied. The chances of having a wide spectrum of mutant types are very good when dealing with populations containing 1×10^8 or more individuals per cubic centimeter. Further, selective forces operate quite strongly under the conditions in which most adaptation experiments are performed. In the absence of decisive supplementary information, no definite conclusions as to mechanism can be drawn from any adaptation experiments in which the change in enzymatic pattern is achieved during the growth of the organisms in a medium containing the adapting substrate.

It is of some importance to recognize that, contrary to the views of most authors, easy reversibility of a particular enzymatic adaptation cannot be accepted as an adequate criterion for the absence of mutation and selection. Thus, it has been generally assumed that if a culture acquires a particular enzymatic capacity after one or two passages in a certain well-defined medium, and if this capacity is lost quickly by growth in a medium lacking the adapting conditions, the modification of enzyme constitution being considered is not preceded by mutation and subsequent selection. While such unrestrained alternations between adapted and unadapted populations are certainly unexpected in cases involving mutational changes, it is by no means impossible that they obtain. All that is required is that the "deadapting" medium be selective for the original type, and that the back mutation to this type be of the same order of magnitude as the forward mutation. Situations quite analogous to this have been observed and are particularly well illustrated by the instance investigated by Ryan and his coworkers of the instability of a histidineless strain of *E. coli*.[20,21]

We may then well ask what method is to be employed in adaptations that do not occur unless growth of the culture takes place during exposure to the adapting medium. This is an extremely important problem since this situation obtains in many instances of adaptation, particularly among the bacteria. There exists, fortunately, at least one approach which we owe to the ingenious efforts of Luria and Delbrück[22] who solved an analogous problem in their now classic investigations on bacteriophage. These authors were concerned with the nature of the change leading to bacteriophage resistance by cultures of *E. coli* exposed to these bacterial viruses. The principle of the method depends upon two circumstances. One is that the

[20] F. J. Ryan and Lillian K. Schneider, *J. Bact.* **56**, 699 (1948).
[21] F. J. Ryan and Lillian K. Schneider, *Genetics* **34**, 72 (1949).
[22] S. E. Luria and M. Delbrück, *Genetics* **28**, 491 (1943).

growth of microbial populations is clonal and the other is that mutations are random and relatively rare events.

We may demonstrate the use of this method by describing its application to an instance of adaptation by yeast cultures to the fermentation of galactose. A series of tubes are seeded containing a medium lacking in galactose which will, however, support the growth of the organism. The size of the inoculum is so adjusted that the chance of seeding a positive galactose-fermenting cell in any of the tubes is negligible. After the appearance of a sufficient number of generations, an estimate is made of the number of organisms in each tube that can give rise to clones adapted to the fermentation of galactose by using a suitable test plate containing galactose. The statistical variance of the number of such organisms found in the different tubes of the series is compared with that of the sampling procedure, obtained by taking an equal number of samples from one tube. If the adaptation is fundamentally mutational in character and can occur in the absence of the adapting substrate, the variance of the samples derived from the separate tubes will be much greater than the variance observed in an equal number of samples from the same tube. This conclusion follows from the random nature of mutations which can occur at any time in the development of the culture. Should a mutant appear early in the history of a given culture, a large number of mutant types would be counted since it and all its progeny would be estimated on the test plate. On the other hand, if the mutation occurs toward the end of the growth, relatively few positive clones would be found on the test plate. For the majority of the tubes, the last mentioned situation will be true. However, if the number of separate tubes seeded is large enough, there will be a few in which an inordinately large number of positives will be found. The variance of the number of positives found in such a series of tubes will thus be much greater than the variance of the sampling procedure.

It is clear that this method detects primarily heritable modifications which can occur independently of the substance used to determine the presence or absence of the character concerned. If the analysis of any adaptation experiment by this device yields a high variance, one must conclude that one is dealing with a mutation which can occur in the absence of substrate. It is important to realize that the method is in no way invalidated by the fact that even when mutation is involved, the change in enzyme patterns cannot appear or be detected unless the cells are exposed to the adaptive substrate. Indeed the method was devised to overcome precisely this methodological difficulty.

The application of this method may not be simple in some instances, and care must be exercised to be certain that the experimental conditions conform to and satisfy the basic assumptions underlying the analysis of Delbrück and Luria.[22] Two pitfalls in particular must be guarded against.

The medium employed for the separate tubes, which lack the adaptive substrate, must not be selective to any extent for either type. Further, the test plate must not permit significant growth of the negative type. Unless both of these criteria are satisfied, the absence of large variance signifies nothing.

2. Enzymatic Adaptation in Resting Cells

The decision as to the nature of the biological mechanism operating is greatly simplified in cases where the adaptation can occur in the absence of significant cell division. Here there can be no question of selection of mutant types, and one must in such cases conclude that the presence of the substrate sufficiently modifies the enzymatic activity in a high enough proportion of the existing cells to be detectable by a suitable measurement. The definitive nature of such an experiment in deciding this important biological issue was recognized early in the history of the problem. Actually the first apparent successful attempt to obtain emzymatic adaptation in the absence of cell division was accomplished by Dienert,[23] who was unfortunately unaware of the genetic implications of his results, a naivete that was hardly surprising in view of the year in which his experiments were performed. The technique he used depended primarily on adapting populations of very high density under the assumption that cell division would not occur during adaptations under such conditions. This method is obviously not completely free of possible error. Dienert concluded that it was possible for nondividing yeast cells to adapt to the fermentation of galactose.

Kluyver's paper in 1914[24] initiated a long series of experimental attacks on the question with a clear understanding of the implications involved. He attempted to obtain adaptation at temperatures high enough to prevent cell division; his results were completely negative, in the sense that such cells did not adapt. Employing the same basic principles, Euler and Nilsson[25] and subsequently Euler and Jansson[26] tried without success to obtain adaptation in the presence of concentrations of phenol which inhibited cell division. The evidence of these carefully controlled experiments seemed to be against Dienert's conclusion and the possibility that enzymatic adaptation represented a direct induction by the substrate of a new enzyme activity in the cytoplasm of existing cells. Such negative evidence is, however, not conclusive. One cannot ignore the possibility that in cultures so treated the physiological state of the cells is such that their ability to synthesize new enzymes has been lost along with their capacity to divide.

Another approach used by Sohngen and Coolhaas,[27] in the case of adapta-

[23] F. Dienert, *Ann. Inst. Pasteur* **14**, 139 (1900).

[24] A. J. Kluyver, Biochemische Suiberbepalingen proef schrift Delft., 1914, p. 91 (cited by Sohngen and Coolhaas, 1924).

[25] H. von Euler and R. Nilsson, *Z. physiol. Chem.* **143**, 89 (1925).

[26] H. von Euler and B. Jansson, *Z. physiol. Chem.* **169**, 226 (1927).

[27] N. Sohngen and C. Coolhaas, *J. Bact.* **9**, 131 (1924).

tion to galactose fermentation by yeast, consisted of a careful comparison of the appearance of enzyme activity with cell counts in growing cultures. The supposition underlying these experiments was that if adaptation was not a consequence of the cell division of a new type being selected, no simple correlation between cell count and enzyme activity was to be expected. On the other hand, if selection were occurring, a relatively simple relation should obtain between the formation of new cells and the increase in enzymatic activity of the culture. Their results seemed once again to decide the issue in favor of selection from a mixture of biotypes as the determining mechanism. The whole question was, however, reopened by the experiments of Stephenson and Yudkin,[28] who concluded from their results that adaptation to galactose was possible in the absence of cell division.

The fundamental work of Winge and his collaborators[29,30,31] and of the Lindegrens[32,33,34] on the genetics and life cycle of the yeasts made the reexamination of this problem profitable. Experiments with known haploid and diploid strains of *S. cerevisiae* made it clear that the contradictory results cited above were inherent not in the mechanism of enzymatic adaptation, but in the nature of the stocks employed.[35] It was possible to duplicate both types of findings by the proper selection of strains.[36] Thus, by the use of diploid stocks, possessing the proper genetic constitution, it was established beyond doubt that yeast cells could adapt without cell division. Thoroughly washed cells of some stocks could adapt to galactose or maltose within a few hours while suspended in a phosphate buffer solution of the proper substrate. Adaptation of other strains, however, depended on the selection of the proper mutant type and were incapable of adapting in the absence of cell division.

While in the majority of instances, bacterial cultures do not adapt unless active division is occurring, there are now several instances of enzymatic adaptations with "resting" suspensions.[37,38,39,40]

[28] M. Stephenson and J. Yudkin, *Biochem. J.* **30**, 506 (1936).
[29] O. Winge and O. Laustsen, *Compt. rend. trav. lab. Carlsberg sér. physiol.* **22**, 99 (1937).
[30] O. Winge and O. Laustsen, *Compt. rend. trav. lab. Carlsberg sér. physiol.* **22**, 235 (1938).
[31] O. Winge and O. Laustsen, *Compt. rend. trav. lab. Carlsberg sér. physiol.* **22**, 337 (1939).
[32] C. C. Lindegren, *Bact. Revs.* **9**, 111 (1945).
[33] C. C. Lindegren, and G. Lindegren, *Cold Spring Harbor Symposia Quant. Biol.* **11**, 115 (1946).
[34] C. C. Lindegren, The Yeast Cell. Educational Publishers, Saint Louis, Mo., 1949.
[35] S. Spiegelman, C. C. Lindegren and L. Hedgcock, *Proc. Natl. Acad. Sci.* **30**, 13 (1944).
[36] S. Spiegelman and C. C. Lindegren, *Ann. Missouri Botan. Garden* **31**, 219 (1944).
[37] M. Stephenson and L. H. Stickland, *Biochem. J.* **26**, 712 (1932).
[38] M. Stephenson and L. H. Stickland, *Biochem. J.* **27**, 1528 (1933).
[39] R. Knox and M. R. Pollock, *Biochem. J.* **37**, 476 (1943).
[40] M. R. Pollock, *Brit. J. Exptl. Path.* **27**, 419 (1946).

It was clearly of the utmost importance to establish conclusively that enzymatic adaptation could appear in the absence of cell division. From a theoretical point of view, such experiments possessed widespread implications. They suggested that the phenotype of a cell at its most elementary level, in terms of patterns of enzymatic activity, was flexibly determined by the environmental conditions. More immediately, they established the reality of the phenomenon of enzymatic adaptation and provided the conditions which for the first time permitted biologists to study the factors, other than genes, controlling the appearance and maintenance of enzyme activity patterns in cells.

IV. Enzymatic Constitution and Enzymatic Adaptation

The next question to consider is the physiological and biochemical significance of enzymatic adaptation. The usual implication in the literature has been that enzymatic adaptation involves the actual formation of new enzymes. Although this is certainly the most interesting possibility, it is not *a priori* obvious in any instance of adaptation that this is necessarily the case and that other mechanisms are not involved.

The relation between the enzymatic constitution of a cell and the pattern of enzyme activity it exhibits need not be a simple one. Not every increase in enzyme activity need necessarily imply an increase in the amount of the corresponding enzyme. All that is observed in any adaptation experiment is a period of increasing enzyme activity. This increase may start either at zero level (i.e., nondetectable) or from a measurable but small amount of enzyme. Such lags preceding the onset of maximum activity may be due to a variety of reasons, none of which need be directly connected with enzymatic constitution. Factors which might be invoked to explain a delay in the onset of maximal enzyme activity in intact cells are:

1) penetration of the adaptive substrate into the cell,

2) formation by existent enzyme systems of special intermediates unique to the metabolism of the adaptive substrate,

3) the time required to raise the concentration of existent cofactors to the levels required for the metabolism of the adaptive substrate,

4) the gradual release of the enzyme system concerned from some regulatory inhibition imposed by the cell,

5) formation of an enzyme system in response to the adaptive substrate.

There are relatively few cases of enzymatic adaptation in which a choice amongst the above possibilities can be made on the basis of adequate experimental data. It is clearly a matter of the utmost difficulty to attempt a decision on the basis of work confined solely to the intact cell. There are several criteria which must be satisfied before the solution can be regarded as satisfactory. In the first place, the genetic criteria mentioned previously

must be adhered to. It would not be particularly relevant to the points at issue to demonstrate the formation of a new enzyme system in a population of unknown genetic constancy. The fact that two cells differing in genotype can exhibit distinctive patterns of enzyme activity is interesting but not directly pertinent to the problems raised uniquely by enzymatic adaptation. Secondly, it is necessary from an enzymological point of view to demonstrate that the enzyme system involved can be extracted from adapted cells and is unobtainable from unadapted preparations.

These and related questions have been most carefully analyzed in the case of galactose adaptation in yeast.

It is possible, on various grounds, to eliminate penetration as playing any significant role in adaptation to galactose. In the first place, it has been demonstrated[41,42] that galactose enters the cell immediately and is metabolized by a purely aerobic mechanism before the adaptive fermentation enzymes make their appearance. Moreover, whereas cell-free extracts prepared from adapted cells are able to ferment galactose, comparable preparations from unadapted cultures are completely inactive.[43] Such experiments demonstrate that something possessing galactose fermenting capacity can be extracted from cells after adaptation which was not present before. They also prove conclusively that penetration is not the critical factor since it obviously cannot be involved in experiments with cell-free extracts.

Such extract experiments do not, however, permit a decision amongst the other possibilities mentioned. The first attempt to settle this question was made by von Euler and his collaborators.[44] Dried cells of adapted and unadapted cultures were washed to rid them of cofactors and intermediates. The resulting preparations were tested for galactose fermenting capacity. They concluded from their results that adaptation to galactose resulted in a modification of the apoenzyme. The methods used by these workers for preparing apoenzymes, however, were such that the activities obtained were quite low as compared with their control preparations. Consequently, their results were not sufficiently conclusive to support definitive conclusions on this question.

In view of the importance of the issues involved, the problem was reexamined[45] with the aid of more modern procedures for making apoenzyme preparations, using cell-free extracts prepared from adapted and unadapted cells. Apoenzyme and coenzyme fractions were prepared from both types of cultures and were combined in all possible ways. The results obtained may be summarized by the following:

1) adapted apoenzyme + unadapted coenzyme = active enzyme

[41] S. Spiegelman, *J. Bact.* **49**, 108 (1945).
[42] J. M. Reiner and S. Spiegelman, *J. Gen. Physiol.* **31**, 51 (1947).
[43] A. Harden and R. V. Norris, *Proc. Roy. Soc. London* **B82**, 645 (1910).
[44] H. von Euler and G. Jansson, *Z. physiol. Chem.* **169**, 226 (1927).
[45] S. Spiegelman, J. M. Reiner and I. Morgan, *Arch. Biochem.* **13**, 113 (1947).

2) adapted apoenzyme + adapted coenzyme = active enzyme
3) unadapted apoenzyme + unadapted coenzyme = inactive enzyme
4) unadapted apoenzyme + adapted coenzyme = inactive enzyme.

It is apparent that the capacity to ferment galactose is invariably associated with the apoenzyme derived from adapted cells. These data lead to the conclusion, therefore, that it is the apoenzyme or protein moiety of the enzyme complex which is modified during the course of the adaptation. That only this modification is involved follows from the ability of unadapted coenzyme preparations equivalently to replace adapted coenzyme in activating adapted apoenzyme to galactose fermentation.

Further evidence that adaptation leads to the appearance of a new enzyme which can be extracted from adapted cells but not from unadapted ones, is furnished by some experiments on bacterial adaptation. Doudoroff and his collaborators[46] have demonstrated that sucrose phosphorylase can only be found in cells which have been exposed to the substrate sucrose. Similarly, a recent investigation by Monod and Torriani[47] identified an amylomaltase as the adaptive enzyme in *E. coli* formed in response to maltose. They showed that this enzyme cannot be obtained from cells which have not been adapted to maltose. Finally, one may cite the remarkable recent contributions of Stanier,[48,49] on adaptive utilization of cyclic compounds. His results indicate complete correspondence between the enzymatic activity of dried-cell preparations and the previous adaptive history of the cells from which they were derived.

These findings indicate that at least in some cases of enzymatic adaptation, one can satisfy the enzymological criteria necessary to conclude that adaptation leads to a real and detectable modification in the protein pattern of the cell associated with specific enzymatic activity. It is necessary to emphasize that the existence of these cases by no means permits one to conclude that all cases of apparent enzymatic adaptation are of a similar nature. It is highly desirable that each case be examined independently before it is used as a tool for the study of enzymatic adaptation in the sense of protein modification.

A word may be said here about the use of the phrase "enzyme formation (or synthesis)" in connection with enzymatic adaptations. Even in cases where an apoenzyme modification can be exhibited, there is no certainty that the process involves protein synthesis in the sense in which the latter is usually understood. Precision in our concepts concerning these matters will come only when we are in possession of the details of the mechanisms involved, and when presumably the employment of such phrases as "en-

[46] M. Doudoroff, N. Kaplan and W. Z. Hassid, *J. Biol. Chem.* **148**, 67 (1943).
[47] J. Monod and A. M. Torriani, *Compt. rend.* **227**, 240 (1948).
[48] R. Y. Stanier, *J. Bact.* **55**, 477 (1948).
[49] R. Y. Stanier and M. Tsuchida, *J. Bact.* **58**, 45 (1949).

zyme formation" will no longer be necessary. For the present, such phrases should be understood as having only operational connotations in which the adaptations being described have satisfied the biological and enzymological criteria previously noted.

All we can be reasonably certain of is that we are, in such cases, studying transformations which yield active enzyme. The most significant feature of the phenomenon of enzymatic adaptation, and the establishment that a real modification in apoenzyme is involved, lies precisely in the fact that it provides a tool with which such transformations can be studied. An experimental analysis of the factors governing the appearance, maintenance and disappearance of active enzyme becomes possible.

V. The Kinetics of Enzymatic Adaptation

Adequate information on the kinetics of adaptation could provide important clues as to the nature of the process involved. In particular, as has been pointed out[3,50] it would permit a test of the mass-action hypothesis first proposed by Yudkin.[51] This hypothesis has suggested that the enzyme and its precursor are essentially in equilibrium with the position far over on the side of the precursor. The substrate, it is presumed, by combining with the enzyme shifts the reaction towards further enzyme formation. This hypothesis makes possible a rather definite prediction with respect to the kinetics of the appearance of enzyme in the presence of substrate. The time-activity curve should be one essentially exponential in character and concave to the time axis.

The use of growing cultures in enzymatic adaptation experiments complicates any attempts at analyzing the kinetics of the process. It is difficult under such circumstances to dissociate the growth of a new cell from the appearance of enzyme activity, and the time-activity curves obtained would be primarily determined by the growth characteristics of the culture.

The possibility of testing the Yudkin hypothesis in a relatively simplified system was provided by adaptations with yeast cells, which can adapt in the resting state while suspended in simple buffer media containing the substrate. When such kinetic studies were made[3,50] on two adaptive enzyme systems, (galactozymase and maltozymase) an S-shaped curve was obtained. The initial part of the curve is characterized by a rising rate of enzyme formation. This is then followed by a declining rate portion and ultimately a maximal level is achieved. Similar curves have been obtained in the case of adaptation to formic acid in *E. coli*[52] and more recently to tetrathionate and nitrate in a coliform organism.[39,40] It is clear that these

[50] S. Spiegelman, *Ann. Missouri Botan. Garden* **32**, 139 (1945).

[51] J. Yudkin, *Biol. Revs. Cambridge Phil. Soc.* **13**, 93 (1938).

[52] M. Stephenson, Bacterial Metabolism. Longmans Green and Co., London, 1939, 391 pp.

findings on the kinetic aspects of adaptation are not consistent with the simple mass-action mechanism suggested for the role of substrate.

A more detailed analysis of the time-activity curve reveals that the experimental points could be fitted quite accurately to a curve derived on the basis that the primary rate limiting process in enzymatic adaptation is an autocatalytic one. This implies only that the rate of enzyme formation is a function of the amount of enzyme present. There are several possible mechanisms that have been offered to explain this particular finding. They may be listed as follows:

1) Enzymatic adaptation consists of an autocatalytic activation of inactive protein analogous to the pepsinogen to pepsin transformation.

2) The adaptive substrate is the energy source for synthetic activity required for the formation of enzyme.

3) The distribution of adaptive capacities within the population of cells being adapted is such that an S-shaped curve would be obtained when total activity is plotted against time.

4) The process is inherently autosynthetic.

The first hypothesis suggested is self-explanatory. Under the second hypothesis, it may be argued that it is hardly surprising to find autocatalytic kinetics if the adapting substrate is ultimately the source of the energy employed by the cell for all synthetic activities including enzyme formation. Under such circumstances obviously the more enzyme present, the greater would be the rate of substrate utilization, the greater therefore the rate of energy generation, and consequently the more rapid the transformation of precursor protein into enzyme. As a consequence the kinetics of enzyme appearance would be autocatalytic no matter what the details of the synthetic mechanism of enzyme formation.

The third hypothesis presupposes that a normal distribution of rates of adaptation exists in the population being adapted. Such distributions, if of the proper numerical type, could give the S-shaped curve observed for the activity of the population as a whole. The fourth hypothesis assumes that the enzyme-forming system in the cytoplasm is a self-duplicating one.

No definitive decision in favor of any one of these hypotheses can be made at present. The available information to be detailed in subsequent sections makes some of them unlikely. In particular, the first hypothesis is inconsistent with experiments demonstrating competitive interaction between enzyme forming systems. Two experimental findings more or less vitiate the explanatory value of the second hypothesis which depends on energy supply by adaptive metabolism of the substrate. One is the autocatalytic nature of adaptations to substrates yielding very little free energy (e.g., the hydrogenylase system). The second is that autocatalytic

curves are found even when endogenous reserves are the primary energy source as in the case of aerobic adaptation to galactose by yeast.[53]

VI. The Relation of Enzymatic Adaptation to Cellular Metabolism

Interpretation of the relative synthetic complexity of enzymatic adaptation would clearly be greatly influenced by the answer to the question of whether or not the process requires energy. The fact that in many cases in the bacteria adaptations do not occur in the absence of growth indicates a rather close link between synthetic capacities and enzymatic adaptation. Such results are, however, not conclusive since one cannot be certain that this correlation may not be a secondary consequence of the conditions which obtain in an actively growing cell. Actual utilization of the energy-generating mechanism of the cell for the specific adaptation cannot be demonstrated under such circumstances. Here again, the employment of systems which exhibit the phenomenon of adaptation in resting cells possesses obvious advantages for the design of experiments intended to investigate such problems. In addition, it is clearly important to use an adaptive enzyme system for which the evidence is reasonably certain that the appearance of enzyme activity is associated with apoenzyme modification.

In the usual experimental procedures employed in the study of enzymatic adaptation in yeasts, the cells are suspended in a buffer solution containing the adaptive substrate. Under such conditions therefore, except for endogenous reserves, the adaptive substrate is the sole source of carbon energy. Early investigations have demonstrated[54] that under such conditions the presence of oxygen greatly stimulates the adaptive process and in some cases is essential to its occurrence. Presumably then, some type of aerobic metabolism is the primary source of energy in these instances. A subsequent investigation[55] of the galactose system revealed that this requirement for oxygen was confined primarily to the initial period of adaptation. If an adequate amount of adaptive enzyme is built up by the previous aerobic incubation, subsequent anaerobic contact with substrate leads to the formation of more enzyme. This was interpreted to indicate that sufficient enzyme had been formed in the presence of oxygen to permit the cells to utilize the adaptive substrate anaerobically as a source of energy for the formation of more enzyme.

The rather marked stimulatory effects of oxygen on the onset of galactose fermentation by baking yeasts led early investigators to the conclusion

[53] S. Spiegelman, Mechanisms of Enzymatic Adaptations in Yeast. Thesis, Washington University, St. Louis, Mo., 1944.

[54] A. S. Schultz, L. Atkin and C. N. Frey, *J. Am. Chem. Soc.* **62**, 2271 (1940).

[55] S. Spiegelman, *J. Cellular Comp. Physiol.* **25**, 121 (1945).

that this effect is due primarily to an "activation" of the galactozymase system. The mechanism presumably invoked is the oxidation of some component necessary for the functioning of the galactose fermenting system. Such an explanation must perforce leave the mechanism of activation vague as to the nature of the component activated. In addition it ignores completely the role of substrate.

A more satisfactory solution to the problem of why aerobiosis is quite generally required to initiate the adaptive process in yeast was suggested by the finding that intact yeast cells of certain strains cannot utilize their carbohydrate reserves anaerobically.[56,57] These reserves can, however, be readily oxidized aerobically. Assuming that the adaptive process does require energy, it is not surprising to find that adaptation is difficult when initiated under anaerobic conditions, since under these circumstances neither the exogenous adaptive substrate nor the endogenous reserves are utilizable as sources of energy. Aerobically, however, the cells can metabolize the endogenous carbohydrate and use the resulting energy to form the adaptive enzyme.

This suggestion led to a more extensive investigation into the relation between the level of aerobic endogenous respiration and adaptability to galactose and maltose fermentation.[58] It further suggested the desirability of determining whether the aerobic endogenous metabolism could be replaced as an initiator of adaptation by supplying a suitable substrate which could be utilized as a source of energy under anaerobic conditions. It was found that the addition of any fermentable substrate such as glucose led to rapid anaerobic adaptation to either galactose or maltose. Fermentability was the sole criterion of usefulness for an exogenous substrate in aiding the adaptive process anaerobically.

A seeming contradiction to this general point of view was found in the case of galactose adaptation. Cells which were depleted of their endogenous reserves by prior dissimilation under aerobic conditions were still found to be capable of adapting to galactose aerobically. Since the respiratory levels achieved in these experiments were extremely low, the question immediately arose as to what was the primary source of energy in these cases; presumably the adaptive substrate itself could not be used as a source of energy. Thorough analysis of this apparent discrepancy led to the discovery[41,42,53] that the adaptation to galactose consists primarily in building up an enzyme system required for its *fermentative* utilization. The unadapted cell possesses an enzymatic apparatus permitting immediate aerobic utilization of the carbohydrate. In the absence of effective endogenous respiration, this pre-adaptive utilization of the substrate provides the en-

[56] T. J. B. Stier and J. N. Stannard, *J. Gen. Physiol.* **19**, 479 (1935).
[57] S. Spiegelman and M. Nozawa, *Arch. Biochem.* **6**, 303 (1945).
[58] S. Spiegelman, J. M. Reiner and R. Cohnberg, *J. Gen. Physiol.* **31**, 27 (1947).

ergy required for the formation of the fermentative adaptive system. One consistent feature in all these experiments was the fact that none of the endogenous and exogenous energy sources which were investigated would facilitate the adaptation unless the adaptive substrate was present while these sources were being utilized.

A more subtle demonstration of the connection between enzymatic adaptation and metabolism involves the use of metabolic poisons capable of dissociating synthetic activities from the energy-generating mechanisms of the cell. Care must, however, be exercised in such experiments to avoid the use of inhibitors which suppress the activity of the adaptive system being studied. Thus for example, the fact that it was demonstrated[59] that such inhibitors as sodium fluoride and iodoacetic acid prevented aerobic adaptation to galactose fermentation in yeast is not particularly informative, since their presence would prevent the expression of the adaptive enzyme system even if it were formed. Another case in point is the recent demonstration[60] of an apparent inconsistency in the response of galactose adaptation to arsenate and azide. A re-investigation[61] of this phenomenon revealed that arsenate acted as a specific inhibitor of the galactose metabolizing system.

Similarly, in view of the results cited, it would not be particularly useful to employ inhibitors which interfere with over-all metabolic processes. Thus, for example, the demonstration[59] that sodium azide (NaN_3), or NaCN inhibit the aerobic adaptation to galactose does not tell us any more than that active respiratory activity is necessary for the onset of the process in the absence of a fermentable substrate.

The first indication that it was possible to dissociate metabolic activity from enzymatic adaptation appeared in the classic investigations of Dienert[23] into the nature of galactose adaptation in yeast. He demonstrated that borate, although not inhibitory to the adaptive system once formed, was capable of preventing its appearance. The exact interpretation of this result must await a more detailed examination into the mechanism of action of borate. Monod[62] employed 2,4,-dinitrophenol (DNP) to demonstrate that adaptation of *E. coli* to lactose and xylose could be dissociated from aerobic respiration. Unfortunately, a clear-cut interpretation of these results is complicated by the fact that Monod's investigations employed systems in which the adaptation could not occur in the absence of cell division. It is not completely certain that the effect of DNP on the adaptive system may not be an indirect consequence of its effect upon the capacity for growth.

[59] J. M. Reiner, *Proc. Soc. Exptl. Biol. Med.* **63**, 81 (1946).
[60] J. M. Reiner, *Arch. Biochem.* **19**, 218 (1948).
[61] M. Sussman and S. Spiegelman, In manuscript, (1950).
[62] J. Monod, *Ann. Inst. Pasteur* **70**, 381 (1944).

Early investigations[63,64] with sodium azide demonstrated that this substance can block the anaerobic assimilation of glucose and of ammonia at concentrations which do not suppress the over-all fermentation rate. In addition, it has been demonstrated[65] that this compound inhibits embryonic developmental processes which do not depend upon aerobic respiration. Similarly, azide inhibits anaerobic adaptations to galactose and maltose by yeast.[66] Attempts[67] to elucidate the mechanism of action of azide led to the conclusion that it functioned by interfering with the transfer of the energy-rich phosphate formed by the coupled oxidation of phosphoglyceraldehyde to diphosphoglycerate. Under anaerobic conditions this represents the primary energy generating mechanism of the yeast cell and it is not surprising therefore to find that this agent interferes with all synthetic activities that have thus far been investigated, including enzymatic adaptation. A reinvestigation[61] of the effect of arsenate, which also acts at the same enzymatic locus, revealed that it had the same capacity as azide to dissociate anaerobic glycolysis from enzymatic adaptation.

Monod and Wollman[68] have performed an interesting and suggestive experiment which exhibits a more complicated case of dissociation of synthetic activities of a specific kind from those involved in enzymatic adaptation. They demonstrated that *E. coli* which were infected with bacterial viruses immediately lost their capacity to form adaptive enzymes. This again may, however, be connected with loss of capacity for growth since virus-infected bacteria cannot undergo cell division subsequent to the infection.

In general, all these results are consistent with the conclusion that enzymatic adaptation requires not merely the operation of an intact over-all metabolism but in particular a functional and utilizable energy-generating mechanism. In fact, a recent investigation,[69] which sought to establish the energy levels required for various synthetic processes, revealed that enzymatic adaptation was the most sensitive of those investigated. Increasingly severe interference with the energy-generating step of the glycolytic cycle resulted in a loss of the capacity to form adaptive enzymes before the assimilation of nitrogen and carbon were seriously curtailed.

That some relation exists between enzymatic adaptation and nitrogen metabolism is indicated by the fact that the majority of enzymatic adaptations will not take place unless the cells are in a complete medium con-

[63] R. J. Winzler, *Science* **99**, 327 (1944).
[64] R. J. Winzler, D. Burk and V. du Vigneaud, *Arch. Biochem.* **5**, 25 (1945).
[65] S. Spiegelman and F. Moog, *Biol. Bull.* **89**, 122 (1945).
[66] S. Spiegelman, *J. Cellular Comp. Physiol.* **30**, 315 (1947).
[67] S. Spiegelman, M. D. Kamen and M. Sussman, *Arch. Biochem.* **18**, 409 (1948).
[68] J. Monod and E. Wollman, *Ann. Inst. Pasteur* **73**, 937 (1947).
[69] M. Sussman, Dissociation of Biosynthesis from Glycolysis in the Intact Yeast Cell. Thesis, University of Minnesota, Minneapolis, Minnesota, 1949.

taining a source of available nitrogen. A more direct demonstration was made possible by the use of yeasts which can adapt in the absence of exogenous nitrogen. Thus it was shown[70] that the level of enzymatic activity attained and the rate at which it was reached were considerably increased in the presence of an exogenous source of nitrogen. In addition, it was observed that any agent or condition which stopped the assimilation of nitrogen simultaneously interfered with the capacity of the exogenous nitrogen to stimulate the adaptive process.

VII. Interactions between Enzyme-Forming Systems

The existence of enzymatic adaptation presents immediately the possibility of inquiring into the nature of the precursor transformed into active apoenzyme. There are three cardinal facts which may be employed as a point of departure for the further experimental analysis of this question:

1) The process requires energy.
2) Exogenous nitrogen stimulates the rate and extent of adaptation.
3) Exogenous nitrogen is not necessary in certain instances.

The last situation implies the presence within the cell of material which can, under the influence of substrate, be converted into active apoenzyme. The question, of course, centers around the nature of this material, and there are several possibilities which can be entertained. There may be a storage of enzymatically inactive protein capable of being transformed into a variety of different active apoenzymes. On the other hand, this inactive protein may be already so highly specified that it can be transformed only into one particular enzyme analogous to the pepsinogen to pepsin transformation. The third possibility is that no such storage of enzymatically inactive protein exists, but that the protein of existing cellular enzymes is employed under certain conditions for the formation of a new enzyme.

The first indication of what may actually be involved emerged from Monod's[18] brilliant investigations into the nature of the growth curve of bacterial cultures. This investigator was primarily concerned with elucidating the factors which determine the various phases of the growth cycle, and in particular the lag phase. His experiments clearly indicated that at least under certain circumstances the determining process involves the formation of a particular enzyme.

Monod was able to exhibit a successive appearance of enzymes in cultures exposed to a mixture of two or more carbohydrates in limiting concentrations. A new enzyme system appeared when the first substrate attacked became exhausted. The lag period between the exhaustion of the first substrate and the appearance of enzymatic activity against the second is the basis for his "diauxie" phenomenon. It is quite evident that diauxie would not be observed if the cells were capable of making simultaneously

[70] S. Spiegelman and R. Dunn, *J. Gen. Physiol.* **31**, 153 (1947).

the enzymes required for the metabolism of both carbohydrates. It is important to note for later reference that these experiments were done with growing cultures in which exogenous nitrogen was obviously present and that even under such circumstances, the severity of the effect amounted to almost a mutual exclusion phenomenon. Only one enzyme system appears to be formed at a time.

Monod interpreted these findings in terms of competition between the two adaptive substrates for the same precursor protein molecules which he called "pre-enzymes." The basic assumption was that a pre-enzyme could be converted into a variety of enzymes depending upon the sub-

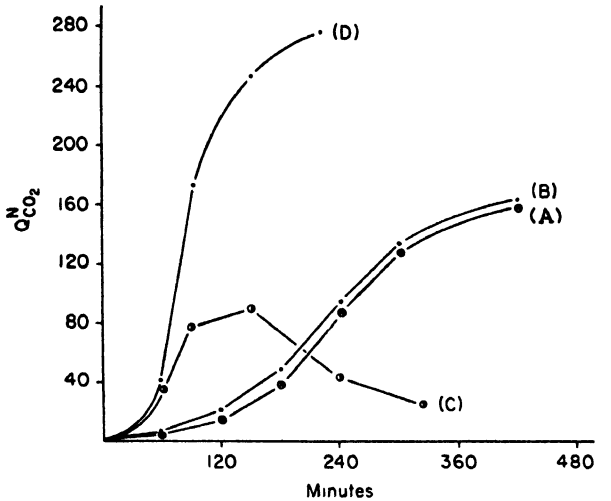

FIG. 1. Simultaneous adaptation to galactose and maltose. *B* is the curve obtained of galactozymase activity when the suspension is incubated in the presence of galactose only. *D* is the corresponding control curve for maltozymase activity. *A* and *C* are curves of galactozymase and maltozymase activities respectively when both are being induced simultaneously.

strate with which it combined. These views were later modified[1] to conform more closely to interpretations of results obtained with yeasts to be described presently.

The existence of interactions between adaptive enzymes similar to those described by Monod was discovered independently in the course of an investigation[3,70] with yeasts designed to elucidate the nature of the precursor. The phenomenon can be illustrated by the results of a typical experiment in which an attempt was made to adapt cells simultaneously to two substrates, galactose and maltose. Curves B and D of Fig. 1 represent the control suspensions in which the adaptation was occurring in the presence of only one of the two substrates, B representing the galactose curve and D the maltose one. Curves A and C record the increasing galactozy-

mase and maltozymase content respectively of the experimental flasks in which the cells were in contact with both substrates.

From the comparison of Curves A and B, it can be seen that the presence of maltose has little, if any, effect on the progress of the adaptation to galactose. However, the presence of galactose has a severely inhibitory effect on the formation of maltozymase as may be seen from a comparison of Curves C and D. While some maltozymase can form in the galactose-maltose mixture in the early part of the curve, the level observed is far

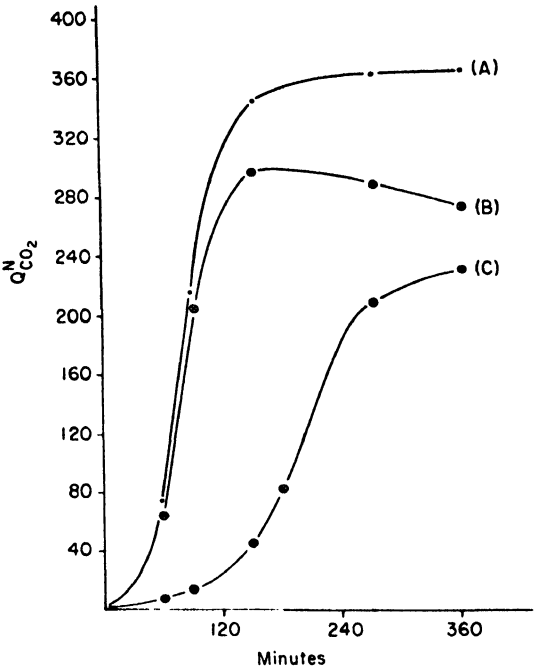

FIG. 2. Simultaneous adaptation to galactose and maltose in the presence of exogenous nitrogen. *A* and *C* are control curves of maltozymase and galactozymase respectively when each is being induced separately. Curve *B* is the maltozymase activity attained in a cell in which galactozymase is simultaneously being induced.

lower than that obtained in the control suspension which is adapting only to maltose. Further, the progressive increase in galactozymase content is paralleled by a sharp drop in the maltozymase activity.

Some indication of the basic nature of this interaction may be gained from experiments examining the effect of exogenous nitrogen on its severity. A typical result is illustrated in Fig. 2. Curve A of this figure is the control curve for maltose adaptation in the presence of maltose with added nitrogen. Curve B represents the maltozymase measurements in the experimental culture in which the cells were in contact with the two substrates in the presence of added nitrogen. Comparing Curve B of Fig. 2 with Curve

C of Fig. 1, we see that the availability of an exogenous source of nitrogen has several striking effects. It raises considerably the attainable level of maltozymase in a culture which is simultaneously adapting to galactose. It also prevents considerably, although not completely, the destruction of the maltose-fermenting system which usually accompanies the appearance of active galactose-fermenting capacity in the cell.

These experiments indicate that the interaction between the two adaptive enzyme systems is one based primarily on the competition for some nitrogenous component. The existence of such competitive interactions between adaptive enzymes suggested the possibility that such interactions

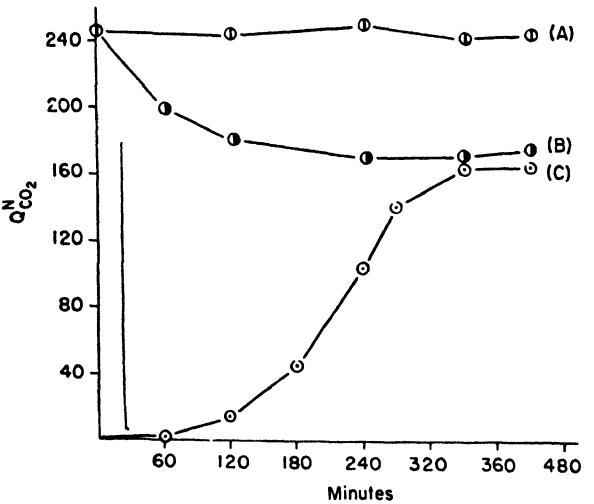

FIG. 3. Interaction between galactozymase and glucozymase during adaptation to galactose in the absence of an exogenous source of nitrogen. Curve *A* is glucozymase in an unadapting control; curve *B* is the glucozymase during adaptation to galactose; curve *C* shows the appearance of the galactozymase enzyme.

may not be peculiar to this particular class of enzymes, and might well be observed with the so-called "constitutive enzymes." If this were the case, it would explain the capacity of some cells to form new enzymes in the absence of an exogenous nitrogen source since, under such circumstances, the cell could draw upon existing enzymes as a source of protein.

Experiments designed to test this reveal that a situation of this kind does indeed exist.[70] Figure 3 illustrates some typical results. In this experiment, the effect on the glucozymase system of adaptation to galactose is examined. The experiment was performed by incubating yeast cells in a phosphate solution of galactose, and at intervals removing samples for simultaneous determinations of glucozymase and galactozymase. It is evident from Curve B of Fig. 3 that the appearance of galactozymase is accompanied by a fall in the glucozymase activity. That this interaction

effect is quite similar to those observed with the adaptive enzymes and involves the formation of a nitrogenous compound is strongly supported by the fact that if the same experiment is performed in the presence of exogenous nitrogen, the fall in glucozymase activity is not observed.

These and similar results would appear to offer fairly definite information relative to the question of protein source of the formation of enzyme. If an external source of nitrogen is present, it will be employed. In its absence, existing enzymes are drawn upon as a source of protein to form the enzyme being induced by substrate. Further supporting evidence that the phenomenon of enzymatic adaptation is closely connected with the protein content of the cell is provided by recent experiments reported by Virtanen and DeLey.[71] Enzymatic activity of intact cells and the ease of enzyme formation were found to be closely correlated with the protein content of the cell.

VIII. Consequences of Competitive Interactions between Enzyme-Forming Systems

The observations reported by Monod and those stemming from investigations of enzymatic adaptation in the yeasts establish that competitive interactions do exist between enzyme forming systems. The results with yeasts would appear to indicate that the competition is for some nitrogenous compound. There are several immediate consequences of some theoretical value for the further experimental analysis of the phenomenon.

1. The Interpretation of the Kinetics of Adaptation

As was pointed out in the section which discussed the kinetics of enzymatic adaptation, one perhaps easily accepted view of the fact that the process follows an autocatalytic law is that it is akin to the transformation of pepsinogen to pepsin. On the basis of this assumption, one would suppose that, for example, in the adaptation to galactose there existed a certain amount of inactive precursor of the galactozymase system. In the presence of galactose, this precursor would presumably be transformed autocatalytically into active enzyme with the aid of energy supplied by cellular metabolism. A transformation of this kind would, however, necessarily involve a highly specific precursor protein which could hardly be assumed to have other enzymatic function or be transformable into any other enzyme but galactozymase. Consequently, its conversion into active galactozymase would scarcely be expected to influence the activity levels of other enzyme systems. Neither would it be expected that one would observe the kind of competitive interactions which would seem to be occurring during *simultaneous* adaptations to maltose and galactose. The existence of these interactions would appear therefore to preclude any

[71] A. I. Virtanen and J. DeLey, *Arch. Biochem.* **16**, 169 (1948).

hypothesis which involves the transformation of protein molecules from inactive units which are already so highly specified that their potential activities are rigidly determined. It seems reasonable therefore to eliminate the pepsinogen to pepsin conversion as a plausible generalized model for enzymatic adaptation or its kinetics. This, of course, does not imply that it is impossible that apparent enzymatic adaptations may not be found which would conform to this particular kind of model.

2. On the General Nature of the Interaction

It is at present impossible to say at what level the actual competitive interaction exists. That the nature of the interrelation between the enzymes of a cell is complex is certainly to be expected. The available data on the interaction emphasizes the fact that the amount of existent enzyme activity which disappears in a given interval bears no simple quantitative relation to the amount of new enzyme which is formed during the same interval of time. This fact would certainly rule out any hypothesis which assumes simple conversion of one enzyme molecule into another. A simple one to one relation of this kind between the disappearance of one enzyme and the appearance of another would only be expected under conditions where, for example, two enzymes have the same prosthetic group which was being transferred from one to the other.

The most probable situation to be expected would be one in which the formation of a particular enzyme would involve interactions with a whole series of enzymes, the component parts of which are utilizable in its construction. Presumably certain enzymes would be preferentially used and as these fall below certain critical values others would be drawn upon to supply the material required for the formation of the enzyme being induced. If sufficient information were available, it should in principle be possible to provide an ordered classification of the enzymes within a cell based on the presence or absence of their interaction and its extent. This could well provide an enzyme classification of greater biological significance than that based on substrate specificity alone.

The severity of interaction between two given enzyme systems should depend not only upon the similarity of their constitutions but also on the comparative ability of the systems which form them to compete with each other for material. It is well to remember that the synthesizing and competitive ability of a particular enzyme-forming system might well be expected to vary from one strain to another.

3. Constitutive and Adaptive Enzymes

It was of no little theoretical importance to find that interaction effects could also be observed with one of the so-called "constitutive" enzymes.

Karström[72] designated as "adaptive" those enzymes which are produced as a specific response to the presence of an homologous substrate. Such enzymes were differentiated from the "constitutive" ones which are always formed by the cells of a given species regardless of the presence or absence of their homologous substrates. It has repeatedly been pointed out[1,3] that it seems doubtful whether classification into adaptive and constitutive enzymes is particularly valid, implying as it does some sort of qualitative difference in origin and function between the two. Accumulation of data on enzymatic variation in microorganisms puts increasing strain on the applicability of this kind of classification. Enzymes which have been labeled constitutive have been found to undergo wide fluctuations in the presence and absence of their substrates.

In connection with the general problem of classifying enzymes on the basis of their response to the presence or absence of their homologous substrates, there are certain important considerations which must be borne in mind. When we say that a given enzyme system, for example, galactozymase, is purely adaptive, we mean operationally that no galactozymase is found in the cell unless galactose is put into the medium. This is to be compared with the situation in the case of glucozymase in which the addition of glucose as such to the medium is not necessary for the appearance of glucozymase activity in the cell. It is clear however that this distinction may lose much of its meaning if the cell can manufacture glucose from the nonglucose compounds that we add. Actually, all the biochemical evidence indicates that this is precisely the condition that prevails in the case of galactose fermentation. Polysaccharides of glucose and intermediates of glucose metabolism have been found in cells which have been metabolizing galactose. Thus, even if glucozymase were a completely adaptive system, it would not be expected to disappear when the cell is metabolizing galactose, since the substrates of the glucozymase system are always present. To conclude therefore that glucozymase is a constitutive enzyme (that is to say, its formation is independent of the presence or absence of substrate) because it is found in cells which are metabolizing galactose, maltose, or some other carbohydrate, is not particularly meaningful.

In general then, whether an enzyme is classified as adaptive or constitutive may have little to do with any inherent capacities of the enzyme itself. The critical factor may well be whether or not the cell can synthesize the homologous substrate. Thus, in operational terms, a particular enzyme could be classified as adaptive only if the experimenter were in a position to control the presence or absence of the homologous substrate in the system. To do this, one must either deal with substrates which the cell cannot

[72] H. Karström, *Ergeb. Enzymforsch.* 7, 350 (1937).

synthesize, or one must be able differentially to prevent the appearance of some substrate normally formed by the cell. The first of these two conditions has been the one thus far employed in the study of adaptive enzymes. It is not an accident that these studies have, for the most part, concerned themselves with enzymes not involved in catalyzing a step in the middle of a cyclic process. Enzymes so employed have their substrates made for them by the preceding step in the cycle. However, with enzymes like maltozymase or galactozymase, which introduce their substrates into a cyclic process, precise experimental control of concentration levels of substrate is possible.

There is a further consequence of these concepts which is of no little importance. If one provides the cell with a substrate A, which has to undergo several reactions before it can be introduced into an enzyme cycle which is already established, then clearly, several adaptations have to occur. This is undoubtedly the case with adaptation to galactose fermentation. The evidence is very strong that at least three enzymatic steps must occur before galactose is converted to an intermediate which is common to the glucozymase complex. Consequently, the adaptation probably involves the formation of three enzymes. As has been pointed out,[50] this raises no real difficulty. Galactose will induce the enzyme which will convert it to the next intermediate. The latter can then act as an adapting substrate to stimulate the formation of the second enzyme in the chain, and so on. Thus, the addition of one substrate could ultimately provide a whole series of substrates for the induction of a whole series of enzymes.

Stanier[73] recently published an ingenious application of these concepts to a method for examining metabolic pathways. As developed by Stanier, the basic idea underlying this procedure may be summarized as follows:

1) If the dissimilation of a given substance A proceeds through a series of intermediates, B, C, D, E, F, G, etc., and if the individual steps in this chain of reactions are under adaptive enzymatic control, then growth on a medium containing A will produce cells that are simultaneously adapted to A, B, C, D, E, F, G, etc.

2) If growth on A fails to adapt the cells to a postulated intermediate X, then X cannot be a member of the reaction chain.

3) Growth on E will adapt the cells to F, G, etc., but not necessarily to A, B, C, and D. The probability that growth on E will adapt the cells to some intermediate preceding it in the reaction chain decreases with the number of intervening steps, that is to say, simultaneous adaptation to D is more probable than to A.

This method of simultaneous adaptation has been used successfully in the study of the bacterial oxidation of aromatic compounds,[48,49] the participation of a tricarboxylic acid cycle in the oxidation of acetate by *Azo-*

[73] R. Y. Stanier, *J. Bact.* **54**, 339 (1947).

tobacter agilis,[74] the feasibility of suggested intermediates in nitrogen fixation,[75] and finally to investigate the utilization of uronic acids by *E. coli*.[76]

4. Factors Controlling Stability of Enzymatic Pattern

It is natural when speculating on the nature of the factors which determine the maintenance or modification of enzymatic constitution to suppose that one which must be considered is the relative stability of the individual enzyme molecule. This viewpoint is often extended to explain how certain agents can modify enzyme content. Thus, for example, most attempts to explain both the inducing and stabilizing effect of substrate in the phenomenon of enzymatic adaptation have assumed that the enzymes involved are unstable in the absence of substrate. Thus, the presence of substrate is necessary to allow the cell to accumulate the appropriate enzyme, and on the removal of substrate the accumulated enzyme molecule is presumed to spontaneously break down with a consequent loss of activity. However, if such explanations are not to remain merely restatements of the experimental findings, a more precise concept of enzymatic stability must ultimately be provided.

In view of the existence of competitive interactions, it is no longer *a priori* obvious that one is discussing the chemical stability of the enzyme molecule involved when the statement is made that a particular enzymatic activity disappears on removal of its substrate. Enzymatic stability in the cell and its dependence on substrate may well be determined primarily by competitive interrelations amongst the protein-modifying systems which are involved in forming enzymes. The inherent chemical stability of the enzyme as an organic molecule may have little to do with the question. The latter characteristic can be adequately studied only when the enzyme has been isolated in the pure state. Even then, it is by no means certain that the data obtained from such studies would be particularly relevant for the determination of those factors which control the behavior of this enzyme in the intact cell.

We may, perhaps, make the point at issue more concrete by putting it in terms of a specific instance. The presence of galactose in the medium induces the appearance of a galactozymase system and the removal of the galactose under the usual conditions results in its disappearance. Two alternative hypotheses may be offered to explain these phenomena.

1) The enzymatic component formed in response to the addition of galactose is a highly unstable protein molecule when uncombined with substrate.

[74] J. L. Karlsson and H. A. Barker, *J. Biol. Chem.* **175**, 913 (1948).
[75] R. H. Burris and P. W. Wilson, *J. Bact.* **52**, 505 (1946).
[76] S. S. Cohen, *J. Biol. Chem.* **177**, 607 (1949).

2) The protein molecule is stable but the enzyme-forming system involved in its synthesis is a poor competitor for protein material in the absence of the substrate. This results in a loss to other synthesizing systems.

The significance of the second hypothesis and its difference from the first can perhaps most clearly be seen in terms of an analogy to predator-prey relationships encountered in the ecology of higher organisms. The prey may be a perfectly stable biological unit so long as it is not forced to coexist and compete in the same biological space as the predator. It must be emphasized that these are not mutually exclusive alternatives since both mechanisms can, and probably do, function simultaneously in determining cellular enzymatic constitution.

IX. The Stabilization of Enzymatic Patterns in the Absence of Substrate

In order to assess the relative importance of competitive interation as compared with molecular stability of enzymes in the maintenance of enzymatic patterns, an examination was made of the stability of enzyme under conditions in which competitive interactions were either completely abolished or greatly minimized.[77] Since effective competitive interaction presumably would occur only between actively synthesizing systems it would be expected that suppression of interaction would be attained with the aid of any agent or condition which inhibits enzyme formation to any considerable extent.

One such condition, as has been mentioned previously, is fulfilled by suspending yeast cells under anaerobic conditions in the absence of a fermentable substrate. It will be recalled that adaptive enzyme formation, under these conditions, either does not occur at all, or does so at a very low rate, from which it follows that competitive interaction among enzyme-forming systems should be sharply reduced. If such interactions are quantitatively important in determining enzymatic stability, adaptive enzymes in the absence of substrate should be relatively stable under anaerobic conditions, provided no utilizable exogenous substrate is available.

A comparison of this kind, between the aerobic and anaerobic stability of galactozymase in the absence of its substrate is shown in Fig. 4. Curve N records the behavior under anaerobic conditions, whereas Curve A was obtained in an aerobic experiment. It is clear from the comparison of the two curves that, in the absence of active metabolism, the presence of substrate is not necessary for the maintenance of the enzyme content of the cell.

Another, and perhaps more significant, method of attaining a similar situation is provided by the use of NaN_3 which dissociates the energy-generation of anaerobic glycolysis from the energy-utilization for synthetic purposes. Again, if competitive interaction, rather than inherent molecular

instability, is the determining factor in maintaining enzyme pattern, it would be expected that azide should prevent the loss of existent enzyme activity. Furthermore, the stability obtained under these conditions should be independent of the substrate present in the environment.

Figure 5 records the results of an experiment designed to test this expectation. The large circles in Fig. 5 represent the galactozymase activity values in controlled suspensions fermenting glucose in the absence of azide. The smaller circles show the behavior of the galactozymase in cells fermenting glucose subsequent to the addition of azide. It is evident that in every instance the addition of the azide prevents any further enzyme disappearance and the suspensions maintain the activity reached at the time of the axide addition.

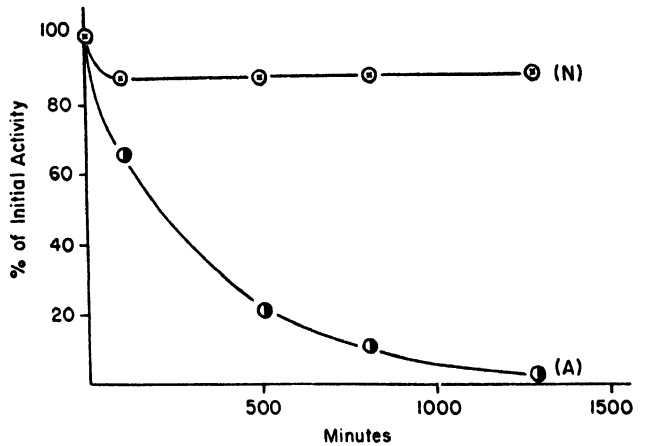

FIG. 4. The stabilization of galactozymase by anaerobiosis in the absence of substrate. The rapid disappearance of activity under aerobic conditions (curve *A*) contrasts with the marked stability appearing during incubation under N_2 (curve *N*).

These results have been repeated with the maltose adaptive system, which behaves in a similar manner.[61] Both anaerobiosis and azide completely prevent or markedly delay the disappearance of the adaptive maltozymase system in the absence of maltose. Reiner[60] recently published an instance which is in apparent contradiction to these findings. Arsenate is known to be capable of inhibiting synthetic activities. Reiner found, however, that the presence of arsenate, far from stabilizing galactozymase, apparently actually accelerated its disappearance. He concluded that energy was necessary both for the formation and maintenance of the adaptive enzyme. This apparent discrepancy was resolved by a re-examination[69] of this system which revealed that arsenate can specifically inhibit galactose metabolism.

Evidently then, the same agents or conditions which effectively inhibit

the formation of new enzymes can, under certain conditions, also effectively prevent the loss of existent enzymatic capacities. It may be concluded from these results that the property of molecular stability can play a relatively minor role in controlling the maintenance of enzyme patterns in the case of certain enzymes in particular strains of yeasts. The extent, severity, and kind of competition, however, can determine the types and amounts of enzymes found in the cytoplasm. Whatever generalities these results may possess, it remains nevertheless true that theories of gene action or substrate action which seek to explain how these factors can exert control over enzymatic constitution must provide a mechanism whereby these agents can perform their functions by influencing the out-

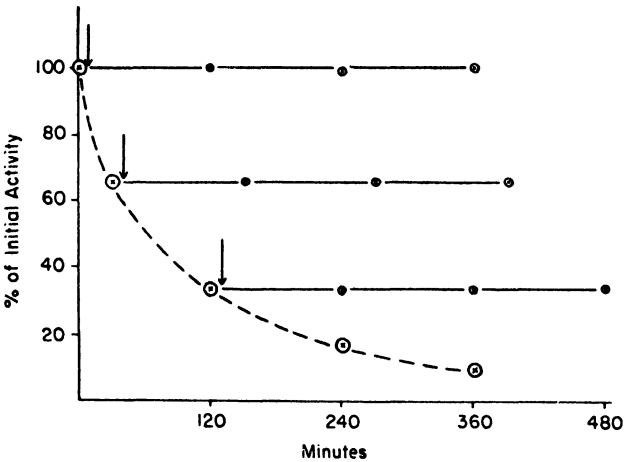

FIG. 5. The stabilization of galactozymase by NaN_3 during the anaerobic fermentation of glucose. The arrows indicate the activities at which NaN_3 was added. The small circles (solid lines) show that the addition of NaN_3 prevented further enzyme breakdown no matter at what level of enzyme activity the agent was added. The large circles (broken curve) show the loss of enzyme which occurs in the control suspension.

come of the competitive interactions amongst the enzyme-forming systems of the cell.

X. The Role of Substrate

The demonstration that substrate is unnecessary for the maintenance of the corresponding enzyme in the absence of competitive interaction forces one to consider less naive interpretations of the role of substrate. It suggests that the effect of substrate on the formation and stabilization of enzyme, during periods of active synthesis, may result from a capacity to *augment* the comparative synthetic capabilities of the enzyme-forming system involved. This would suggest that the presence of substrate is not a necessary condition for the formation of the corresponding adaptive

enzyme. Under the usual conditions in which such adaptations are carried out, the formation of adaptive enzymes in the absence of the corresponding substrate is not usually detected. However, if competitive conditions between the various enzyme-forming systems could be minimized, then in principle, each could form the enzyme of which it is capable, even in the absence of the corresponding substrate.

The problem, from an experimental point of view, was to find the correct condition for minimal competitive interaction in order to subject this concept to experimental test. Theoretically, one would suppose that such minimal conditions would be most likely to be obtained under conditions which were optimal for growth. The very fact that growth is occurring at a maximal rate suggests that all essential protein-synthesizing systems are functioning effectively, which in turn implies a suppression of inhibitory competitive interactions. If this be the case, it might be expected that the capacity of growing cells to maintain and form enzymes, in the absence of their corresponding substrate, would be observably greater than that of resting cells.

Experiments bearing on this point were performed.[77] The ability to form galactozymase in the absence of galactose was compared in cells which were permitted to increase at various rates. The results obtained are given in Fig. 6, in which are plotted the ratios of galactozymase activity observed to initial values against time. There are two measurements here of primary interest. One is the total enzyme activity indicated by a circle enclosing a T, and the other is the enzyme activity per cell, indicated by a circle enclosing an inclined line. The numbers placed next to each curve indicate the amount of increase which occurred in cell number in the corresponding suspensions during the course of the experiment.

It is seen that for both the slowly and rapidly growing suspensions, the enzyme activity *per cell* decreased markedly. The decrease is more marked in the case of a culture which experienced an 8.6-fold increase in cell number. However, the *total amount of enzyme* remained absolutely constant in the more slowly growing culture, and actually increased almost twofold in the suspension which was permitted to grow more rapidly. These are to be compared with total activity curves of the nondividing suspension in which rapid disappearance of the enzyme occurred. We have here, then, a remarkable instance of adaptive enzyme formation in the absence of substrate and strong support for the view that competitive interactions are dominant determinants of enzyme patterns.

Of the experiments illustrating the dispensability of substrate in the formation and maintenance of adaptive enzymes when competitive interactions are absent or greatly ameliorated, no one is completely conclusive in itself. Taken together, however, they constitute a rather critical test

[77] S. Spiegelman and J. M. Reiner, *J. Gen. Physiol.* **31**, 175 (1947).

for any proposed mechanism of substrate action in the process of enzymatic adaptation. It is clear that any proposed mechanism must explain not only the inducing capacity of substrate on enzyme formation, but must also explain the disappearance of the enzyme upon removal of substrate. In view of the experiments cited, any hypothesis of substrate action which has as its prime requisite the presence of substrate as a *sine qua non* for the formation or maintenance of the corresponding enzyme molecule, is inadequate to explain all the available facts. Hypotheses depending on

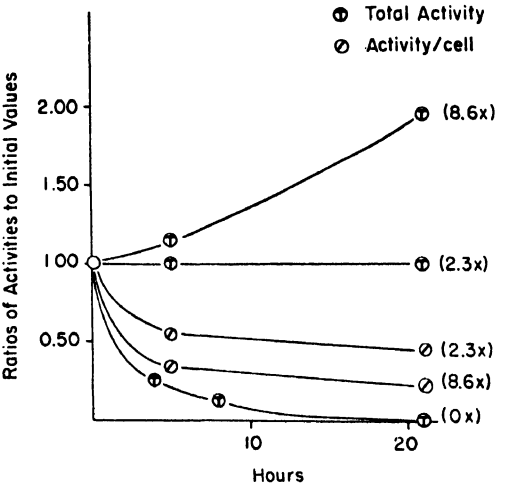

FIG. 6. The formation and maintenance of adaptive galactozymase by growing cells in the absence of galactose. Glucose was the carbohydrate source, and growth occurred in a complete medium (yeast extract-peptone broth). The designations (2.3 ×) and (8.6 ×) give the factor by which the initial number of cells increased during the experiment. The lowest curve shows the fall of enzyme activity in the absence of growth (medium: buffer plus glucose). The next two curves show the loss of activity *per cell* during increase of cell number; the more rapidly growing culture showed the most loss. The two uppermost curves show the *total activity* of the culture. They demonstrate the ability to maintain (slowly growing) and even to increase (rapidly growing) the initial enzyme activity in the absence of substrate under favorable conditions.

direct conversion or activation by substrate of a precursor molecule into enzyme are thus made unlikely. Similarly, the mass-action hypothesis, requiring the presence of substrate to shift the equilibrium position towards active enzyme, loses much of its explanatory attractiveness.

There is one other point worthy of note, which is illustrated by the last experiment cited. Referring back to Fig. 6, it may be noted that in the two instances in which there was either no loss of enzyme, or an actual net formation of enzyme, there was nevertheless in both cases a loss of enzyme activity per cell. This would indicate that though enzyme was being

formed, its rate of formation was not adequate to keep up with the rate of new cell formation, which led to a dilution of the enzyme in the cytoplasm of the new cells being produced. The existence of this asynchrony between enzyme formation and cell duplication should be borne in mind in interpreting the results of enzymatic adaptation in growing cultures. It is conceivable that situations may arise wherein enzymatic adaptation cannot occur in the lag phase of the growth cycle simply because of the existence of this dilution effect. If this be the case, adaptation would be observed in such cultures only when the stationary phase is reached and the enzyme-forming systems can begin to catch up with the formation of new cells.

Recently, Wainwright and Pollock[78] reported an instance of loss of enzymatic activity per cell by a simple dilution effect. Such results do not necessarily indicate that there is no interaction between the adaptive enzyme, nitratase, and the other enzyme systems of the cell. It is not impossible that the conditions under which their experiments were performed were analogous to those obtaining in the last set of experiments cited with galactozymase, an enzyme system for which one can show severe interaction under the proper conditions.

XI. Specificity Relations in Enzymatic Adaptation

One characteristic of the phenomenon of enzymatic adaptation which has been greatly emphasized is the specificity of the response to a particular substrate. Thus, if one exposes yeast cells to galactose under normal conditions, the enzyme system which is induced is the one concerned with the metabolism of this particular substrate. The investigations of Dubos, already noted, indicate that adaptations can be even more specific in the details of the reactions involved than those met with in immunological reactions. Mirick's[17] investigations offer another indication that only substances upon which the enzyme can act are capable of inducing its formation.

Not all cases of enzymatic adaptation are quite so rigidly specific. Dienert[23] was the first to offer a concrete instance in which a substance other than the homologous substrate possessed the capacity to induce a particular enzyme. He demonstrated that yeast cells adapted to galactose were simultaneously adapted to lactose. The reverse situation was also found to be true: cells adapted to lactose were also adapted to galactose. Monod[79] found a similar situation in the case of lactose and galactose adaptation in *E. coli*. The fact that lactose-grown cells are adapted to galactose is not too surprising and may represent an instance of simultaneous adaptation in the sense of Stanier,[73] providing the first step of lactose fermentation is a hydrolytic cleavage. The ability of galactose to induce the formation of

[78] S. D. Wainwright and M. R. Pollock, *Brit. J. Exptl. Path.* **30**, 190 (1949).
[79] J. Monod, *Colloques Intern. Centre Natl. Recherche Sci.* **8**, 181 (1949).

the adaptive lactose system is usually explained in terms of the capacity of a product to combine with the enzyme involved in its production. This combination is presumed to be sufficient to induce the production of more enzyme. This is obviously not a necessary conclusion since one can suppose that the cells can manufacture lactose from galactose by a mechanism other than by a reversal of the lactose-adaptive system.

The possibility, however, that galactose can directly induce the lactose system raises an interesting question for it presupposes that a compound which is not utilizable or upon which the enzyme cannot act is capable of inducing the enzyme. This possibility was tested in the case of maltose fermentation by *S. cerevisiae*. In agreement with previous investigators,[80] it was found that fully adapted cultures possessed no measurable capacity to ferment maltose at pH values greater than 8.1. Accordingly, experiments were performed[81] in which maltozymase activity was measured subsequent to various periods of incubation with maltose at pH 8.5. In all cases, it was possible to demonstrate that the incubation period with the maltose under these conditions in which the maltose could not be utilized resulted in the appearance of enzyme as measured at a lower pH. The generality of this phenomenon was extended by experiments which demonstrated that unutilizable analogs can be used to induce specific enzymes. An example of this is the ability of α-methyl glucoside to induce the maltozymase system, the normal substrate of which is maltose. The analog, α-methyl glucoside is completely nonutilizable by the intact cell under the conditions of the experiment. Subsequent isolation[82] of the enzyme involved confirmed the fact that the adaptive enzyme formed in response to maltose has no capacity to split α-methyl glucoside. However, the fact that it, in common with maltose, contains the α-glucosidic link is apparently sufficient to enable it to combine and induce further synthesis of the maltozymase enzyme. It would appear, therefore, that the specificity of enzymatic adaptation can be broader than the specificity of substrate utilization.

XII. Genetic Implications and "Long-Term Adaptation"

A basic assumption of modern biology is that genes function by controlling enzyme synthesis. From this point of view it is obvious that enzymatic adaptation has profound implications for one of the central themes of biological thinking.

The experiments which established that an apoenzymatic change can occur without cell division signify that a modification of enzyme patterns can take place against a constant genetic background. The fact that a cell

[80] J. Leibowitz and S. Hestrin, *Biochem. J.* **36**, 772 (1942).
[81] S. Spiegelman, J. M. Reiner and M. Sussman, *Federation Proc.* **6**, 209 (1947).
[82] S. Spiegelman, M. Sussman and B. Taylor, *Federation Proc.* in press, (1950).

possesses the gene required for the formation of a particular enzyme does not thereby guarantee that the corresponding enzyme will be found in the cytoplasm of the cell. Théoretically then, no matter how complete our knowledge of the genotype of the cell may be, it is not possible on the basis of this information alone to predict the phenotype of a cell in terms of its enzymatic pattern. Furthermore, if such a prediction is impossible, prediction of phenotype from genotype on any other basis is equally uncertain.

With this situation in mind, the statement "genes control enzymes" does not, without further qualification, describe the actual situation. In view of the existence of the phenomenon of enzymatic adaptation, this statement should be rephrased to read, "genes determine the potentiality for enzyme formation." Whether or not the enzyme is actually formed in the cytoplasm is apparently determined by other factors of which the presence of substrate can obviously be one.

It is, of course, tempting to go beyond such general conclusions and endeavor to arrive at a more particularized concept of the mechanism of gene action with the aid of the information gained from the study of enzymatic adaptation. Such temptations were not long withstood. Using a term which had existed in the biological literature, Spiegelman proposed the "plasmagene" theory of gene function.[2,3] The basic assumption was the existence of self-duplicating units (plasmagenes) in the cytoplasm which were concerned with enzyme formation. These cytoplasmic units are presumed to be generated from the genes continuously. Accordingly, the enzyme patterns exhibited by a cell are determined by the competitive interactions which exist between these cytoplasmic units and the factors which influence the extent and severity of these interactions. The theory provides the requisite flexibility required to explain the cardinal fact of enzymatic adaptation, namely, that the gene is a necessary but not a sufficient condition for initiating the formation of the corresponding enzyme. It further provides a mechanism for the function of substrate consistent with available facts and explains the kinetics of the adaptive process. The competitive interactions between enzyme forming systems which have been experimentally exhibited receive a natural explanation in terms of the plasmagene concept. Monod[1] has proposed a modification of this theory which is designed to avoid the necessity of postulating a self-duplicating unit in the cell other than the gene. The available evidence does not as yet make it possible to be certain whether the self-duplicating model or some more restricted modification of it is the most adequate description of the situation. In view of its future importance it is desirable to examine the nature of the attempts which have been made to solve this aspect of the problem.

If enzyme synthesis is mediated by cytoplasmic self-duplicating units, enzyme formation should, in principle, be observable in the absence of the corresponding gene. Experiments were performed which were designed

primarily to test this prediction. The inheritance of the capacity to ferment melibiose in the segregants from a diploid derived from mating a genetic negative and a genetic positive were used in such studies. Some indications were obtained[83] that the presence of substrate during the segregation disturbed the normal 1:1 Mendelian ratios to be expected from such a heterozygote. Since only two out of every four such segregants presumably possessed the gene, the fact that more than 50% were capable of fermenting melibiose and of transmitting this capacity to their progeny implied enzyme formation in the absence of the corresponding gene. The conclusiveness of these experiments was, however, disturbed by the fact that substrate was neither a necessary nor sufficient condition for the appearance of non-Mendelian inheritance.[32,33,34,84]

Subsequent to these investigations Winge and Roberts[85] published a paper which confirmed many of the peculiarities observed in transmission of the capacity to form enzymes in yeasts. They concluded that their results confirmed the apparent ability of substrate to modify the Mendelian ratios expected in the segregation of a heterozygote.

Winge and Roberts, however, proposed an entirely different explanation for these discrepant ratios, one which would appear to avoid completely the necessity for invoking the existence of any cytoplasmic determinants of enzyme formation. They point out that their own results, as well as those cited previously, are consistent with the concept that in the yeasts, crosses between negatives and positives with respect to the fermentation of particular carbohydrates need not necessarily involve pure genetic negatives and positives (in the sense of complete absence of functional genes for the former and presence of fully functional activity for the latter). They would assume rather that one is dealing here with an instance in which a mating is being made between "slow" (the negative strain) and "fast" (the positive strain) genes. On this basis, after sufficient time has elapsed it would be impossible to distinguish the two strains since both would exhibit full enzymatic activity.

One may well question the necessity and the validity of the sharp distinction drawn by these authors between what they call "biochemical adaptation" and "long-term adaptation." Thus they state that the phenomenon they are studying "is not a question of enzymatic adaptation in the general biochemical sense, but involves the phenomenon which we call long-term adaptation and which, according to our opinion, is due to a

[83] S. Spiegelman, C. C. Lindegren and G. Lindegren, *Proc. Natl. Acad. Sci.* **31**, 95 (1945).

[84] C. C. Lindegren, S. Spiegelman and G. Lindegren, *Proc. Natl. Acad. Sci.* **30**, 346 (1945).

[85] O. Winge and C. Roberts, *Compt. rend. trav. lab. Carlsberg sér. physiol.* **24**, 263 (1948).

gradually increasing production and accumulation of galactozymase in the cells when galactose is present in the substrate."[85a]

It is evident from the above quotation that their concept of the mechanism underlying "long-term adaptation" is no different from that proposed previously by many authors to explain what Winge and Roberts would call "biochemical adaptation." It has generally been assumed that the period preceding the appearance of full enzyme activity represents the time required to accumulate sufficient enzyme for the expression of full activity. The only difference therefore between the observations as well as the interpretations of Winge and Roberts and those previously noted is a purely quantitative one involving the time required for the enzyme formation.

Disregarding the question of terminology, there is no doubt that Winge and Roberts have made an observation of great importance and one which raises many problems. The first question which comes to mind is whether in their long-term adaptation, which involves growth and can take anywhere from 7 to 13 days, one is perhaps not dealing with mutation and selection. Winge and Roberts examined this aspect of the problem, but unfortunately the experiments they performed were inadequate for a decision.

Lindegren[34] examined this question and came to the conclusion that mutation and selection were involved in such long-term adaptations. The basic evidence on which he based his conclusion consisted of the demonstration of heterogeneity within the population with respect to the capacity to give rise to adapted clones on galactose test plates. Such a situation would, however, obtain if a small proportion of the cells in the population were capable of adapting and of giving rise to adapted progeny. Variation in physiological capacities amongst a population homogeneous in genotype is not impossible. Consequently his evidence cannot be accepted as conclusive.[85b]

A Delbrück-Luria analysis of this situation performed recently in the author's laboratory indicates that no significant variance is obtained (unpublished experiments). The experimental conditions were such as to insure the meaningfulness of the absence of variance. It appears, therefore,

[85a] O. Winge and C. Roberts, *Compt. rend. trav. lab. Carlsberg* 298 *sér. physiol.* **24**, (1948).

[85b] Mundkur and Lindegren (*Am. J. Botan.* **36**, 722, 1949) present a Delbrük-Luria analysis of long term adaptation to galactose. None of the conditions required for the adequate performance of this experiment were met by these authors. Calculation of the variance of the data presented reveals that it is almost identically equal to the mean, indicating therefore no more variance than would be expected from errors of sampling. Their data consequently are completely at odds with the conclusion derived by these authors that mutation and selection are involved in the adaptation they analyzed.

that an adaptation is involved, at least in the sense that the *heritable modification* does not occur in the absence of the adaptive substrate. It is however a slow adaptation not because of the reasons supposed by Winge and Roberts, namely that a slow gene is functioning in all the cells of the population, but principally because only a small proportion of the starting population is capable of undergoing the adaptation, and time is required for the growth of these cells before the biochemical characteristics of the population change.

It is of interest to note that the hypothesis of Winge and Roberts is actually inconsistent with some of the data which they themselves present. They distinguish between what they call a "slow" gene and a "fast" gene on the basis of the time required for a completely unadapted culture to adapt to a particular substrate. Operationally, by adaptation they mean the exhibition of active fermentation in an Einhorn tube.

Let us take a particular case for discussion and consider that we have a culture which requires seven days for the completion of the adaptation. Winge and Roberts would then presumably say that it took that period of time for the gene controlling galactozymase formation to mediate the synthesis of sufficient enzyme per cell so that full activity could be expressed. Let us assume, for the purpose of further analysis, that the presence of 170 molecules of enzyme per cell is necessary for the expression of full enzyme activity. This would mean, then, that the slow gene is capable of forming one enzyme molecule per hour. When such a fully adapted culture is now transferred into a fresh medium again containing the galactose substrate, it will begin to divide. For ease of calculation, we may take the generation time to be 1 hour. A bud receives roughly one tenth of the cytoplasm of the cell from which it is derived. If we assume random distribution of cytoplasmic components, a bud formed from the cell fully adapted to galactose would receive on the average 17 molecules of the galactozymase enzyme. On the hypothesis of slow gene action, this bud could synthesize only one enzyme molecule per hour. Consequently, by the time it is in turn ready to generate a bud, only 18 enzyme molecules would be available for distribution, and the second generation bud would receive only one or two enzyme molecules. It is clear that under such circumstances, the vast majority of cells produced would, in very few generations, contain few enzyme molecules. It might be added that, with these assumptions, this situation would eventually prevail irrespective of the absolute values assumed for rate of enzyme production and number of molecules required for full enzyme activity.

We see then that the hypothesis of slow gene action in a simple and unmodified form would lead one to predict that transfer of the fully adapted culture to fresh adapting medium would inevitably result in de-adaptation.

Further, one should never be able to either obtain or maintain the adapted state in a rapidly growing culture.

This condition is, of course, contrary to the facts as described by Winge and Roberts. They point out that once a slow-adapting strain has become adapted, its subsequent behavior on transfer to the same medium is indistinguishable from that of a fast-adapting culture. Furthermore, this property can be maintained indefinitely. In order for the fully adapted culture to maintain its activity per cell during active cell division, it is evident that the rate of enzyme formation must be greater than that observed during the adaptation.

One must apparently draw the conclusion from these results that two changes have occurred in the cells during the 7-day adapting period. One is, of course, that sufficient enzyme has accumulated for the exhibition of full enzymatic activity. The other, which is probably more significant, is that the rate of enzyme formation has been greatly increased as a result of the adaptation, since this augmentative capacity for enzyme formation is inherited.

The problem is, of course, where to look for explanations for this strikingly different ability to synthesize enzymes, which is apparently passed on from one cell generation to the next. If a genic change is unlikely, as appears to be true in this case, it seems difficult to avoid implicating a cytoplasmic modification as the basis for the explanation, thereby implying the existence of cytoplasmic determinants controlling enzyme formation. On the contrary, the results of Winge and Roberts lead to precisely the same conclusions as those derived from data obtained in the study of the fast-adapting strains of yeasts.[2,3] Both types of adaptation can be understood if one makes the basic assumption that the capacity to form a particular enzyme is determined by cytoplasmic conditions. In particular, the rate of formation of a given enzyme is an autocatalytic function of the amount of that particular enzyme present in the cytoplasm.

XIII. Conclusion

It is evident from the data summarized that relatively little can be said as yet concerning the biochemical details of the transformations underlying enzymatic adaptation. The identification of the chemical nature of the components connected with conversion to active apoenzyme remains to be accomplished. Some hints of a preliminary nature do exist. Thus, it is known as a result of tracer experiments with yeast that a marked stimulation of ribonucleic acid fraction accompanies enzymatic adaptation.[86,87] Further it has been possible to demonstrate the existence of

[86] S. Spiegelman and M. D. Kamen, *Science* **104**, 581 (1946).

[87] S. Spiegelman and M. D. Kamen, *Cold Spring Harbor Symposia Quant. Biol.* **12**, 211 (1947).

substances, ("adaptins"), associated with the ribonucleoproteins which stimulate adaptation.[3] Further analysis along such lines should eventually enable us to specify concretely the nature of the precursor and the mechanism of its conversion to active enzyme.

The results reported in the preceding section provide some general information concerning certain enzyme forming systems. On the basis of the available data we can be reasonably certain that at least in particular instances of enzymatic adaptation we are dealing with processes which

a) can occur without a preceding change in genotype,
b) lead to a modification in a protein component of the cell,
c) require energy,
d) are related to nitrogen metabolism,
e) possess kinetics of the autocatalytic type,
f) can competitively interact with other systems for nitrogenous compounds, or energy, or both.

The above characteristics of enzymatic adaptation define the nature of the problem being studied in experiments on adaptive enzymes, providing the biological and enzymological criteria previously detailed are satisfied. Under these conditions enzymatic adaptation becomes an experimental tool which can extend the inquiries of enzymology beyond the questions of mere composition which constitute the primary goal of classical biochemistry. With the aid of this phenomenon it now becomes possible to inquire into the mechanism of the formation of the enzymes themselves.

CHAPTER 7

Enzyme Inhibition

BY L. MASSART

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I. Introduction. Specific and Nonspecific Inhibitions, Competitive and Noncompetitive Inhibition

The knowledge of the chemical structure of enzymes in general and the kinetics of enzymatic reactions enables one to establish a classification of the types of inhibitions of enzymes and to understand in many cases the mechanism of these inhibitions. According to the theory of Michaelis and Menten,[1] the most general in this field, an enzymatic reaction proceeds in

[1] L. MICHAELIS AND M. L. MENTEN, *Biochem. Z.* **49**, 333 (1913).

two stages:

(*1*) One molecule of the enzyme unites with one molecule of the substrate to form the enzyme–substrate complex; this complex is in equilibrium, according to the law of mass action, with its constituents:

$$E + S \rightleftharpoons ES$$

The result of this union is, presumably, that the substrate molecules become more chemically reactive by some intramolecular changes. We say that the substrate is activated by the enzyme. This activation determines the second stage of the enzymatic reaction.

(*2*) The unstable intermediate complex gives rise to the end products of the enzymatic reaction and the free enzyme molecule:

$$ES \rightleftharpoons E + R + R_1$$

It is generally admitted that of these two stages the rate of formation of the enzyme–substrate complex is more rapid than its decomposition into the free enzyme and the reaction products R and R_1.

The theory of Michaelis and Menten has lead to the definition of the so-called Michaelis constant, which in fact measures the affinity of an enzyme for its substrate and which is found in the Michaelis equation:

$$V = \frac{V_{max}[S]}{K_m + [S]} \tag{1}$$

in which K_m is the Michaelis constant, V the over-all velocity of the enzymatic reaction, V_{max} the maximum value of this velocity, and [S] the substrate concentration. K_m is in fact the dissociation constant of the enzyme–substrate complex and is numerically equal to the substrate concentration for which the velocity is half the maximum value. We shall represent it in what follows by K_s.

A more useful form of equation (1) has been introduced by Lineweaver and Burke,[2] who pointed out that when one plots the reciprocal of velocity against the reciprocal of substrate concentration a straight line is obtained according to equation (2):

$$\frac{1}{V} = \frac{K_s + [S]}{V_{max}[S]}$$

or:

$$\frac{1}{V} = \frac{1}{V_{max}} + \frac{K_s}{V_{max}} \frac{1}{[S]} \tag{2}$$

As results from Fig. 1 the slope of this straight line is K_s/V_{max} and its intercept on the $1/V$ axis is equal to $1/V_{max}$, whereas the slope/intercept equals K_s, the dissociation constant of the enzyme–substrate complex.

[2] H. LINEWEAVER AND D. BURK, *J. Am. Chem. Soc.* **56**, 658 (1934).

It has been proved that the kinetic data for many enzyme reactions corroborate the theory of Michaelis and Menten, but definite exceptions have been described. However, for most purposes equations derived from this theory are very useful and this is also the case when one aims at a better understanding of inhibition phenomenona.

When one admits that a substrate combines with an enzyme by means of the "essential groups" or "active centers" of this enzyme, it is easily understood that any substance reacting with these essential groups will inhibit the enzyme. This will be due to the fact that there is no possibility for the enzyme to combine with or to activate its substrate.

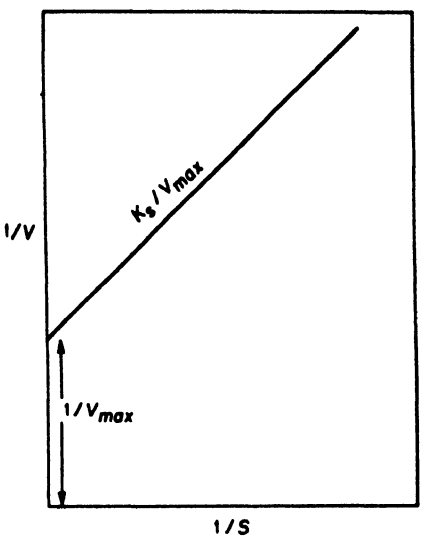


FIG. 1. Variation of reciprocal of velocity with reciprocal of substrate concentration.

Since enzymes are proteins, certain groups such as free carboxyl, amino, and sulfhydryl groups will be common to many enzymes and the blocking of these groups will of course give rise to a rather nonspecific inhibition, although it might yield interesting results as to the nature of essential groups in enzymes. If the blocking or inhibiting agent is well chosen a reversible "nonspecific inhibition" will be obtained. If the agent causes irreversible denaturation or destroys the enzyme the resulting irreversible inhibition will of course give little valuable information.

In considering "specific inhibitions" we shall pay attention to those substances blocking those groups conferring specificity on an enzyme, that is to say, to the active centers on which specificity is based and the coenzyme or prosthetic group responsible in general for the type of chemical reaction catalyzed by the enzyme. A study of these reversible and specific inhibi-

tions has revealed that at least two definite types should be distinguished, the "competitive" and the "noncompetitive."

II. Kinetics of Specific Inhibitions

1. Competitive Inhibition

A well known case of competitive inhibition is shown by some substrate analogs of succinic acid. Since in certain cases the specificity of an enzyme is only relative, the enzyme is capable of binding substances structurally related to the substrate or to the coenzyme, *e.g.*, malonic acid instead of succinic acid[3] and pyridinesulfonic acid instead of cozymase by lactic dehydrogenase.[4] The enzyme, *i.e.*, succinic dehydrogenase, however is not able to activate these substances and the resulting inhibition is due to the fact that access to the active centers is hindered. A competition exists between the substrate (or coenzyme) and the structurally related substances.

Many enzymes are inhibited by the products of their own action because these reaction products have a structural analogy with the substrate and compete with it for the active centers of the enzyme molecule. The kinetics of enzyme inhibitions we are considering, are based on the theory of Michaelis and Menten.[1] Different authors have treated this aspect of the enzyme problem.[2,5–11]

In competitive inhibition two reversible reactions are to be considered:

(*1*) The formation of the enzyme–substrate complex:

$$E + S \rightleftharpoons ES$$

(*2*) The formation of the enzyme–inhibitor (structurally related substance) complex:

$$E + I \rightleftharpoons EI$$

If [S] is concentration of substrate, [I] the concentration of the inhibitor, [e] the concentration of the enzyme, [p] the concentration of the enzyme-substrate complex, [q] the concentration of the enzyme-inhibitor complex, K_s the dissociation constant of the enzyme-substrate complex, and K_i the dissociation constant of the enzyme-inhibitor complex, application of the

[3] J. H. Quastel and W. R. Wooldridge, *Biochem. J.* **22**, 689 (1928).
[4] H. von Euler, *Ber.* **75**, 1876 (1942).
[5] J. B. S. Haldane, Enzymes. Longmans, Green, London, 1930.
[6] P. W. Wilson, *in* Elvehjem and Wilson, Respiratory Enzymes. Burgess, Minneapolis, 1939.
[7] A. Hunter and C. E. Downs, *J. Biol. Chem.* **157**, 427 (1945).
[8] E. R. Ebersole, C. Guttentag, and P. W. Wilson, *Arch. Biochem.* **3**, 399 (1944).
[9] A. Goldstein, *J. Gen. Physiol.* **27**, 529 (1944).
[10] L. Hellerman, A. Lindsay, and M. R. Bovarnick, *J. Biol. Chem.* **163**, 553 (1946).
[11] T. S. Work and E. Work, Basis of Chemotherapy. Oliver and Boyd, Edinburgh, 1948.

law of mass action yields the following equations:

$$[S]([e] - [p] - [q]) = K_s[p]$$
$$[I]([e] - [p] - [q]) = K_i[q]$$

and by eliminating [q]:

$$K_i = \frac{[I]K_s}{[S]\left(\frac{[e]}{[p]} - 1\right) - K_s} \tag{3}$$

The last equation gives no information as to the velocity of the reaction in the presence or in the absence of the inhibitor. Now it is clear that in both cases this velocity will be proportional to the concentration of the enzyme–substrate complex and, thus, if

v_i is the velocity of the reaction in the presence of the inhibitor, v the velocity of the reaction in the absence of the inhibitor, and $[p_0]$ the concentration of the enzyme–substrate complex in the absence of the inhibitor:

$$\frac{v}{v_i} = \frac{[p_0]}{[p]} \quad \text{and} \quad [p_0] = [p]\frac{v}{v_i} \tag{4}$$

Now another value for $[p_0]$ can easily be found. Indeed if we consider the reaction in the absence of inhibitor, we can write:

$$E + S \rightleftharpoons ES$$

If [e] is the concentration of the enzyme, [S] the concentration of the substrate, [p] the concentration of the enzyme–substrate complex:

$$K_1([e] - [p])[S] = K_2[p]$$
$$([e] - [p])[S] = (K_2/K_1)[p]$$
$$([e] - [p])[S] = K_s[p]$$

In the last equation, K_s is the dissociation constant of the enzyme–substrate complex. From it follows that:

$$[p] = \frac{[e][S]}{K_s + [S]}$$

If we substitute this last value of [p] for $[p_0]$ in equation (4) we find:

$$\frac{[e][S]}{K_s + [S]} = \frac{v}{v_i}[p]$$

whence:

$$\frac{[e]}{[p]} = \frac{v}{v_i}\frac{K_s + [S]}{[S]}$$

Substituting this value of [e]/[p] in equation (3) we obtain:

$$K_i = \frac{[I]K_s}{[S]\left(\frac{v}{v_i}\frac{K_s + [S]}{[S]} - 1\right) - K_s}$$

and:

$$K_i = \frac{[I]K_s}{(K_s + [S])\left(\frac{v}{v_i} - 1\right)} \tag{5}$$

whence:

$$\frac{v}{v_i} = 1 + \frac{K_s}{K_i}\frac{[I]}{K_s + [S]} \tag{6}$$

A useful transformation of equation (5) has been introduced by Hunter and Downs.[7] These authors substitute the value of v_i/v (the fractional activity) by α. Doing this, we obtain:

$$K_i = \frac{[I]K_s}{(K_s + [S])\left(\frac{1}{\alpha} - 1\right)}$$

and finally:

$$[I]\frac{\alpha}{1 - \alpha} = K_i + \frac{K_i}{K_s}[S] \tag{7}$$

If we now plot $[I]\frac{\alpha}{1 - \alpha}$ against [S] we obtain, as shown in Fig. 2 a straight line, its intercept on the $[I]\frac{\alpha}{1 - \alpha}$ axis being equal to K_i and the slope having the value K_i/K_s. This figure makes possible calculation of K_i and K_s. Equation (7) shows that $[I]\frac{\alpha}{1 - \alpha}$, which is numerically equal to the concentration of inhibitor producing an inhibition of 50%, depends on the concentration of the substrate, the values of K_i and K_s being constant.

2. Noncompetitive Inhibition

In this case there is no competition between the substrate and the inhibitor and the degree of enzyme inhibition is dependent solely on the amount of enzyme combined with the drug. The inhibitor attacks E and ES with equal affinity at a point not attacked by the substrate:

$$E + I \rightleftharpoons EI$$

and:

$$ES + I \rightleftharpoons ESI$$

ESI is inactive and the dissociation constant $K_{esi} = K_i$. The inhibitor combines with a grouping not essential for the formation of the enzyme–substrate complex but necessary for the substrate activation.

In this case, α being the fractional activity, v_i/v, Hunter and Downs[7] arrive at the equation:

$$[I] \frac{\alpha}{1 - \alpha} = K_i \tag{8}$$

which in fact can be derived directly by applying the law of mass action to reaction:

$$E + I \rightleftharpoons EI$$

Assuming that the amount of inhibitor combined with the enzyme is negligible compared with the total amount of inhibitor present and if

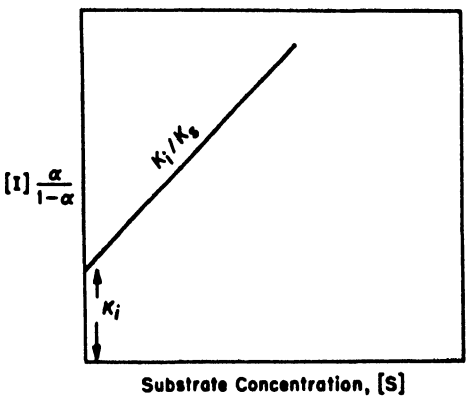


FIG. 2. Competitive inhibition

[e] is the initial concentration of the enzyme, [q] the concentration of EI, and [q]/[e] is i, the fractional inhibition:

$$[I] ([e] - [q]) = K_i[q]$$

$$[I] \frac{([e] - [q])}{[q]} = K_i$$

Since $[q] = [e]i$:

$$[I] \frac{(1 - i)}{i} = K_i$$

Since $i = 1 - \alpha$:

$$[I] \frac{\alpha}{1 - \alpha} = K_i$$

If we plot [I] $\frac{\alpha}{1-\alpha}$ against [S] we obtain a straight line (as seen in Fig. 3). The intercept on the [I] $\frac{\alpha}{1-\alpha}$ axis equals K_i. The line runs parallel with the [S] axis, showing that the inhibition does not depend on substrate concentration. From the figure the value of K_i can be deduced.

3. Practical Determination of Type of Inhibition

Umbreit, Burris, and Staufer[12] draw the attention to the fact that "the mere observation of the constancy of inhibition at one inhibitor concentration and with varying substrate concentration should not be relied upon to

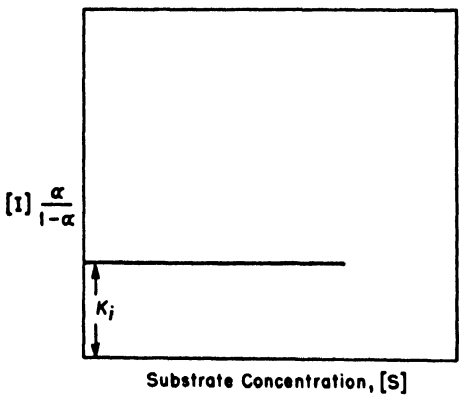


Fig. 3. Noncompetitive inhibition

establish the competitive or noncompetitive nature of the inhibitione." They consider the application of the equations derived by Lineweaver and Burk[2] to be a more reliable method. For competitive inhibitions these authors obtain:

$$\frac{v}{v_i} = 1 + \frac{K_s}{K_i}\frac{[I]}{K_s + [S]} \tag{9}$$

This equation is the equivalent of equation (6). The velocity equation is, according to Ebersole, Guttentag, and Wilson[8]:

$$\frac{1}{v_i} = \frac{1}{V^0} + \left(1 + \frac{[I]}{K_i}\right)\frac{K_s}{[S]}\frac{1}{[S]} \tag{10}$$

where v_i is velocity at substrate concentration [S] in presence of the inhibitor and V^0 maximum velocity in presence of the inhibitor. This equation is obtained from equations (2) and (6).

[12] W. W. Umbreit, R. H. Burris, and J. F. Stauffer, Manometric Techniques and Related Methods for the Study of Tissue Metabolism. Burgess, Minneapolis, 1947, p. 122.

As we have seen in Fig. 1 when one plots the reciprocal of the velocity constant $1/V$ against the reciprocal of the substrate concentration (in the absence of an inhibitor) a straight line is obtained with intercept on the $1/V$ axis equal to $1/V_{max}$, the slope being K_s/V_{max} and the slope/intercept equaling K_s.

As results from equation (9) in competitive inhibition, the intercept remains constant, but the slope is increased by $1 + ([I]/K_i)$. The slope/intercept or apparent K_s is increased by the same factor, *i.e.*, $1 + ([I]/K_i)$.

In noncompetitive inhibitions:

$$\frac{v}{v_i} = 1 + \frac{[I]}{K_i}$$

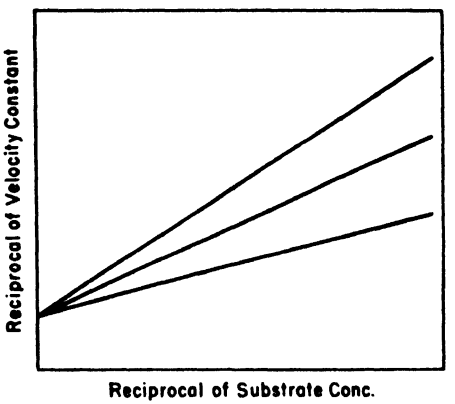


FIG. 4. Representation of competitive inhibition at different inhibitor concentrations.

and the velocity equation is[8]:

$$\frac{1}{v_i} = \left(1 + \frac{[I]}{K_i}\right)\left(\frac{1}{V^0} + \frac{K_s}{V^0}\frac{1}{[S]}\right)$$

V^0 in this case equals $V_{max}\,(1 + [I]/K)$, so that when plotting the reciprocal of the velocity constant against the reciprocal of the substrate concentration a straight line is obtained but here the slope and the intercept are both increased by $1 + [I/K_i)$ and the slope/intercept equals K_s.

Figs. 4 and 5 represent competitive and noncompetitive inhibition at different inhibitor concentrations.

According to Burris, for practical purposes the velocity of the reaction has to be determined over as wide a range of substrate concentrations as is practical and at two or more concentrations of the inhibitor. The examination of the graph $1/V$ *vs.* $1/[S]$ will reveal the nature of the inhibition. Of course the method of Hunter and Downs,[7] plotting $[I]\,\frac{\alpha}{1-\alpha}$ against con-

centration of the substrate, is also reliable. Fig. 6 represents the results obtained by these authors concerning the inhibition of arginase by ornithine

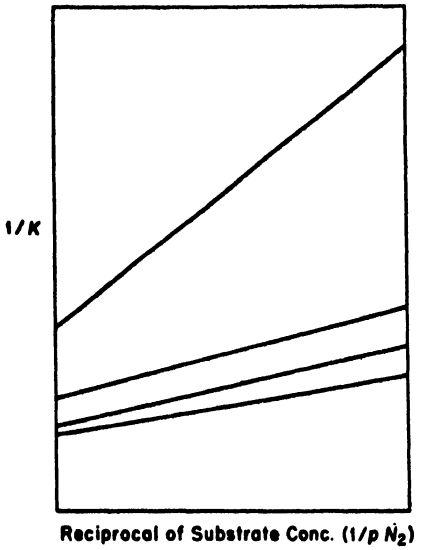


FIG. 5. Carbon monoxide as a noncompetitive inhibitor of nitrogen fixation by *Azobacter vinelandii* at different concentrations of inhibitor.[8]

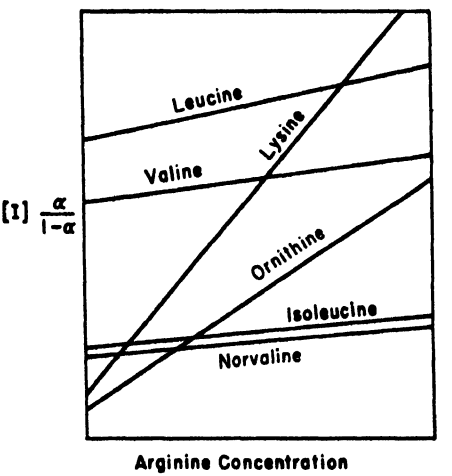


FIG. 6. Inhibition of arginase by ornithine and lysine (competitive) and by leucine, valine, isoleucine, and norvaline (noncompetitive).[7]

and lysine on the one side and leucine, valine, norvaline, and isoleucine on the other. The diamino acids produce a competitive and the monoamino acids a noncompetitive inhibition.

4. Other Types of Specific Inhibition

Ebersole, Guttentag, and Wilson[8] refer to Burk, who distinguishes between two other types of inhibition in which the percentage inhibition also depends on concentration of substrate, but which differ from the strictly competitive type.

In the "uncompetitive inhibition" the inhibitor combines with ES but not with E and the kinetic equation is:

$$\frac{1}{v_i} = \left(1 + \frac{[I]}{K_i}\right)\frac{1}{V^0} + \frac{K_s}{V^0}\frac{1}{[S]}$$

An example of this type is furnished by the combination of azide with the oxidized form of the *Atmungsferment* but not with its reduced form.[13]

A "quadratic inhibition" is also discussed but is of less interest for the present section.

III. Compilation of Examples

As pointed out by Work and Work[11] in their excellent book, it is rather easy to distinguish between specific and nonspecific inhibitions, but only careful experiments in most cases allow one to decide if a specific inhibition belongs to the competitive or to the noncompetitive type. In many cases the necessary data obtained with pure enzymes are lacking and in the following compilation of examples we shall not make a categoric distinction between the two types. From the considerations it will, however, appear to which type the examined inhibitions belong.

1. Inhibition by Cations (*Inorganic and Organic*)

The toxicity to enzymes of cations, more especially of heavy metals, and of basic dyes and large organic cations, too, has been described in many cases.

For heavy metals, Bersin[14] reports inactivation of amylase,[15] and dehydrases of *Escherichia coli*[16]; for silver ions he cites the inactivation of aminopeptidase,[17] asparaginase,[18] dipeptidase,[19] flavoprotein,[20] pancreas and liver lipase,[14,21] and urease.[22,23]

[13] R. J. Winzler, *J. Cellular Comp. Physiol.* **21**, 229 (1943).
[14] T. Bersin, *in* Handbuch der Enzymologie. Vol. 1, Akademische Verlagsgesellschaft, Leipzig, 1940, p. 154.
[15] K. Myrbäck, *Z. physiol. Chem.* **159**, 1 (1926).
[16] Y. Yudkin, *Enzymologia* **2**, 161 (1937).
[17] E. Abderhalden, *Fermentforschung*, **14**, 27 (1933); and **15**, 1 (1936).
[18] W. Grasmann and W. Mayr, *Z. Physiol. Chem.* **214**, 185 (1933).
[19] E. Waldschmidt-Leitz and A. Schäffner, *Z. physiol. Chem.* **151**, 31 (1926).
[20] R. Kuhn and P. Desnuelle, *Z. physiol. Chem.* **251**, 23 (1938).
[21] I. A. Parfentjev, W. Devrient and B. F. Sokoloff, *J. Biol. Chem.* **92**, 33 (1931).
[22] M. Jacoby, *Biochem. Z.* **262**, 181 (1933).
[23] J. B. Sumner and K. Myrbäck, *Z. physiol. Chem.*, **189**, 218 (1930).

Quastel *et al.* studied the influence of basic dyes on bacterial dehydrogenases[24–26], on fumarase, urease, and saccharase.[27] Haas[28] found an inhibition of cytochrome reductase by atabrine and quinine. The action of acridine drugs and quinine was examined on different enzymes by Hellerman *et al.* These investigations will be reconsidered in more detail. The inhibiting activity of quinine was measured by Rona *et al.* on saccharase,[29] serum lipase,[30] stomach lipase,[31] and pancreatic lipase.[32] Heavy metals are of course able to cause precipitation and denaturation of proteins; they can bind thiol groups and as will be shown bring about reversible inhibitions.

Myrbäck,[33] for instance, made a detailed investigation on the action of silver ions on saccharase. He found that silver combined with a carboxyl group in saccharase to form a feebly dissociated salt. With von Euler[34] he proved that the curve representing the degree of inhibition as a function of the concentration of the inhibitor takes the typical form of a dissociation curve. Working at varying pH values, Myrbäck[33] observed that the inhibition depends on the pH, being stronger in alkaline than in acid medium. Myrbäck[33] explains the action of the silver ion as due to a competition with the hydrogen ion and, since the latter is indispensable for the catalytic activity, an inactivation results. This inactivation is completely reversed by hydrogen sulfide. According to the author the following formula expresses the equilibrium:

$$\frac{[\mathrm{Ag}][\mathrm{E}^-]}{\mathrm{AgE}} = K_{\mathrm{A_g}}$$

In this equation E^- is the anion of the enzyme. $K_{\mathrm{A_g}}$ was found to have a value of $10^{-7.4}$.

Experimenting with other metals, copper, zinc, cadmium, and lead, Myrbäck[33] noted the same facts as for silver. We can add to this list barium and lanthanum which in our experiments behaved like silver.[35] Myrbäck[33] pointed out that mercury behaves in quite a different way. Mercury combines with a basic group of saccharase to form an inactive compound. This inhibition is reversed by hydrogen sulfide. Whereas in the case of silver the inhibition is not influenced by substrate concentration, with mercury on the contrary the inactivation depends on substrate concentration, so that it

[24] J. H. Quastel, *Biochem. J.* **25**, 898, 1121 (1931).
[25] J. H. Quastel and A. H. M. Wheatley, *Biochem. J.*, **25**, 629 (1931).
[26] J. H. Quastel, *Biochem. J.* **26**, 1685 (1932).
[27] J. H. Quastel and E. D. Yates, *Enzymologia*, **1**, 60 (1936).
[28] E. Haas, *J. Biol. Chem* **155**, 321 (1944).
[29] P. Rona and E. Block, *Biochem. Z.* **118**, 185 (1921).
[30] P. Rona and D. Reinecke, *Biochem. Z.* **118**, 213 (1921).
[31] P. Rona and M. Takata, *Biochem. Z.* **134**, 118 (1923).
[32] P. Rona and R. Pavlovic, *Biochem. Z.*, **134**, 108 (1923).
[33] K. Myrbäck, *Z. physiol. Chem.* **158**, 4 (1926).
[34] H. von Euler and K. Myrbäck, *Z. physiol. Chem.* **121**, 177 (1921).
[35] L. Massart, unpublished results.

may be concluded that the substrate-binding group of saccharase is affected by mercury.

Returning to the researches of Quastel *et al.*[24–27] we note that in their experiments on dehydrogenases these authors observed that at pH 7.4 basic dyestuffs are highly toxic, that specificity of action occurs among the dyes, molecular structure playing an important role, and that some dissociation of the combination between enzyme and dye may occur. The same observations were made for fumarase, but here the combination between enzyme and dye seemed to be irreversible. It was however found that the substrate (fumarate) and substrate analogs (succinic, malic, and aspartic acids) protect against the inhibition. About the same facts were noted for urease; there was observed an apparent irreversibility of combination of the dyes with the enzyme. Protection was found to be afforded to urease by the presence of substances which possess basic amino or imino groups, the latter substances combining reversibly with the enzyme, presumably at the negatively charged groups which attract the basic dyes.

With saccharase, however, a complete reversibility of the combination between dye and enzyme was noted. At the same time it was found that not only sucrose but also glucose competes with the basic dyestuffs. These results in a certain way amplify those of Myrbäck[33] obtained with silver ions. Indeed the reversible action of dyes on saccharase is expressed, according to Quastel and Yates,[27] by the formula:

$$\frac{1}{[D]}\frac{I}{1-I} = K$$

where I is fractional inhibition and D the molar concentration. This formula is the same as that found by Myrbäck[33] for the action of silver ions on invertase. It is the expression of a reversible reaction between the dye and the active group of the enzyme, provided that the drug is present in excess.

Quastel and Wheatley[27] also drew attention to the fact that increase in pH augments the inhibition by basic dyes. As we have already seen this is also the case for the inhibition of saccharase by silver ions.[33] It holds[35] also for Ba^{2+} and La^{3+}. The accompanying figure (Fig. 7) illustrates the results of Quastel and Wheatley. When the pH varies, the concentration of the basic dye being kept constant, the following relation between pH and degree of inhibition is obtained:

$$\mathrm{pH} - \log\frac{I}{1-I} = \text{constant}$$

When both pH and dye concentration are varied, the concentrations causing 50% inhibition were found to be expressed by the following formula:

$$\mathrm{pH} - \log\frac{1}{[D]} = \text{constant}$$

Evidently we have here a simple example of antagonism. Since pH = log 1/[H], we can write:

$$\log 1/[H] - \log 1/[D] = \text{constant}$$

or:

$$\log [D] - \log [H] = \text{constant}$$

whence:

$$[D]/[H] = \text{constant}$$

From these results and those obtained in experiments with acid dyes, Quastel and Wheatley[27] conclude that saccharase is a zwitterion which is enzymatically active. This zwitterion forms an anion, $OH \cdot E^-$, combining

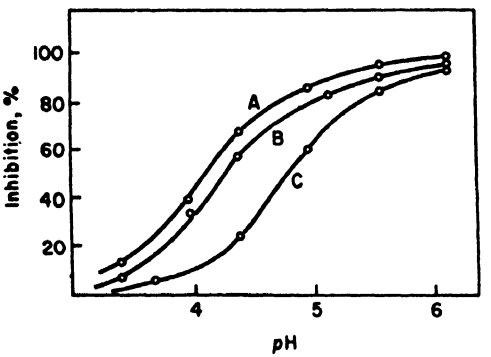


FIG. 7. Effect of pH on inhibition of saccharase by basic dyes.[27] (A) Safranine 8.6×10^{-4} *M*. (B) Ethyl violet 2.03×10^{-4} *M*. (C) Janus green 2.07×10^{-4} *M*.

with the basic dyes and binding the glucose part of the sucrose molecule and a cation, HE^+, binding the acid dye and the fructose part of the sucrose molecule. This hypothesis is supported by the fact that, as we said above, sucrose and glucose protect against the basic dyes, and that sucrose and fructose were proved to antagonize the acid dyes.

Another explanation for the inhibition by some basic dyes was suggested by experiments of Haas[28] and Wright and Sabine.[36] Wright and Sabine indeed observed a lowering of the atabrine inhibition of tissue respiration and of D-amino acid oxidase activity by flavin-adenine-dinucleotide (FAD) and Haas[28] noted similar facts for cytochrome oxidase. These observations were extended by Hellerman *et al.*[10] to the effect of atabrine and related compounds. Some of their results are given in Table I. From the results of

[36] C. J. WRIGHT AND J. C. SABINE, *J. Biol Chem.* **155**, 315 (1944).

Hellerman *et al.*[10] it appears that the FAD-inhibitor antagonism is not specific for atabrine; it is nearly as marked with quinine, plasmoquine, and different quinolines examined in this respect, and the authors, conclude that all these compounds share the property of combining with numerous proteins. When the protein is an enzyme, the combination may or may not result in inhibition depending upon whether the portion of the protein molecule affected is essential for activity. When the grouping affected takes part in the binding of the prosthetic group by the enzyme, the result is a competitive inhibition. If the grouping affected is not indispensable

TABLE I

EFFECT OF CONCENTRATION OF FLAVIN-ADENINE-DINUCLEOTIDE ON INHIBITION OF D-AMINO ACID OXIDASE BY ATABRINE AND RELATED COMPOUNDS[10]

FAD added, moles $\times 10^{-7}$	Control rate, μl. O_2/20 min.	Inhibitor, *M*	Inhibition, %	Temp., °C.
1.1	23	Atabrine, 0.001	80	30
4.1	99	0.001	54	30
11.0	150	0.001	4	30
0.4	35	0.001	70	37
2.9	96	0.001	36	37
27.5	144	0.001	8	37
0.4	43	Quinine, 0.001	61	37
2.9	113	0.001	28	37
27.5	179	0.001	10	37
0.4	43	0.003	72	37
2.9	113	0.003	44	37
27.5	179	0.003	21	37
0.4	43	Plasmochin, 0.003	71	37
2.9	113	0.003	39	37
27.5	179	0.003	5	37

for this combination, but still essential to activity, a noncompetitive inhibition results. Such an inhibition by basic compounds was found by the authors in the case of Straub's[37] flavoprotein from heart muscle (diaphorase)

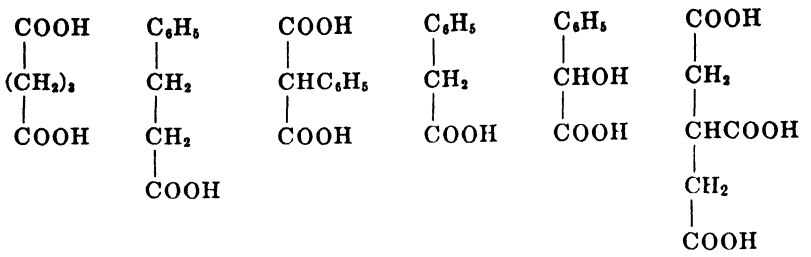
The results of Hellerman *et al.*[10] prove that testing inhibitions on an insufficient number of enzymes may lead to misinterpretations. They show also that competitive inhibition is not confined to substrate analogs.

2. INHIBITION OF SUCCINIC DEHYDROGENASE BY SUBSTRATE ANALOGS

We owe the classical example of competitive inhibition to Quastel and Wooldridge,[8] who proved that malonic acid and other substrate analogs

[37] F. B. STRAUB, *Biochem. J.* **33**, 787 (1939).

(shown in the accompanying formulas) inhibit succinic dehydrogenase:



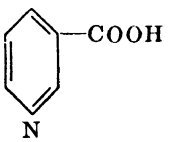
Potter and Dubois[38] have measured the affinity of succinic and malonic acid to succinic dehydrogenase and found that the inhibition–substrate ratio for 50% inhibition is 1:50.

Franke and Siewardt[39] report a progressive change in inhibitory activity with increasing chain length when experimenting with alkylated succinic acids.

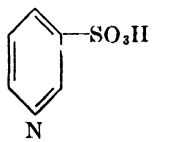
Klotz and Tietze[40] have found that 1,2-ethanedisulfonic and β-sulfopropionic acid in concentrations between 0.0033 and 0.02 *M* inhibit succinic dehydrogenase almost as much as does malonate.

3. Inhibition of Lactic Dehydrogenase by Pyridinesulfonic Acid

In the inhibition of lactic dehydrogenase by pyridinesulfonic acid, described by von Euler,[4] a competitive competition takes place between cozymase and the chemical analog of the essential molecule of this dehydrogenase. The inhibition of the lactic dehydrogenase exerted by pyri-



Nicotinic acid



Pyridine-3-sulfonic acid

dine-3-sulfonic acid disappears completely in the presence of adequate amounts of cozymase. This is seen in Fig. 8. Von Euler[4] has extended his results to glucose dehydrogenase from cow's liver with the same results. He proved also that the inhibition caused by salicylic acid or by adenosine and adenylic acid is not reversed by cozymase.

4. Amine Oxidase and Diamine Oxidase

Interesting instances of competitive inhibition are found in the investigations of Zeller and of Blaschko and Duthie on amine and diamine oxidase.

[38] V. R. Potter and K. P. DuBois, *J. Gen. Physiol.* **26**, 391 (1943).
[39] W. Francke and D. Siewerdt, *Z. physiol. Chem.* **280**, 76 (1944).
[40] I. M. Klotz and F. Tietze, *J. Biol. Chem.* **168**, 399 (1947).

Zeller[41] notes that all derivates of ammonia when present in a sufficient concentration are able to compete with diamines for the diamine oxidase. Monoamines, such as methyl- and amylamine, have a low affinity, choline[42] and ephedrine[43] have moderate affinity, and guanidine[44] and imidazole[45] a high affinity for the enzyme. In simple guanidines the inhibition increases from guanidine to methyl- and *asym*-dimethylguanidine.[46] The inactivation by the diguanidines arcain and synthalin is very large.[46] Vitamin B_1 also brings about a strong inhibition.[41]

In two more recent papers Blaschko and Duthie[47,48] examined the influence of different monoamines, diamines, and mono- and diamidines on the activity of amine oxidase and diamine oxidase. Of monamines of the type $CH_3(CH_2)_nNH_2$ the 12-carbon compound acted as a substrate for monoamide oxidase but the 18-carbon compound did not. With diamines

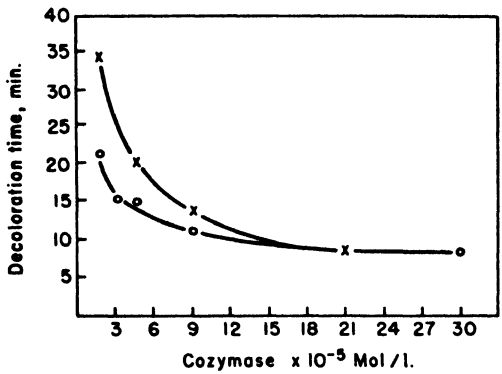


FIG. 8. Influence of cozymase concentration on inhibition of lactic dehydrogenase.[4]

of the types $NH_2(CH_2)_nNH_2$ it was observed that compounds with short chains up to C_8 were not oxidized by monoamine oxidase; compounds with longer chains (C_{14}–C_{18}) however acted as substrates for monoamine oxidase. So it can be concluded that the second amino group of the diamines interferes with the activity of monoamine oxidase when the chains are short; with longer chains this interference disappears. Conversely, short chains (up to C_8) appear to be necessary for substrates of diamine oxidase; indeed in this case diamines with longer chains (C_{14}, C_{16}, C_{18}) are not oxidized by this enzyme.[47]

[41] E. A. ZELLER, *Advances in Enzymol.* **2**, 93 (1942).
[42] E. A. ZELLER, *Helv. Chim. Acta* **23**, 1502 (1940).
[43] E. A. ZELLER, R. STERN, AND M. WENK, *Helv. Chim. Acta* **23**, 3 (1940).
[44] H. BLASCHKO, *J. Physiol.* **95**, 30P (1939).
[45] E. A. ZELLER, *Helv. Chim. Acta*, **24**, 539 (1941).
[46] E. A. ZELLER, *Helv. Chim. Acta*, **21**, 1645 (1938).
[47] H. BLASCHKO AND R. DUTHIE, *Biochem. J.* **39**, 347 (1945).
[48] H. BLASCHKO AND R. DUTHIE, *Biochem. J.* **39**, 478 (1945).

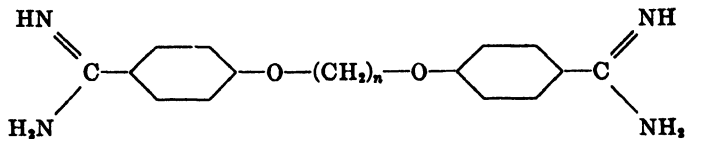
The inhibitory influence on the activity of amine oxidase by different amidines was measured.[47] These compounds were monoamidines of the type:

$$CH_3(CH_2)_nC(=NH)NH_2$$

diamidines of the type:

$$NH_2(NH=)C(CH_2)_nC(=NH)NH_2$$

diamidines of the type:



The authors were able to draw the conclusion that in each series there is an increase in inhibitory activity with an increasing number of CH_2 groups

TABLE II[48]

INHIBITION OF AMINE OXIDASE BY MONOAMIDINES[a] OF THE TYPE $CH_3(CH_2)_nC(=NH)NH_2$

n	Inhibition, %	n	Inhibition, %
3	15	10	61
4	42	14	8
8	50.5	16	7.5

[a] Inhibitor concentration 10^{-3} *M*.

until a certain chain length is reached; beyond this point a further increase in chain length causes a fall in inhibitory activity. For monoamidines the critical chain length is C_{10} as appears from Table II.

For the alkyl diamidines of the straight-chain type the inhibitory activity increases up to C_{12} and then falls. For the second type of diamidines (with aromatic group) the C_3 and C_5 compounds were the strongest inhibitors; the C_{10} compound was much less active.

It is remarkable that among the alkyl diamidines the increased formation of enzyme–inhibitor complex which is absorbed when the chain length is increased up to C_{12} is paralleled by increased affinity of substrate for enzyme among the related alkyl diamines. Blaschko and Duthie[47] studied also the inhibitory activity of mono- and diguanidines and diisothioureas. Zeller[41] and Blaschko and Duthie[48] give valuable discussions on the arrangement of the molecule for oxidation by diamine oxidase.

In addition to these investigations, Heegaard and Alles[49] have observed that the oxidation of amylamine by amine oxidase is inhibited by secondary

[49] E. V. HEEGAARD AND G. A. ALLES, *J. Biol. Chem.* **147**, 503 (1943).

and tertiary amines, the magnitude of the effect being related to the size of the molecule.

5. Inhibition of Sulfhydryl Enzymes

Sulfhydryl groups are common to many enzymes and thus inhibitors of sulfhydryl enzymes bring about a nonspecific inhibition. The first experiments on this type of inhibition were performed by Dickens,[50] Hellerman *et al.*[51] and Bersin.[52]

Dickens[51] has shown that iodoacetate reacts stoichiometrically with sulfhydryl-containing compounds such as glutathione. Hellerman *et al.*[51] observed that crystalline urease is inactivated in air by catalytic traces of heavy metals in the solution and Bersin[52] explained the activating effect of reducing agents on papain by reduction of disulfide linkages to the sulfhydryl form.

The general study of sulfhydryl groups in proteins has brought forward the division of these groups into two types: freely reactive sulfhydryl groups, which are oxidized by mild oxidants and give the nitroprusside test when the protein is in the native state, and sluggish sulfhydryl groups, which are not oxidized and do not give the nitroprusside test except after unfolding of the polypeptide chain by denaturation.

From the numerous investigations on sulfhydryl enzymes, the following items are perhaps the most interesting:

a. Wide Distribution of Sulfhydryl Enzymes

This item has been reviewed by Bersin[53] up to 1935. Dixon[54] found that of 12 dehydrogenases, the alcohol dehydrogenase of yeast was the most sensitive to iodoacetamide and was inhibited by concentrations as low as 0.0003 *M*.

Succinic dehydrogenase was studied by a number of authors. Libbrecht and Massart[55] found that oxygen under pressure inactivates this enzyme. This fact was confirmed by Dickens[56] and later by Stadie, Riggs, and Haugaard.[57] Hopkins *et al.*[58,59] recognized the essential role of sulfhydryl groups in succinic dehydrogenase and studied the inhibition by oxidized

[50] F. Dickens, *Biochem. J.* **27**, 1141 (1933).
[51] L. Hellerman, M. E. Perkins, and W. M. Clark, *Proc. Natl. Acad. Sci. U. S.* **19**, 855, (1933).
[52] T. Bersin, *Z. physiol. Chem.* **222**, 177 (1933).
[53] T. Bersin, *Ergeb. Enzymforsch.* **4**, 68 (1935).
[54] M. Dixon, *Nature* **140**, 406, 806 (1940).
[55] W. Libbrecht and L. Massart, *Compt. rend. soc. biol.* **124**, 299 (1937).
[56] F. Dickens, *in* Aspects actuels de l'Enzymologie. Desoer, Liège, 1947, p. 142.
[57] W. C. Stadie, B. C. Riggs, and N. Haugaard, *J. Biol. Chem.*, **161**, 153, 1945.
[58] F. G. Hopkins, E. Y. Morgan, and C. Lutwak-Mann, *Biochem. J.* **32**, 1829, 1938.
[59] F. G. Hopkins and E. Y. Morgan, *Biochem. J.* **32**, 611 (1938).

glutathione, alloxan, copper, maleic acid, and iodoacetic acid. Von Euler and Hellström[60] established the inhibition of succinic dehydrogenase by $Fe(CN_6)^{3+}$ and the reactivation by $Na_2S_2O_4$. Horecker[61] and Bernheim[62] experimented with copper as the inactivating agent and Barron and Kalnitsky[63] with heavy metals. Potter and Dubois[39] conclude from their experiments that succinic dehydrogenase is inhibited by a large number and variety of compounds, the only resemblance among them being their ability to react with thiol groups. These compounds are thiol reagents: Cu, SeO_3^{2-}, AsO_3^{3-} and compounds with quinonoid or potential quinonoid structure. Bergstermann and Stein[64] observed an inhibition by benzoquinone.

Experiments of von Euler and Adler[65] suggested the presence of sulfhydryl groups in triosephosphate dehydrogenase. Rapkine[66] proved the dependence of phosphoglyceraldehyde mutase on sulfhydryl groups and Morgan and Friedman[67] observed the inhibition of glyoxalase by maleic acid. Experiments of Lehman[68] indicated the presence of sulfhydryl groups in phosphoglucomutase and Iri[69] found an inactivation of hexokinase by iodoacetic acid. Mims *et al.*[70] proved that pteroylglutamic acid conjugase is a sulfhydryl enzyme.

After a thorough investigation of sulfhydryl enzymes in carbohydrate, fat, and protein metabolism, Barron and Singer,[71] making use of iodoacetamide, *p*-chloromercuribenzoic acid, and organic arsenicals, conclude that the following enzymes are to be considered sulfhydryl enzymes: pyruvate dehydrogenase, transaminase, yeast alcohol dehydrogenase, stearate oxidase (liver and bacterial), oleate oxidase (bacterial), pancreatic lipase, β-hydroxybutyrate oxidase (heart), acetylcholinesterase, human serum esterase, hog liver esterase, and pancreatic esterase.

According to the same authors the following enzymes are not affected by thiol reagents: polyphenoloxidase, arginase, citric dehydrogenase, uricase, catalase, lactic dehydrogenase, liver alcohol dehydrogenase, histaminase, potato phosphorylase, carbonic anhydrase, acid phosphatase, peanut fat oxidase, pepsin, cytochrome oxidase, and flavoproteins.

[60] H. VON EULER AND H. HELLSTRÖM, *Arkiv Kemi Mineral. Geol.* **13B**, 1 (1939).
[61] B. L. HORECKER, *Enzymologia*, **7**, 331 (1939).
[62] F. BERNHEIM, *J. Biol. Chem.* **133**, 485 (1940).
[63] E. S. G. BARRON AND G. KALNITSKY, *Biochem. J.* **41**, 346 (1947).
[64] H. BERGSTERMANN AND W. STEIN, *Biochem. Z.* **317**, 217 (1944).
[65] E. ADLER, H. VON EULER, AND G. GÜNTHER, *Skand. Arch. Physiol.* **80**, 1 (1938).
[66] L. RAPKINE, *Compt. rend. soc. biol.* **207**, 301 (1938); *Biochem. J.* **32**, 1729 (1938).
[67] E. Y. MORGAN AND E. FRIEDMAN, *Biochem. J.* **32**, 862 (1938).
[68] H. LEHMAN, *Biochem. J.* **33**, 241 (1939).
[69] S. IRI, *J. Biochem. Japan* **30**, 217 (1939).
[70] V. MIMS, M. E. SWENSEID, AND O. D. BIRD, *J. Biol. Chem.* **170**, 367 (1947).
[71] E. S. G. BARRON AND T. P. SINGER, *Science*, **97**, 358 (1943); *J. Biol. Chem.* **157**, 221, 241 (1945).

b. *Hopkins Phenomenon*

Hopkins *et al.*[58,59] discovered that previous addition of malonate or succinate protects succinic dehydrogenase from inhibition by sulfhydryl inhibitors. These experiments and those of Rapkine,[66] who proved that phosphoglyceraldehyde dehydrogenase is protected to some extent by its coenzyme, together with those of Hellerman, Lindsay, and Bovarnick,[10] who observed that the inhibition of D-amino acid oxidase by *p*-chloromercuribenzoate is partly prevented by the presence of an excess of flavinadenine-dinucleotide, explain the role of sulfhydryl groups in enzymes. Indeed, since in the above cases combination of the enzyme with either coenzyme or substrate is prevented by the inhibition, it is probable that here sulfhydryl groups play an essential role in the formation of the enzyme–substrate complex.

Potter and Dubois[38] and Barron and Singer[71] extended the experiments of Hopkins *et al.* to other enzymes and other thiol reagents.

c. *Variation in Reactivity of Sulfhydryl Groups*

The discovery of Hellerman *et al.*[72] of the existence in urease of sulfhydryl groups which are not of the same importance to the enzymatic activity has been a very interesting contribution. These authors proved that in urease at least two types of sulfhydryl groups should be distinguished: those which are easily available and not necessary for enzyme activity, and others —groups which are less readily available and on the contrary, essential for enzyme activity. Indeed, when crystalline urease is treated with one mole of *p*-chloromercuribenzoate per 21,300 g. urease no loss in activity takes place; when a second mole of reagent is added an inhibition occurs, although only two out of a total of five sulfhydryl groups are then combined. The remaining three sulfhydryl groups are only attacked after denaturation of urease.

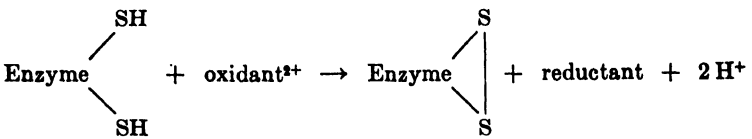
The existence of a variation in reactivity of sulfhydryl groups in enzymes had before been brought forward by experiments of Balls and Lineweaver,[73] who gave proof of the fact that papain is inactivated by iodoacetate and that only one of the ten sulfhydryl groups of this enzyme is alkylated by the reagent.

d. *Different Types of Sulfhydryl Reagents*

Barron and Singer[71] give the following classification of sulfhydryl reagents: oxidizing agents, alkylating agents, and trivalent organic arsenicals. Following up these authors it is interesting to observe with them that different oxidizing agents such as ferricyanide and porphyrindin are widely

[72] L. HELLERMAN, F. CHINARD, AND V. R. DEITZ, *J. Biol. Chem.* **147**, 443 (1943).
[73] A. K. BALLS AND H. LINEWEAVER, *J. Biol. Chem.* **130**, 669 (1939).

used for the titration of sulfhydryl groups in proteins (*cf.* Anson[74]), although they are less effective in inhibiting and detecting sulfhydryl enzymes. These agents oxidize the sulfhydryl groups to the disulfide:



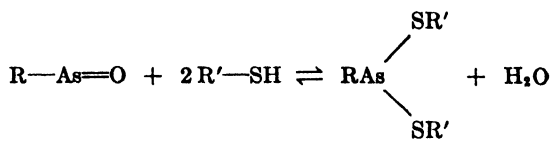
Ferricyanide, *e.g.*, has no effect on urease[75] and porphyrindin does not inhibit papain.[76] These results are to be considered in the light of the discussion above (Sect. *c*). Of the alkylating reagents iodoacetate has been the most used; iodoacetamide has also been employed extensively. It is assumed that iodoacetate reacts by substitution of the hydrogen of sulfhydryl by the carboxymethyl group[50]:

$$\text{Enzyme—SH} + \text{CH}_2\text{ICOOH} \rightarrow \text{enzyme—SCH}_2\text{COOH} + \text{HI}$$

However Michaelis and Schubert[77] called attention to the fact that acid halides combine with amino groups. Under definite conditions, however, the alkylation of sulfhydryl groups is much more rapid, although this does not preclude reaction with amino groups.

Barron and Singer[71] conclude from their experiments that some alkylating reagents (iodoacetamide) combine with more sulfhydryl groups than do the oxidizing agents, but still leave some unattacked. They admit that *p*-chloromercuribenzoate combines with all the available sulfhydryl groups in the native protein. This should also be the case for the organic arsenicals.

The trivalent organic arsenicals were first used as sulfhydryl reagents by Bersin.[53] They combine reversibly with sulfhydryl groups.[78,79]



The addition compounds thus formed readily dissociate on increase of the pH.

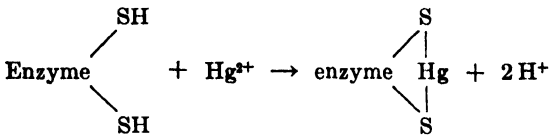
Ions of heavy metals, cystine, oxidized glutathione, and maleic acid have also often been used to detect sulfhydryl enzymes.

The reversion after inhibition has occurred has been the subject of a large number of investigations. As reversing agents reducing substances such as

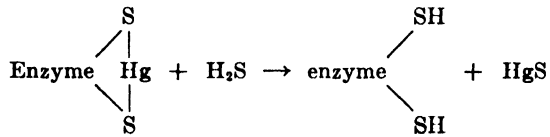
[74] M. L. Anson, *Science*, **90**, 142 (1939).
[75] L. Hellerman, *Physiol. Rev.* **17**, 454 (1937).
[76] J. P. Greenstein, *J. Biol. Chem.* **125**, 501 (1938).
[77] L. Michaelis and M. P. Schubert, *J. Biol. Chem.* **106**, 331 (1934).
[78] C. Voegtlin, H. A. Dyer, and C. S. Leonard, *Public Health Repts.* **38**, 1911 (1923).
[79] A. Cohen, H. King, and W. Y. Strangeways, *J. Chem. Soc.* **1931**, 3043.

vitamin C, hydrogen sulfide, cyanide, and thiols such as cysteine, glutathione, and BAL (British Anti-Lewisite) were used. Here follow some instances:

As Jacoby[80] observed, urease is inactivated by Hg^{2+} ions. These ions probably combine with reactive sulfhydryl groups to form mercaptide linkages:



This inactivation is reversed by substances forming insoluble or sparingly soluble compounds with the toxic ion. This was shown to be the case by Bersin[53] for hydrogen sulfide:



Reactivation of the inhibition brought about by *p*-chloromercuribenzoate and organic arsenicals is seen from Table III, showing some results of an investigation of Barron and Singer.[71]

Reactivations by BAL or 2,3-dimercaptopropanol have been extensively investigated by Stockin and Thompson[81] and Barron *et al.*[63] in the case of inhibition of sulfhydryl enzymes by Lewisite and the inhibition of succinic dehydrogenase by heavy metals.

Before finishing this item it is perhaps worth while to refer to a paper of Lewis,[82] who attributes the activity of methyl bromide to reaction with sulfhydryl groups.

Interesting developments in the field of sulfhydryl enzymes have been made during and since the war by investigations on the biochemical activity of warfare agents and of antibacterials.

In 1936 Peters[83] noted that traces of the vesicant mustard gas sulfone inactivate the pyruvate oxidase of brain, but during the war immense progress in this field has been made by the school of Peters *et al.*, Dixon *et al.*, Gilman and Philips, and Bacq *et al.* Among the first experiments on known sulfhydryl enzymes we cite those obtained by collaborators of Bacq, Massart, and Peeters,[84] who found a strong inhibition of succinic dehydrogenase by vesicants and lachrymators. The actual status of the

[80] M. JACOBY, *Biochem. Z.* **181**, 194 (1927).
[81] L. A. STOCHEN AND R. H. S. THOMPSON, *Biochem. J.* **40**, 535 (1946).
[82] S. E. LEWIS, *Nature*, **161**, 692 (1948).
[83] R. A. PETERS, *Nature* **138**, 327 (1936).
[84] L. MASSART AND G. PEETERS, *Acta biol. belg.* **1**, 42 (1941).

problem has been reviewed in an excellent publication of Peters.[85] Here we shall give only the conclusions of this author:

Vesicants which are trivalent organic arsenicals, such as Lewisite ($CHCl:CHAsCl_2$) have a definite affinity for sulfhydryl enzymes. Pyruvate oxidase is particularly sensitive to this reagent. In this case monothiols are poor reversers, but good effects are obtained with the dithiol BAL (2,3-dimercaptopropanol) because the arsenic in the

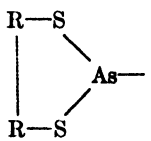
TABLE III

REACTIVATION OF SULFHYDRYL ENZYMES INHIBITED BY MERCAPTIDE-FORMING SUBSTANCES[a]

Enzyme	Inhibitor	Inhibition, %	Reactivator	Reactivation, %
Succinoxidase	Arsenical I, 5×10^{-5} *M*	80	Glutathione, 1×10^{-3} *M*	Complete
		80	Cysteine, 1×10^{-2} *M*	25
	Benzoquinone, 5×10^{-5} *M*	80	Glutathione, 2×10^{-3} *M*	Complete
Pyruvate oxidase (gonococci)	Arsenical IV, 1×10^{-4} *M*	94	1×10^{-3} *M*	15
	Arsenical I, 5×10^{-5} *M*	40	1×10^{-3} *M*	77.5
Pyruvate oxidase (ground, washed chicken liver)	*p*-Chloromer-curibenzoate, 5×10^{-4} *M*	92	2.5×10^{-3} *M*	39
Adenosinetriphosphatase (myosin)	*p*-Chloromercuribenzoate, 1×10^{-3} *M*	Complete	1×10^{-2} *M*	Complete

[a] Arsenical I, *p*-carboxyphenylarsine oxide. Arsenical IV, *p* carbamylphenylarsine oxide. From E. S. G. Barron and T. P. Singer, *J. Biol. Chem.* **157**, 234 (1945).

enzyme is in a ring, which can be represented as follows:



The ring form of arsenic with BAL is somewhat more stable than the one of arsenic with the enzyme. This explains why BAL is an excellent reverser of the effects produced by Lewisite.

Mustard gas combines with sulfhydryl groups and also with carboxyl and amino groups. It cannot be concluded that vesication in general is always induced by an

[85] R. A. PETERS, *Symposia Soc. Exptl. Biol.* **3**, 36 (1949).

attack upon an sulfhydryl enzyme and enzyme damage is not necessarily the first step in vesication.

Concerning lachrymators it is generally admitted that they are inhibitors of sulfhydryl enzymes and Dixon even regards lachrymators as the test agents par excellence for sulfhydryl enzymes.

The work on the biochemical activities of chemical warfare substances has also been reviewed by Peters, Stocken, and Thompson,[86] Waters and Stock,[87] Dixon and Needham,[88] Gilman and Phillips,[89] Peters,[90] and Bacq.[91,92]

Interesting results on sulfhydryl enzymes have also been obtained in the field of antibacterials. Indeed many quinones are potent antibacterials and quinones are known to inactivate a number of sulfhydryl enzymes, *e.g.*, urease and succinic dehydrogenase.[38]

Cysteine and thioglycolate inhibit the antibacterial activity of 2-methyl-1,4-naphthoquinone.[93] Unsaturated lactones react with sulfhydryl groups to add the RS to the carbon β to the lactone in $\Delta^{\alpha,\beta}$- and $\Delta^{\beta,\gamma}$-lactones.[94] It has however been pointed out by Hoffman, Ostenhof, and Lee[95] that the inhibition of urease activity by various antibiotics shows no relationship to the bacteriostatic effect. The strongest antibacterial substances such as 4-methoxytoluquinone as well as a series of unsaturated lactones belonging to the patulin series show no inhibition of urease activity.

Cavallito and Haskel[94] and Glock *et al.*[96] note that different antibacterials are unsaturated α,β-ketones. The latter authors observed that the α,β-unsaturated ketones which react most completely with cysteine are also the ones which are the most strongly antibacterial.

Cysteine reacts also with penicillin.[97] The question of whether sulfhydryl enzymes are the first point of attack of these different antibacterials are still very conflicting. More details will be found in this book in Chapter 4.

6. Inhibition by Fluoride

In the chapter on enzyme inhibitions Bersin[14] refers to fluoride as inhibitor of different enzymes: yeast phosphatase,[98] glucosulfatase,[99] acid

[86] R. A. Peters, R. A. Stocken, and R. H. S. Thompson, *Nature*, **156**, 616 (1945).
[87] R. L. Waters and C. Stock, *Science*, **102**, 601 (1945).
[88] M. Dixon and D. M. Needham, *Nature*, **158**, 432 (1946).
[89] A. Gilman and F. S. Phillips, *Science*, **103**, 409 (1946).
[90] R. A. Peters, *Nature* **159**, 149 (1947).
[91] Z. M. Bacq, Actualités biochimiques: Travaux récents sur les toxiques de guerre-Ed. Desoer, Liège, 1947. Z. M. Bacq, *Experientia*, **2**, 349 (1946).
[92] Z. M. Bacq and V. Desreux, *Exposés annuels de Biochimie Médicale*, **8**, 67 (1948).
[93] C. A. Colwell and M. Mc Call, *J. Bact.* **51**, 659 (1946).
[94] C. J. Cavallito and T. H. Haskell, *J. Am. Chem. Soc.* **67**, 1991 (1945).
[95] O. Hoffman-Ostenhof and W. H. Lee, *Monatsh.* **76**, 180 (1946).
[96] G. E. Glock, R. H. Thorp, J. Ungar, and R. Wien, *Biochem. J.* **39**, 308 (1945).
[97] C. J. Cavallito and J. H. Bailey, *Science* **100**, 390 (1944).
[98] E. Aughagen and Grzycki, *Biochem. Z.*, **265**, 217 (1934).
[99] T. Soda and F. Egami, *J. Chem. Soc. Japan*, **55**, 1164 (1934).

phosphatase,[100] and hydrogenlyase.[101] Various other enzymes can be added to this list: *e.g.*, enolase.[102]

Massart and Dufait[103] compared the activations by bivalent metals in the esterase group and the inhibitions by fluoride and arrive at the results given in Table IV.

As explanation of this inhibition two main hypotheses have been advanced: elimination of activating Mg^{2+} or Ca^{2+} ions and the special position of fluoride in the Hofmeister series. The first hypothesis grew in importance for the enzyme enolase when Ohlmeyer and Dufait[111] succeeded in inactivating this enzyme by electrodialysis and in restoring the activity by addition of magnesium salts. The real explanation and the intimate mechanism of this inhibition were however revealed by the experiments of Warburg and Christian.[112] These authors were able to isolate and crystallize the enolase of yeast. They proved it to be a magnesium protein in which magnesium can be replaced by zinc and manganese. These last metals have to be

TABLE IV

ACTIVATORS AND INHIBITORS OF ESTERASES[103]

Enzyme	Activators	Inhibitors
Alkaline phosphatase	Mg[104]	NaF[98]
Acid phosphatase	Ca[105]	NaF[105]
Lipase	Ca[106] Mn[107]	NaF[108]
Cholinesterase	Ca, Mg, Mn[103]	NaF[109]
Chlorophyllase	Ca[110]	?

used in low concentrations however, since otherwise they poison the enzyme.

Warburg and Christian[112] observed that the inhibition of enolase by fluoride is influenced by the presence of phosphate: *i.e.*, 10^{-3} *M* phosphate does not inhibit, 10^{-6} *M* phosphate + 1.67×10^{-3} *M* fluoride does not inhibit, but 10^{-3} *M* phosphate + 1.67×10^{-3} *M* fluoride brings about an inhibition of 71%.

[100] A. CONTARDI AND ROVAZONNI, *Rend. ist. lombardo Sci.* **68**, 363 (1935).
[101] M. STEPHENSON AND STICKLAND, *Biochem. J.* **26**, 712 (1932).
[102] K. LOHMANN AND O. MEYERHOF, *Biochem. Z.*, **273**, 60 (1934).
[103] L. MASSART AND R. DUFAIT, *Enzymologia*, **6**, 282, 1938.
[104] H. ERDTMANN, *Z. physiol. Chem.* **172**, 182 (1928).
[105] S. BELFANTI, A. CONTARDI, AND A. ERCOLI, *Biochem. J.* **29**, 842 (1935).
[106] R. WILLSTÄTTER AND F. MEMMEN, *Z. physiol. Chem.* **133**, 229 (1934).
[107] C. NEUBERG, *Biochem. Z.* **11**, 400 (1908).
[108] A. S. LOEWENHART AND Y. W. PRICE, *J. Biol. Chem.* **11**, 397 (1910).
[109] N. MATTES, *J. Physiol.* **70**, 338 (1938).
[110] T. BERSIN, Kurzes Lehrbuch der Enzymologie. Akademische Verlagsgesellschaft, Leipzig, 1938, p. 52.
[111] P. OHLMEYER AND R. DUFAIT, *Natuurw. Tijds.* **23**, 77 (1941).
[112] O. WARBURG AND W. CHRISTIAN, *Biochem. Z.* **310**, 384 (1942).

They also proved that in $M/20$ phosphate buffer the inhibition depends upon the concentration of magnesium salt, as is seen from Table V. The fluoride concentration necessary to cause an inhibition of 50% decreases when the magnesium salt concentration rises. From these results the authors[112] conclude that the cause of the inhibition is not a fall of the free magnesium sulfate in the solution. The fluoride inhibition is due to a magnesium fluoride compound which forms a dissociating compound with the enzyme protein. Working with bicarbonate buffer, the authors[112] observed that additions of small quantities of phosphate (10^{-5} M) cause a strong increase of the fluoride inhibition.

TABLE V

INHIBITION OF ENOLASE BY VARYING CONCENTRATIONS OF MAGNESIUM SULFATE[112]

$MgSo_4$, M	Fluoride concn. for 50% inhibition, M
1.0×10^{-3}	3.9×10^{-4}
2.7×10^{-3}	2.0×10^{-4}
2.7×10^{-2}	0.6×10^{-4}

From experiments in which the concentration of phosphate, magnesium sulfate, and fluoride were varied they calculate that the value:

$$C_{Mg} C_{PO_4} C^2_{\text{fluoride}} \times \frac{\text{fractional activity}}{\text{inhibition}}$$

is constant and has a value of 3.2×10^{-12} when the concentrations are expressed in moles per liter. They conclude that the fluoride inhibition is due to a magnesium fluorophosphate, which competes with the magnesium of the magnesium enolase:

$$\text{Mg-fluorophosphate} + \text{Mg-enolase} \rightleftarrows \text{Mg-fluorophosphoenolase} + \text{Mg salt}$$

Warburg and Christian[112] themselves stress that fluoride inhibition is not always due to the formation of inactive magnesium fluorophosphate enzyme and proved that, in the case of carboxylase, phosphate does not alter the inhibition by fluoride.

Borei[113] and Runnström *et al.*,[114] after a critical investigation of the inhibition of yeast respiration by fluoride, come to the conclusion that the point of attack of fluoride is a carrier intermediate between the cytochromes and the dehydrogenases.

[113] H. BOREI, *Arkiv Kemi Mineral. Geol.* **13A**, No. 23 (1940).

[114] J. RUNNSTRÖM, H. BOREI, AND E. SPERBER, *Arkiv Kemi. Mineral. Geol.* **13A**, No. 22 (1940).

7. Inhibition by Carbonyl Reagents

Different inhibitions of this type are mentioned by Bersin.[14] Werle and Heitzer[115] observed an inhibition of histidine decarboxylase by carbonyl reagents. Zeller[46] reports inhibition of diamine oxidase by these reagents and Werle[116] corroborated these results. Lawrence and Smythe[117] have found that the enzyme which produces hydrogen sulfide from cysteine in rat liver is affected by carbonyl reagents. This enzyme was named cysteine desulfurase by Fromageot *et al.*[118] who observed also that this enzyme is inhibited by potassium cyanide. Massart[119] confirmed this inhibition by cyanide and also found inactivation by other carbonyl reagents. Fromageot and Grand[120] came to the same conclusion. Schales *et al.*[121] studied the inhibition of enzymatic proteolysis by carbonyl reagents. In this connection is also to be mentioned the inactivation of uricase and xanthine oxidase observed by Keilin and Hartree.[122]

8. Inhibition of Carboxylase

Buchman, Heegaard, and Bonner[123] report the inactivation of the carboxylase system by thiazole pyrophosphate. According to these authors this inactivation is probably due to combination of the inhibiting compound with the carboxylase protein. Sevag *et al.*[124] have made an extensive investigation of the inhibition of carboxylase by sulfonamides.

According to Warburg and Christian[112] the fluoride inhibition of carboxylase is due to the fact that this enzyme is a magnesium protein. Green *et al.*[125] found also that carboxylase is a magnesium protein.

Karrer and Visconti[126] have observed that the following compounds exert a more or less strong inhibiting action on the activity of carboxylase: patulin, β-ionone, acetophenone, *p*-hydroxyacetophenone, phenol, quinol, resorcinol, phloroglucinol, acetaldehyde, acrolein, benzaldehyde, furfurole, and flavonol.

Kuhn and Beinert[127] state that quinones affect carboxylase in a specific

[115] E. Werle and K. Heitzer, *Biochem. Z.* **299**, 420 (1938).
[116] E. Werle, *Biochem. Z.* **304**, 201 (1940).
[117] J. M. Lawrence and C. V. Smythe, *Arch. Biochem.* **2**, 225 (1943).
[118] C. Fromageot, E. Woockey, and P. Chaix, *Enzymologia* **9**, 198 (1940–1941).
[119] L. Massart, *Verhandel Koninkl.* VI. Academie vr Geneeskunde 1943.
[120] C. Fromageot and R. Grand, *Enzymologia* **11**, 81 (1943).
[121] O. Schales, A. M. Suthon, R. M. Roux, E. Lloyd, and S. S. Schales, *Arch. Biochem.* **19**, 119 (1948).
[122] D. Keilin and E. F. Hartree, *Proc. Roy. Soc. London* **B119**, 114 (1936).
[123] E. R. Buchman, E. Heegaard, and J. Bonner, *Proc. Natl. Acad. Sci. U. S.* **26**, 561 (1940).
[124] M. G. Sevag, M. Shelburne, and S. Mudd, *J. Gen. Physiol.* **25**, 805 (1942).
[125] D. E. Green, D. Herbert, and V. Subrahmanyan, *J. Biol. Chem.* **138**, 327 (1941).
[126] P. Karrer and W. Visconti, *Helv. Chim. Acta* **30**, 268 (1947).
[127] R. Kuhn and H. Beinert, *Ber.* **76**, 904 (1943); **80**, 101 (1947).

way. They have observed parallelism between the anticarboxylase action of quinones and their antibacterial activity. The most active compounds are quinones and naphthazarine in which substitution by halogens has taken place. An inhibition of 50% was exerted by 2-bromonaphthazarine at the dilution 1/2,000,000; seven molecules of the inhibitor are bound by one molecule of the enzyme. Kuhn and Beinert state that pyruvic dehydrogenase too is very sensitive to quinones; at a concentration of 1×10^{-5} the degradation of pyruvic acid by gonococci is inhibited 37% and at a concentration of 3×10^{-5} as much as 96%.

According to Massart *et al.*[128,129] carboxylase is strongly inhibited by dinitrophenols.

9. Inhibition of Carbonic Anhydrase by Sulfonamides

Mann and Keilin[130] report that even at concentrations as low as 2×10^{-6} *M* sulfanilamide exerts an inhibition of carbonic anhydrase. They proved that the SO_2NH_2 grouping is responsible for this inhibition. Sulfamic acid (NH_2SO_2OH) and sulfamide ($NH_2SO_2NH_2$) are less active. The inhibiting group probably reacts with the prosthetic group of the enzyme.

Making use of this remarkable inhibition, different authors have studied the role of carbonic anhydrase in physiological phenomena: gastric secretion,[131] transport of carbon dioxide and chloride shift,[132] and acid–base equilibrium in the oviduct.[133]

Krebs[134] examined the inhibitory action of 25 sulfonamides on carbonic anhydrase. This author gives data for the concentration causing an inhibition of 50% at 0 and 15° in dilute enzyme solution; more sulfonamide is needed at the higher temperature and in the presence of tissue homogenates. The suitability of various sulfonamides for inhibiting experiments and the relations between chemical constitution and inhibiting power are discussed. Krebs confirmed the earlier finding[130] that compounds with the group SO_2NH_2 are much more inhibitory than these of the type RSO_2NHR'.

10. Inhibition by Dinitrophenols

Krahl, Keltch, and Clowes[135] report that diamino acid oxidase and flavoprotein of heart muscle (diaphorase) are inhibited by dinitrophenol. Haas, Harrer, and Hogness,[136] after stating that cytochrome reductase is

[128] L. Massart and L. Vandendriessche, *Naturwissenschaften* **28**, 781 (1940).
[129] L. Vandendriessche, *Enzymologia* **10**, 69 (1941).
[130] T. Mann and D. Keilin, *Nature* **146**, 164 (1940).
[131] W. Feldberg, D. Keilin, and T. Mann, *Nature* **146**, 651 (1940).
[132] D. Keilin and T. Mann, *Nature* **148**, 493 (1941).
[133] R. Benesch, N. S. Barron, and C. A. Mawson, *Nature* **153**, 138 (1944).
[134] H. A. Krebs, *Biochem. J.* **43**, 525 (1948).
[135] M. E. Krahl, A. K. Keltch, and G. H. A. Clowes, *J. Biol. Chem.* **136**, 563 (1940).
[136] E. Haas, C. J. Harrer, and T. R. Hogness, *J. Biol. Chem.* **143**, 341 (1942).

inactivated by 2,4-dinitro-*O*-cyclohexylphenol, come to the conclusion that this compound is not a specific inhibitor of flavoproteins.

Massart *et al.*[128] have proved that the induction phase of fermentation by Lebedew juice is shortened by 2,4- and 2,5-dinitrophenols, but not by 2,6-dinitrophenol. The same authors[129,137] have proved that carboxylase and pyruvodehydrase are strongly inhibited by the same compounds. Succinic dehydrogenase is as heavily affected as is respiration. The 2,6-dinitrophenol was always without inactivating action.

Wuff and Ionesco[138] have found that oxidative decarboxylation of malic acid is affected by 2,4-dinitrophenol. Reasons are advanced for considering this oxidative decarboxylation to be associated with phosphorylation.

11. Inhibition of Phosphatases

Gould[139] has found that free amino groups are essential for the activity of alkaline phosphatase. Roche[140] has reviewed and discussed the activations and inhibitions of metallo–proteins, more especially of alkaline phosphatases. The activators and inhibitors of phosphatases and pyrophosphatases have been the subject of extensive investigations of Roche *et al.*[141–146]

Cloetens[147] divides the alkaline phosphatases into two groups: group 1 is inhibited by fluoride and not by potassium cyanide and thiols (*e.g.*, liver phosphatase); group 2 is inhibited by potassium cyanide and thiols and not by fluoride (*e.g.*, kidney phosphatase).

Hove, Elvehjem, and Hart[148] report that zinc ions inhibit alkaline phosphatase, but in the presence of amino acids these ions activate. According to Massart and Vandendriessche[149] glycine inhibits alkaline phosphatase. These authors corroborate the activating effect of the system glycine–Zn^{2+} reported by Hove *et al.*

Bodansky[150] has observed that glycine exerts a noncompetitive inhibition on rat bone phosphatase. Inhibition of rat intestinal phosphatase is both

[137] L. Massart and L. Vandendriessche, *Enzymologia* **10**, 244 (1942).
[138] A. L. Wuff and H. Ionesco, *Compt. rend.* **225**, 263 (1947).
[139] B. S. Gould, *J. Biol. Chem.* **156**, 365 (1944).
[140] J. Roche, *Actualités de Biochemie Médicale* **6**, 93 (1946).
[141] J. Roche, S. De Laromigniere, and A. Laurens, *Bull. soc. chim. biol.* **25**, 1019 (1943).
[142] J. Roche and Nguyen-Van Thoai, *Bull. soc. chim. biol.* **24**, 1237 (1942).
[143] Nguyen-Van Thoai, *Bull. soc. chim. biol.* **24**, 1077 (1942).
[144] J. Roche, Nguyen-Van Thoai and O. Mimhan, *Bull. soc. chim. biol.* **25**, 1217 (1943).
[145] J. Roche and Nguyen-Van Thoai, *Bull. soc. chim. biol.* **25**, 1365 (1943).
[146] J. Roche, Nguyen-Van Thoai, and M. Roger, *Bull. soc. chim. biol.* **26**, 1047 (1944).
[147] R. Cloetens, *Biochem. Z.* **307**, 252 (1941); **308**, 37 (1941); **310**, 42 (1941).
[148] E. Hove, C. A. Elvehjem, and E. B. Hart, *J. Biol. Chem.* **134**, 425 (1940).
[149] L. Massart and L. Vandendriessche, *Enzymologia* **11**, 265 (1945).
[150] O. Bodansky, *J. Biol. Chem.* **165**, 605 (1946).

competitive and noncompetitive, the relative amounts of each varying with glycine concentration; *i.e.*, at a glycine concentration of 0.0625 *M* inhibition due to the noncompetitive compound is 74% of the total, while at a concentration of 0.0012 *M* of glycine there is an increase to 91%. According to the same author the inactivation is dependent on the presence of unsubstituted amino and carboxyl groups in glycine; inhibition by glycine ethyl ester, monomethylglycine, and dimethylglycine amounts, respectively to 50, 10, and 2% of that shown by glycine.

Acid phosphatases are inhibited by molybdate.[151,152] Courtois[153,154] has studied the influence on acid phosphatase by products arising from the oxidation of vitamin C by copper.

12. Inhibitors of Tyrosinase

Tennenbaum and Jensen[155] report that tyrosinase is inactivated by potassium cyanide, sodium diethyldithiocarbamate, and ethyl xanthate. The inhibition is reversed by Cu^{2+}. Wisansky *et al.*[156] have found that *p*-aminobenzoic acid is an inhibitor of tyrosinase, but that sulfanilamide causes no inactivation. Bernheim and Bernheim[157] observed an inhibition by phenylthiourea. In a more extensive investigation DuBois and Erway[158] confirmed the inhibition by phenylthiourea and found thiourea and derivatives of thiourea and thiouracil to be inhibitors of tyrosinase.

Chodat and Duparc[159] examined the influence of thiourea on the blackening and the respiration of potatoes. Potatoes impregnated with a 0.1% solution of thiourea do not blacken, but the respiration is little or not affected at this concentration. *p*-Cresol is not oxidized in the presence of thiourea, the presence of one molecule of thiourea preventing the enzymatic oxidation of one molecule of *p*-cresol. Enzyme oxidation starts again when the molecular concentration of *p*-cresol is higher than the concentration of thiourea.

13. Inhibitors of Cholinesterase and Choline Acetylase

Perhaps no other enzyme has been the subject of so many inhibition experiments as cholinesterase. The situation has been somewhat confused because in the earlier experiments the existence of different cholinesterases was not taken into account. Very detailed research on these enzymes in-

[151] L. Massart and K. Vermeyen, *Naturwissenschaften* **30**, 170 (1942).
[152] J. Courtois and M. Bossard, *Bull. soc. chim. biol.* **26**, 464 (1944).
[153] J. Courtois and J. Rigaud-Maneuvrier, *Bull. soc. chim. biol.* **25**, 211 (1940).
[154] J. Courtois, *Bull. soc. chim. biol.* **25**, 363 (1943).
[155] L. E. Tenenbaum and H. Jensen, *J. Biol. Chem.* **147**, 27 (1943).
[156] W. A. Wisansky, G. J. Martin, and S. Ausbacher, *J. Am. Chem. Soc.* **63**, 1771 (1941).
[157] F. Bernheim and M. L. C. Bernheim, *J. Biol. Chem.* **145**, 231 (1942).
[158] K. P. DuBois and W. F. Erway, *J. Biol. Chem.* **165**, 711 (1946).
[159] F. Chodat and G. Duparc, *Helv. Chim. Acta* **27**, 334 (1944).

cluding inhibition experiments and giving about 700 references was made by Augustinsson[160] in Myrbäck's laboratory. This author divides the inhibitors of cholinesterases in the following groups:

(*a*) *Urethans.* The most potent of these compounds is physostigmine, which owes its activity, according to Stedman and Stedman,[161] to the urethan group. Prostigmine, also called neostigmine, is also a strong inhibitor.

(*b*) *Quaternary and Tertiary Ammonium Bases.* In general all quaternary ammonium bases are strong inhibitors.[162] Basic dyes, such as methylene blue, exert a powerful inactivation, while the corresponding leuco compound is without activity.[163–165] Tertiary amines are less powerful inhibitors and show a stronger inactivating activity against nonspecific esterase.[162]

(*c*) *Amines and Amides.* In this group the activity of phenanthrene amino alcohols should be noted. Both red cell cholinesterase and plasma cholinesterase were tested by Wright.[166] Considerable differences in response to the inhibitor were observed. Among a series of phenanthrene-9-amino alcohols, the progressive increase of size of the dialkylamino group caused an increased inactivation of plasma cholinesterase. A maximum was noted for dipropylamino and was followed by a rapid diminution of inhibiting activity. These results are to be compared with those of Blaschko on monoamine and diamine oxidase.[47,48]

(*d*) *Nitrogen-Heterocyclic Compounds.* Alkaloids were also the subjects of extensive investigations. A very strong inhibitor is the alkaloid from *Tabernanthe iboga.*[167]

(*e*) *Alkyl Fluorophosphates.* Considerable attention has been paid in recent years to these compounds, the first of which was investigated by Adrian *et al.*[168] The inhibition is progressive and irreversible[169] and said to be selective for the nonspecific esterase.[170,171]

(*f*) *Alkyl Polyphosphates.* These compounds seem to be still more powerful inhibitors than the fluorophosphates. Their activity was proved by Dubois and Mangun[172] and by Chadwick and Hill.[173]

[160] K. B. Augustinsson, *Acta Physiol. Scand.* **15,** Suppl. 52, 182 (1948).
[161] E. Stedman and E. Stedman, *Biochem. J.* **25,** 1147 (1931).
[162] M. C. Sanz, *Helv. Physiol. et Pharmacol. Acta* **3,** C14 (1945).
[163] L. Massart and R. Dufait, *Enzymologia* **9,** 364 (1941).
[164] E. Rentz, *Arch. exptl. Path. Pharmakol.* **196,** 148 (1940).
[165] W. Reichert and E. Schmid, *Arch. exptl. Path. Pharmakol.* **199,** 66 (1942).
[166] C. J. Wright, *J. Pharmacol. Exptl. Therap.* **87,** 109 (1946).
[167] D. Vincent and J. Sero, *Bull. soc. chim. biol.* **24,** 1352 (1942).
[168] E. D. Adrian, W. Feldberg, and B. A. Kilby, *Nature* **158,** 625 (1946).
[169] A. Mazur and O. Bodansky, *J. Biol. Chem.* **163,** 261 (1946).
[170] R. D. Hawkins and B. Mendel, *Brit. J. Pharmacol.* **2,** 173 (1947).
[171] O. Bodansky, *Ann. N. Y. Acad. Sci.* **47,** 521 (1946).
[172] K. P. DuBois and G. H. Mangun, *Proc. Soc. Exptl. Biol. Med.* **64,** 137 (1947).
[173] L. E. Chadwick and D. L. Hill, *J. Neurophysiol.* **10,** 235 (1947).

(*g*) *Thiol Reagents.* It was shown by Nachmansohn and Lederer[174] that cholinesterase is to be considered a sulfhydryl enzyme. Different thiol reagents, such as iodoacetic acid, are inhibitors of the enzyme.

(*h*) *Other Inhibitors.* Alcohols, phenols, aldehydes, acids, vitamins, hormones, anions, metal salts, etc. For more details and interesting results and discussions the publication of Augustinsson[160] should be consulted.

A recent investigation of Harpur and Quastel[175] reveals the inhibitory effects of glucose and fructose on acetylcholine synthesis. In the same paper inhibition by D-glucosamine and relief by *N*-acetylglucosamine are treated.

14. MISCELLANEOUS

Various other examples of enzyme inhibitions are discussed in the literature: Inhibition of pyrocatechol oxidase by dihydroxymaleic acid.[176] Inhibition of succinic dehydrogenase by concentrations of cozymase higher than the physiological ones.[177] Inhibition of carbonic anhydrase by potassium cyanide, hydrogen sulfide, and sodium azide.[178] Inhibition of enzymatic synthesis of glycogen from glucose phosphate.[179] Inhibition of catalase and cytochrome oxidase by hydroxyphenothiazine and hydroxysulfanilamide.[180] Reversible inhibition of L(−)-cysteic acid carboxylase by potassium cyanide.[181] Inhibition of lipoxidase by antioxidants and heavy metals.[182] Inhibition of the copper protein laccase.[183] Inhibitors of penicillinase.[184] Identification of trypsin inhibitor of egg white with ovomucoid.[185] Effect of sodium arsenate on phosphoesterase from calf intestinal mucosa.[186] Effect of adrenal cortex, anterior pituitary extract, and insulin on hexokinase reaction.[187] Effects of estrogens on malic dehydrogenase of rat liver.[188] Inhibition of rat liver succinic dehydrogenase by synthetic estrogens and related substances.[189] Inhibition of hexokinase by amidone, 2-dimethylamino-4,4-diphenylheptan-5-one hydrochloride.[190] Inhibition of the oxidation of succinic acid by tissue preparations.[191] Inhibition of xanthine oxidase and related enzymes by 6-pteridyl aldehyde.[192] Inhibition of nucleotidase, with adenylic acid as substrate, by glutathione, arsenite, and oxalate.[193] Competitive inhibition by glu-

[174] D. NACHMANSOHN AND E. LEDERER, *Bull. soc. chim. biol.* **21**, 797 (1939).
[175] R. P. HARPUR AND J. H. QUASTEL, *Nature* **164**, 779 (1949).
[176] M. FLORKIN AND G. DUCHATEAU, *Bull. soc. chim biol.* **21**, 1204 (1939).
[177] V. R. POTTER, *Arkiv. Kemi Mineral. Geol.* **13B**, No. 7 (1939).
[178] D. KEILIN AND T. MANN, *Biochem. J.* **34**, 1163 (1940).
[179] G. T. CORI AND C. F. CORI, *J. Biol. Chem.* **135**, 733 (1940).
[180] H. B. COLLIER, *Can. J. Research* **18B**, 345 (1940).
[181] H. BLASCHKO, *Biochem. J.* **36**, 571 (1942).
[182] H. SÜLMANN, *Helv. Chim. Acta* **26**, 1114 (1943).
[183] D. KEILIN AND T. MANN, *Nature* **143**, 23 (1939).
[184] R. J. HENRY AND R. D. HOUSEWRIGHT, *J. Biol. Chem.* **167**, 553, 571 (1947).
[185] H. LINEWEAVER AND C. W. MURRAY, *J. Biol. Chem.* **171**, 565 (1947).
[186] C. A. ZITTLE, L. A. WELLS, AND W. G. BATT, *Arch. Biochem.* **13**, 395 (1947).
[187] S. P. COLOWICK, G. T. CORI, AND M. W. SLEIN, *J. Biol. Chem.* **168**, 583 (1947).
[188] W. F. ERWAY, R. K. MEYER, AND W. H. MCSHANN, *Proc. Soc. Exptl. Biol. Med.* **66**, 291 (1947).
[189] E. M. CASE AND F. DICKENS, *Biochem. J.* **42**, 1 (1948); **43**, 481 (1948).
[190] M. E. GREIC, *Arch. Biochem.* **17**, 129 (1948).
[191] D. KEILIN AND E. F. HARTREE, *Biochem. J.* **41**, 503 (1947).
[192] H. KALCKAR, *J. Biol. Chem.* **174**, 771 (1948).
[193] N B. DAS, *Arkiv. Kemi Mineral. Geol.* **13A**, No. 7 (1939).

cose of the phosphorylase synthesizing glycogen from glucose phosphate.[194] Inhibition of glutamine-synthesizing system of *Staphylococcus aureus* by penicillin, crystal violet, and methionine sulfoxide.[195] Inhibition of succinic dehydrogenase by sulfuric acid derivatives[196] and surface-active agents.[197] Inhibition of hyaluronidase by suramin.[198] Suramin as enzyme inhibitor.[199, 200] Inhibitors of ribonuclease by mono- and tetranucleotides,[201] adenylic and guanylic acids,[202] copper, zinc, and iodoacetate,[203] by compounds such as ninhydrin and formaldehyde, attacking the amino group,[204] and by streptomycin.[205] Free amino groups are not essential for pepsin and chymotrypsin, while free tyrosine hydroxy groups are essential.[206-210] Free amino groups are essential for pancreatic lipase.[211]

IV. Influence of Radiations on Enzymes

Under this heading we shall consider only those experiments performed with purified enzymes.

Dale[212] admits that enzyme molecules are not directly affected by ionizing radiations, but indirectly through collision with a labile product resulting from the ionization of water. This "activated solvent" hypothesis was suggested by Risse[213] and developed by Fricke.[214]

According to Weiss[215] the products first formed when water is irradiated with X-rays are the positive ion H_2O^+ and an electron:

$$H_2O \xrightarrow{\text{Radiation}} H_2O^+ + e$$

The reaction $H_2O^+ \rightarrow H^+ + OH$ is highly exothermic and probably occurs soon after ionization. The electron set free at the ionization reacts

[194] C. F. Cori, G. T. Cori, and A. A. Green, *J. Biol. Chem.* **151**, 39 (1943).
[195] W. H. Elliot and E. F. Gale, *Nature* **161**, 129 (1948).
[196] D. Hockenhull, *Nature* **162**, 813 (1948).
[197] D. Hockenhull, *Nature* **162**, 850 (1948).
[198] J. M. Beiler and G. J. Martin, *J. Biol. Chem.* **174**, 31 (1948).
[199] B. W. Town, E. D. Wills, and A. Wormall, *Nature* **163**, 735 (1949).
[200] E. D. Wills and A. Wormall, *Biochem. J.* **44**, 39 (1949).
[201] C. A. Zittle, *J. Biol. Chem.* **160**, 527 (1945).
[202] C. A. Zittle, *J. Biol. Chem.* **162**, 287 (1946).
[203] C. A. Zittle, *J. Biol. Chem.* **163**, 111 (1946).
[204] C. A. Zittle, *J. Franklin Inst.* **246**, 266 (1948).
[205] L. Massart, G. Peeters, and A. Lagrain, *Arch. intern. pharmacodynamie* **76**, 72 (1948).
[206] R. M. Herriott and J. M. Northrop, *J. Gen. Physiol.* **18**, 35 (1934).
[207] R. M. Herriott, *J. Gen. Physiol.* **19**, 283 (1935); **20**, 335 (1936).
[208] J. H. L. Philpot and P. A. Small, *Biochem. J.* **32**, 542 (1938).
[209] I. W. Sizer, *J. Biol. Chem.* **160**, 547 (1945).
[210] C. H. Li, *J. Am. Chem. Soc.* **67**, 1065 (1945).
[211] J. E. Little and M. L. Caldwell, *J. Biol. Chem.* **147**, 229 (1943).
[212] W. M. Dale, *Biochem. J.* **34**, 1387 (1940).
[213] O. Risse, *Ergeb. Physiol.* **30**, 242 (1930).
[214] H. Fricke, *Cold Spring Harbor Symposia Quant. Biol.* **2**, 241 (1934).
[215] J. Weiss, *Nature* **153**, 748 (1944); **157**, 584 (1946).

with water to produce H_2O^-:

$$H_2O + e \longrightarrow H_2O^-$$

Decomposition of the H_2O^- ion gives

$$H_2O^- \longrightarrow H + OH^-$$

Considering another series of possible reactions, it appears that four powerful oxidizing agents are formed: the free radicals OH and O_2H, atomic oxygen, and H_2O_2. These oxidizing agents may react with $-$ SH groups of enzymes according to the following scheme:

$$2RSH + 2OH \rightleftharpoons RS{-}SR + 2OH^- + 2H^+$$

$$2RSH + 2O_2H \rightleftharpoons RS{-}SR + 2H_2O_2$$

$$2RSH + H_2O_2 \rightleftharpoons RS{-}SR + 2H_2O$$

$$2RSH + 2O \rightleftharpoons RS{-}SR + H_2O_2$$

Barron *et al.* [216] tested the hypothesis that when X-rays act on solutes dissolved in water a number of oxidations by the products of the ionization of water, such as oxidation of sulfhydryl groups of enzymes, result. They observed a reversible inhibition of sulfhydryl enzymes on irradiation with X-rays, and a further and irreversible inhibition when the X-ray dose was increased. The sulfhydryl enzymes tested were phosphoglyceraldehyde dehydrogenase, adenosine triphosphatase, and succinic dehydrogenase. The reactivation experiments were made with glutathione. The X-ray dose was kept below 5000 r. The irreversible inhibition observed when the dose is higher than the one causing reversible inhibition is attributed to protein denaturation. The inhibition of the nonsulfhydryl enzymes, trypsin, catalase, and ribonuclease, which requires larger amounts of X-rays than the inhibition of $-$SH enzymes, is also attributed to protein denaturation.

Barron *et al.*[216] thus bring evidence of the fact that inhibition of enzymes by ionizing radiations is due to the indirect action of the products of irradiated water and not to direct ionization of the enzyme through collision with the ionizing radiation.

In another paper Barron and Dickman[217] have examined the influence of α-rays from Po, β-rays from Sr_{89} and γ-rays from Ra on phosphoglyceraldehyde dehydrogenase and urease. After stating that Northrop[218] reported that the inactivation of crystalline pepsin requires large amounts of β- and γ-rays from radium, these authors communicate that they observed

[216] E. S. G. BARRON, S. DICKMAN, J. A. MUNTZ, AND T. P. SINGER, *J. Gen. Physiol* **32**, 595 (1948–1949).

[217] E. S. G. BARRON AND S. DICKMAN, *J. Gen. Physiol.* **32**, 595 (1948–1949).

[218] J. H. NORTHROP, *J. Gen. Physiol.* **17**, 359 (1933–1934).

an inhibition of the two — SH enzymes studied. Partial reactivation of the enzymes by the addition of glutathione was observed after inhibition with α-rays. Evidence that these inhibitions are due to oxidation of the — SH groups of the enzymes is given by the irradiation of the mercury-mercaptide urease with γ-rays. Indeed, this irradiated complex is completely reactivated by glutathione, as is the nonirradiated enzyme.

The authors state also that the ionic efficiency of all these ionizing radiations on the inhibition of phosphoglyceraldehyde dehydrogenase was similar.

CHAPTER 8

Enzymes and Immunology

BY J. R. MARRACK

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I. Introduction

Definitions of antigens and antibodies are difficult to devise satisfactorily. The definitions given by Topley and Wilson[1] run: An *antigen* is any substance which, when introduced parenterally into the animal tissues, stimulates the production of an antibody, and which, when mixed with that antibody, reacts with it in some observable way. An *antibody* is any substance which makes its appearance in the blood serum or body fluids of an animal, in response to the stimulus provided by the parenteral introduction of an antigen into the tissues, and reacts specifically with that antigen in an observable way.

In the first place this is definition in a circle, but no definitions seem to have avoided this objection; the objection is less serious in practice than might appear at first sight. But to conform with modern knowledge and practice the definitions must be extended and modified. The so-called antibody formed in response to injection of antigen may in some cases not react with the antigen in a way that is observable unless very special methods of observation are used; these antibodies, which are called *incomplete* antibodies, combine with antigen, but there the reaction may end. The specific polysaccharides of the pneumococci will act as true antigens when injected into some animals; but it is usual to obtain antibodies to these polysaccharides by injecting whole pneumococci. When the reactions of the polysaccharides with the antibodies produced are studied it is usual to speak of the polysaccharides as antigens. Antibodies may appear in the serum without any previous introduction of antigen. The group-specific agglutinins in human serum, which agglutinate the red corpuscles of incompatible groups, seem to be part of the hereditary makeup of the individual and not an acquired character; but they do not seem to differ essentially from other agglutinins that are antibodies in the sense of the definition.

The essential characteristic of antibodies is that they combine specifically with antigens. This specificity is the feature common to the reactions between antibody and antigen and between enzyme and substrate. The main contribution that immunology can make to the study of enzymes lies in the information that it may give about this specific combination.

[1] W. W. C. Topley and G. S. Wilson, The Principles of Bacteriology and Immunity. Edwin Arnold, London, 1936, p. 137.

II. Reactions between Antibodies and Antigens

A short description of certain aspects of the reactions between antibodies and antigens is needed in order to make the subsequent discussion clear.

1. The Nature of Antibodies

An animal that has been treated with antigen is said to be immunized. A serum which contains an antibody is called an antiserum. It is customary to use whole antisera or the globulin fractions of antisera rather than the isolated antibodies. The antibodies are globulins. They have the electrophoretic mobility of γ-globulin or a mobility between these of β- and γ-globulin. The antibodies in rabbit sera, with one exception, have the same molecular weight (about 160,000) as the major component of the globulin (Kabat,[2] Paič[3]). The antibodies in horse sera that react with proteins have a molecular weight of the same order; those that react with polysaccharides may have a molecular weight of about 900,000, equal to that of a minor component of normal horse serum or may have molecular weights intermediate between 900,000 and that of the main globulin component (Heidelberger and Pedersen,[4] Kabat,[2]).

2. Formation of Precipitates

The simplest reaction between antigen and antibody is the formation of a precipitate when solutions of antigen and antibody are mixed. This reaction lends itself to chemical study, as one reagent, the antigen, and sometimes the antibody, can be used in a pure state and the products of the reaction can be measured. If the antigen is fixed on the surface of particles, these particles run together into clumps; this is called agglutination and the antibody involved is then called an agglutinin. This reaction is strictly comparable to precipitation; the difference lies in the physical state of the antigen; the same antibody can both form a precipitate with an antigen in solution and agglutinate particles coated with antigen.

A point that is of primary importance and has a special interest in relation to anti-enzymes is that these precipitates consist of antibody and antigen. They do not adsorb other proteins; an exception to this rule is the fixation of complement, which is discussed later. The precipitates are very slightly soluble at a temperature near 0°C. The ratio of antigen to antibody in a precipitate depends on the proportions in which antigen and antibody are mixed and not on their concentrations in the mixture. The precipitates are, therefore, highly insoluble compounds of antigen and antibody.

The relation between the amount of antigen added and the amount of

[2] E. A. Kabat, *J. Exptl. Med.* **69**, 103 (1939).
[3] M. Paič, *Bull. soc. chim. biol.* **21**, 412 (1939).
[4] M. Heidelberger and K. O. Pedersen, *J. Exptl. Med.* **65**, 393 (1937).

antibody precipitated and the composition of the precipitate formed, when variable amounts of antigen are added to a constant amount of antibody, is shown by a *precipitation curve* (Fig. 1).

In a zone A to B, which is called the equivalence zone, neither antigen nor antibody can be detected in the supernatant fluid after the precipitate

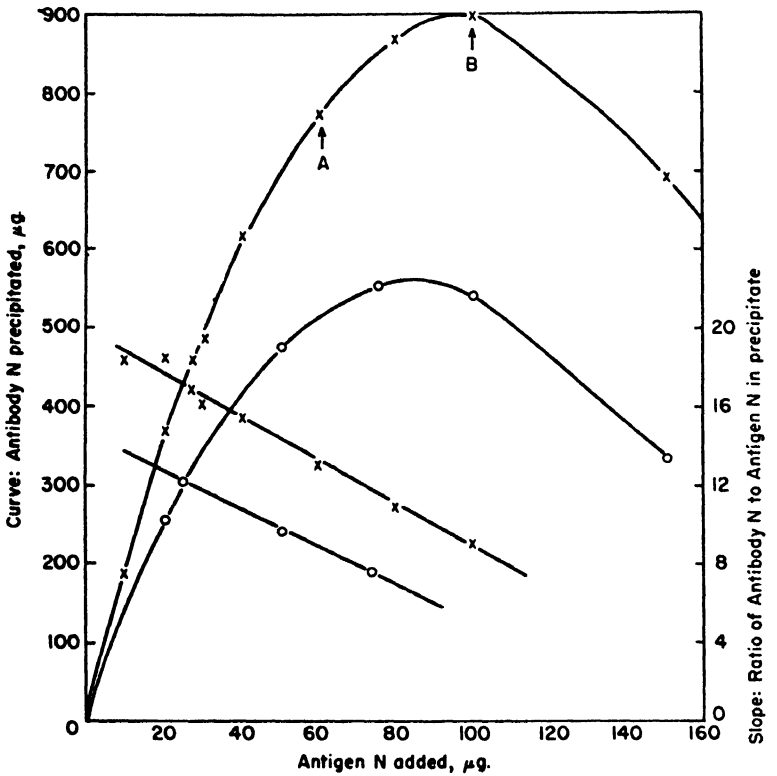


FIG. 1. Amount of antibody N precipitated from a constant amount of anti-hen-egg albumin serum by hen-egg albumin and duck-egg albumin and ratio of antibody to antigen in the precipitates (A. G, Osler and M. Heidelberger; *J. Immunol.* **60**, 327, 1948.)

—X—X—X— Hen-egg albumin
—O—O—O— Duck-egg albumin

has been separated. In the zone OA, the zone of antibody excess, antibody can be detected in the supernatant: in the zone to the right of B, the zone of antigen excess, antigen can be detected in the supernant. As the amount of antigen rises, in the zone of antigen excess, the amount of precipitate gradually falls to zero; both antigen and antibody remain in solution.

The molecular compositions of the precipitates formed by certain pro-

tein antigens of different molecular weights with the corresponding antibodies are given in Table I.

The curve shown in Fig. 1 is the typical curve obtained with rabbit antisera, both with protein and polysaccharide antigens. With horse antisera and polysaccharide antigens similar curves are obtained. The curve (Fig. 2) given by diphtheria toxin and horse serum antibody (diphtheria antitoxin) is typical of the reactions between horse antisera and protein antigens. No precipitate is formed when either antigen or antibody are slightly in excess. It is possible that the difference between the two types of horse antisera depends on the route of immunization and not on the nature of the antigen.

This curve given by diphtheria toxin with the horse antitoxin is of special interest in relation to the work that has been done on the hydrolysis of antitoxin by proteases. Antitoxin is measured in arbitary units. Toxin is

TABLE I

MOLECULAR COMPOSITION OF ANTIGEN-ANTIBODY PRECIPITATES

Antigen	Molecular weight	Composition of precipitate			
		At extreme antibody excess	At A	At B	At extreme antigen excess
Egg albumin	44,000	A_5G	A_3G	A_3G_2	A_2G
Horse serum albumin	70,000	A_6G	A_4G	A_2G	A_2G
Diphtheria toxin	72,000		A_2G	AG	
Thyreoglobulin	630,000	$A_{40}G$	$A_{14}G$	$A_{10}G$	A_2G

A = Antibody
G = Antigen.

measured in Lf (flocculating units); 1 Lf of pure toxin contains 0.46 μg. of N (2.87 μg. of protein). One Lf of toxin is neutralized by approximately one unit of antitoxin.

In Fig. 2 the segment of the curve between A and B is straight and rises by 0.465 μg. for each flocculating unit of toxin added; this is very nearly equal to the N in 1 Lf of toxin. It is inferred that all the antitoxin (300 units) and all the toxin added are precipitated in this range and that 300 units of antitoxin contain 470 to 480 μg. of N, the difference between the N in the precipitate and that in the toxin added; that is, about 1.6 μg. of antibody N per unit. However Pope and Healey[5] think that some of the compound of toxin and antitoxin remains in suspension and that antitoxin contains 60–90,000 units per gram of protein; this estimate gives 2.7 to 1.8 μg. of N per unit.

[5] C. G. Pope and M. Healey, *Brit. J. Expil. Path.* **20**, 213 (1939).

3. Rate of Combination

Antigens and antibodies combine rapidly on mixing. Heidelberger, Treffers and Mayer[6] concluded that combination between egg albumin and antibody was complete in 20 seconds even at 0°C. The combination is reversible even after a precipitate has formed. Antibody can be recovered from the precipitate by mild treatment such as extraction with strong salt solu-

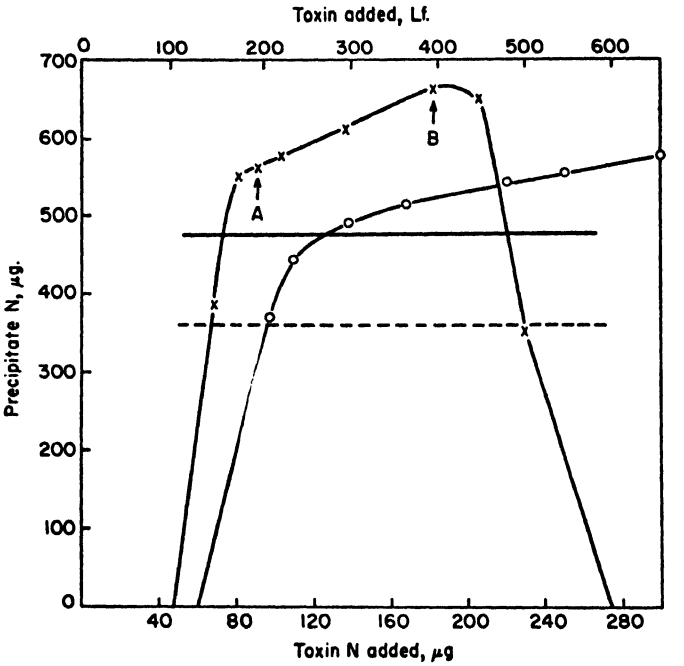


Fig. 2. Amount of precipitate N (micrograms) formed by diphtheria toxin with antitoxic serum (300 units, upper curve) and with digested diphtheria antitoxin (300 units, lower curve).
Unbroken horizontal line: Total undigested antitoxin in mixtures
Broken horizontal line: Total digested antitoxin in mixtures (Data from A. M. Pappenheimer and E. S. Robinson, *J. Immunol.* **32**, 291, 1937, and J. Bourdillon, *Arch. Biochem.* **5**, 385, 1944.)

tion (Heidelberger and Kendall[7]). If, after a precipitate has formed in a mixture of antigen and antibody, a further instalment of antigen or antibody is added, the eventual amount and composition of the precipitate is the same as it would be if the antigen and antibody were mixed in one stage in the final proportions; the final state is reached but slowly.

[6] M. Heidelberger, H. Treffers, and M. Mayer, *J. Exptl. Med.* **70**, 271 (1940).
[7] M. Heidelberger and F. E. Kendall, *ibid.* **61**, 559 (1935).

4. Cross Reactions

A substance B, which can act as an antigen, may form a precipitate with an antiserum that has been made by immunizing an animal with an antigen A, which closely resembles B. Such interactions between similar antigens and the corresponding antisera are known as cross-reactions. The type of precipitation curve usually given by a heterologous antigen is illustrated in Fig. 1. The maximum amount of precipitate formed with the heterologous antigen, duck-egg albumin, is less than that formed with the homologous antigen, hen egg-albumin. The heterologous antigen does not precipitate all the antibody; if hen-egg albumin is added to the supernatant fluid after the maximum precipitate with duck-egg albumin has been separated, a further precipitate of hen-egg albumin and antibody is formed.

The amounts of precipitate formed by antigens with antisera are often expressed semiquantitatively by a number of + signs. Care must be taken in interpreting the results. In the first place the amount of antigen added may not be that which will form the maximum amount of precipitate with the antibody in the serum. In the second place, antisera differ; comparisons should be made between the amounts of precipitate formed by two antigens with the same antisera, not between the amounts of precipitate formed by one antigen with antisera to two different antigens; in Table III the horizontal rows should be compared, not the vertical columns. Also, an antiserum that contains little antibody may form no precipitate with a heterologous antigen; whereas an antiserum that contains more antibody may form a precipitate with this antigen. Some of the antisera made by immunizing with enzymes are weak; the failure of similar enzymes, derived from different species, to form precipitates with such antisera is evidence that the two enzymes are not identical, but it cannot be inferred that they are not immunologically related.

5. Anaphylaxis

If a small amount of antigen is injected into a guinea pig, a second injection, at best some three weeks later, is followed by a condition called anaphylactic shock. The most important features of this state are contraction of the muscles of the bronchioles and a fall of the blood pressure. Other animals, dogs for example, are liable to anaphylactic shock, though the manifestations are different. The shock is due, indirectly, to the reaction of antigen with antibody fixed in the tissues. Like the precipitation reaction, it is specific. A still more specific method was introduced by Dale. The uterus of a sensitized female guinea pig is suspended in a bath of Ringer's solution; on addition of the homologous antigen the uterus contracts. By this method Dakin and Dale[8] were able to demonstrate a dif-

[8] H. D. Dakin and H. H. Dale, *Biochem. J.* **13**, 248 (1919).

ference between the albumins of hens' and ducks' eggs, which they had not distinguished by a qualitative precipitin test and by the anaphylactic reaction of the intact animal. The method is particularly useful in dealing with antigens which do not readily form antibodies. Ten Broeck[9] used this method to distinguish between the trypsin of pigs and cattle and between chymotrypsin and chymotrypsinogen.

6. Foreign Proteins

Proteins of an animal, which differ in composition, differ immunologically. There is no 'species specificity' common to all the proteins of animals of the same species. In the immunological sense a foreign protein is one which will stimulate the formation of antibodies in an animal or sensitize an animal to further injection of the same protein. The sense in which Northrop[10] (p. 57) uses the expression—"the sense that it" (a protein) "is immunologically distinct from the serum proteins"—is unusual.

III. Specific Combination of Antigen and Antibody

1. Relation to Composition and Structure

a. Determinant, Groups

The chief evidence of the specific combination of antigen and antibody is derived from the use of artificial antigens made by attaching some new group to a protein. In most of the work that has been done on these lines the new group was attached by an azo-link; for example *m*-aminobenzene sulfonic acid (D′) can be diazotized and then coupled with a protein, e.g., horse serum albumin (P′)—forming the azo-protein P′D′. Rabbits immunized with P′D′ form precipitates not only with P′D′, but also with similar compounds formed with another protein (P″)—for example, egg albumin—coupled with *m*-aminobenzene sulfonic acid; also with P″ coupled with some closely related compound (D″) such as *o*-aminobenzene sulfonic acid, though in this case the amount of precipitate will be much less; but not with P″ coupled with some less closely related diazo compound (B) such as *p*-aminobenzenearsonic acid (Table II); these reactions are specific. Such groups, that confer a specificity on the proteins to which they are attached are called *determinant groups.*

b. Haptenes

Compounds made by coupling D′ with relatively small molecules such as phenol (T) do not, as a rule form precipitates with antisera to P′D′, but,

[9] C. Ten Broeck, *J. Biol. Chem.* **106**, 724 (1934).
[10] J. H. Northrup, Crystalline Enzymes. Columbia University Press, New York, 1939.

if in sufficient concentration, they inhibit the formation of a precipitate by P″D′ and the antiserum to P′D′; this inhibition, again, is relatively specific. TD″, also, may inhibit, but a higher concentration is needed. The inhibition of formation of a precipitate is an example of competitive inhibition; the TD′ combines with antibody and blocks the approach of P″D′. As the formation of a precipitate has little relation to the nature of the protein P″ the specificity of the reaction lies in the combination of the determinant group D′ with the antibody and not in the formation of a precipitate. Simple compounds TD′ that contain a determinant group are called *haptenes*.

The reactions of these artificial antigens afford an excellent opportunity for the study of the relation of biological specificity to composition and structure. Suitable determinant groups can be chosen to decide questions that may arise and rabbits may be induced to make antibodies to corre-

TABLE II

AMOUNT OF PRECIPITATE FORMED ON ADDITION OF HOMOLOGOUS AND HETEROLOGOUS ANTIGENS TO AN ANTISERUM

(K. Landsteiner and J. van der Scheer, *J. Exptl. Med.* **63**, *325, 1936.*)

Antiserum to *m*-amino benzene sulfonic acid coupled to horse serum protein. Test antigens prepared from chicken serum proteins.

Determinant group of test antigen	Position of acid group in determinant group of test antigen		
	Ortho	Meta	Para
Benzenesulfonic acid	+±	+++	±
Benzenearsonic acid	0	+	0
Benzoic acid	0	+	0

spond; whereas we have to take enzymes as we find them. The relation of specific activity to composition and structure can be demonstrated; (a) by the amount of precipitate formed by a heterologous compound (P″D″) with antiserum to P′D′ (Table II), (b) by the degree of inhibition of the formation of a precipitate by antiserum (anti-P′D′) and homologous antigen (P″D′ by heterologous haptene (TD″) (Table III); or by the concentration of heterologous haptene required to inhibit.

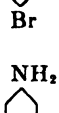
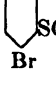
The examples given illustrate the effects of changes in elementary composition (—AsO_3H_2, —SO_3H, —COOH). Changes in the nature of small neutral substituents attached to the benzene ring have little effect; —Cl, —Br and —CH_3 are almost equivalent. Acid groups have a predominant effect. Antisera to antigens in which D′ is *p*-toluidine form precipitates with the compounds P″D″, in which D″ is *p*-chloro- or *p*-bromoaniline or *p*-aminobenzoic acid methyl ester; but not with that in which D″ is *p*-amino-

benzoic acid (Lansteiner and van der Scheer[11]). The immunological difference between optical isomers is illustrated by experiments with antigens formed from tartranilic acid (Landsteiner and van der Scheer[12]). The antiserum to the levo-antigen formed no precipitate with the dextro-antigen, which differs from the homologous antigen in the configuration at both asymmetric C atoms, and a slight precipitate with the meso-antigen, from which it differs in the configuration at one C atom. Similarly the antiserum to the dextro-antigen formed no precipitate with the levo-antigen and a slight precipitate with the meso-antigen.

TABLE III

INHIBITION OF PRECIPITATE FORMATION WITH HOMOLOGOUS TEST ANTIGEN BY HOMOLOGOUS AND HETEROLOGOUS HAPTENES

(J. Jacobs, *J. Gen. Physiol.* **20**, 353, 1937.)

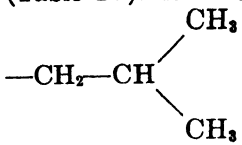
Antiserum to antigens prepared from	Haptenes						Control
							
	F. tr.	+±	0	+±	±	+±	++±
	+±	F. tr.	+±	0	++	±	++++

c. *Polypeptide Determinant Groups*

Experiments that are most important in relation to the specificity of natural proteins are those of Landsteiner and van der Scheer.[13,14,15] In the latest of these they diazotized the *p*-aminobenzoyl compounds of pentapeptides, coupled these with proteins and immunized rabbits with these azo-proteins. The antisera obtained were tested with azo-proteins, similarly made with single amino acids and with di-, tri-, tetra- and pentapeptides attached. Inhibition tests were made with nitrobenzoylated amino acids

[11] K. Landsteiner and J. van der Scheer, *J. Exptl. Med.* **45**, 1045 (1927).
[12] K. Landsteiner and J. van der Scheer, *ibid.* **50**, 407 (1929).
[13] K. Landsteiner and J. van der Scheer, *ibid.* **55**, 781 (1932).
[14] K. Landsteriner and J. van der Scheer, *ibid.* **59**, 769 (1934).
[15] K. Landsteiner and J. van der Scheer, *ibid.* **69**, 705 (1939).

and peptides. The results gave no indication of the presence in the antisera of special antibodies for parts of the peptide molecule; antibodies appeared to be specific for the whole pentapeptide. The specificity depended both on the nature of the amino acids and on order of their sequence (Table IV). It is remarkable, on the one hand, that the difference of a



group at the end of the peptide should have a considerable effect on specificity and, on the other hand that single benzoylated amino acids should inhibit to some extent.

TABLE IV

INHIBITION BY POLYPEPTIDE HAPTENES OF THE FORMATION OF PRECIPITATES BY POLYPEPTIDE ANTIGENS ON ADDITION TO HOMOLOGOUS SERUM

(K. Landsteiner and J. van der Scheer, *J. Exptl. Med.* **69**, 705, 1939.)

Anti-serum	Haptene									
	L	G_2	LG_2	G_3L	G_4L	G_2LG_2	G_5	Glut. G_2	Tyr. G_2	Control
G_4 L	++	−	−	±	T_2	+++	+++±	−	−	+++I
G_5	−	+±	+±		+±	+±	0	++	++	+++
G_2 LG_2	−	++±	++		+++±	0	++±	++±	+++	+++±

Antisera of rabbits immunized with azo-proteins prepared from stromata of red blood corpuscles. Test antigens prepared from chicken serum proteins.

Nitrobenzoylated peptides as haptenes.

G = glycine; L = leucine; Glut. = glutamic acid; Tyr. = tyrosine; I. G_2 = *p*-nitrobenzoyl-leucyl-glycyl-glycine, etc.

Haptenes are less efficient inhibitors when they differ in composition from the determinant group of the antigen used for immunization (Table III). The efficiency of inhibitors is better shown by the relation of the amount of precipitate obtained to the concentration of haptene. Pauling and his colleagues (Pauling *et al.*,[16] Pressman *et al.*,[17] Pauling *et al.*[18]) have calculated the relative bond-strength constants for the haptene-antibody bond. Even if the various assumptions and approximations involved in these calculations are justified the quantitative results cannot be accepted, as no allowance has been made for the binding of the haptenes by the albumin of the sera used; but the results illustrated the variation of efficiency with configuration and composition. These, like those of Erlenmeyer and Ber-

[16] L. Pauling, D. Pressman, D. H. Campbell, and C. Ikeda, *J. Am. Chem. Soc.* **64**, 3003 (1942).

[17] D. Pressman, D. H. Brown, and L. Pauling, *ibid.* **64**, 3015 (1942).

[18] L. Pauling, D. Pressman, and A. L. Grossberg, *ibid.* **66**, 784 (1944).

ger,[19] show that the efficiency of benzene-acid haptenes is increased when the H atom at the site of attachment of the —N=N— group in the homologous azo-protein antigen is replaced by some larger atom or group. For example tyrosine-azo-benzene sulfonic acid is a more effective inhibitor than benzene sulfonic acid. The area of the determinant group that combines with antibody is not therefore limited to the acid group, nor to the benzene ring. The superiority of the larger haptenes may be greater than appears. For the albumin of the antisera used probably combines with a higher proportion of the larger than of the smaller haptenes (cf. Klotz[20]) and, consequently, less of the larger haptene is free to combine with antibody and inhibit precipitation.

d. Comparison with Growth Factors

The method of attaching determinant groups to proteins used in most of these experiments involved the use of substituted anilines. The type of compound used in the studies was, as a rule, unrelated to the substrates of enzymes. However the effects of changes on composition and structure on the specificity of these compounds are like the effects on the activity of growth factors which are essential parts of coenzymes; examples are given in Table V. The inhibition of precipitation by haptenes, in particular, recalls inhibitions such as the inhibition of growth by pyridine-3-sulfonic acid which is reversed by nicotinic acid.

e. Carbohydrate Determinant Groups

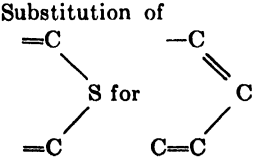
The importance of the bacterial polysaccharides in relation to immunity led to the use of carbohydrate derivatives as artificial antigens. The specificity of the reactions of these antigens with antibodies is, in some respects, comparable to the specificity of the reactions of enzymes with carbohydrate substrates. The method used was to attach the carbohydrate by a glucoside link to *p*-nitrophenol; to reduce the nitro group, diazotize and couple to a protein. A monosaccharide S_1 or a disaccharide S_1—S_2 might be used (S_2 being attached to the phenol). The antigens used differed in four particulars: (1) the α or β form at the attachment of the terminal monosaccharide S_1 to S_2 or phenol; (2) the configuration at C atoms other than No. 1; (3) the use of a mono- or disaccharide; (4) the point of attachment of the S_1—S_2 link. The work of Avery, Goebel, and Babers[21] and Goebel, Avery, and Babers[22] show the influence of these differences on specificity by methods (a) and (b) above: for example, antiserum to β-glucoside antigen forms more precipitate with the β-glucoside compound than with the

[19] H. Erlenmeyer and E. Berger, *Biochem. Z.* **262**, 196 (1933).
[20] I. M. Klotz, *J. Am. Chem. Soc.* **68**, 2299 (1946).
[21] O. T. Avery, W. F. Goebel, and F. H. Babers, *J. Exptl. Med.* **55**, 769 (1932).
[22] W. F. Goebel, O. T. Avery, and F. H. Babers, *ibid.* **60**, 559 (1934).

α-glucoside compound (difference 1) or with the β-cellobioside and β-gentiobioside compounds (difference 3) and none with the β-maltoside com-

TABLE V

EFFECTS OF MODIFICATIONS OF ANTIGENS ON IMMUNOLOGICAL REACTIONS AND OF MODIFICATIONS OF GROWTH FACTORS ON THEIR EFFICIENCY

Modification	Immunological	Growth
Change of position of —COOH group	*o*-Benzoic acid antigen with *m*-benzoic acid antiserum, no precipitate.[a]	Picolinic acid does not replace nicotinic acid for bacteria (p. 123).[b]
Change of position of $-CH_3$ group	*o*-Toluidine and *m*-toluidine antigens with *p*-toluidine antiserum, less precipitate.[c]	Transfer from C2 to C6 in pyridine ring of thiamine (isothiamine) 1/10,000 efficiency (p. 130).[b]
Substitution of —Cl for $-CH_3$	*p*-Chloroaniline and *p*-toluidine equivalent.[c]	Dichloroflavin inhibits riboflavin (p. 218).[b]
Substitution of CH_3 for —H	Toluidine antigens with aniline antiserum, less precipitate.[c]	Substitution at C2 in thiazole ring inactivates thiamine for *Staph. aureus* (p. 129).[b]
Substitution of $-SO_3H$ for —COOH	*m*-Sulfonic acid antigen with *m*-benzoic acid antiserum, much less precipitate (Table II).[d]	Pyridine-3-sulfonic acid inhibits nicotinic acid (p. 215).[b]
Substitution of 	Homologous antigen more precipitate than heterologous.[e]	Pyrithiamin inhibits growth (p. 219).[b]
Substitution of O for S	Homologous antigen more precipitate than heterologous.[e]	Oxybiotin can replace biotin for yeasts and *L. casei* (p. 143).[b]

[a] K. Landsteiner, The Specificity of Serological Reactions. C. C. Thomas, Springfield, Illinois, 1936, p 106.

[b] Page numbers refer to T. S. Work and B. Work, The Basis of Chemotherapy. Oliver and Boyd, London, 1948.

[c] K. Landsteiner and J. van der Scheer, *J. Exptl. Med.* **45**, 1045 (1927).

[d] K. Landsteiner and J. van der Scheer, *J. Exptl. Med.* **63**, 325 (1936).

[e] B. Berger and E. Erlenmeyer, *Helv. Chim. Acta* **16**, 731 (1933).

pound (difference 1 and 3); and no precipitate with the β-galactoside (difference 2) or β-lactoside antigen (difference 2 and 3). The antiserum to the

β-cellobioside antigen forms more precipitate with the β-cellobioside antigen than with the β-gentiobioside compound (difference 4). These immunological specificities resemble the specificities of enzymes. Thus α and β-glucosidases are specific (difference 1); glucosidases do not split galactosides (difference 2).

f. Bacterial Polysaccharides

The structure of some of the bacterial polysaccharides is known well enough for specificity to be related to it. Thus the polysaccharide of Type III pneumococci has the structure —(Gn—Gl)—(Gn—Gl)—(Gn—Gl)— where Gn is a glucose residue and Gl a glucuronic acid residue (Hotchkiss and Goebel[23]); Type VIII polysaccharide appears to have the structure.

—(Gn—Gl)—Gl—Gl—(Gn—Gl)—Gl—Gl—(Gn—Gl)—

About one-third of the antibody in a horse antiserum to Type VIII pneumococci and less of the antibody in rabbit antiserum is precipitated by Type III polysaccharide (Heidelberger, Kendall and Mayer[24]). The polysaccharide of Type II pneumococci also contains glucuronic acid; that of Type I pneumococci contains galacturonic acid. The horse antisera to both Type II and Type III pneumococci form precipitates with certain gums that contain glucuronic acid and with synthetic compounds containing glucuronic acid; Type I antiserum does not form precipitates with these gums and synthetic compounds. The formation of such precipitates with Type II and Type III antisera is inhibited by glucuronic acid but not by galacturonic or mannuronic acid (Goebel,[25] Woolf, Marrack and Downie,[26] Marrack[27]). The acid group of the uronic acid is essential, for synthetic compounds containing glucose in place of glucuronic acid are not precipitated by Type III antisera; glucose will not inhibit; the methyl ester of Type I polysaccharide does not form a precipitate with Type I antiserum.

Although the correct position of the —OH group at the C 4 atom is essential, the —OH, as such, seems to be of less importance in relation to the specificity of Type III polysaccharide than in enzyme reactions. Methylated Type III polysaccharide forms a precipitate with Type III antiserum, though this is considerably less than that formed by the untreated polysaccharide (Heidelberger and Kendall[7]); the introduction of methyl groups into an —OH and a —NH_2 group of Type I polysaccharide does not prevent precipitation with Type I antiserum (Chow and Goebel[28]). However, dimethyl-methylglucuronide does not inhibit the formation of a pre-

[23] R. D. Hotchkiss and W. F. Goebel, *J. Biol. Chem.* **121**, 495 (1937).
[24] M. Heidelberger, E. A. Kabat, and M. Mayer, *J. Exptl. Med.* **75**, 35 (1942).
[25] W. F. Goebel, *J. Bact.* **31**, 66 (1936).
[26] B. Woolf, J. R. Marrack, and A. W. Downie, *J. Soc. Chem. Ind.* **55**, 156 (1936).
[27] J. R. Marrack, *Proc. Intern. Congr. Microbiol.* p. 425 (1937).
[28] B. F. Chow and W. F. Goebel, *J. Exptl. Med.* **62**, 179 (1935).

cipitate by gums (containing glucuronic acid) with Type II antipneumococcal serum whereas glucuronides do inhibit (Marrack[27]).

g. Natural Proteins

The immunological specificity of natural proteins is much more remarkable than that of the polysaccharides and more difficult to explain. Antisera to ox serum albumin form little or no precipitate with human serum albumin. These two proteins differ very little in physical properties and in composition (see Edsall[29]). As the immunological resemblance gets less the chemical or physical differences become greater. Turkey egg albumin forms a considerable precipitate with antiserum to hen egg-albumin; the electrophoretic mobilities of the two albumins at pH 5.2 are nearly the same (Landsteiner, Longsworth, and van der Scheer[30]). The mobility of duck egg albumin, which forms less precipitate with anti-hen-albumin serum, is definitely higher.

h. Differences between Antibodies

Moreover, no constant differences have been found between antibodies and other serum globulins, or between antibody to one antigen and antibody to another, that will account for their specific activities. In horse sera the molecular weights of some of the antibodies are in the neighborhood of 170,000 while the molecular weights of others are heavier. Here there is some relation to the nature of the antigen, for the antibodies to bacterial polysaccharides fall in the second group and antibodies proteins in the first group. But both groups include antibodies to diverse antigens. In rabbit sera all the known molecular weights of antibodies, with one exception (hemolysins, Paič[3]) are of the same order.

Antibodies, being globulins, will act as antigens; if antibodies formed in an animal of one species are injected into an animal of another species they will give rise to antisera containing anti-antibodies. It might be expected that the characteristics that confer their specific properties as antibodies on globulin molecules would also confer antigenic differences. However, the quantitative experiments of Treffers and Heidelberger[30a] show that antisera, formed by immunizing rabbits with horse antibodies to the pneumococcal polysaccharides, will distinguish between the horse antibodies to protein antigens (egg albumin and diphtheria toxin) and antibodies to bacterial polysaccharides; but these antisera will not distinguish between antibodies to the different polysaccharides of pneumococcus Type I, Type II, Type VIII and of *H. influenzae*. Antisera formed in chickens will not distinguish between rabbit antibodies to pneumococcal polysaccharides

[29] J. T. Edsall, *Advances in Protein Chem.* **3**, 465 (1947).
[30] K. Landsteiner, L. G. Longsworth, and J. van der Scheer, *Science* **88**, 83 (1938).
[30a] H. P. Treffers and M. Heidelberger, *J. Exptl. Med.* **73**, 125 (1941).

and rabbit antibodies to egg albumin (Treffers and Heidelberger[31]). The antibodies to diverse antigens are no better distinguished by their antigenic specificity than they are by differences of molecular weight.

2. Effects of Modification of Amino and Tyrosine Groups of Antigens, antibodies, and Enzymes

Antibodies and antigens have been treated with various reagents with the object of finding out what groups are involved in the specific combination rather than of introducing a new determinant group, as in the examples already given. The group attached should not be so large that its bulk will prevent the approach to other parts of the molecule. A treated antibody may combine with antigen but fail to precipitate or agglutinate it. Such an antibody will inhibit or delay precipitation or agglutination by untreated antibodies, and, if a particulate antigen is used, will change the surface properties of the antigen; it may also protect animals against the homologous toxin or virulent bacteria. A treated antigen, that combines with antibody, but is not precipitated, will inhibit precipitation or agglutination by untreated antibody. Unfortunately, this distinction between failure to combine and failure to agglutinate or precipitate has not always been made. It is also possible that aggregation of serum albumin with the globulin (which contains the antibodies), such as is found when sera are heated (Kleckowski[32]) or photoirradiated (Smetana and Shemin[33]) may follow treatment with the reagents used; such a change may lead to loss of ability to precipitate while ability to combine is not affected.

a. Amino Groups

The most important studies have used reagents that affect the free amino groups and the phenol group of tyrosine. Formaldehyde combines with the primary amino groups and affects other groups when higher concentrations are used and the action is prolonged. Antibodies treated with formaldehyde, neutralize toxins as well or nearly as well as untreated antitoxins (Eisler and Löwenstein[34]) but do not form precipitates with the toxins (Eagle[35]). Mudd and Joffe[36] showed that agglutinins, treated with formaldehyde combined with the antigens as they inhibited agglutination by untreated serum and altered the surface changes of the antigens. Chow and Goebel[28] found that purified antibody to Type I pneumococci, treated with formalde-

[31] H. P. Treffers and M. Heidelberger, *ibid.* **73**, 293 (1941).
[32] A. Kleckowski, *Brit. J. Exptl. Path.* **22**, 192 (1941).
[33] H. Smetana and D. Shemin, *J. Exptl. Med.* **73**, 223 (1941).
[34] M. von Eisler and E. Lowenstein, *Zentr. Bakt. Parasitenk. Abt. I. Orig.* **261**, 63 (1912).
[35] H. Eagle, *J. Exptl. Med.* **67**, 495 (1938).
[36] S. Mudd and E. W. Joffe, *J. Gen. Physiol.* **16**, 947 (1933).

hyde, did not form precipitates with Type I polysaccharide. Eagle[35] did not find that formaldehyde abolished the ability of antibody to precipitate when purified antibody was used, but Heidelberger, Grabar, and Treffers[37] confirmed the results of Chow and Goebel and concluded that Eagle treated the antibody at a hydrogen ion too high for the formaldehyde to attack the amino groups. Chow and Goebel suggest that the combination of antibody and polysaccharide antigen, the specificity of which is determined by an acid group, is due to the attraction between the —COOH groups of the polysaccharide, and suitably spaced —NH_2 groups of the antibody. However, Eagle[35] found that the antibodies of whole sera treated with formaldehyde still combined with the polysaccharide.

Ketene had a vogue as a reagent that combines with amino groups. Chow and Goebel[28] found that purified Type I antipneumococcal antibody, which had been treated with ketene and contained one acetyl group for every primary amino group present in the original molecule, would not form a precipitate with the Type I polysaccharide. Goldie and Sandor[38] found that diphtheria antitoxin no longer flocculated with diphtheria toxin when 17 to 19% of the amino groups had been acetylated, but still neutralized (and therefore combined with) toxin when over 25% of the amino groups were acetylated. It appears that the amino groups of antibodies, with the possible exception of antibodies to acid antigens, are not involved in combination with antigens. The amino groups of antigens seem to play an even less important part in the specific reactions of protein antigens. Kleckowski[39] found that horse serum globulin and egg albumin treated with phenylisocyanate precipitated all the antibody from antiserum to the untreated proteins. In the globulin the free amino groups, estimated with nitrous acid, has been reduced 61% with formaldehyde and 60% with phenylisocyanate; in the egg albumin by 47 and 70%, respectively. However, Haurowitz, Sarafian, and Schwerin[40] found that when about 100 acetyl groups per molecule were introduced into horse serum pseudoglobulin the product would no longer form a precipitate with antiserum.

Pappenheimer[41] found that the number of free amino groups blocked when purified diphtheria toxin was treated with formaldehyde in low concentration or with ketene for a short time corresponded closely with the number of ϵ-amino groups of lysine; the number of flocculating units was not affected by this treatment. Further treatment with ketene acetylated the —OH groups of tyrosine; the treated toxin then no longer flocculated with antitoxin.

[37] M. Heidelberger, P. Grabar, and H. P. Treffers, *J. Exptl. Med.* **68**, 913 (1938).
[38] H. Goldie and G. Sandor, *Compt. rend. soc. biol.* **128**, 974 (1938).
[39] A. Kleckowski, *Brit. J. Exptl. Path.* **21**, 1 (1940).
[40] F. Haurowitz, K. Sarafian, and P. Schwerin, *J. Immunol.* **40**, 391 (1941).
[41] A. M. Pappenheimer, Jr., *J. Biol. Chem.* **125**, 201 (1938).

b. Diphtheria Toxoid

In the customary method of converting toxin to toxoid, which is non-toxic, the time of exposure to formaldehyde is much longer than in the usual treatment of proteins by which the groups affected are mainly the amino groups. Recently Woiwod and Lingwood[42] separated by two-dimensional paper chromatography the amino acids of such toxoid hydrolyzed with acid, and found that tyrosine was absent.[42a] Holden and Freeman[43] and Brown[44] have shown that formaldehyde reacts with tyrosine to give a product that has no amino groups; this product resists acid hydrolysis. In spite of the absence of tyrosine such toxoid flocculates with antitoxin and gives rise to antitoxin on injection.

The number of flocculating units of diphtheria toxin treated with formaldehyde or after acetylation of 33 to 54% of its amino groups with ketene is the same as that of the untreated toxin (Goldie[45]).

c. Tyrosine Groups

Treatment of antigens with iodine has more effect on their reactions with antibodies. Kleckowski[46] found iodination of horse serum globulin in alkaline solution, which reduced the color value with Folin's phenol reagent by about 70% led to complete loss of ability to form a precipitate with antiserum to native horse serum globulin. When 30% of the color value was lost, after treatment with iodine in neutral solution, the globulin formed a heavy precipitate with antiserum to native globulin. Haurowitz and his colleagues[40] also found that the amount of precipitate formed by iodinated globulin with antiserum was reduced when the amount of iodine rose to 5.48% or 80 iodine atoms per molecule and a trace only of precipitate was formed when the amount of iodine rose to 238 atoms per molecule. To iodinate all the tyrosine 144 iodine atoms per molecule would be needed. When proteins are treated with iodine H atoms of the tyrosine residues are replaced by iodine. As Folin's reagent also reacts with tryptophan, all the tyrosine residues may have been iodinated in Kleckowski's globulin that retained 30% of its Folin color value.

d. Comparison with Modification of Enzymes

These effects of treatment of antigens and antibodies on their specific reactions may be compared with similar treatment of enzymes. Herriott and Northrop[47] first found that acetylation of the amino groups of pepsin

[42] A. J. Woiwod and F. V. Lingwood, *Nature*, **163**, 218 (1949).
[42a] The compound formed with lysine would be broken down in acid.
[43] H. F. Holden and M. Freeman, *Australian J. Exptl. Biol. Med. Sci.* **8**, 189 (1931).
[44] A. E. Brown, *J. Am. Chem. Soc.* **68**, 1011 (1946).
[45] H. Goldie, *Compt. rend. soc. biol.* **126**, 974 (1937).
[46] A. Kleckowski, *Brit. J. Exptl. Path.* **21**, 98 (1940).
[47] R. M. Herriott and J. H. Northrop, *J. Gen. Physiol.* **18**, 35 (1934).

did not reduce its activity; later Herriott[48] found that acetylation of the two to three tyrosine residues in the enzyme molecule led to loss of 40 to 50% of its activity. Hollander[49] also found that acetylation of the amino groups did not reduce the activity of pepsin. The activity of chymotrypsin also is not affected when the amino groups are blocked by treatment with formaldehyde or ketene (Sizer[50]); but this treatment destroys the activity of pancreatic amylase (Little and Caldwell[51]). Pepsin is almost completely inactivated when 35 to 40 iodine atoms are introduced per molecule (Herriott[52]); not less than 80% of the iodine is attached to tyrosine residues.

Apart from the suggestion that NH_2 groups of antibodies may be involved in the combination with acid antigens and that the tyrosine groups of some antigens and antibodies may be essential, these studies give no indication that any of the amino acids are specially involved in the combination of antigens and antibodies. On the other hand the great similarity in the amino acid contents of antigens and of antibodies that have different immunological specificities and the effect of the order of arrangement on the specificity of antigens containing peptides as determinant groups, quoted above (Table IV), are strong arguments in favor of the theory that the ability to combine specifically with antigen and antibody, respectively, depends on the space pattern of the constituent amino acids. The specificity of the patterns may be due to slight variations in the numbers of residues of different amino acids in these proteins, to variations in the order of arrangement of these residues in peptide chains, or to variation in the coiling of the peptide chains in the molecules.

3. Bergmann's Theory of Binding Faces

Bergmann[53] suggested, as an explanation of the failure of dipeptidase to split peptides containing dextro isomers of the amino acids, that this enzyme contains more than two different atoms or groups of atoms, fixed in space with regard to one another; these groups enter, during catalysis, into opposition with a similar number of atoms or atomic groups on the substrate. These atoms or atomic groups are arranged in what Bergmann calls the 'binding plane' which must be approached from the correct side; otherwise the spatial distribution of the atoms or atomic groups on enzyme and substrate, that are brought into opposition, will not correspond—hence the need to postulate more than two atoms or atomic groups. When one of the amino acids of a dipeptide is a dextro isomer, D-leucine for example, the hydrocarbon chain sticks up above the binding plane, obstructs

[48] R. M. Herriott, *ibid.* **19**, 283 (1935).
[49] V. Hollander, *Proc. Soc. Exptl. Biol. Med.* **53**, 179 (1943).
[50] I. W. Sizer, *J. Biol. Chem.* **160**, 547 (1945).
[51] J. E. Little and M. L. Caldwell, *ibid.* **147**, 229 (1943).
[52] R. M. Herriott, *J. Gen. Physiol.* **20**, 335 (1936–37).
[53] M. Bergmann, *Harvey Lectures* **31**, 37 (1936).

the approach of the enzyme and prevents catalysis. The essence of this theory is that specific combination depends on a pattern in space of both enzyme and substrate. A similar theory must be invoked to explain the specific combination of antibody with antigen or haptene. No other theory will account for the specific difference between the optical isomers of tartranilic acid or between the peptide and the carbohydrate antigens already cited. It will explain the specific differences between natural protein antigens and between antibodies that differ little in composition.

4. Forces Involved in Specific Combination

In deciding what forces are involved in the specific combination of antigens and antibodies the following features must be taken into account:

1. The patterns in space of antigen and antibody determine their specificity.
2. Combination is rapid at temperatures below 40°C.
3. Combination is reversible.
4. Determinant groups that differ in their atomic composition may combine with the same antibody.

The rapidity of combination at relatively low temperatures, the ease of reversibility and the indifference to the atomic composition are incompatible with the formation of covalent bonds.

As their combination is determined by spatial arrangement the molecules must be held together by short range forces; long range forces would hold molecules together even when their fit was not exact. Pauling, Campbell, and Pressman[54] consider that, since electrostatic interactions are very much smaller in water than in a medium of low dielectric constant, electric forces are of major importance only when an isolated or essentially isolated electric charge is involved. The ionized groups of artificial antigens and of the specific polysaccharides contribute to the strength of combination of these antigens with the corresponding antibodies; so may attractions between ionized groups of natural protein antigens and suitably spaced ionized groups of antibodies. But the attraction between these groups alone would not account for the strength of the specific combination. Other forces that are involved are van der Waals attractions, which operate between every pair of molecules, and hydrogen bonds between two electronegative atoms, which in most of the antigens and antibodies must be oxygen and nitrogen. These forces are not specific and are weak; but two molecules will be firmly bound together if these forces can operate between a large number of their constituent atoms. "A molecule would hence show strong attraction for another molecule which possessed complete complementariness in surface configuration and distribution of active electrically charged and hydrogen-bond forming groups, somewhat weaker attraction

[54] L. Pauling, D. H. Campbell, and D. Pressman, *Physiol. Revs.* **23**, 203 (1943).

for molecules with approximate but not complete complementariness to it, and only very weak attraction for other molecules" (Pauling, Campbell, and Pressman[54]).

IV. Effects of Disorganization of Molecules of Antigens and Antibodies

If the specificities of protein antigens and of antibodies are due to specific arrangements of amino acids in the peptide chains it might be possible to find fractions, after hydrolysis with enzymes or with acids, which contained relatively few amino acids but still combined with antibody or antigen. On the other hand if the specific pattern that insures combination is due to the mode of coiling of the peptide chains in the molecule it might be expected that specificity would be lost in the early stages of hydrolysis and when the structures of the molecules were altered by denaturation or by spreading in thin films.

1. Hydrolysis of Antigens and Antibodies

Peterman[55] has suggested that there may be two types of digestion of proteins by enzymes.

"*All or none.*" The protein molecules are broken down rapidly to small dialyzable fragments; the large molecules that remain are unchanged. Tiselius and Ericksson-Quensel[56] found that pepsin digested egg albumin in this way.

"*Piece-meal.*" Light dialyzable fragments are split off, leaving heavy particles, but no unchanged protein. This type was found by Annetts[57] when egg albumin was digested by papain.

a. Antigens

Tiselius and Ericksson-Quensel[56] found that pepsin breaks egg albumin down to fractions of molecular weight about 1,000. Even when most of the albumin was broken down the remainder was almost unchanged; its electrophoretic mobility and sedimentation and diffusion velocities were nearly the same as those of native egg albumin. It formed a precipitate with antiserum to egg albumin. This large fraction may have been the same as or similar to the plakalbumin of Linderstrøm-Lang and Ottesen[58] (also Eeg-Larsen, Linderstrøm-Lang and Ottesen[59]). This is formed by the action of a protease *B. subtilis*. About 6 or 7 amino acids are split off per molecule of albumin. The molecular weight estimated from the sedimentation velocity and osmotic pressure is very little below that of native egg albumin. The amino acids split off are glycine, alanine, valine, aspartic

[55] M. Peterman, *J. Phys. Chem.* **46**, 183 (1942).
[56] A. Tiselius and I. Ericksson-Quensel, *Biochem. J.* **33**, 1752 (1939).
[57] M. Annetts, *ibid.* **30**, 1807 (1936).
[58] K. Linderstrøm-Lang and M. Ottesen, *Nature* **159**, 807 (1947).
[59] N. Eig-Larsen, K. Linderstrøm-Lang, and M. Ottesen, *Arch. Biochem.* **19**, 340 (1948).

and glutamic acids. Kaminska and Grabar[60] found that plakalbumin gave a precipitation curve with antiserum to native hen-egg albumin similar to that given by duck albumin (Fig. 1), with this antiserum. The maximum amount of precipitate formed by plakalbumin was, however, about $\frac{1}{8}$ of the maximum amount formed by native albumin with the same serum.

Further stages of digestion were studied by Holiday[61] and Kleckowski.[62] Holiday digested highly purified crystalline horse serum albumin with pepsin at pH 2.1. In 5 minutes the split product was homogeneous in the ultracentrifuge ($S_{20} = 1.95 \times 10^{-13}$), but two components with different electrophoretic mobilities were found; less than 10% of the nitrogen was dialyzable and the dialyzable fraction contained over 90 % of the tryptophan of the albumin. At this stage the digest formed rather less precipitate with an antiserum to the serum albumin than was formed by the undigested albumin. After 30 minutes digestion the split product was again homogeneous in the ultracentrifuge ($S_{20} = 1.14 \times 10^{-13}$); two components with different mobilities were present. This 30 minute digest formed no precipitate with antiserum to horse serum albumin and partially inhibited the formation of a precipitate by undigested albumin with this antiserum. Holiday considered that the 5 minute digest contained molecules of one-fourth and the 30 minute digest molecules of one-eighth the weight of the original albumin molecules. Kleckowski found that after 5 minutes digestion of human and horse albumin with pepsin at room temperature and pH 2.6, and after longer periods at higher pH, the digest no longer formed a precipitate with antiserum but inhibited precipitation by undigested albumin and antiserum. His results do not conflict with those of Holiday, as Holiday used undiluted antiserum in the tests, while Kleckowski used antiserum diluted 1/30 or 1/35; with antiserum diluted 1/10 Kleckowski's 5 minute digests formed some precipitate, though the precipitate was smaller and formed more slowly than that formed by undigested albumin. However, Kleckowski found that after 5 minutes digestion at pH 2.6 the digest contained a fraction whose diffusion coefficient did not differ appreciably from that of undigested albumin. Even after long digestion, when the digest apparently did not react with antiserum, it was still possible, by precipitation with trichloracetic acid or ammonium sulfate, to concentrate a material that inhibited.

Kleckowski considered that all the products still able to combine with antibodies were probably contained in the material that was precipitated by 2% trichloracetic acid or by two-thirds saturation with ammonium sulfate. He based this conclusion on the inhibiting titer of material, recovered from the digest by precipitation with trichloracetic acid, resolu-

[60] M. Kaminska and P. Grabar, *Bull. Soc. Chim. Biol.* **21**, 684 (1949).
[61] E. R. Holiday, *Proc. Roy. Soc. London* **B127**, 40 (1939).
[62] A. Kleckowski, *Brit. J. Exptl. Path.* **26**, 24 (1945).

tion, and reprecipitation with ammonium sulfate. This conclusion, if correct, would rule out inhibition by peptides containing relatively few amino acids. However, Landsteiner and Chase[63] got evidence of inhibition by smaller fractions. They digested serum proteins with pepsin for 3 hours and precipitated heteroalbumose with 33% of alcohol or half saturation with ammonium sulfate. Antisera made by immunizing rabbits with heteroalbumose formed precipitates with heteroalbumose. Precipitation was inhibited by fractions that would pass through a collodion membrane. Landsteiner[64] also found that peptides containing 8 to 12 amino acids, made by hydrolysis with acid would inhibit precipitation by a product of silk and the corresponding antiserum. However, in this case the specific pattern of the amino acids cannot have been formed by coiling of the peptide chains in the antigen molecule, since the peptide chains of silk are not coiled.

The effect of digestion on the reactions of globulins as antigens has been studied mainly in connection with the preparation of digested diphtheria antitoxin for therapeutic purposes, which is fully discussed later. The fraction of the antibody molecule obtained in this way appears to have about half the molecular weight of the original antibody and to be formed by splitting along a plane at right angles to the long axis of the molecules. Weil, Parfentjev, and Bowman[65] immunized a rabbit with the globulin of horse diphtheria antitoxin serum. They found that the digested antitoxin formed very much less precipitate with this antiserum than was formed by the native globulin. This reaction was not due to the presence of traces of unaltered globulin in the digested product, for the maximum precipitate formed with very small amounts; if the formation of the precipitated had been due to such traces relatively large amounts would have been needed. Northrop's[66] crystalline product, obtained by digestion with trypsin, did not form a precipitate with antiserum to horse serum globulin. The digested antibody did not shock guinea pigs sensitized with undigested horse serum; but guinea pigs sensitized with digested antibody were shocked by undigested serum. Coghill and colleagues[67] found similar results with antitoxin digested with the protease of taka-diatase. Kass, Scherago, and Weaver[68] found that guinea pigs sensitized with digested antitoxin were shocked by digested antitoxin if the dose were sufficiently large. As judged by test with anaphylaxis, digested antibodies were closely allied, whether prepared with *Aspergillus oryzae* diastase, malt-diastase, or pepsin.

[63] K. Landsteiner and M. W. Chase, *Proc. Soc. Exptl. Biol. Med.* **30**, 1413 (1932–1933).
[64] K. Landsteiner, *J. Exptl. Med.* **75**, 269 (1942).
[65] A. J. Weil, I. A. Parfentjev, and K. L. Bowman, *J. Immunol.* **35**, 399 (1938).
[66] J. H. Northrup, *J. Gen. Physiol.* **25**, 465 (1942).
[67] R. D. Coghill, N. Fell, M. Creighton, and G. Brown, *J. Immunol.* **39**, 207 (1940).
[68] E. H. Kass, M. Scherago, and R. H. Weaver, *J. Immunol.* **45**, 87 (1942).

It seems, therefore, that most of the determinant groups of the antitoxin globulins, of which there must be at least 5 per molecule, are lost with the non-antitoxin half of the molecule, that is removed by this process of digestion and of separation of the antitoxic half. It seems that the antitoxic receptor groups are confined to one half of the molecule and the determinant groups to the other half. On the other hand, Treffers and Heidelberger[30] found that horse antibody to the specific polysaccharide of Type I pneumococci retained its determinant groups after digestion with pepsin; this antibody also is split into molecules of molecular weight about 100,000 (Peterman and Pappenheimer[69]).

b. Antibodies

Most of the investigations into the digestion of antibodies have been made with the object of getting an active product, as free as possible from inert protein, that will not cause unpleasant reactions when injected. Such investigations could, however, tell us much about the structure of proteins and about the active part of antibodies. For an antibody fraction that is precipitated by antigen can be picked out in the precipitate. As the terminal amino acids of the antigen, if it is a protein, can be determined separately and as the amount of antigen in the precipitate is less than the amount of antibody the terminal amino acids of the antibody could be determined by the method of Sanger.[70]

It has been claimed by many investigators that antibodies were more resistant to digestion by proteases than other proteins were. Rosenheim[71] found that O-agglutinins to *B. typhosus* in horse serum were rapidly destroyed by pepsin and trypsin. The H-agglutinins after a first course of immunization also were rapidly destroyed; those formed after several courses of immunization were not appreciably destroyed under similar conditions. This difference in susceptibility to proteases may be related to other properties. Deutsch and colleagues[72] found that the mobility of the γ-globulin of human serum that contains the O-agglutinins to *B. typhosus* is higher than that of the fraction that contains the H-agglutinins. As immunization of horses with diphtheria toxin proceeds the proportion of diphtheria antitoxin in a fraction that has a mobility higher than that of ordinary horse γ-globulin rises (Kekwick and Record[73]). When sera containing the O-type of agglutinins are heated at 75°C. for 10 minutes they lose their power to agglutinate, while H-type agglutinins do not. Kleckowski[37] has shown that the O-type of agglutinins form complexes with the

[69] M. Peterman and A. M. Pappenheimer, Jr., *Science* **93**, 457 (1941).
[70] F. Sanger, *Biochem. J.* **39**, 507 (1944).
[71] A. R. Rosenheim, *ibid.* **31**, 54 (1937).
[72] H. F. Deutsch, R. A. Alberty, L. J. Gostling, and J. W. Walker, *J. Immunol.* **56**, 183 (1947).
[73] R. A. Kekwick and B. R. Record, *Brit. J. Exptl. Path.* **22**, 29 (1940).

albumin in the serum on heating; these complexes fail to agglutinate, but appear to combine with the antigens, as they inhibit agglutination by unheated serum.

After Parfentjev[74] has introduced a successful method of preparing digested antitoxin for therapeutic purpose, Pope made a thorough investigation into the action of proteases on diphtheria antitoxin. He showed[75] that, when pseudoglobulin from normal or antitoxic serum was heated at 59°C. in the presence of salt at pH 4.2 for 30 minutes, about 94% of the protein was coagulated; when pseudoglobulin from antitoxic serum was used about 93% of the antitoxin was removed from solution. But if proteases were allowed to produce a degree of hydrolysis of pseudoglobulin from antitoxic serum, which could not be detected as a change in total protein content, and the solution was then heated under identical conditions, part of the protein was coagulated and part, containing the antitoxin, remained in solution. If, instead of pseudoglobulin from antitoxic serum, pseudoglobulin from normal horse serum was used nearly all the pseudoglobulin was coagulated on heating after digestion. When the pseudoglobulin was derived from sera that contained relatively little antitoxin the rate of loss of antitoxin ran parallel to the rate of digestion of protein; whereas with high titer plasma over 80% of the antitoxin might remain when 80% of the protein had been digested to a stage that would not coagulate on heating (Pope[76]). Besides the antitoxin some of the inert pseudoglobulin of antitoxic sera was split by pepsin into a heat labile and heat resistant fraction. The proportion of the heat resistant fraction that was made up of antitoxin varied with the titer of the serum. Pope concluded that when horses are immunized a new type of pseudoglobulin, of which little is present in normal horse serum, is formed; part of this, the proportion depending on the antitoxic value of the serum, is antitoxin. After short hydrolysis of this new pseudoglobulin by protease the molecules are split into two parts, one of which is easily denatured by heat, while the other bearing the antitoxic properties is less easily denatured. The appearance of this fraction in serum may be associated with the physical changes of the globulin fractions already mentioned.

The essential difference between the method finally evolved by Pope[77] and that of Parfentjev[74,78,79] is that in Parfentjev's method the serum, or globulin from it, is digested for several hours until proteoses are formed; these and the remaining pepsin are removed by adsorption. In Pope's method the globulin is digested at pH about 3 for a short time; the inac-

[74] I. A. Parfentjev, U. S. Patent 2,065,196 (1936).
[75] C. G. Pope, *Brit. J. Exptl. Path.* **19**, 245 (1938).
[76] C. G. Pope, *ibid.* **20**, 132 (1939).
[77] C. G. Pope, *ibid.* **20**, 201 (1939).
[78] I. A. Parfentjev, U. S. Patent 2,123,198 (1938).
[79] I. A. Parfentjev, U. S. Patent 2,175,090 (1939).

tive fraction is removed by heating at pH 4.2 in the presence of ammonium sulfate. The Parfentjev method depends on the resistance of the antitoxin fraction to farther hydrolysis by pepsin and the Pope method on the resistance of this fraction to denaturation by heat. Other proteases that split antitoxic globulins into two fragments, differing in heat stability and resistance to further digestion are: fibrinolysin and papain (Pope[76]), trypsin (Pope[76] and Northrop[66]), and the protease of taka-diatase (Coghill *et al.*[67]). Digestion with trypsin is very slow unless the globulin is first denatured by heating in acid solution (Pope[76]). The split antitoxin obtained in these ways forms a precipitate with toxin or toxoid more rapidly than undigested antitoxin does. The precipitation curve also differs (Fig. 2): the ratios of antibody N to antigen N in the precipitates formed are lower than with undigested antitoxin. At the equivalence point 1 Lf (0.46 μg. of N) of toxin or toxoid combines with 1.6 μg. of undigested antitoxin N[79a] and with 1.16 μg. of digested antitoxin N (Pappenheimer and Robinson[80]). These developments led to further studies of the process of digestion of serum globulins and of the molecular weights of the fractions. Peterman and Pappenheimer[81] digested pseudoglobulin, prepared from horse diphtheria antitoxin, with pepsin at pH 4.2 for 30 minutes at room temperature and heated to coagulate inert protein. They found that the molecular weight of the soluble product was about half that of the undigested pseudoglobulin (184,000).[82] By further digestion of floccules formed by purified toxoid and pepsin-digested antitoxin, followed by fractional precipitation they got a more homogeneous product, 77% of which was precipitated by toxoid. They calculated that the molecules of this product were about 60% as heavy (mol. wt. 113,000) and 70% as long as those of the undigested antitoxin. They considered that the antitoxin molecule had been split at right angles to its major axis. At the neutral point, the native diphtheria antitoxin (mol. wt. 184,000) combined with $15,800 \times 10^6$ Lf (44,300 g. of toxin protein or 0.62 moles) of toxin per mole and the 'half' molecule (mol. wt. 113,000) with $15,255 \times 10^6$ (42,800 g. of toxin or 0.59 mole) per mole. The fragment therefore had as many receptor groups for toxin per molecule as the original globulin had. Pope and Healey[5] also had obtained a specially purified fragment of the antitoxin molecule by digesting the floccules with pepsin and found that 1 mg. of this was equivalent to 140 Lf of toxin (0.39 mg.), or 113,000 g. of antitoxic fragment to 43,500 g. of toxin. All of the carbohydrate of the antitoxin remained in the heat resistant fraction. Peterman and Pappenheimer agreed with Pope in finding

[79a] As noted in Section II, Pope and Healey[5] considered that this figure is too low.

[80] A. M. Pappenheimer, Jr. and E. S. Robinson, *J. Immunol.* **32**, 291 (1937).

[81] M. Peterman and A. M. Pappenheimer, Jr., *J. Phys. Chem.* **45**, 1 (1941).

[82] This estimate of the molecular weight of the antitoxin is higher than that previously calculated by A. M. Pappenheimer, Jr., H. P. Lundgren, and J. M. Williams, *J. Exptl. Med.* **71**, 247 (1941).

that heat resistant fragments were formed from some of the inert pseudoglobulin of antitoxic sera; the molecular weights of these inert fragments were the same as those of the antitoxic fragments. As Peterman[55] also found that normal cow pseudoglobulin is split into fragments by pepsin in the same way this course of digestion with pepsin is not peculiar to antibodies.

These studies of the digestion of antibodies have led to studies of the digestion of normal serum pseudoglobulin with pepsin. Peterman[55] found that molecules with sedimentation constants in the neighborhood of 5, 3, and 2 $\times$ 10^{-13} were formed by digestion of pseudoglobulin of normal ox serum. The 5 $\times$ 10^{-13} fraction was the most homogeneous in the ultracentrifuge and seems to have been formed by splitting the original molecules into halves; the next lighter fraction probably consisted of quarter molecules. The largest proportion of half molecules was formed by digestion at pH 4. Bridgeman[82a] digested human serum globulin fractions which consisted largely of γ-globulin. Fractions with sedimentation constants in the neighborhood of 6 and 3 $\times$ 10^{-13}, corresponding to half and quarter molecules, were found. The highest proportion of half molecules was found when the protein was digested with less than 0.05 hemoglobin units of pepsin at pH 3.5 for 3 or 4 days. Antibodies to influenza Type A, diphtheria, and scarlet fever antitoxins and typhoid H-agglutinin survived digestion; typhoid O- aggluinin was destroyed. Deutsch, Peterman, and Williams[83] found that, at a pH well removed from the optimum of pepsin, an apparent equilibrium was reached with relative proportions of whole, half, quarter, and smaller molecules; the equilibrium state was characteristic of the globulin used and of the pH.

Pope[76] found that active antitoxin could be recovered after horse diphtheria antitoxin had been digested with papain. Using Annetts'[57] method Peterman[84] again found that fractions with sedimentation constants about 5 and 3 $\times$ 10^{-13} were formed. The heavier fraction was homogeneous with a molecular weight of about 90,000; it was not, therefore, formed by progressive splitting of small fragments from the original molecules.

Free diphtheria toxin is destroyed rapidly by trypsin. It might be expected that, when trypsin is mixed with a suspension of the precipitate formed by diphtheria toxin and horse antitoxin, the toxin would be destroyed and that antitoxin, which is not readily digested, would be set free. Actually nonprotein nitrogen is set free slowly, but no appreciable amount of antitoxin is liberated. If, however, the precipitate is dissolved in acid, the solution then neutralized and trypsin added, about one-third

[82a] W. B. Bridgeman., *J. Am. Chem. Soc.* **68**, 857 (1946).
[83] H. F. Deutsch, M. L. Peterman, and J. H. Williams, *J. Biol. Chem.* **164**, 93 (1946).
[84] M. Peterman, *ibid.* **144**, 607 (1942).

of the antitoxin is set free (Northrop[66]). If, after neutralization, the solution is left at pH 7.2 for several hours and trypsin is then added, no antitoxin can be recovered. Apparently the structure of the antibody molecule suffers some reversible change on exposure to acid, which makes it more susceptible to digestion by trypsin. This change may be compared to the increased susceptibility to digestion by trypsin shown by serum albumin after denaturation and regeneration (Bernheim *et al.*[35]).

By this method of digesting precipitates and fractional precipitation Northrop obtained a small amount of crystalline material. From the sedimentation and diffusion constants Rothen [86] calculated that the molecular weight of this material was 90,500. The precipitation curve of the digested antibody and crude diphtheria toxin was wider than the curve with undigested antibody; with purified toxin precipitates formed when either toxin or antitoxin were in great excess (Northrop[66]).

Peterman[55] suggested that the "all-or-none" course of digestion is characteristic of globulin, whatever the enzyme, and that half and quarter molecules were found because the reaction was slowed down. However, Deutsch, Peterman, and Williams[83] found that at 0°C. and pH 4.4 (a pH further removed from the optimum than that at which they found half and quarter molecules) increasing amount of dialyzable material were formed from human γ-globulin, without the formation of appreciable amounts of half molecules.

Investigations of diphtheria antitoxin and similar proteins have dealt mainly with pseudoglobulin, soluble in water. The horse-antibodies to pneumococcal polysaccharides are euglobulins, insoluble in water when the salt concentration is below a certain level. Grabar[87] digested concentrated antibodies from horse anti-pneumococcal sera with pepsin at 0°C. pH 4.5. After 5 days 82% of the original protein was still precipitable by trichloroacetic acid but only 45% of the antibody protein remained. At the equivalence point the ratio of pneumococcal polysaccharide to antibody protein in the precipitates formed by the digested protein with polysaccharide was approximately double that in the precipitates formed by the undigested solution. Peterman and Pappenheimer[69] repeated this work; they also precipitated the digested antibody with polysaccharide and dissociated the antibody from the precipitate with a strong solution of sodium chloride (Heidelberger and Kendall[88]). The sedimentation constant of the dissociated antibody was 5.2×10^{-13}; the molecular weight was probably below 100,000. In one of the sera the antibody molecules were not of uniform molecular weight; their sedimentation constants were

[35] F. Bernheim, H. Neurath, and J. O. Erickson, *ibid.* **144**, 259 (1942).
[86] A. Rothen, *J. Gen. Physiol.* **25**, 487 (1942).
[87] P. Grabar, *Comp. rend.* **207**, 807 (1938).
[88] M. Heidelberger and F. E. Kendall, *J. Exptl. Med.* **64**, 161 (1936).

11, 18, and 30 $\times$ 10^{-18}. The size of the molecules of the antibody fraction formed by digestion was independent of the size of the molecules of the original antibody molecules. This fits the suggestion of Treffers, Moore, and Heidelberger[89] that these long antibody molecules are formed by end to end polymerization of globulin molecules of normal molecular weight.

All that has come out of these investigations, so far, with regard to the structure and location of the combining sites of antibodies, is that certain antibody molecules, particularly those of diphtheria antitoxin, are asymmetric about a transverse axis; the receptor sites (probably 2) and the carbohydrate are in one half. The possibility that the quarter molecules and smaller fragments combine with antigen, but do not form precipitates, has not been specifically investigated, but the evidence suggests that they do not.

The absorption of antibodies from the colostrum by new-born lambs, calves, and foals raises an interesting question in relation to the digestion of antibodies. The absorption of antibodies is accompanied by an increase in the γ-globulin in the plasma of new-born animals (Jameson *et al.*[90] and San Clemente and Huddleston[91]). Dent and Schilling[92] have shown by paper chromatography of ultrafiltrates from portal blood that dog plasma proteins disappear from the intestines of dogs without the rise of amino acids in the portal blood that accompanies the absorption of other proteins. It is suggested that plasma proteins may be absorbed intact. However, Hansen and Phillips[93] showed that the increase in γ-globulin in the serum of calves after feeding with colostrum occurs only during the first 24 hours of life. The absorption of the antibody as such cannot be explained solely by the resistance of the antibody to digestion. Nor can it be explained solely by absence of proteases from the digestive tracts of new-born animals; for the casein of the colostrum does not appear in the plasma after digestion. Nor can it be ascribed to a special resistance of the antibodies in colostrum, for Smith[94] found that calves can absorb antibodies from cow's serum, taken by mouth.

c. The Synthesis of Antibodies

There are two possible concepts of the mode of synthesis of proteins from amino acids. The first—the "conveyor-belt" theory—supposes that the growing protein molecule is passed along a series of enzymes, of selec-

[89] H. P. Treffers, D. H. Moore, and M. Heidelberger, *J. Exptl. Med.* **75**, 135 (1942).
[90] E. Jameson, C. Alvarez-Tostado, and H. H. Sortor, *Proc. Soc. Exptl. Biol. Med.* **51**, 162 (1942).
[91] C. L. San Clemente and I. F. Huddleston, *Mich. Agr. Expt. Sta. Bul.* **182**, 3 (1943).
[92] C. E. Dent and J. A. Schilling, *Biochem. J.* **42**, XXIX (1947).
[93] R. G. Hansen and P. H. Phillips, *J. Biol. Chem.* **171**, 223 (1947).
[94] T. Smith, *J. Exptl. Med.* **51**, 473 (1930).

tive synthetic ability, that add the appropriate amino acids from a choice that is offered; the second—the "template" theory—supposes that the amino acids are fixed by some mechanism in the appropriate pattern and then synthesized into a protein molecule by enzymes. The controlling factor, according to the first concept, is the enzyme system; according to the second it is the previous arrangement of the amino acids. Two theories of the way in which receptor sites are formed on antibody molecules correspond with these two concepts. That of Burnet[95] supposes that certain proteinases, in the cells that form antibodies, are modified as they are engaged in destroying antigen. These modified enzymes synthesize the antibodies. The "template" theory supposes that the determinant groups of antigen molecules, present in antibody-forming cells, attract amino acids in a pattern resembling that of the receptor site of an antibody molecule, and that these amino acids are then joined by appropriate enzymes in this pattern. This theory has been advocated by Breinl and Haurowitz,[96] Alexander,[97] and Mudd.[98]

Pauling[99] defers the differentiation of antibodies from ordinary serum globulin to a later stage in the construction of the molecules. He suggests that the final coiling up to two ends of a peptide chain may be modified by the presence of an antigen to fit the determinant groups of the antigen. It does not seem possible, however, that the different ways of coiling of the ends of the peptide chains would offer a sufficient variety of receptor sites to fit all the different determinant groups. Pauling and Campbell[100] claimed to have made antibodies from normal globulins by denaturing the globulins and then slowly reversing the denaturation in the presence of antibody. Their experiments seem to have been uncontrolled and have been criticized on various grounds. If denaturation and regeneration, in the presence of antigen, adapts globulin molecules to the determinant groups of the antigen, denaturation and regeneration of antibodies in the absence of antigen should destroy the specificity of the antibodies. Erickson and Neurath,[101] however, denatured antibodies to pneumococcal polysaccharide, Type I, with sodium thiocyanate and regenerated them in the absence of antigen; the regenerated antibody still formed a precipitate with homologous polysaccharide.

Whatever theory of the actual process of synthesis of antibodies is

[95] F. M. Burnet, The Production of Antibodies, MacMillan, Melbourne, Australia, 1941.
[96] F. Breinl and F. Haurowitz, *Z. physiol. Chem.* **192**, 45 (1930).
[97] J. Alexander, *Protoplasma*, **14**, 296 (1932).
[98] S. Mudd, *J. Immunol.* **23**, 423 (1932).
[99] L. Pauling, *J. Am. Chem. Soc.* **62**, 2643 (1940).
[100] L. Pauling and D. H. Campbell, *J. Exptl. Med.* **76**, 211 (1942).
[101] J. D. Erickson and H. Neurath, *Science* **98**, 284 (1943).

adopted we are still left with the difficulty of explaining the long duration of the effects of immunization. We believe that anaphylaxis is due to the formation of antibodies; a guinea pig sensitized by a single injection of protein remains sensitized, to some degree, for many months, possibly for the rest of its life. We should expect that all traces of the antigen would disappear in a few weeks. Stacey[102] adopting the "conveyor belt" theory, has suggested that the chromosome system of the cells in which antibodies are formed may be altered by antigens, as those of one type of pneumococci appear to be alterable by desoxyribonucleic acid of another type (Avery *et al.*[103]) and that this alteration is self-perpetuating.

2. Denaturation

Various qualitative investigations have shown that proteins, denatured by various methods, form precipitates with antisera to native proteins. Quantitative investigations (MacPherson and Heidelberger[104]) showed that denatured egg albumin formed precipitates with antiserum to native egg albumin. About ten times as much of the denatured albumin as of the native albumin was needed to form a given amount of precipitates with antiserum to native albumin. This may have been due to occlusion of determinant groups in the aggregates that were formed (McPherson, Heidelberger, and Moore[105]). The amount of precipitate formed and, consequently, the amount of antibody precipitated by the denatured albumin was, in most cases, considerably less than that formed by the native albumin. The adaptation of the determinant groups of the antigen to the combining groups of the antibody was reduced but not destroyed by the distortion of the molecules of albumin in the course of denaturation.

Serum albumin denatured by guanidine and regenerated differs from native serum albumin in the shape of the molecules, in electrophoretic mobility and in resistance to digestion by trypsin. Erickson and Neurath[106] have shown by quantitative methods that the amount of antibody precipitated by the regenerated albumin from one antiserum to native albumin was less than that precipitated by native albumin. The determinant groups seem to have been slightly affected by this treatment; but, as the ratio of regenerated antigen to homologous antibody at the equivalence point was much the same as the ratio of native albumin to homologous antibody, the number of determinant groups on the antibody molecule was probably unchanged.

[102] M. Stacey, *Quart. Rev. Chem. Soc.* **1**, 179 (1948).
[103] O. T. Avery, C. M. MacLeod, and M. McCarty, *J. Exptl. Med.* **79**, 137 (1944).
[104] C. F. C. MacPherson and M. Heidelberger, *J. Am. Chem. Soc.* **67**, 585 (1945).
[105] C. F. C. MacPherson, M. Heidelberger, and D. H. Moore, *ibid.* **67**, 578 (1945).
[106] J. D. Erickson and H. Neurath, *J. Exptl. Med.* **78**, 1 (1943).

3. Specific Activities of Films

The effects of spreading in films on the specific activities of antigens and antibodies and of enzymes may be compared. A distinction must be made between thin films of thickness of about 10 A. in which the molecules of protein have been unrolled or split into layers of the thickness of one amino acid, and films whose thickness is about 40 A. in which the molecules may be distorted but little or not at all. Retention of specificity by molecules in the thicker films tells nothing about the relation of the determinant and receptor groups to the structure of the molecules. Thin films of urease are inactive as enzymes (Langmuir and Schaefer[107]); unpublished experiments (Rothen[108]) indicate that trypsin also loses its activity on spreading in thin films. Langmuir and Schaefer[107] found that catalase retains some of its activity when spread in films 23 A. thick; but films 55 A. thick had only one-fifth to one-tenth the activity of the unspread enzyme (Harkins, Fourt, and Fourt[109]). As the smallest diameter of a calatase molecule (reckoned as a prolate ellipsoid) must be well over 23 A. the molecules in the thinner of these films must have been distorted.

Danielli, Danielli, and Marrack[110] could find no evidence of combination between Type II pneumococcal polysaccharide and the homologous antibody prepared from horse serum when this antibody was spread as a thin film at a water-oil interface. Various experiments have been made on films of antibodies and antigens transferred to slides by Blodgett's[111] method. An increase in the thickness of the film after a drop of a solution of antibody is applied to a film of antigen is regarded as a measure of combination of antibody with the antigen molecules in the film. Similarly an increase of the thickness of a film of antibody after application of a solution of antigen is evidence of combination of antigen with the molecules of antibody in the film. Caution must be used in interpreting the results. In the first place the combination is less specific than is precipitation from solution. Films of proteins absorb proteins nonspecifically (Porter and Pappenheimer[112]) though the resulting increase in thickness of the films is less than the increase due to specific combination. Also it does not seem reasonable to expect that the molecules of antigen and antibody remain as separate layers after combination. It might be expected that the specific attraction between antigen and antibody molecules would lead to a rearrangement and formation of a layer of the antigen-antibody aggregate. It cannot be assumed that, when a solution of antibody is put on a solution

[107] I. Langmuir and V. J. Schaefer, *Chem. Revs.* **24**, 181 (1939).
[108] A. Rothen, *Advances in Protein Chem.* **3**, 127 (1947).
[109] W. D. Harkins, L. Fourt, and P. C. Fourt, *J. Biol. Chem.* **132**, 111 (1940).
[110] D. Danielli, M. Danielli, and J. R. Marrack, *Brit. J. Exptl. Path.* **38**, 19 (1938).
[111] K. B. Blodgett, *J. Am. Chem. Soc.* **56**, 495 (1934).
[112] E. F. Porter and A. M. Pappenheimer, Jr., *J. Exptl. Med.* **69**, 755 (1939).

of antigen, the increase in thickness of the film is equal to the thickness of a layer of antibody molecules. This is the natural explanation of the effect of the number of layers of antigen on the increase in thickness when antibody is added (Rothen[113]).

The number of antibodies that can be used to test the specific activity of films of antibodies is limited as it is possible to get sufficiently pure preparations of only a few kinds of antibody. Porter and Pappenheimer[112] found that horse antipneumococcal antibodies formed films about 45 A. thick on slides. When a drop of a solution of polysaccharide was put on the film of antibody an increase of thickness due to the addition of a layer of polysaccharide to the antibody was too small to detect. But combination of the homologous polysaccharide with the antibody in the film could be demonstrated, for if a drop of a solution of homologous antibody was added after the polysaccharide the thickness of the film increased by about 40 A. (Fig. 3). If the polysaccharide added to the primary antibody film or if the antibody, added after the polysaccharide, was heterologous the thickness of the film increased by 11 A. or less. The thickness of the primary

FIG. 3. Diagram of formation of layers by antipneumococcal antibody, homologous polysaccharide and a second application of homologous antibody.

layer of antibody was about equal to the lesser diameter of the antibody molecules, so there was no evidence that the molecules was distorted. When Porter and Pappenheimer used antipneumococcal antibodies from rabbit sera the results were less convincing. The thickness of primary films of antibody was about 20 A.; secondary layers of the same order of thickness were found with two of the preparations of antibody used; but when the antibody to Type II pneumococcus was used the increase of thickness produced by the second application of antibody was not more than 10 A. Rothen and Landsteiner[114] also made films of rabbit pneumococcal antibodies; these however were only from 8 to 12 A. thick. After an application of the homologous polysaccharide they found that the application of homologous antibody led to an increase of thickness of over 40 A. whether horse or rabbit antibody was used. Porter and Pappenheimer[112] made films (average thickness 23 A.) from a preparation of pseudoglobulin from horse antiserum to diphtheria toxin; 35% of this pseudoglobulin was precipitable by toxin. They found no evidence that these films would combine with toxin. It appears that rabbit antibodies when spread in layers of thickness equal to half the lesser diameter of the molecules in solution and even in layers one amino acid thick, may, in some cases, still combine with the homologous antigen; but this is not found in all cases.

[113] A. Rothen, *Science*, **102**, 446 (1945).

On the other hand, antigens spread in thin films still combine with antibodies. This might be expected of the pneumococcal polysaccharides owing to their structure. Thin films of egg albumin (Bateman, Calkins, and Chambers;[115] Rothen and Landsteiner[114]) and nucleoprotein of Type A streptococci (Bateman, Calkins and Chambers[115]) also combine with the corresponding antibodies. Some of the determinant groups of these antigens, therefore, survive distortion of the molecules both by denaturation and by spreading on thin films.

Diphtheria antitoxin combined with layers of purified toxin 33A. thick (Porter and Pappenheimer[112]) and anticatalase with layers of catalase 55A. thick (Harkins, Fourt and Fourt[109]). The thickness of layers of both these antigens differed little from possible lesser diameters of the molecules of the antigens in solution: the molecules were not necessarily distorted. In the case of catalase the increase in thickness when more catalase was added to films containing anticatalase was only 10A. This does not necessarily mean that the later layers of catalase were spread to the thickness of one amino acid, but rather that the molecules in the layers were rearranged, with some addition of catalase.

The increase in thickness when an antibody combines with a layer of antigen is very variable. This may well depend on the extent to which antigen is freed from the surface to form aggregates with the antibody. When serum albumin was used as antigen the increment in thickness, after addition of the homologous antibody, increased with the number of layers of antigen; but not when the antigen was egg albumin (Rothen[113]). It is possible that, owing to the irreversibility of the spreading of the egg albumin molecules, the layer of these molecules remains undisturbed and is not rearranged in an aggregate with antibody when antibody is added.

Rothen[116] used immunological methods to show that crystalline pepsin and trypsin hydrolyze egg albumin and horse serum albumin, spread as thin films. After the action of the enzyme addition of homologous antibody did not lead to the increase in thickness of the film that would be found if the layer of antigen had remained intact. In these and subsequent experiments Rothen[117] applied layers of heterologous proteins, barium stearate, a plastic 'Formvar' or octadecylamine to the layers of antigen *before* treatment with antibody or enzyme. The thickness of the films still increased on treatment with homologous antibody in spite of these layers of inert material. After the film was treated with protease, the antibody did not increase its thickness; the enzymes therefore appear to have digested the antigen in spite of intervening layers of inert material. In the case of

[114] A. Rothen and K. Landsteiner, *J. Exptl. Med.* **76**, 437 (1942).
[115] J. B. Bateman, H. E. Calkins, and L. A. Chambers, *J. Immunol.* **41**, 321 (1941).
[116] A. Rothen, *J. Biol. Chem.* **163**, 345 (1946).
[117] A. Rothen, *ibid.* **168**, 75 (1947).

serum albumin the thickness of the layers of inert material needed to prevent the increase of thickness on application of antibody varied with the thickness of the film of serum albumin.

It seems from these experiments that antibody may be attracted by antigen and enzyme hydrolyze its substrate through layers of inert material over 100A. thick. There are two possible explanations: either the substances applied to the slides are not arranged in neat layers one above the other, or the mutual attraction of antigens and antibodies and the activity of enzyme are due to long range forces. It is difficult to reconcile the great dependence of the specificity of combination of antigen and antibody on patterns in space with the second explanation. Rothen[117] suggests that antigen and antibody interact as resonating oscillators; this interaction between *large* molecules built on the principle of repetition of pattern would be interpreted by a field action, rather than by specific chemical reacting groups. But specific combination also occurs between antibodies and *small* molecules of appropriate pattern. The first explanation seems incompatible with an observation of Rothen.[111] If a layer of antigen, after treatment with antibody, is washed with a 5 to 10% solution of sodium chloride, its thickness diminishes by 46 to 63A.—presumably owing to the extraction of antibody (Heidelberger and Kendall[88]). If a layer of formvar is applied, after the antibody, subsequent washing with sodium chloride solution does not reduce the thickness of the film; the layer of formvar protects the film. Final judgment on the significance of these results must await fuller investigation of the structure of these films.

V. Enzymes as Antigens

Since the early days of immunology much work has been done on the preparation of antisera to enzymes; a comprehensive account is given by Sevag.[118] For years the formation of antibodies by enzymes themselves was questioned; the main objections were given by Bayliss.[119] Since his monograph was published the premises on which his arguments were based have proved false. Antisera were prepared by immunizing animals with preparations of enzymes and these enzymes were removed from solution or neutralized by the antisera; but it was argued that the preparations of enzymes used contained some protein, which was not the enzyme, and that the enzymes were carried down with the precipitates formed by this protein and the antibody. One of the earliest demonstrations of the freedom of precipitates of antibody and antigen from nonspecific material was connected with this question. Burnett and Schmidt[120] in 1921 showed

[118] M. S. Sevag, Immuno-catalysis. C. C. Thomas, Springfield, Ill., 1945.

[119] W. M. Bayliss, The Nature of Enzyme Action. Longmans, Green and Co., New York, 1925,

[120] T. C. Burnett and O. L. A. Schmidt, *J. Immunol.* **6**, 255 (1921).

that the formation of a precipitate by human serum and the corresponding antiserum did not remove catalase from solution. With the repeated demonstration that these specific precipitates rarely carry down nonspecific proteins this argument has ceased to be plausible.

Since enzymes have been prepared in a crystalline form it has become possible to study the chemistry of the reactions of enzymes with the corresponding antibodies and to compare these reactions with those between other pure proteins and antibodies. It is remarkable that more such studies have not been made, particularly as the antibodies to urease and catalase can be isolated. Two interesting questions arise: first, do these remarkable proteins react with antisera as other proteins react?, and second, are the determinant groups of enzymes the same as the groups on which the activity as enzymes depends?

1. Quantitative Studies

Few quantitative studies have been made of the reactions of enzymes with antibodies. Adams[121] found that the precipitation curve of tyrosinase resembles that found with other protein antigens (Fig. 4). Ribonuclease (mol. wt. about 15,000, Kunitz[122]) and lysozyme (mol. wt. 15,000 to 18,000 Abraham[123]) are the smallest protein antigens to which antisera have been made. The reaction of ribonuclease with antiserum has been studied quantitatively; the amount of precipitate formed when increasing amounts of antigen are added to constant amounts of antiserum rises to a maximum and then falls (Smolens and Sevag[124]). The ratio of precipitate to antigen added, when the amount of precipitate is maximum, is about 24/1; this, as might be expected from the size of the antigen, is high. The molecular composition of the precipitate, if all the antigen is precipitated at this point, is between A_2G and A_3G.

The ratio of antibody to urease (mol. wt. 480,000) in the precipitate, calculated from the figures of Kirk and Sumner,[125] is 7/11 in the equivalence zone and 1/3 in the zone of antigen excess; the corresponding molecular compositions are about A_2G and AG.

In the zone of antigen excess the amount of catalase precipitated by antiserum varies as the ratio of antiserum to catalase rises (Campbell and Fourt[126]). The system urease-antiurease seems to differ from most systems, as the amount of antiurease precipitated and the ratio of antigen to antibody in the precipitate are approximately constant in the zone of antigen excess up to a ratio of antigen added to antibody present equal to four times that at the equivalence point (Kirk and Sumner[125]).

[121] M. H. Adams, *J. Exptl. Med.* **76**, 175 (1942).
[122] M. Kunitz, *J. Gen. Physiol.* **24**, 15 (1940).
[123] J. Abraham, *Biochem. J.* **33**, 622 (1939).
[124] J. Smolens and M. G. Sevag, *J. Gen. Physiol.* **26**, 11 (1942–43).
[125] J. S. Kirk and J. B. Sumner, *J. Immunol.* **26**, 495 (1934).
[126] D. H. Campbell and L. Fourt, *J. Biol.* **129**, 385 (1939).

2. Independence of Sites Specific for Activity as Enzymes and as Antigens

If the determinant groups and the groups on which the activity as enzymes depends are the same we should expect that (a) an enzyme would cross-react with an antiserum to another enzyme that had the same specific enzyme action and (b) the enzyme activity of an enzyme should be inhibited by an antiserum to this enzyme or to another enzyme with the same specific action.

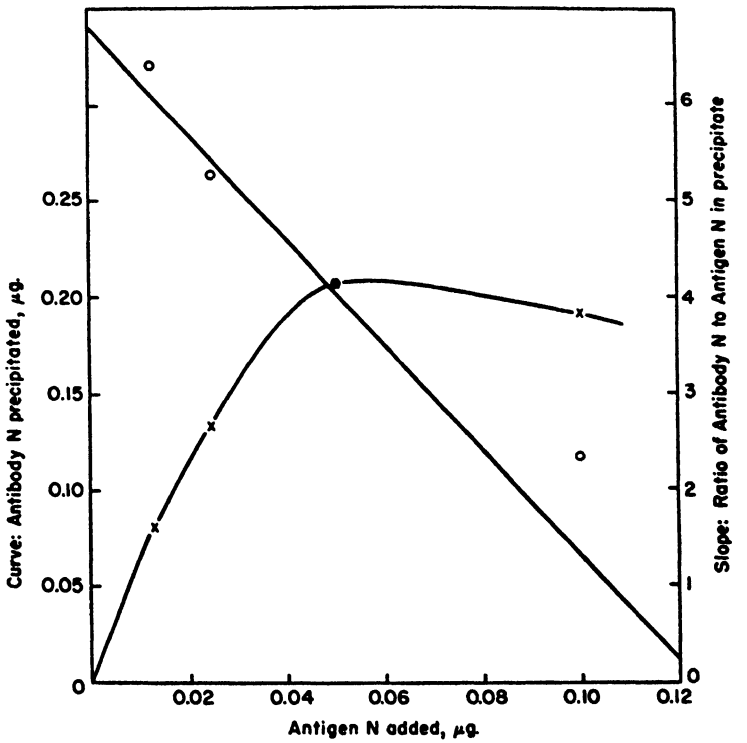


FIG. 4. Amount of antibody N precipitated from a constant amount of antiserum to tyrosinase by tyrosinase and ratio of antibody to antigen in the precipitate. (M. H. Adams, *J. Exptl. Med.* **76**, 175, 1942.)

We have already had examples of the independence of the antigenic determinant groups and specific receptor groups in the case of antibodies. Further examples may be given. In the system: diphtheria toxin (T), horse diphtheria antitoxin (HaT) and rabbit antibody to horse serum globulin (RaH): the combination of (HaT) with (T) is not inhibited by previous combination (HaT) with (RaH), and *vice versa* (Eisler[127] Marrack

[127] M. von Eisler, *Zentr. Bakt. Parasitenk.*, I. Orig. **84**, 46 (1920).

and Smith[128]; Eagle[129]). In the system pneumococcal polysaccharide (S), horse antipolysaccharide antibody (HaS) and rabbit anti-(HaS) antibody (RaHs): Treffers, Moore, and Heidelberger[89] found that the precipitate formed by (HaS) with (S) would combine with as much (RaHs) as free (HaS) would, although for steric reason the precipitation curves were different. Also Grabar and Oudin[130] found that the precipitation curves given with (RaHs) by the soluble compounds formed by (HaS) with (S) in excess were similar to those given by free (HaS) unless the amount of (S) was very large.

a. Cross Reactions

The fact that two enzymes which have the same action cross react does not prove that their antigenic and enzymic groups are identical. Thus horse and donkey hemoglobins cross react (Heidelberger and Landsteiner[131]); but this does not mean that the heme groups of these hemoglobins act as determinant groups. Animals of fairly closely related species make their proteins on somewhat similar patterns; the hemoglobins of animals that are less closely related do not cross-react; although the heme groups are the same, the globins differ. The molecules of both horse and donkey hemoglobins have six peptide chains each ending in a valine residue; whereas the molecule of ox hemoglobin, for example, has four chains, two of which end in a valine residue. (Porter and Sanger[132]). In the second place, even if determinant groups and enzyme-active groups were identical the reactions of two enzymes that have the same enzyme-active groups need not be quantitatively identical. If an antiserum is obtained by immunizing a rabbit with an azo-protein, more antibody is precipitated from this serum by the azo-protein used for immunization than by an azo-protein containing the same determinant group coupled with a second protein; and more antibody is precipitated if the second protein is closely related to the first than if it is not.

In many cases cross-reactions are found, though quantitative studies show that the antisera detect difference between enzymes from different species. Thus Kirk[133] found that 20 times as much antiserum to jackbean urease was needed to precipitate a given number of units of soybean urease as was needed to precipitate the same number of units of jackbean urease. From the figures of Campbell and Fourt[126] it can be inferred that a similar relation holds between the amounts of an antiserum to ox liver catalase needed to precipitate a given amount of horse liver catalase and of ox liver

[128] J. R. Marrack and F. C. Smith, *Proc. Roy. Soc. London* **B101**, 1 (1930).
[129] H. Eagle, *J. Immunol.* **30**, 339 (1936).
[130] P. Grabar and J. Oudin, *Ann. inst. Pasteur* **69**, 394 (1945).
[131] M. Heidelberger and K. Landsteiner, *J. Exptl. Med.* **38**, 561 (1923).
[132] R. R. Porter and E. Sanger, *Biochem. J.* **42**, 287 (1948).
[133] J. S. Kirk, *J. Biol. Chem.* **100**, 667 (1933).

catalase. The amount of precipitate with horse catalase was roughly one-half that with ox catalase at the same ratio of antigen to serum. Tria[134] also found that, with antiserum to ox liver catalase the amounts of precipitate formed by lamb and horse liver catalase were less than that formed by ox liver catalase. These cross reactions are comparable to that between hen and duck egg albumin (Fig. 1). Bonnichsen[135] found that the same amounts of horse liver or blood catalase were precipitated by anti-horse liver catalase serum when the catalases were added in excess; the two enzymes have the same amino acids, but differ in that liver catalase has a verdohematin in place of a hematin group. No precipitates were formed with this antiserum by human blood catalase or horse hemoglobin. Seastone and Herriott[136] found that the amount of precipitate formed by ox and by guinea pig pepsin with anti-pig pepsin serum was considerably less than that formed by pig pepsin with this antiserum; but after addition of sufficient ox or guinea pig pepsin to this antiserum it no longer formed a precipitate with pig pepsin. This cross reaction differs from the hen-duck egg-albumin system, as duck egg albumin will not thus inhibit the reaction of hen egg albumin with antiserum to hen egg albumin. On the other hand, no cross reactions may be found between enzymes that catalyze the same reaction; thus tyrosinase of *Lactarius piperatus* does not form a precipitate with antiserum to tyrosinase of *Psalliota campestris* (Adams[121]). Other examples of enzymes with the same specific activity that do not cross react are given later in the section on toxic enzymes.

The independence of antigenically determinant and enzyme-active groups is further illustrated by the fact that urease treated by irradiation or mild oxidation no longer breaks down urea, but still cross reacts with antiserum to urease (Pillemer *et al.*[137]). In the same way, diphtheria toxin treated with formaldehyde is not toxic but still combines with the antibody, diphtheria antitoxin; the toxic groups and the determinant groups on the toxin molecule are independent.

b. Inhibition by Antisera

Some enzymes retain their activity, little reduced, when precipitated in combination with antibody. Examples of the activity of the enzyme-antibody compound expressed as a percentage of the activity that the enzyme contained would have if free, are: Urease-antiurease precipitate, in a salt solution of strength just sufficient to keep the compound flocculent, 80% (Kirk[138]); catalase-anticatalase precipitate resuspended to form an

[134] E. Tria, *ibid.* **129**, 377 (1939).
[135] R. K. Bonnichsen, *Arch. Biochem.* **12**, 83 (1947).
[136] C. V. Seastone and R. M. Herriott, *J. Gen. Physiol.* **20**, 797 (1937).
[137] L. Pillemer, E. E. Ecker, V. C. Myers, and E. Muntwyler, *J. Biol. Chem.* **123**, 365 (1938).
[138] R. Haas, *Biochem. Z.* **305**, 280 (1940).

opalescent suspension, 32 to 73% (Campbell and Fourt[126]); tyrosinase-antityrosinase precipitate, 100% (Adams[121]); papain-antipapain precipitate, 30% (Haas[138]); ribonuclease-antiribonuclease precipitate resuspended, 70 to 78% (Smolens and Sevag[124]). It is remarkable that the enzyme-antibody precipitates should retain the activity of the free enzyme, even when the substrate molecules are not relatively small; it is difficult to understand how the large molecules of ribonucleic acid can penetrate into the aggregate formed by ribonuclease and the antibody so rapidly that a high rate of catalysis is maintained. For in order to keep up this rate of breakdown the molecules of substrate would have to keep on penetrating a complex in which each small enzyme molecule is surrounded by three antibody molecules, ten times its size; it would not have merely to penetrate the complex once and for all, as when rabbit antibody to horse globulin penetrates the complex formed by horse-antipneumococcal antibody with the pneumococcal polysaccharide. The activity of the enzyme-antibody complex is tested in the presence of excess of substrate. The substrate and antibody may compete for combination with the enzyme and the antibody may be displaced.

In one case the competition between substrate and antibody for the enzyme molecule has been demonstrated. If anti-lecithinase serum is added to a mixture of lecithinase and lecithin the anti-lecithinase combines with the lecithinase, prevents the combination with lecithin and inhibits the action of the enzyme (Zamechnik and Lipman[139]). This may be compared to the displacement of Type III polysaccharide from combination with antibody to Type VIII polysaccharide (Mayer and Heidelberger[140]). However, the rate of combination of the lecithinase and antilecithinase seems to be slower than that of the combination of Type III polysaccharide and antibody and of other antigens and antibodies (Boyd[141]).

In the cases in which antisera inhibit the action of the enzyme it cannot be inferred that the sites of combination with antibody and substrate are the same. The large antibody molecules, although combined at separated sites, may obstruct the approach of substrate to enzyme; the larger the substrate the greater the probability of inhibition.

3. Precursors of Enzymes

Ten Broeck[9] showed by the guinea pig uterus method that trypsinogen and trypsin, and chymotrypsinogen and chymotrypsin are immunologically different. According to Jacobsen[142] 4 peptide links are opened in the conversion of chymotrypsinogen to chymotrypsin. The effect on the structure of

[139] P. C. Zamecknik and F. Lipman, *J. Exptl. Med.* **85**, 395 (1947).
[140] M. Mayer and M. Heidelberger, *J. Biol. Chem.* **143**, 567 (1942).
[141] W. C. Boyd, *J. Exptl. Med.* **74**, 369 (1941).
[142] C. F. Jacobsen, *Compt. rend. trav. lab. Carlsberg. Ser. chim.* **25**, (No. 14) 327 (1947).

the molecule may be more profound than that in the formation of plakalbumin from egg albumin,

4. Purified Antienzymes

Advantage has been taken of the denaturation of urease (Kirk and Sumner[125]) and catalase (Tria[134]) by acid to recover the antibodies from the precipitates formed by the enzymes with antisera. The precipitates were washed and dissolved in dilute hydrochloric acid; the pH of the solution was then raised to over 5 and the denatured enzyme centrifuged down. The number of anticatalase units per gram of solid was about 50 times as high in anticatalase prepared in this way as in the original serum. The preparation must have been nearly pure antibody. After the process of precipitation of antiurease with urease and recovery has been repeated twice the final product retained 70 to 80% of the original activity of the antiserum. The antiurease was not attacked by trypsin; even after incubation with activated trypsin for 24 hours at 37°C. the antiurease solution formed a precipitate with urease. It was rapidly inactivated by pepsin at pH 4.3 or less, by inactivated papain and by papain-H_2S at pH 5.0; slowly by papain-H_2S at pH 7.0.

This method of dissociation of precipitates has been used to recover several kinds of antibodies. Usually the dissociation is incomplete. Thus, part of the antibodies can be recovered from the precipitates formed by azo-proteins (Haurowitz *et al.*[143]) with the corresponding antisera; but an insoluble fraction is left behind containing antigen and some of the antibody. The degree of dissociation varies. Various workers have recovered diphtheria antitoxin by treatment of toxin-antitoxin precipitates with acid. But Pappenheimer, Lundgren and Williams [144] found that this precipitate, dissolved in acetate buffer of pH 3.6 was not separated into two fractions by electrophoresis.

VI. Toxic Enzymes

Some toxins are enzymes and others appear to be related to enzymes. The reactions of these enzymes with antisera are an aspect of immunity in the common sense of resistance to disease.

1. Toxicity of Urease

Urease poisons animals that excrete urea as the main end product of the metabolism of nitrogen; it is harmless to birds that have little urea in their body fluids (Howell[145]). The harmful action is secondary to the forma-

[143] F. Haurowitz, S. Tekman, M. Bilen, and P. Schwerin, *Biochem. J.* **41**, 304 (1947).
[144] A. M. Pappenheimer, Jr., H. P. Lundgren, and J. W. Williams, *J. Exptl. Med* **71**, 247 (1940).
[145] S. F. Howell, *Proc. Soc. Exptl. Biol. Med.* **29**, 759 (1932).

tion of ammonia from urea (Kirk and Sumner[146]). Although urease is, in itself, harmless, the way animals are protected against it serves as an example of the way in which antibodies may act. Rabbits that have been immunized with urea and have formed antibodies are able to withstand 100 times the dose of urease that is fatal to untreated rabbits. Serum from a rabbit that has been immunized will protect a rabbit against a fatal dose of urease, even if it is injected 1½ to 2 hours after the urease (Kirk and Sumner[146]). This protection cannot be due merely to the combination of urease and antibody, for the antibody does not inhibit the activity of the urease with which it is combined even when a precipitate is formed. Possibly the compound of urease and antibody is removed from the body fluids more quickly than the free urease is and the urease in combination is rapidly destroyed. Diphtheria toxin is not destroyed when it combines with antitoxin; it is not possible to test whether antitoxin neutralizes its direct action because its direct action is not known. Neutralization is not due to precipitation of the toxin, for the compound formed by toxin with a large excess of antitoxin is soluble but is not toxic. The neutralization of toxin by antiserum, like that of urease, may be due to its rapid removal and destruction when combined with antibody.

2. Neutralization and Cross-Reactions

Among the toxic enzymes are hemolysins which act by splitting lecithin. The hemolytic venoms of snakes, scorpions, bees, wasps, and spiders contain lecithinase A, which splits lecithin at the junction between glycerol and a fatty acid. Snake venoms split off one unsaturated fatty acid, leaving lysolecithin, which is itself hemolytic. Wasp venom may carry the reaction further and split off two fatty acids per molecule (Belfanti[147]), like the lecithinase B of rice bran. The hemolytic α-toxin of *Cl. welchii* was shown by Macfarlane and Knight[148] to split lecithin to phosphoryl choline and a diglyceride. This reaction is the cause of the opalescence formed by the toxin with serum (Nagler[149]) and with clear emulsions of lecithovitellin (Macfarlane, Oakley, and Anderson[150]). This type of enzyme is activated by Ca ions. Enzymes with this type of action are found in the toxins of *Cl. oedematiens* including the β and γ toxins (Oakley *et al.*[151]) and of *Cl. histolyticum* (Macfarlane[152]). Some other process besides the splitting of lecithin is involved in hemolysis by these toxins, as they differ

[146] J. S. Kirk and J. B. Sumner, *J. Biol. Chem.* **94**, 21 (1931).
[147] S. Belfanti, *Z. Immunitätsforsch.* **56**, 449 (1928).
[148] M. G. Macfarlane and B. C. J. G. Knight, *Biochem. J.* **35**, 884 (1941).
[149] F. P. O. Nagler, *Brit. J. Exptl. Path.* **20**, 473 (1939).
[150] M. G. Macfarlane, C. L. Oakley, and C. G. Anderson, *J. Path. Bact.* **52**, 99 (1941).
[151] C. L. Oakley, G. H. Warrack, and P. H. Clarke, *J. Gen. Microbiol.* **1**, 91 (1947).
[152] M. G. Macfarlane, *ibid.* **2**, XXIV (1948).

in their ability to hemolyze different red blood corpuscles. The β- and γ-toxins of *Cl. oedematiens* attack horse red blood corpuscles, but hardly affect those of sheep; the α-toxin of *Cl. welchii* has little effect on horse corpuscles and hemolyzes those of sheep. The hemolysin of spiders acts strongly on the red corpuscles of rabbit and man, but has practically no effect on those of guinea pig and horse (Kyes[152a]).

Another type of hemolysin, which is characterized by being made inactive by oxidation and reactivated by reduction, is found in the toxins of *Cl. welchii* (θ-toxin), *Cl. tetani*, streptococcus (streptolysin O) and pneumococcus (Todd[153]) and *Cl. oedematiens* (δ-toxin, Oakley, Warrack, and Clarke[151]). The mode of action of these hemolysins is not known, but it is noticeable that the oxygen-labile hemolysin of *Cl. tetani* hemolyzes horse blood corpuscles preferentially.

The immunological specificity of these toxins varies. There are considerable immunological differences between the bacterial lecithinases. Antisera to *Cl. welchii* α-toxin do not neutralize other lecithinases. *Cl. oedematiens* forms three lecithinases, α-, β- and γ-, with enzyme actions similar to that of *Cl. welchii* α-toxin; these are distinct immunologically. Antisera to the β-toxin of *Cl. oedematiens* also neutralize a hemolytic toxin of *Cl. haemolyticum*, which has similar enzyme action (Macfarlane[152]).

The oxygen-labile hemolysins differ less immunologically. All those mentioned are neutralized by antiserum to streptolysin O; antiserum the *Cl. welchii* θ-toxin neutralizes streptolysin (Todd[153]). However these toxins are not immunologically identical. Strong antisera are needed for neutralization of heterologous toxins and the amounts of antiserum required to neutralize homologous and heterologous toxins are not proportional to their hemolytic activity. The snake venoms are species specific; the venoms of various American rattlesnakes are almost immunologically identical; but antisera are ineffective against venoms of less closely related snakes (Boyd[154]).

Macfarlane and MacLennan[155] showed that *Cl. welchii* produces a protease, collagenase, which breaks down connective tissue. This enzyme is immunologically distinct from the α- and θ-toxins of *Cl. welchii* (Oakley, Warrack, and von Heyningen[156]). Streptococci produce a proteolytic enzyme, streptokinase, which activates the precursor of fibrinolysin (Christensen and MacLeod[157]); the activated fibrinolysin can break down a wall of

[152a] Kyes, P. *Z. physiol. Chem.* **41**, (1904).
[153] E. W. Todd, *Brit. J. Exptl. Path.* **22**, 172 (1941).
[154] W. C. Boyd, Fundamentals of Immunology. Interscience Publishers, New York, 1947.
[155] R. G. Macfarlane and J. D. MacLennan, *Lancet* **2**, 328 (1945).
[156] C. L. Oakley, G. H. Warrack, and W. E. van Heyningen, *J. Path. Bact.* **58**, 229 (1946).
[157] L. R. Christensen and C. M. MacLeod, *J. Gen. Physiol.* **28**, 559 (1945).

fibrin formed round a focus of infection; antibodies to streptokinase specifically inhibit the activation of fibrinolysin.

Snake venoms contain two types of proteolytic enzyme that coagulate blood (Eagle[158]). (1) A protease that coagulates pure fibrinogen without calcium ions; it is not inhibited by antithrombin; (2) does not coagulate pure fibrinogen; converts prothrombin to thrombin without the presence of Ca ions or tissue-extract. The immunological specificity of these enzymes does not appear to have been studied, but the generalization that antisera in the venoms of a species do not neutralize the toxins of species that are not closely related applies to these enzymes.

Hyalonuridase is an enzyme that depolymerizes hyalonuric acid. The breakdown of intracellular hyalonuric acid in the skin allows a wider spreading of substances including bacteria and toxins. Hyalonuridases are produced by *Cl. welchii*, *Vibrion septique* and pneumococci; they are also found in the venoms of snakes and bees and in leech extracts (Chain and Duthie[159]). The bacterial hyalonuridases are immunologically specific; those formed by *Cl. welchii* and *Vibrion septique* are inhibited by homologous and not by heterologous antisera (McClean and Hale[160]).

As with other enzymes, some degree of immunological cross reaction may be found between toxic enzymes that have the same enzyme action but are formed by different species. On the other hand one species may form toxins that have different or even similar enzymic actions but are immunologically distinct. Thus *Cl. oedematiens* strain A forms two distinct lecithinases, with an action similar to that of *Cl. welchii* α-toxin, and also forms the oxygen-labile δ-toxin, another hemolysin (γ-toxin) and a lipase (the ϵ-toxin), all of which are immunologically distinct. There is no species specificity in the sense of a common immunological factor, shared by different toxins of the same species.

3. The Classical Exotoxins

The classical exotoxins, diphtheria toxin, tetanus toxin and botulinus toxin have, during recent years, been prepared in a crystalline form. The toxins are homogeneous in electrophoresis and in the ultracentrifuge. Extraordinarily small amounts of these toxins are fatal. The minimal lethal doses for a mouse are approximately; diphtheria toxin, 7 μg., tetanus toxin, 8×10^{-5} μg., botulinus toxin 3×10^{-5} μg. The molecular weight of botulinus toxin is about 900,000. Approximately 20 million molecules will kill a mouse; this is about one-sixth the number of molecules of hemoglobin in one mouse red blood corpuscle. The amino acids of diphtheria toxin and botulinus toxin have been estimated fairly completely; nothing

[158] H. Eagle, *J. Exptl. Med.* **65**, 613 (1937).
[159] E. Chain and E. S. Duthie, *Brit. J. Exptl. Path.* **21**, 324 (1940).
[160] D. McClean and C. W. Hale, *Biochem. J.* **35**, 159 (1941).

is known of their composition that will account for their toxicity. After injection of one or two minimal lethal doses into a susceptible animal, no symptoms can be observed for 18 or more hours. Pappenheimer[161] considers that it does not appear likely that these toxins act by direct inhibition of essential enzymes or that they are enzymes themselves; he suggests that they may act by blocking the synthesis of some essential cellular enzyme. Although antitoxin will save an animal if it is injected before or with the toxin, enormous doses have no effect if injected after the toxin. It seems that irreversible damage is done before the symptoms appear.

Both tetanus and botulinus toxin seem to affect the acetyl-choline mechanism of the myoneural junctions; nothing is known of their functions or metabolism in the bacteria that form them. Diphtheria toxin seems to be connected with the formation of catalase b. The maximum amount of toxin is produced when the concentration of iron in the culture medium is about 0.1 mg. per liter; a porphyrin is then found in the culture medium in proportion to the amount of toxin. When the concentration of iron is above the optimum for production of toxin, 4 molecules of porphyrin and 1 molecule of toxin fail to appear. The extra iron is taken up quantitatively by the bacteria and as the iron accumulates an iron containing porphyrin compound resembling cytochrome b increases in the cells. It was suggested by Pappenheimer and Hendee[162] that the toxin is the protein component or precursor of the protein component of the cytochrome b of the bacteria. The toxin may act by interfering with the synthesis of cytochrome b by susceptible animals. However, the contents of the bacterial cells form very little precipitate with diphtheria antitoxin. If the toxin is present in the cytochrome b of the cells it must be somewhat changed or its determinant groups must be blocked.

VII. Complement

If the serum of a rabbit that has been immunized with red blood corpuscles of another species of animal is heated at 56°C. for ½ hour and then mixed with a suspension of red blood corpuscles of the species from which those used for immunization were derived, the corpuscles are agglutinated but the hemoglobin remains in the corpuscles. If fresh unheated serum (which may be that of an unimmunized rabbit though serum of guinea pigs is most commonly used) is added the hemoglobin diffuses out of the corpuscles. The corpuscles are said to be hemolyzed and the process is known as lysis. There is no evidence of lysis, in the sense of a breakdown of the molecules; the framework of the corpuscles is not broken up; it seems that the structure of surface membrane is so altered that it becomes permeable to hemoglobin. Similarly, other cells, such as certain bacteria,

[161] A. M. Pappenheimer, Jr., *Advances in Protein Chem.* **4**, 123 (1948).
[162] A. M. Pappenheimer, Jr., and E. D. Hendee, *J. Biol. Chem.* **171**, 701 (1942).

are lyzed by the joint action of antibody and some other agent present in fresh serum. This other agent is called complement. It is nonspecific; the same complement is involved in the lysis of the red blood corpuscles of various species and in the lysis of bacteria. Lysis of red blood corpuscles that have been sensitized by antiserum is the standard method of demonstrating the presence of this complement.

Complement combines with the complexes formed by rabbit and human antisera and the homologous antigens. This *fixation* of complement can be shown by the failure to hemolyze sensitized red blood corpuscles. Earlier workers (Marrack and Smith,[128] Haurowitz and Appel[163]) could not detect any difference between the amounts of precipitate formed by antigen and antibody in the presence of complement and the amount formed without complement. The nature of complement remained a mystery. But in 1941 Heidelberger[164] showed that the fixation of complement may lead to a considerable increase of the total nitrogen of an antigen-antibody precipitate, if enough of the serum that supplies the complement is used. For example, the nitrogen of the precipitate formed by 1 ml. of rabbit antipneumococcal serum and 1 ml. homologous polysaccharide, complement absent, was 0.389 mg.; the nitrogen of the precipitate formed by the same amounts of antiserum and polysaccharide in the presence of 5 ml. of unheated guinea pig serum was 0.565 mg. Allowing for a small increase in the presence of heated guinea pig serum, the increase that can be attributed to the fixation of complement was 0.16 mg.—rather under half the antibody nitrogen.

Four components of complement are recognized. Two, C′1 and C′2, are destroyed by heating at 56°C. for half an hour; C′1 is insoluble in distilled water and is a globulin; C′2 is soluble in distilled water and is a globulin that contains a high proportion of carbohydrate. C′3 and C′4 are not destroyed by heating at 56°C. for half an hour; C′3 is absorbed by yeast and C′4 is destroyed by ammonia.

Heidelberger[165] calculated that 600 molecules of rabbit antibody, together with complement, are sufficient to lyse one sheep red blood corpuscle; he assumed that the molecular weight of this antibody is 900,000 as found by Paic.[3] These molecules of antibody would cover only 0.7% of the surface of the corpuscle. The number of molecules of nonspecific lytic agents that are needed for lysis is very much higher. Thus 5×10^6 molecules of sodium tetradecyl sulfate, one of the most efficient, are needed to change the disc shape of one human red corpuscle to a sphere, the stage that precedes lysis (Ponder[166]).

We may suppose that some constituent of the cell membrane of a corpuscle combines with antibody and that subsequent combination with com-

[163] F. Haurowitz and G. Appel, *Z. Immunitätsforsch.* **95**, 200 (1939).
[164] M. Heidelberger, *J. Exptl. Med.* **73**, 681 (1941).
[165] M. Heidelberger, *Advances in Enzymol.* **8**, 71 (1948).

plement leads to a disorganization of the arrangement of the molecules and makes a hole in the membrane. If the area of hole formed is of the same order as the surface of an antibody molecule the hemoglobin of the corpuscles might diffuse out as rapidly as is found. For Ponder[166] quotes Fricke[167] to the effect that 90% of the hemoglobin molecules could diffuse out in 4 seconds through 100 holes 70 A. in diameter, which together would make up only 1/30,000th of the surface of the corpuscle. It is assumed that the hemoglobin molecules are free in the interior of the corpuscle and not arranged in any structure. Also the disorganization of the arrangement of molecules round one antibody molecules may displace some keystone of the surface membrane and lead to collapse of the structure over a wide area. It is therefore not necessary to assume that hemolysis by antibody and complement resembles the action of an enzyme.

Heidelberger[165] points out that the amount of C'4 involved in hemolysis is minute and suggests that, if any component of complement acts as an enzyme, it is C'4.[168] The concentration of magnesium ions has a specific effect on the lytic activity of complement (Mayer *et al.*[169]). This may be compared with the influence of magnesium ions on enzyme actions. However, in both cases the special effect may be due to the formation of unionized compounds of protein and magnesium.

[166] E. Ponder, Haemolysis and Related Phenomena. Grune and Stratton, New York, 1948, p. 249.

[167] H. Fricke, *J. Gen. Physiol.* **18**, 103 (1934).

[168] Mayer and Croft (M. Mayer and C. C. Croft, *Federation Proc.* **7**, 308, 1948) suggest that the action of hemolytic antibody resembles that of any enzyme, while the complement appears to play the role of a cofactor that is used up in the process. Complement may furnish energy that enables the antibody to fulfill its hemolytic function.

[169] M. Mayer, A. G. Osler, O. G. Bier, and M. Heidelberger, *J. Exptl. Med.* **84**, 535 (1946).

CHAPTER 9

Enzymes Hydrolyzing Fats and Esters*

(Lipases, esterases proper, acetylesterase, tropinesterases, cholesterol esterase, chlorophyllase, tannase)

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I. Classification of the Esterases and Remarks on the Organization of the Field

The esterases catalyze the reaction:

$$R\cdot COOH + HO\cdot R' \rightleftharpoons R\cdot COOR' + H_2O$$

This equation already indicates the hydrolyzing and synthesizing actions of these enzymes. The chemical nature of the alcohol and acid or of the

* Translated by Erich Hirschberg, McArdle Memorial Laboratory, The University of Wisconsin, Madison, Wisconsin.

radicals R and R′ bound in the ester causes special types of specificity among the esterases.

If simple, common esters, e.g., methylbutyrate, are involved, we speak of *esterases proper*. Recently, *acetylesterase* has been separated from this group; the latter hydrolyzes esters of acetic acid with particular specificity, while the nature of the alcohol has no effect. If the alcohol is glycerol and the acid a true fatty acid, we are dealing with the *lipases*. If glycerol is replaced by cholesterol, *cholesterol esterase* functions in the formation and hydrolysis of the esters. The hydrolysis of esters which contain *N*-alcohols and *N*-substituted alcohols is attributed to the *azolesterases*; this group comprises the *cholinesterases*, the *benzoylcholinesterase*, *atropinesterase*, *cocainesterase* and *tropacocainesterase*, as well as other esterases which have not been named more precisely. *Tannase* is an enzyme which hydrolyzes tannin and also gallic acid methyl ester. *Chlorophyllase* is also classified among the esterases although it causes not only a hydrolysis but also an alcoholysis. Esterases are, moreover, involved in the hydrolysis of the lecithins (*lecithinases A and B*) and of the pectins (*pectase*).

The two large groups, the *phosphatases* and *sulfatases*, should be considered as esterases in the true sense of the word. However, these enzymes act to break oxygen-phosphorus or oxygen-sulfur bonds, so that these two groups of enzymes fall outside the scope of the esterases which have been mentioned and which always hydrolyze an oxygen-carbon bond.

Cholinesterase and benzoylcholinesterase have been treated by Augustinsson (Chapter 10) and pectase by Kertesz (Chapter 21), the lecithinases have been discussed by Zeller (Chapter 30), the sulfatases by Fromageot (Chapter 13), and the phosphatases by Roche (Chapter 11) (cf. also Ref. 1).

II. Brief Survey of Methods of Determination and of Esterase Preparations

1. The Determination of Esterase Activity

At this point, some general remarks are in order. Detailed descriptions of the procedures used for the determination of the various esterases and for the preparation of enzyme samples may be found in *Die Methoden der Fermentforschung* by Bamann and Myrbäck.[2] Some indications of methods of determination are also given in the treatment of the individual esterases.

In order to determine esterase activity in the direction of synthesis as well as of hydrolysis, the amount of acid being used up or produced is very often measured. Omitting older procedures, the titrimetric determinations of Willstätter *et al.*[3] may

[1] Among recent reviews on esterases the following may be mentioned: D. Glick, *Ann. Rev. Biochem.* **11**, 51 (1942); J. B. Sumner, *Ann. Rev. Biochem.* **17**, 35 (1948); J. B. Sumner and G. F. Somers, Chemistry and Methods of Enzymes. 2nd ed., Academic Press, New York, 1947; H. Tauber, *Ann. Rev. Biochem.* **10**, 47 (1941).

[2] Lipases and esterases: E. Waldschmidt-Leitz and A. Schäffner, *in* Bamann-Myrbäck, Die Methoden der Fermentforschung. Thieme, Leipzig. 1941, p. 1547; Tannase: O. Th. Schmidt, *ibid.* p. 1590; Chlorophyllase: R. Lambrecht, *ibid.* p. 1599.

[3] R. Willstätter, E. Waldschmidt-Leitz, and F. Memmen, *Z. physiol. Chem.* **125**, 93 (1923).

be mentioned as the first method. The principle consists of a determination of the degree of saponification of olive oil, which serves as substrate, produced by the enzymatic hydrolysis. At the same time, the olive oil substrate may be improved by the addition of buffers and suitable activators (calcium chloride, albumin, and oleate). The acid which is produced is titrated with alcoholic KOH. The hydrolysis of esters other than fats was followed by Willstätter and Memmen.[4] Esters of low water solubility may be advantageously maintained in fine dispersion by an emulsifying agent, e.g., gum arabic.[5] Knaffl-Lenz[6] determined the saponification of esters on the basis of the amount of alkali required to keep the hydrogen ion concentration constant as shown by an added indicator. In a similar manner, Willstätter *et al.*[7] followed the hydrolysis of esters in solutions free of buffer by titration with the aid of bromthymol blue.[8] Bamann and Schmeller[9] have pointed out the very considerable sources of error inherent in the use of indicators in esterase determinations, since indicators may sometimes have a significant inhibitory effect on the enzyme-substrate system; this has also been shown for gum arabic by Fodor.[10] Rona and Ammon[11] employed the electrotitrimetric method which checks on the formation of acid by continuous pH measurements and maintains a constant pH by the addition of alkali. Glick[12] used the glass electrode for this purpose (as described for cholinesterase). The electrotitrimetric procedures are carried out in the absence of buffers. If for certain experimental reasons it is desired to use a buffer, it is necessary to determine the influence of the latter in calibration curves, as described by Galwialo and Simina[13] and Rona and Ammon.[14] Special methods for the titrimetric determination of the acid remaining in the nonesterified state in enzymatic synthesis have not been described. The investigations and remarks on methods by Bodenstein and Dietz,[15] Rona *et al.*[16] Sym,[17,18] Rona, Ammon[14] and Fabisch[19] may be listed. Finally, the work of Sym[20] with simultaneous determinations of the acid by titration and of the alcohol disappearing during synthesis according to Tschugaeff and Zerewitinoff deserves mention. Singer and Hofstee[21] studied the hydrolysis of esters by plant lipases with the aid of steam distillation whereby the free fatty acids distill over. Linderstrøm-Lang and Holter[22] and Linderstrøm-Lang and Glick[23] have described elegant methods for microdetermination in small portions of tissue (enzymatic histochemistry).

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[4] R. Willstätter and F. Memmen, *Z. physiol. Chem.* **133**, 229 (1924); R. Willstätter, R. Kuhn, O. Lind, and F. Memmen, *ibid.* **167**, 303 (1927); see also G. Scoz and L. Guzzi, *Klin. Wochschr.* **19**, 1014 (1940); G. Scoz, *Boll. soc. ital. biol. sper.* **15**, 1132 (1940); *Riv. fisiol.* **15**, 99 (1942)/*C.A.* **37**, 6296[1] (1943).

[5] J. R. Koch and S. M. D. Duellman, *Oil & Soap* **18**, 86 (1941); R. M. Archibald, *J. Biol. Chem.* **165**, 443 (1946); P. J. Fodor, *Nature* **158**, 375 (1946).

[6] E. Knaffl-Lenz, *Arch. exptl. Path. Pharmakol.* **97**, 242 (1923).

[7] R. Willstätter, R. Kuhn, and E. Bamann, *Ber.* **61**, 886 (1928).

[8] See also F. Walker, *Am. J. Physiol.* **139**, 343 (1943).

[9] E. Bamann and M. Schmeller, *Z. physiol. Chem.* **194**, 1 (1931).

[10] Cf. ref. 5; P. J. Fodor, *Nature* **158**, 375 (1946).

[11] P. Rona and R. Ammon, *Biochem. Z.* **181**, 49 (1927).

[12] D. Glick, *Compt. rend. trav. lab. Carlsberg. Sér. chim.* **21**, 225 (1935–1938).

[13] M. J. Galwialo and L. J. Simina, *Biochem. Z.* **238**, 24 (1931).

[14] P. Rona and R. Ammon, *Biochem. Z.* **249**, 446 (1932).

[15] M. Bodenstein and W. Dietz, *Z. Elektrochem.* **12**, 605 (1906).

[16] P. Rona, R. Ammon, and H. Fischgold, *Biochem. Z.* **241**, 460 (1931).

[17] E. A. Sym, *Biochem. Z.* **230**, 19 (1931).

[18] E. A. Sym and W. Śviatkovska, *Enzymologia* **2**, 79 (1937–1938).

[19] W. Fabisch, *Biochem. Z.* **259**, 420 (1933).

[20] E. A. Sym, *Biochem. J.* **30**, 609 (1936); *Enzymologia* **1**, 156 (1936–1937).

[21] Th. P. Singer and B. H. J. Hofstee, *Arch. Biochem.* **18**, 229 (1948).

[22] K. Linderstrøm-Lang and H. Holter, *Ergeb. Enzymforsch.* **3**, 309 (1934).

[23] K. Linderstrøm-Lang and D. Glick, *Compt. rend. trav. lab. Carlsberg. Sér. chim.* **22**, 300 (1938).

Besides the titrimetric procedures used for studies of hydrolysis and synthesis, use is made of the following methods involving mainly 1) stalagmometry, 2) the Warburg apparatus, 3) dilatometry, 4) colorimetry, and 5) nephelometry.

Rona and Michaelis[24] developed the stalagmometric method which makes use of the strongly surface-tension-reducing action of tributyrin. As tributyrin disappears during hydrolysis, the surface tension value approaches that of pure water. A special drop pipette is used for the measurement of surface tension. Willstätter and Memmen[25] introduced some modifications of this method. One of the important steps is a purification of the tributyrin. Measurements of surface tension with the most varied modifications in the determination of lipase activity were employed by Krijgsman,[26] Wwedenski and Dobrowitzky,[27] Lejhanec,[28] and recently Lagerlöf.[29] Bullo and Poli[30] did not count the drops but rather weighed them on the torsion balance.

Rona and Lasnitzki[31] have recommended the gasometric method based on the procedure developed by Warburg[32] for studying glycolytic reactions in surviving tissue. Ammon[33] employed the same principle for the determination of cholinesterase. The gasometric method involves the liberation and measurement of carbonic acid which is equivalent to the acid liberated from the ester by the enzyme. The Warburg apparatus is also suitable for the determination of the tropinesterases and of acetylesterase.

Dilatometry has also been used for the measurement of reactions catalyzed by esterases. However, Rona and Ammon[14] have pointed out the relatively slight utility of the dilatometer for esterases. A special dilatometer described by Ammon and Bartscht[34] makes it possible to obtain data on the volume changes which take place during synthesis and hydrolysis.

The use of colorimetric methods (with the Pulfrich or photoelectric colorimeter) may be illustrated by the methods of Huggins and Lapides[35] and Gad[36] which employ *p*-nitrophenylbutyrate as the substrate.

A nephelometric lipase determination based on the measurement of the turbidity of fat has been described by Rona and Kleinmann.[37]

2. Preparation of Esterase Samples

The specific details for this section may also be found in *Die Methoden der Fermentforschung* by Bamann and Myrbäck. Only a brief survey of the most essential fundamentals which lead to the preparation of various esterase samples is intended here. A few remarks on suitable enzyme preparations are presented in the discussion of the individual esterases.

In the very simplest instance, the esterases may be demonstrated directly in the

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[24] P. Rona and L. Michaelis, *Biochem. Z.* **31**, 345 (1911).
[25] R. Willstätter and F. Memmen, *Z. physiol. Chem.* **129**, 1 (1923).
[26] B. J. Krijgsman, *Natuurw. Tijdschr. Belg.* **10**, 137 (1928).
[27] N. Wwedensky and P. J. Dobrowitzky, *Biochem. Z.* **188**, 448 (1927).
[28] G. Lejhanec, *Compt. rend. soc. biol.* **113**, 1231 (1933).
[29] H. O. Lagerlöf, *Acta Physiol. Scand.* **13**, 301 (1947).
[30] E. Bullo and E. Poli, *Diagnost. e tec. lab. Napoli, Riv. mens.* **7**, 1 (1936).
[31] P. Rona and A. Lasnitzki, *Biochem. Z.* **152**, 504 (1924).
[32] O. Warburg, *Biochem. Z.* **142**, 317 (1923); *ibid.* **152**, 51 (1924).
[33] R. Ammon, *Arch. ges. Physiol.* (*Pflügers*) **233**, 486 (1933).
[34] R. Ammon and K. Bartscht, *Biochem. Z.* **268**, 331 (1934).
[35] C. Huggins and J. Lapides, *J. Biol. Chem.* **170**, 467 (1947).
[36] I. Gad, *Dansk Tids. Farm.* **23**, 1 (1949).
[37] P. Rona and H. Kleinmann, *Biochem. Z.* **174**, 18 (1926).

body fluids such as saliva, gastric juice, blood, urine, serum, or plasma; no preparative treatment is then needed. It is thus feasible in many experiments to determine esterases directly in serum or in urine. If the samples are kept in the refrigerator, the esterases of serum, for example, have a relatively high stability. But in general it is desirable to employ stable enzyme preparations. The simplest possibility for testing tissues for enzyme activity is the press juice; while it is not stable—it may be kept for some time under toluene in the refrigerator—it gives an indication of the enzymatic capacity of a tissue and consequently leads to the preparation of more suitable samples. Glycerol may be used in a simple manner as an extractant for tissue breis. Such glycerol extracts, e.g., embryo extract, are distinguished by a high stability, especially if they are maintained in the refrigerator. They also have the advantage of containing only relatively low levels of protein and of being resistant to extensive bacterial growth. A significant advance in the preparation of stable tissue powders and extracts is by means of adsorption and elution. The so-called acetone-ether powders of tissues may be prepared according to Willstätter and Waldschmidt-Leitz.[38] The tissues are first defatted as completely as possible by crude mechanical means and put through a meat grinder. The tissue brei is shaken several times with acetone, followed by a single treatment with a mixture of ether and acetone and finally by several treatments with ether. In this way, the tissue brei is largely freed of water and fat. Following air-drying for several hours until the odor of ether has disappeared, the powder is ground in a ball mill and put through a sieve.

Highly active enzyme solutions may be prepared from such powders by treatment with either 87% glycerol or $N/40$ ammonia. Glycerol extracts are quite stable, as stated earlier. Ammonia extracts must be neutralized with acetic acid before further use; after centrifugation to remove the precipitate which is formed, this aqueous extract may be kept for some time in the refrigerator under toluene. This ammonia extract may also be concentrated under reduced pressure according to Willstätter *et al.*[39]

Bamann[40] prefers to remove the precipitate formed by the addition of acetic acid to the ammonia extract and then to dialyze the enzyme solution in mutton caeca. Such solutions exhibit a high stability if maintained under toluene in the refrigerator. They are distinguished by the absence of a large portion of the buffering impurities which may disturb the effect of added substances or even the titrimetric determination.

Willstätter and Waldschmidt-Leitz[41] have developed adsorption and elution methods for the preparation of purified pancreatic lipase solutions; these methods lead to preparations which are not only much more active but also largely free from the other pancreatic enzymes. The principle of the purification consists of adsorption of the enzyme, after extraction with glycerol, from dilute acetic acid on a specially prepared aluminum hydroxide.[42] The enzyme is eluted by a specified solution of ammonium phosphate which contains glycerol to stabilize the enzyme. If further adsorption and elution is to be carried out, it is necessary first to precipitate the phosphoric acid as magnesium ammonium phosphate; then adsorption and elution proceeds as indicated. If further purification is desired, the enzyme may be adsorbed

[38] R. Willstätter and E. Waldschmidt-Leitz, *Z. physiol. Chem.* **125**, 132 (1923); *ibid.* **142**, 217 (1925).

[39] R. Willstätter, J. Graser, and R. Kuhn, *Z. physiol. Chem.* **123**, 1 (1922).

[40] E. Bamann, *Ber.* **62**, 1538 (1929).

[41] See ref. 38; see also p. 135, Zur Geschichte der Adsorption von Enzymen.

[42] R. Willstätter and H. Kraut, *Ber.* **56**, 149 (1923).

on kaolin[43] or on tristearin or cholesterol.[44] If the latter two substances are used, the lipase is isolated by dissolving out the lipid in benzene. In the case of liver esterase it is better to start with an extraction with $N/40$ ammonia and to adsorb the enzyme on kaolin from a dilute acetic acid solution[45]; ammonia is again used for the elution. A further purification can be achieved by electrodialysis according to Willstätter and Schneider.[46] Similar, though somewhat modified procedures have been applied to stomach lipase, which has been purified by Willstätter *et al.*[47] According to Rona and Petow[48] serum lipase may be removed by ammonium sulfate precipitation on the globulin precipitates which are then taken up in glycerol. It is indicated below that these procedures for the preparation of the enzyme sample fail with castor oil lipase, since this enzyme complex is quite different. According to Willstätter and Waldschmidt-Leitz[49] the starting material is the cream obtained by triturating castor beans with water. Chromatography may also be successfully employed for the separation of mixtures of esterases. According to Ruffili,[50] beef pancreas preparations may be separated with the aid of Al_2O_3 into fractions which have a different hydrolytic action on olive oil and methylbutyrate.

The more recent findings on lyo- and desmo-enzymes have thrown a different light on the isolation of the enzyme from the tissues in the case of the esterases as well. The treatment of the tissues with acetone or ether which has been described indeed produces profound changes in cell structure, so that after such treatment quite different enzymes go into solution. Liver esterase, e.g., may be isolated rapidly and quantitatively from fresh liver by allowing the minced brei to autolyze for several days in an alkaline medium, as described by Bamann *et al.*[51] Largely purified esterase preparations may be obtained from liver extracts by salt precipitation according to Falconer and Taylor.[52] In their experiments on pancreatic lyo- and desmo-lipases. Bamann and Laeverenz[53] further reported that they had succeeded in preparing a crystallized lipase protein. Safwat Mohamed[54] recently reported the exact conditions for the preparation of a crystallized esterase from horse liver.

Finally, the preparation of the "pheron" and "agon" of esterase according to Kraut and Pantschenko-Jurewicz[55] should be briefly mentioned. These investigators were able to show that liver esterase may be brought to a higher degree of purity in a few purification steps by adsorption on lead phosphate. If the liver extract which has first been adsorbed on lead phosphate is treated with siliceous earth, a preparation rich in esterase pheron is obtained. The simplest method for the preparation of a sample rich in pancreatic lipase agon consists of dilution of glycerol extract with water and removal of the flocculent precipitate by centrifugation. The solution contains the lipase coenzyme (agon).

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[43] See ref. 38, p. 182. [44] See ref. 38, p. 195.

[45] R. Willstätter and F. Memmen, *Z. physiol. Chem.* **138**, 216 (1924).

[46] R. Willstätter and K. Schneider, *Z. physiol. Chem.* **133**, 193 (1924).

[47] R. Willstätter and F. Memmen, *Z. physiol. Chem.* **133**, 247 (1924); R. Willstätter, F. Haurowitz, and F. Memmen, *ibid.* **140**, 203 (1924).

[48] P. Rona and H. Petow, *Biochem. Z.* **146**, 144 (1924).

[49] R. Willstätter and E. Waldschmidt-Leitz, *Z. physiol. Chem.* **134**, 161 (1924).

[50] D. Ruffili, *Boll. soc. ital. biol. sper.* **19**, 239 (1944).

[51] E. Bamann, J. N. Mukherjee, and L. Vogel, *Z. physiol. Chem.* **229**, 15 (1934).

[52] J. S. Falconer and D. B. Taylor, *Biochem. J.* **40**, 831 (1946); *ibid.* **40**, 835 (1946).

[53] E. Bamann and P. Laeverenz, *Z. physiol. Chem.* **223**, 1 (1934).

[54] M. Safwat Mohamed, *Acta Chem. Scand.* **2**, 90 (1948).

[55] H. Kraut and W. von Pantschenko-Jurewicz, *Biochem. Z.* **275**, 114 (1935).

III. The Individual Esterases

1. Lipases and Simple Esterases

a. Esterase Models

The possible existence of enzyme models has been discussed in the case of the esterases[56] as of other enzymes. Esterase models involve the phenomenon of the so-called transesterification, i.e., an ester AB (A = alcohol component, B = acid component) forms an equilibrium AB + C $\rightleftarrows$ CB + A with an alcohol C under the catalytic influence of hydroxyl ions. If a suitable alcohol C is used, such that CB is more easily hydrolyzed by water than AB, then C acts as the catalyst:

$$AB + C \rightleftarrows CB + A$$

$$\underline{CB + H_2O \rightarrow C + B}$$

$$AB + H_2O \rightarrow A + B$$

According to Langenbeck, benzoylcarbinol, $C_6H_5 \cdot CO \cdot CH_2OH$, and arylamides of glycolic acid, $CH_2OH \cdot COOH$, are examples of such alcohols.

TABLE I

Hydrolysis of Butyric Acid Methyl Ester at 70°

Catalyst	Moles in 2 cc H_2O	Ester cc.	$N/10$ $Ba(OH)_2$ after 10 min., cc.
Benzoylcarbinol	5×10^{-4}	—	0.00
		0.5	0.44
Naphthoyl-(2)-carbinol	5×10^{-4}	—	0.00
		0.5	0.66
N-2-Naphthylglycolamide	5×10^{-4}	0.5	0.93

It may clearly be seen how the hydrolysis of methylbutyrate, an ester which has been extensively used in studies of the esterases, can be accelerated. Ionescu and Cotani,[57] however, were unable to confirm Langenbeck's findings on the effect of benzoylcarbinol.

Nevertheless, this principle of a more rapid saponifiability of an ester AB in the presence of another ester CB, whereby the hydrolytic cleavage of AB may be catalyzed, appears to be well-founded. Indeed, during the esterase-catalyzed hydrolysis of esters there occurs the formation of the intermediary enzyme-substrate complexes which represent new esters, comparable to CB, which may be more rapidly saponified than the sub-

[56] W. Langenbeck, Die organischen Katalysatoren und ihre Beziehungen zu Fermenten. Julius Springer, Berlin, 1935; G.-M. Schwab and F. Rost, *in* Schwab, Handbuch der Katalyse, III, Biokatalyse. Springer-Verlag, Wien, 1941, p. 545.

[57] C. N. Ionescu and I. Cotani, *Ber.* **71**, 1367 (1938).

strate AB. It appears likely that a similar explanation may be found for the effect of the so-called Twitchell lipolytic reagents,[58] which represent aromatic sulfonic acids.

Mention should also be made of clupein ascorbinate, a further esterase model, which according to Felix and Mager[59] has a slight activity in the hydrolysis of methylbutyrate.

b. General Properties of the Esterases

(pH optima, activators and inhibitors, coenzyme, prolipase and lipokinase, affinity and hydrolysis constants, temperature effects, esterase units)

The *pH optima* of the esterases, which lie between pH 5 and 9 and which are in part presented in the description of the individual enzymes, give only an approximate clue to the behavior of a particular enzyme at the various levels of acidity. It has been found that the optimum is dependent upon the most varied factors. The enzyme complex, with its many possible contaminants which may activate or inhibit, plays an important role; the substrate to be hydrolyzed also may have an effect; it has been shown by Noyes *et al.*[60] that tissue lipases exhibit different pH optima following different pretreatments. Stomach lipase provides a very impressive illustration. With progressing purification of the enzyme preparation the pH optimum moves from the initial acid level (pH 5) toward the alkaline side (pH 8). Gyotoku[61] was unsuccessful in his attempts to attain the acid optimum of stomach lipase by adding back the contaminants which had been removed. By changing the pH value toward the acid side, the hydrolysis of the second alcohol residue in acid esters, e.g. of succinic acid, becomes possible.[62]

Activators and inhibitors. One of the most significant contributions is the so-called compensating activation of Willstätter;[63] according to this principle, it is possible to compare different lipase activities by means of the maximal activation of different lipase preparations. Albumin and calcium oleate are employed as activators. The activating effect of calcium chloride at various pH levels was investigated by Schønheyder and Volqvartz;[64] it was concluded that Willstätter's concept of the formation of complex adsorbates does not appear to be well-founded. Great significance has been attributed recently to the compensating activation of the desmo- and lyolipases of pancreas and liver which may perhaps explain many conflicting

[58] S. N. Basu, *J. Indian Chem. Soc. Ind. & News Ed.* **4**, 113 (1941).
[59] K. Felix and A. Mager, *Z. physiol. Chem.* **249**, 111 (1937); *ibid.* **249**, 126 (1937).
[60] H. M. Noyes, K. Sugiura, and K. G. Falk, *J. Biol. Chem.* **55**, 653 (1923).
[61] K. Gyotoku, *Biochem. Z.* **193**, 18 (1928); *ibid.* **193**, 27 (1928).
[62] E. Bamann and E. Rendlen, *Z. physiol. Chem.* **238**, 133 (1936).
[63] See ref. 4; R. Willstätter and F. Memmen, *Z. physiol. Chem.* **133**, 229 (1924).
[64] F. Schønheyder and K. Volqvarts, *Acta Physiol. Scand.* **10**, 62 (1945).

data of other investigators concerning activation. Bamann and Laeverenz[63] have stated that the lyo-lipase of pancreas is characterized by a 5000–9000% increase in activity upon the addition of compensating activators. The desmo-lipase in the form of the finest suspension of extracted glandular material, on the other hand, exhibits an activation of only a few hundred per cent under these conditions.[65] Salts of bile acids also activate the hydrolytic action of pancreatic lipase. Shoda, [66] Okamura,[67] and Kaziro and Tsuji[68] have investigated the activating effect of bile acids in relation to their constitution. The activating effect of bile acid salts for stomach and pancreatic lipase takes place only upon the attainment of a certain degree of purity.[69] Krähling and Weber[70] studied the influence of contaminants on the hydrolysis of esters by lipases. According to their data, the influence of fatty acid or bile acid salts on the pancreatic hydrolysis of water-soluble esters consists solely in the effect on the rate of decomposition; no effect on enzyme-substrate affinity has been observed. Bamann and Laeverenz[71] reported that sodium oleate not only inhibits strongly the hydrolysis of mandelic acid esters by liver esterase but also changes the optical selectivity of the enzyme. The activation of the lipase effect of the pancreatic enzyme by amino acids is a striking phenomenon. Activations of several hundred per cent may be observed with leucylglycylglycine.[45, 72] Abderhalden and Geidel[73] have established that this effect is shared by a series of other polypeptides, and Gertler[74] was able to demonstrate that the L-form of leucylglycylglycine exerts the greatest influence on pancreatic lipase. Liver esterase (and likewise cholinesterase) are not affected.

Many inhibitory effects have been observed in studies on esterases. The most varied tissue lipases and esterases proved more or less capable of being inhibited by the most manifold drugs.

Broekmeyer[75] reported on the susceptibility of serum and liver esterase to strychnine and cocaine. Rona and Ammon[76] investigated the effect of a few isomeric poisons on various lipases.

It was also established that the sensitivity of the esterases toward some inhibitors depends to a large extent on their degree of purity. Zummo[77]

[65] See also E. Bamann and W. Salzer, *Ergeb. Enzymforsch.* **7**, 28 (1938).
[66] M. Shoda, *J. Biochem. Japan* **6**, 395 (1926); *ibid.* **7**, 505 (1927).
[67] T. Okamura, *J. Biochem. Japan* **8**, 351 (1928).
[68] K. Kaziro and K. Tsuji, *J. Biochem. Japan* **11**, 333 (1930).
[69] R. Willstätter and E. Bamann, *Z. physiol. Chem.* **173**, 17 (1928).
[70] K. Krähling and H. H. Weber, *Biochem. Z.* **298**, 227 (1938).
[71] E. Bamann and P. Laeverenz, *Z. physiol. Chem.* **223**, 185 (1934).
[72] E. R. Dawson, *Biochem. J.* **21**, 398 (1927).
[73] E. Abderhalden and W. Geidel, *Fermentforschung* **13**, 156 (1933).
[74] W. Gertler, *Fermentforschung* **15**, 171 (1938).
[75] J. Broekmeyer, *Klin. Wochschr.* **3**, 874 (1924).
[76] Cf. ref. 11; P. Rona and R. Ammon, *Biochem. Z.* **181**, 49 (1927).
[77] C. Zummo, *Boll. soc. ital. biol. sper.* **3**, 455 (1928).

and Gyotoku[78, 79] again showed that purified pancreatic lipase is less sensitive toward quinine. Conversely, Rona and Gyotoku[80] were able to cause the quinine-resistant stomach lipase to become quinine-sensitive. Wohlgemuth and Nakamura[81] demonstrated in a similar manner that the lipase of the skin, which normally is not sensitive to atoxyl and quinine, can be made sensitive toward these drugs by dialysis. Resistance to atoxyl and quinine appears to be due to the presence of contaminants, since it could again be produced by the addition of nondialyzed extract. Rona and Ammon[76] tested whether alkaloids, such as quinine and quinidine, which inhibit the hydrolysis of mandelic acid ester by hog liver esterase, also have an effect on the optical selectivity; this is not the case, however. But Bamann and Laeverenz[82] were able to report that this effect may be demonstrated in more suitable systems. Strychnine not only activated the hydrolysis of mandelic acid esters under the influence of human liver esterase but also caused a decided change in the stereochemical specificity of the enzyme (cf. p. 414). Rona and Petow[48] believed that it would be possible to use the different behavior of a series of tissue lipases, principally towards atoxyl and quinine, for clinical-chemical diagnosis; that is, to establish whether in pathological conditions the appearance of certain tissue lipases can be proved by a change in the behavior toward the two inhibitors. Indeed, lipases which have not been encountered in normal blood seem to be present in some typical cases (cf. also blood lipase, p. 425). Among the hormones which have been investigated, thyroxine has been shown to be an inhibitor for the enzymes which hydrolyze fats.[83] Among the vitamins, vitamin A has been postulated by Johnson[84] to have a specific effect on the activity of lipase (concerning vitamin C and esterase, cf. below).

Murray and King[85] investigated the influence of optically active alcohols on the hydrolysis of ethylbutyrate and ethylpropionate catalyzed by sheep liver esterase. According to their data, the levorotatory forms of methyl-*n*-hexylcarbinol and methyl-β-phenylethylcarbinol inhibit enzymatic hydrolysis about five times more strongly than the dextrorotatory isomers. When rabbit liver esterase is used, the difference between the alcohol forms is only slight. The inhibition is explained on the basis of an affinity of the enzyme for the alcohols.

Glick and King[86, 87] found definite differences between pancreatic and liver

[78] K. Gyotoku, *Biochem. Z.* **193**, 39 (1928).
[79] K. Gyotoku, *Biochem. Z.* **217**, 279 (1930).
[80] P. Rona and K. Gyotoku, *Biochem. Z.* **167**, 171 (1926).
[81] J. Wohlgemuth and Y. Nakamura, *Biochem. Z.* **175**, 216 (1926).
[82] E. Bamann and P. Laeverenz, *Ber.* **63**, 394 (1930).
[83] O. Mühlbock and C. Kaufmann, *Biochem. Z.* **238**, 377 (1931).
[84] B. L. Johnson, *Iowa State Coll. J. Sci.* **2**, 145 (1928).
[85] D. R. P. Murray and C. G. King, *Biochem. J.* **24**, 190 (1930).
[86] D. Glick and C. G. King, *J. Biol. Chem.* **94**, 497 (1931–1932).
[87] D. Glick and C. G. King, *J. Biol. Chem.* **97**, 675 (1932).

esterase: the former is activated, the latter inhibited by a series of quite different alcohols.[88] Finally, mention should be made of the very strong, but reversible inhibitory action of urethans (miotin) on guinea pig serum lipase.[89] The inhibitory effect of the fluoride ion on lipases may also be noted.[90] The fluorophosphates, which are strongly inhibitory for cholinesterase and acetylesterase, have been shown by Webb[91] to be inhibitors for liver esterase and milk lipase as well.

The strongly activating effect of calcium has already been mentioned. It is of considerable interest that according to Scoz[92] sodium citrate also acts as an effective activator for liver, blood, and adrenal esterase in the hydrolysis of tributyrin. Ruffo,[93] in dialysis experiments with esterase preparations from hog liver, found that a coenzyme-like substance went into the dialyzate; this substance could be replaced by traces of Cu, but not by Mg, Mn, Co, or Fe.

A *prolipase* and a *lipokinase* have been postulated by Freudenberg.[94] Mother's milk is assumed to contain the proenzyme which is converted to the enzyme proper by lipokinase, an activator present in gastric juice. It is doubtful whether the experiments of Freudenberg should be interpreted in this way, since bile acids may exert a strongly activating effect on lipase and since Freudenberg himself found that oxidation products of fatty acids may also replace "lipokinase." The assumption of Pantschenko-Jurewicz and Kraut[95] that ascorbic acid (vitamin C) participates in the esterase complex (as esterase "agon") similarly lacks cogency. Starting with the observation that blood lipase is reduced in scurvy[96], it was found that the esterase content of blood and liver in the guinea pig is raised by the injection of ascorbic acid. This increase in blood esterase following ascorbic acid injections was also found in man by Mosters[97] and somewhat later by Krüger.[98] Pantschenko-Jurewicz and Kraut further established that the co-esterase, which can be removed by dialysis, contains a reducing group. The reduction value of one unit of purified esterase is as large as that of approximately 1 γ ascorbic acid. The fact that ascorbic acid may activate this and other enzymes by virtue of its reducing power[99] should

[88] In reference to the influence of indicator dyes on esterase splitting, cf. E. Bamann and M. Schmeller, *Z. physiol. Chem.* **194**, 1 (1931).

[89] E. Stedman and E. Stedman, *Biochem. J.* **25**, 1147 (1931).

[90] J. H. Kastle and A. S. Loevenhart, *Am. Chem. J.* **24**, 491 (1900); G. Peirce, *J. Biol. Chem.* **16**, 5 (1913–1914).

[91] E. C. Webb, *Biochem. J.* **42**, 96 (1948).

[92] G. Scoz, *Enzymologia* **9**, 1 (1940–1941).

[93] A. Ruffo, *Boll. soc. ital. biol. sper.* **17**, 675 (1942); *Atti reale accad. Italia. Rend. classe sci. fis. mat. e nat.* **4**, 424 (1943).

[94] E. Freudenberg, *Z. Kinderheilk.* **46**, 170 (1928).

[95] W. von Pantschenko-Jurewicz and H. Kraut, *Biochem. Z.* **285**, 407 (1936).

[96] B. N. Ghosh, *Ann. Biochem. and Exptl. Med. India* **2**, 233 (1942).

[97] J. Mosters, *Klin. Wochschr.* **15**, 1557 (1936).

[98] W. Krüger, *Klin. Wochschr.* **18**, 19 (1939).

[99] D. Mihklin and O. Ya. Borodina, *Compt. rend. acad. sci. U.R.S.S.* **31**, 921 (1941).

not be disregarded; but the conclusions of Pantschenko-Jurewicz and Kraut are too far-reaching, as has been pointed out. Kraut and Weischer[100] in a later publication no longer supported this concept. Bersin *et al.*[101] concluded that their experiments with animals poisoned with silver indicate a connection between ascorbic acid and the esterase. The poisoning leads to a *C*-hypovitaminosis and a simultaneous drop of the esterase value is observed. In this connection, the methylbutyrate-hydrolyzing action of clupein ascorbinate, though slight, should be brought to mind.[102] (Concerning the so-called lipase activator cf. p. 397.)

The characteristic action of thiols on certain enzymes suggests the desirability of an investigation of compounds with SH groups on esterases as well. Raabe[103] has been able to show that there are relations of the esterase to glutathione. In rabbits the administration of glutathione caused a pronounced increase in liver esterase, as shown by liver test sections. According to Singer,[104] the inhibitory effect of SH reagents increases with decreasing dissociation constant of the ester-esterase complex (tributyrin, tripropionin, and wheat lipase). Pancreatic lipase has likewise been shown to require the presence of SH groups in the activating protein.

Affinity and hydrolysis constants according to Michaelis and Menten, which have been repeatedly determined with ester-esterase complexes (cf. also stereochemical specificity), have been reported by Sobotka and Glick[105] and more recently by Schønheyder and Volqvartz.[106]

The effect of *temperature* on lipase or esterase activity has recently been determined by Schwartz[107] and Sizer.[108]

Esterase units. The establishment of an exactly defined enzyme unit is of importance for the control of the progress of purification of enzyme preparations and also for a whole series of quantitative questions. A few of the proposed definitions of esterase units are presented at this point.

According to Willstätter *et al.*,[3] the "lipase unit" is the amount of lipase that in 1 hour at 30°C. splits 24% of 2.5 g. of olive oil with a saponification number of 185.5 if it is present in a volume of 13 ml. containing 2 ml. of

[100] H. Kraut and Ä. Weischer, *Biochem. Z.* **305**, 94 (1940); Cf. also the reviews of D. Glick, *Ann. Rev. Biochem.* **11**, 51 (1942) and H. Tauber, *Ann. Rev. Biochem.* **10**, 47 (1941), as well as the experiments of C. J. Harrer and C. G. King, *J. Biol. Chem.* **138**, 111 (1941) and of A. Ruffo, *Ricerca sci.* **13**, 608 (1942).

[101] Th. Bersin, S. Raabe, and H. J. Lauber, *Klin. Wochschr.* **17**, 1014 (1938).

[102] K. Felix and A. Mager, *Z. physiol. Chem.* **249**, 111 (1937); *ibid.* **249**, 126 (1937).

[103] S. Raabe, *Biochem. Z.* **299**, 141 (1938).

[104] Th. P. Singer, *J. Biol. Chem.* **174**, 11 (1948); Th. P. Singer and E. S. G. Barron, *ibid.* **157**, 241 (1945).

[105] H. Sobotka and D. Glick, *J. Biol. Chem.* **105**, 199 (1934); see also: R. Ammon, *in* Nord-Weidenhagen, Handbuch der Enzymologie. Akademische Verlagsgesellschaft, Leipzig, 1940, p. 375, table 4.

[106] F. Schønheyder and K. Volqvartz, *Acta Physiol. Scand.* **7**, 376 (1944); *Enzymologia* **11**, 178 (1943–1945); *Acta Physiol. Scand.* **9**, 57 (1945).

[107] B. Schwartz, *J. Gen. Physiol.* **27**, 113(1943).

[108] I. W. Sizer, *Advances in Enzymol.* **3**, 35 (1943).

NH_3—NH_4Cl buffer at pH 8.9 and 10 mg. $CaCl_2$ and 15 mg. albumin as activators. A "butyrase unit," measured on the basis of the hydrolysis of tributyrin, has been defined by Willstätter and Memmen[109] as the amount of enzyme which at pH 8.6 causes a 20-drop diminution of the number of drops of a saturated solution of tributyrin in 50 minutes in a stalagmometer. Finally, an "esterase unit" is the amount of enzyme, measured by its hydrolytic action on methylbutyrate, which at pH 8.9 splits 25% of the ester in 60 minutes. Triacetin may also be used for the hydrolysis. However, the determination of esterase units with triacetin has the severe disadvantage that the enzyme is inactivated too rapidly. The *lipase value* consists of the number of lipase units in 0.01 g. substance.[110]

c. Specificity

Considerations of specificity may well be prefaced by the observation that the nonsusceptibility of an ester to hydrolysis should not always be interpreted to mean that the substrate and the enzyme have nothing to do with each other. It may well be that the affinity of the esterase for the substrate is appreciable but that the intermediate reaction product breaks down so slowly that the ester practically cannot be hydrolyzed at all. Conversely, a reaction which at first glance indicates good enzymatic catalysis need not always be a sign of a particularly high affinity of the enzyme for the substrate. Certain activators may be present in the solution and their removal may bring about less favorable kinetic conditions.[111]

Among the substrates of the lipases and common esterases, a few esters deserve special mention because they are employed as standard substrates for following and measuring esterase activity. The esters which are involved are those of butyric acid, as methyl- and ethylbutyrate and tributyrin. Among the natural fats, olive, castor, almond, cotton, soya, linseed, and coconut oils are used most frequently for the determination of lipase activity. The characteristic relative specificity may be discussed in connection with this listing of the esters and fats. The esterases which preferentially split the butyric acid esters and which exhibit little activity towards the fats, are the esterases proper, as present in liver. On the other hand, pancreas contains a lipase which has a pronounced activity for the fats and only slight activity for the simple esters. Richter and Croft[112] proposed the name of "aliesterases" for those esterases which hydrolyze simple aliphatic esters and glycerides.

Mention may be made of some of the more recent hydrolysis experi-

[109] R. Willstätter and F. Memmen, *Z. physiol. Chem.* **129**, 1 (1923).
[110] R. Willstätter and F. Memmen, *Z. physiol. Chem.* **138**, 216 (1924).
[111] See experiments by P. Rona, E. Chain, and R. Ammon, *Biochem. Z.* **247**, 113 (1932).
[112] D. Richter and P. G. Croft, *Biochem. J.* **36**, 746 (1942).

ments[113] with a series of esters, with both the alcohol and acid residues being determinants of the specificity relationships. It has been established that the hydrolyzability of glycerides by lipase increases with the number of fatty acid residues on the glyceride and with the chain length of the fatty acids. Unsaturated fatty acids of greater chain length increase the hydrolyzability of the glyceride.[114] Tofte[115] in his experiments determined the hydrolyzability and perhaps also the utilization of whale and soya oils of very different degrees of hardness by pancreatic lipases; the higher the degree of hardness of an oil, the more slowly it was hydrolyzed. Leubner[116] also found that the lipase of duodenal juice saponified triolein about ten times more rapidly than tristearin. Ono[117] who employed a great variety

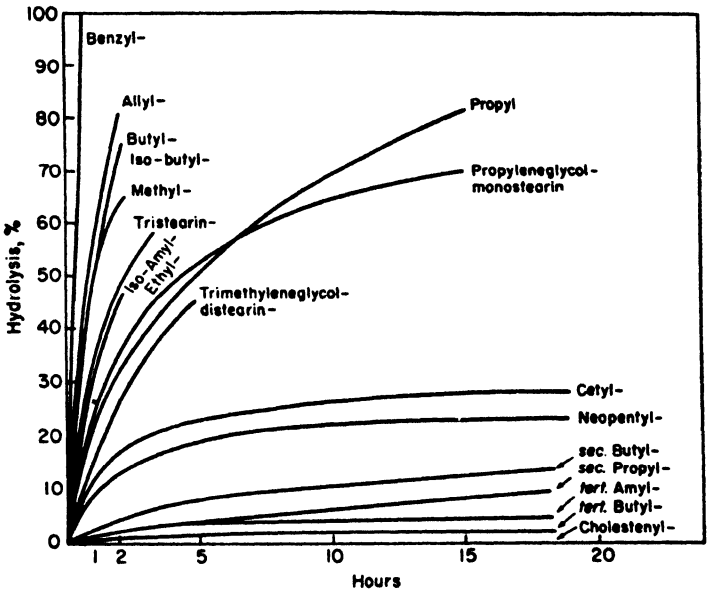


FIG. 1. Hydrolysis of various esters of stearic acid by pancreatic lipase (A. K. Balls and M. B. Matlack, *J. Biol. Chem.* **123**, 679, 1938).

of fats and fatty acid esters as substrates, also established, among other findings, that unsaturated glycerides are more rapidly hydrolyzed. Balls and Matlack[118] investigated the hydrolysis of stearic acid esters by pancreatic lipase; the various alcohols caused appreciable differences in the

[113] Concerning the older data, cf. C. Oppenheimer, Die Fermente und ihre Wirkungen. 5th ed., Georg Thieme, Leipzig, 1925, and Supplement 1, 1935; also the new reviews F. O. Breusch, *Advances in Enzymol.* **8**, 406 (1948) and A. M. Wynne, *Ann. Rev. Biochem.* **15**, 35 (1946).

[114] F. Schønheyder and K. Volqvartz, *Enzymologia* **11**, 178 (1943–1945).

[115] F. Tofte, *Biochem. Z.* **272**, 308 (1934).

[116] H. Leubner, *Deut. Z. Verdauungs- u. Stoffwechselkrankh.* **1**, 155 (1938–1939).

[117] T. Ono, *J. Agr. Chem. Soc. Japan.* **16**, 43 (1940); *ibid.* **17**, 199 (1941).

[118] A. K. Balls and M. B. Matlack, *J. Biol. Chem.* **123**, 679 (1938).

hydrolysis. Figure 1 reproduces these experiments. It may clearly be seen how well e.g., benzylstearate is hydrolyzed; it is even more susceptible than tristearin; the cholesterol ester, on the other hand, is not saponified at all. When a triglyceride, such as triolein, is used, the hydrolysis may yield changing final products, e.g., glycerol, monoglyceride, and diglyceride, depending on the conditions of the experiment (pH, presence and absence of Ca salts).[119]

The hydrolysis of common esters by esterases proper has also been investigated often and from the most different points of view. Lactic acid esters and mandelic acid esters, e.g., are among the hydroxy acids that are hydrolyzed well, as will be discussed in the section on stereochemical specificity.

Among the esterified keto acids, benzoylacetic acid ester is hydrolyzed very rapidly.[120] Concerning benzoylformic acid ester, cf. p. 406 in the section on induction. Only *one* alcohol residue is generally hydrolyzed off from esters of dibasic acids. Accordingly, the esters of oxalic, succinic, phthalic, and fumaric acids are not considered to be hydrolyzable.[121] Among the higher members of this series, however, the diesters as well as the monoesters (adipic acid diethyl and monoethyl ester) are saponified. But Bamann and Rendlen[122] were able to show that the acid esters of lower members of the series of dibasic acids, e.g. succinic and malonic acids, may also be split. Hydrolysis takes place only if the pH of the reaction medium is changed to approximately 5.0. These experiments and findings on the possibility of hydrolyzing ester anhydrides and lactides support the concept formulated already by Bamann, Schweizer, and Schmeller that "the capacity of the ester group of a substrate to combine with the enzyme is decisively influenced by the electrochemical character of the neighboring group."

It is questionable whether cetyl alcohol esterified with higher fatty acids, as found in waxes, causes a special specificity so that the existence of a "cerase" must be assumed. It is true that cerase effects are known. Pertzoff[123] believed that he possessed evidence for a wax-hydrolyzing enzyme in wax moth larvae. However, the experimental work in this study presents appreciable difficulties that greatly complicate or even make impossible an interpretation of such data. Kraut *et al.*[124] were able to show in

[119] P. Desnuelle, M. Naudet and J. Rouzier, *Biochim. et Biophys. Acta* **2**, 561 (1948); A. C. Frazer, and H. G. Sammons, *Biochem. J.* **39**, 122 (1945).

[120] E. Bamann and M. Schmeller, *Z. physiol. Chem.* **188**, 251 (1930).

[121] J. H. Kastle, *Am. Chem. J.* **27**, 481 (1902); A. A. Christman and H. B. Lewis, *J. Biol. Chem.* **47**, 495 (1921); D. A. McGinty and H. B. Lewis, *ibid.* **67**, 567 (1926); A. Lourteig and C. E. Cardini, *Anales farm. y bioquím. Buenos Aires* **18**, 59 (1947); *ibid.* **18**, 54 (1947).

[122] E. Bamann and E. Rendlen, *Z. physiol. Chem.* **238**, 133 (1936).

[123] V. Pertzoff, *Compt. rend.* **187**, 253 (1928).

[124] H. Kraut, H. Burger and W. von Pantschenko-Jurewicz, *Biochem. Z.* **269**, 205 (1934).

their investigations that the hydrolysis of wax which had been described is to be explained by the incompleteness of the methods which had been employed. They further established in their own experiments that larval extracts prepared by different methods exhibited a high activity towards tributyrin and methylbutyrate but no ceratic activity. However, the existence of a cerase need not be unconditionally denied on the basis of these experiments even if positive proof has not been provided for the enzymes as pointed out by Kraut and collaborators. The natural activator of the enzyme may not have been carried over into the extracts, or perhaps "cerase" belongs to the desmoenzymes, i.e., it may be bound so tightly to insoluble protoplasmic components that it cannot be brought into solution by the extraction procedures.

More convincing proof of a ceratic activity has been obtained, however, in wax synthesis. Rona *et al.*[125] carried out the first experiments of this type. Under the conditions which were chosen, synthesis amounting to 19% was found after 5 hours. Fabisch[126] went a step further. He not only showed that cetyl alcohol is esterified with higher fatty acids in aqueous emulsions in the presence of sodium oleate and desoxycholate by ammoniacal extracts of hog pancreas but was also able to isolate the cetyl palmitate which was formed and to identify it chemically.

In connection with specificity considerations mentioned above, the findings of Bamann and collaborators[127] and of Steensholt[128] may now be mentioned. The behavior of esterases toward lactones, which may be considered as inner esters, was investigated. Such compounds, e.g., the lactones of γ-hydroxybutyric acid and of γ-hydroxyvaleric acid, coumarin and santonin, are not hydrolyzed; if they are added to samples of butyric acid methyl ester, they inhibit the hydrolysis of the latter to a considerable degree. It may be concluded from these findings that the esterases combine with the lactones to form complexes. A different situation obtains with certain lactides and esterlike anhydrides. According to Bamann and Schmeller,[127] who studied the lactides of lactic and glycolic acids, diphenylglycolide, benzilide, tetrasalicylide, and polysalicylide, only the first two, and only lactic acid lactide to a significant degree, are hydrolyzed enzymatically. The other compounds, as well as lactyllactic acid, acetylsalicylic acid, and salicylosalicylic acid, are totally unaffected. In these instances, the failure to be hydrolyzed is due to a poor affinity of the tissue lipases for these compounds. Urethans, which may be considered as esters of carbamic acid, again exhibit a different behavior towards esterases:

[125] P. Rona, R. Ammon and H. Fischgold, *Biochem. Z.* **241**, 460 (1931).
[126] W. Fabisch, *Biochem. Z.* **259**, 420 (1933).
[127] E. Bamann and M. Schmeller, *Z. physiol. Chem.* **194**, 14 (1931); E. Bamann, E. Schweizer, and M. Schmeller, *ibid.* **222**, 121 (1933).
[128] G. Steensholt, *Acta Physiol. Scand.* **5**, 71 (1943).

they are split not at all or only very slowly, but they possess a very much greater affinity for the enzyme than does tributyrin.[129]

In this connection, mention should be made of the so-called induction effect. Willstätter and collaborators[130] and later Bamann[131] observed that esterases at first hydrolyze mandelic acid ethyl ester at a very slow rate; normal hydrolysis only sets in after a certain time (cf. Fig. 2). The reason for this finding is a slight admixture of benzoylformic acid ester which

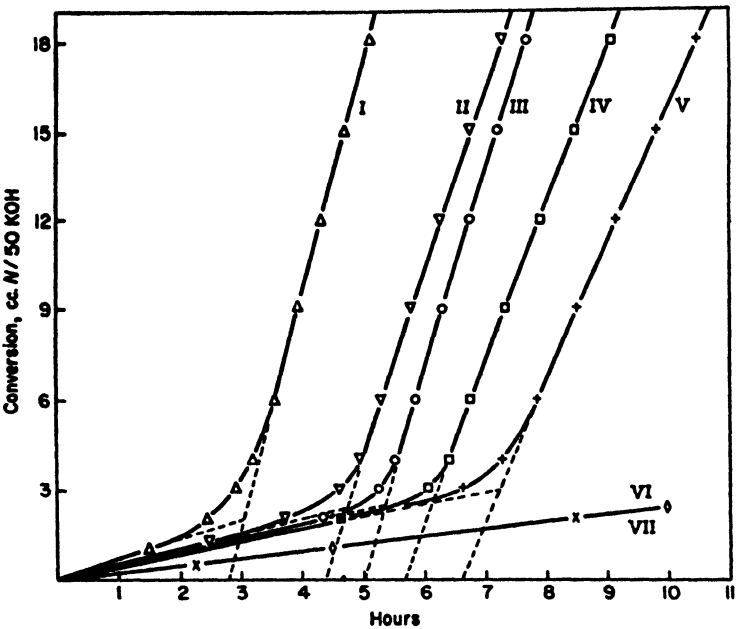


FIG. 2. Induction effect of benzoylformic acid ester in the hydrolysis of mandelic acid ethyl ester by various esterases (E. Bamann and M. Schmeller, *Z. physiol. Chem.* **188**, 251, 1930).

Each sample contained 500 mg. of mandelic acid ethyl ester and approximately 5 mg. of benzoylformic acid ester in 100 cc.

I and II—Experiments with two different human liver esterase preparations.

III to VII—Experiments with the liver esterase of horse (III), dog (IV), rabbit (V), sheep (VI), and hog (VII).

has 5000 times as high an affinity for the enzyme as mandelic acid ethyl ester and which has a very low rate of decomposition. The hydrolyzing enzyme is therefore occupied by the ester until the very slow hydrolysis of the latter is complete. Only then can the saponification of ethylmandelate take place. A highly purified mandelic acid ethyl ester is "induction-free."

[129] E. Stedman and E. Stedman, *Biochem. J.* **25**, 1147 (1931).

[130] R. Willstätter, R. Kuhn, O. Lind, and F. Memmen, *Z. physiol. Chem.* **167**, 303 (1927).

[131] E. Bamann and M. Schmeller *Z. physiol. Chem.* **188**, 251 (1930).

There is no doubt that many observations on the hydrolyzability of substrates by or non-susceptibility to esterases should be thoroughly checked from these points of view before statements on the specificity relationships may safely be made.

Concerning specificity in the synthetic direction, cf. p. 416; concerning stereochemical and *cis-trans* specificity in the hydrolysis and synthesis of esters by esterases, cf. p. 410.

d. Synthetic Activity

The first reports on the synthetic activity of the esterases were by Kastle and Loevenhart[90] and Mohr[132] at the turn of the century. In these studies, the fact that an ester, ethyl butyrate, was formed, was proved by its characteristic fruitlike odor. In later investigations, isolation of triolein[133] was used to give direct proof of the synthesis of esters by pancreas powder; it could be shown that plant esterases also were capable of forming and hydrolyzing triolein.[134] Hamsik[135] investigated the synthetic activity of a series of dry tissue powders from various animals and found that when amyl alcohol or glycerol was used with oleic acid, esterification was promoted particularly by the enzymes derived from pancreas, liver, lung, and intestinal mucosa.

The first study on the equilibria established in hydrolysis and synthesis was reported in 1906. Bodenstein and Dietz[15] investigated the formation and hydrolysis of isoamylbutyric acid ester, with the aid of titrimetric determinations of the formation or disappearance of butyric acid as a measure of esterase activity. Rona and Ammon[14] more recently studied these equilibria with a more uniform ester, namely, *n*-butyl-*n*-butyrate, under otherwise similar conditions and with the use of hog pancreas dry powder. Figure 3 indicates clearly how the equilibrium, catalyzed from both sides, is established.

Rona[16] attempted to set up activity-P_s curves also for the process of enzymatic synthesis by means of suitable examples. Just as the concentration of water may be considered constant in hydrolysis, so the amount of alcohol may be assumed to remain constant in synthetic experiments in view of the large excess and the rate of synthesis may be graphically represented as a function of the amount of acid. In studying the synthetic activity of an esterase, a similar dependence on substrate concentration and on decomposition of the complex is encountered as in studying hydrolysis. An *S*-shaped curve was indeed obtained, but it did not represent a typical activity-P_s curve according to Michaelis and Menten. Rona, Ammon, and Fischgold further investigated the enzymatic esterification

[132] O. Mohr, *Wochschr. Brau.* **19**, 588 (1902).
[133] H. Pottevin, *Compt. rend.* **136**, 767 (1903); *Bull. soc. chim. Paris* **35**, 693 (1906).
[134] Y. W. Jalander, *Biochem. Z.* **36**, 435 (1911); S. Iwanow, *Ber. deut. botan. Ges.* **29**, 595 (1911).
[135] A. Hamsik, *Z. physiol. Chem.* **90**, 489 (1914).

of the geometrically isomeric oleic and elaidic acids with *n*-butyl alcohol. In as far as the curves permitted an evaluation of the affinity relationships, it could be shown that the enzyme has about the same affinity for both acids but that the rate of decomposition of the enzyme-elaidic acid-alcohol complex is considerably higher than that of the corresponding oleic acid complex.

The use of esterase preparations provides an extremely valuable tool for the synthesis of esters of the most varied composition. Preparative and technological chemistry may perhaps draw practical conclusions from this fact. Rona and Mühlbock[136] investigated specificity relationships in numerous experiments on differences in the rate of esterification by hog liver

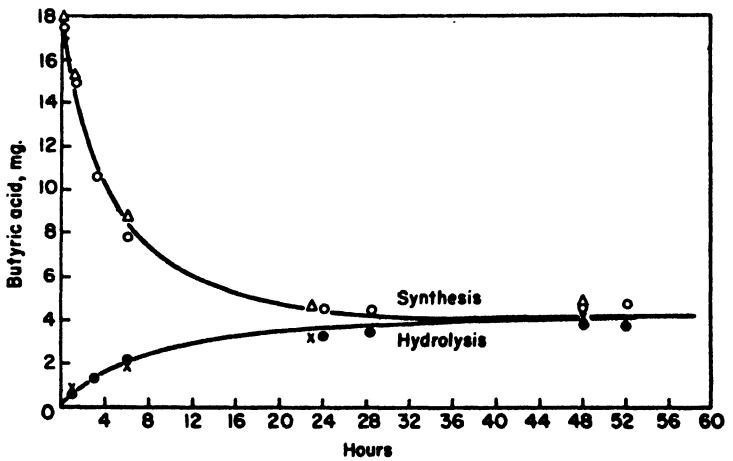


FIG. 3. Formation and hydrolysis of *n*-butyl butyrate by hog pancreas powder (P. Rona and R. Ammon, *Biochem. Z.* **249**, 446, 1932).

and pancreas powders with regard to the constitution of the alcohol and acid. It appears that pancreatic esterase, acting more like a lipase, forms esters of true fatty acids more efficiently than the liver enzyme, which exhibits a greater facility in esterifying short-chain fatty acids. According to Nyrek,[137] cyclic alcohols like cyclohexanol are also esterified with butyric acid. On the other hand, glucose and also the amino acids proved to be refractory to enzymatic esterification.

The described syntheses take place in systems that differ radically from physiological conditions. Sym[138] found benzene to be a particularly suitable solvent for the water-insoluble alcohols and fatty acids. Rona, Ammon, and Fischgold similarly carried out the synthesis of waxes with the aid of pancreas dry powder in a mixture of ether and acetone that contained

[136] P. Rona and O. Mühlbock, *Biochem. Z.* **223**, 130 (1930).
[137] S. Nyrek, *Acta Biol. Exptl. Warsaw* **14**, 157 (1947); *C.A.* **42**, 8844*d* (1948).
[138] E. A. Sym, *Biochem. J.* **30**, 609 (1936).

dissolved palmitic acid and cetyl alcohol.[139] It was, therefore, a great step forward when Fabisch[140] carried out syntheses by means of esterases under conditions approaching physiological ones. He showed that the formation of stearic acid amyl ester by ammoniacal extracts of pancreas powders can proceed quite well in aqueous solutions; however, certain emulsifying agents like sodium oleate or sodium desoxycholate must be added for this purpose. The synthetic and hydrolytic activity of the esterase may also be demonstrated in fresh tissue breis of the livers of rabbits, guinea pigs, and rats.[141]

Itoh[142] described an activator which regulated the hydrolytic as well as the synthetic action of an esterase. According to his data, castor beans contain a substance, the "lipase-activator," which represents a type of redox system and which in its reduced form activates the hydrolysis and inhibits the synthesis mediated by castor bean lipase. The oxidized form, conversely, acts as activator of synthesis and inhibitor of hydrolysis. Cedrangolo[143] also studied the conditions for enzymatic synthesis and hydrolysis of esters and determined the influence of a number of added compounds. In a later publication,[144] this author disagreed with the assertion that oxidation favors the synthetic action of the esterases.

A peculiar phenomenon was observed by Rona *et al.*[145] If the same carbinol with the general formula $C_6H_5 \cdot CHOH \cdot R$ was prepared once by the Grignard method and another time by reduction of the corresponding ketone and then used as a substrate, fundamental differences appeared in the kinetics of the synthesis of the butyric acid ester by hog pancreas powder, although the substrates were apparently identical and differed solely in their method of preparation. The carbinol synthesized by reduction was esterified with butyric acid only to a small extent while the carbinol obtained according to Grignard was esterified very readily. The following explanations for this unusual finding may be considered. The carbinols prepared by the Grignard method may be particularly pure and therefore particularly easily esterified; the carbinols obtained from ketones may contain a contaminant formed during the preparation which inhibits ester synthesis; finally, it may be postulated that the carbinols obtained by reduction are particularly pure and exactly for this reason fail to be esterified because an activator is lacking which is present in the carbinols prepared according to Grignard.

[139] see also: E. A. Sym, *Biochem. Z.* **230**, 19 (1931).
[140] W. Fabisch, *Biochem. Z.* **259**, 420 (1933).
[141] E. S. El Yashkevich, *Biochem. J. Ukraine* **17**, 271 (1941); *C.A.* **39**, 5258[8] (1945).
[142] R. Itoh, *J. Biochem. Japan* **23**, 299 (1936).
[143] F. Cedrangolo, *Enzymologia* **5**, 1 (1938–1939).
[144] F. Cedrangolo, *Atti reale accad. Italia. Rend. classe sci. fis. mat. e nat.* **2**, 78 (1941).
[145] P. Rona, E. Chain, and R. Ammon, *Biochem. Z.* **247**, 113 (1932).

In the paper by Bach and Lovas,[146] evidence was presented for a dependence of the esterase activity of hog pancreas powder, in the direction of synthesis as well as of hydrolysis, on the nutrition of the animal. The pancreas powders derived from fat hogs exhibited a considerably higher enzymatic activity than those derived from lean animals. This finding may be of importance for the industrial preparation of pancreas powders.

Concerning the stereochemical specificity of the esterases in their synthetic capacity, cf. p. 416.

e. Stereochemical and cis-trans Specificity

Stereochemical specificity of the hydrolytic action. The schools of Willstätter and Rona carried out detailed investigations on the stereochemical relationships of the esterases; Bamann in Munich and Ammon in Berlin, with their collaborators, particularly continued along the lines suggested by their teachers and brought the extensive studies on the configuration specificity of the esterases to a certain conclusion.[147]

This development was introduced by the following findings. The first observation of a stereochemical specificity was made by Dakin[148] in 1903. This investigator found that if the hydrolysis of racemic mandelic acid esters by hog liver esterase was stopped before 100% saponification had taken place, and if the acid formed by the enzyme was isolated and tested for optical rotation, then it was seen that the acid was dextrorotatory. Hog liver esterase, therefore, preferred the dextrorotatory ester when allowed to act on the DL-form. If, however, the DL-ester of phenylchloracetic acid was used, it became apparent that the same enzyme hydrolyzed the levorotatory form more rapidly. Dakin's fundamental contribution was given due attention again only twenty years later when the school of Willstätter taught the preparation of purer enzyme samples. Esterases of the most varied origin were tested for their optical selectivity with the aid of such enzyme solutions. Table II summarizes a few of these experiments designed to prove a stereochemical specificity of esterases of different origin.

The most striking result is the pronounced difference between hog pancreas and hog liver esterase in the hydrolysis of mandelic acid esters. This finding is of particular importance because Willstätter and Memmen, using the pure optically active forms of mandelic acid ester, found at first that hog pancreas esterase saponifies the levorotatory ester more rapidly

[146] E. Bach and L. Lovas, *Biochem. Z.* **245**, 345 (1932).

[147] Detailed literature surveys are provided by P. Rona and R. Ammon, *Ergeb. Enzymforsch.* **2**, 50 (1933); R. Ammon, *in* Nord-Weidenhagen, Handbuch der Enzymologie. Akademische Verlagsgesellschaft/Becker & Erler Kom.-Ges., Leipzig, 1940, especially p. 370; E. Bamann und R. Ammon, *in* Bamann-Myrbäck, Die Methoden der Fermentforschung. Georg Thieme, Leipzig, 1941, p. 1704.

[148] H. D. Dakin, *J. Physiol. London* **30**, 253 (1904); *ibid.* **32**, 199 (1905); *Proc. Chem. Soc.* **19**, 161 (1903).

than the dextrorotatory. But when Rona and Ammon[149] investigated the behavior of hog liver esterase toward the pure optically active forms of mandelic acid ester, they also found that the levorotatory form was more rapidly hydrolyzed; this was, however, in direct contrast to the results obtained by hydrolyzing the racemate, which indicated that the dextrorotatory ester was split more efficiently. This contradiction was cleared up by Willstätter *et al.*[150] and by Weber and Ammon[151] after Weber[152] had already suggested the explanation of the optical specificity of the liver esterase which was later confirmed experimentally.

TABLE II

STEREOCHEMICAL SPECIFICITY OF THE ESTERASES

Substrate	Origin of the esterases							
	Human pancreas	Hog			Dog stomach	Guinea pig serum	Carp liver	*Aspergillus oryzae*
		Pancreas	Liver	Stomach				
Mandelic acid methyl ester	−	−	+			−		+
Mandelic acid ethyl ester	−	−	+	+	+		−	+
Phenylchloracetic acid methyl ester		−	−		+			−
Tropic acid methyl ester		+	−	+				−
n-Butyric acid *sec*-butyl ester		−	−					

The − or + sign indicates the rotation of the more rapidly saponified component of the tested racemate.

The two illustrations taken from the publication of Weber and Ammon represent these relationships by means of the activity-P_H curves. The evaluation of these curves yielded the following data for the hydrolysis of mandelic acid ester by the liver enzyme: a comparison of the dissociation constants of the enzyme-substrate complexes $K_{(+)} = 10^{-3.75}$ and $K_{(-)} = 10^{-2.9}$ indicated that the affinity of the enzyme for the (+)-ester is seven times greater than that for the (−)-ester and that the ratio of the hydrolysis constants $k_{(-)}:k_{(+)}$ amounts to 1.75, i.e., that the (−)-ester complex decomposes 1.75 times faster than the (+)-ester complex. The

[149] P. Rona and R. Ammon, *Biochem. Z.* **181**, 49 (1927).
[150] R. Willstätter, R. Kuhn and E. Bamann, *Ber.* **61**, 886 (1928).
[151] H. H. Weber and R. Ammon, *Biochem. Z.* **204**, 197 (1929).
[152] see ref. 149, p. 70.

affinity of pancreatic esterase, on the other hand, is the same for both optically active esters. The ratio of the hydrolysis constants of the two enzyme-substrate complexes is quite similar to that found with liver esterase; it amounts to 1.7, i.e., the (−)-ester complex decomposes 1.7 times more rapidly than the (+)-ester complex. The contradictory result obtained in the enzymatic saponification of the DL-ester is now readily explained: In the racemate, the enzyme is largely removed from the (−)-ester because of the sevenfold higher affinity of the enzyme for the (+)-ester, and the complex with the (+)-ester decomposes more rapidly. If, on the other hand, the optically active esters are separately exposed to the enzyme, this competition for the enzyme does not take place and the rate of ester hydrolysis is determined solely by the more rapid decomposition of the enzyme-(−)-ester complex.

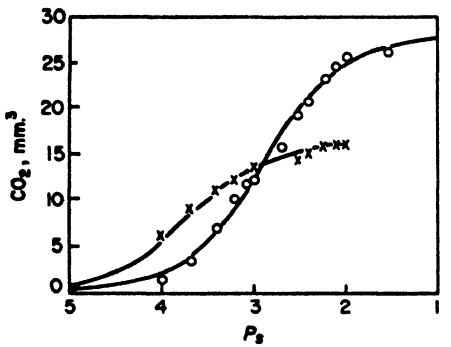


FIG. 4

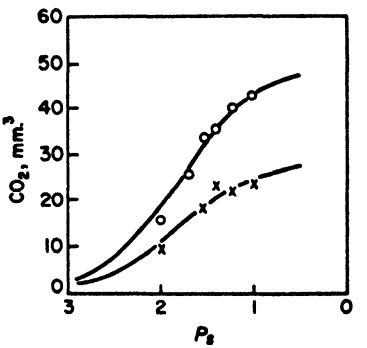


FIG. 5

FIGS. 4 AND 5. Activity-P_s-curve of the hydrolysis of mandelic acid ester modifications by hog liver esterase (Fig. 4) and hog pancreatic esterase (Fig. 5). ×——× (+)-ester, O——O (−)-ester (H. H. Weber and R. Ammon, *Biochem. Z.* **204**, 197, 1929).

The optical selectivity of an enzyme is therefore the resultant of the joint action of the possibly different affinity of the esterases for the optically active forms and the possibly different rates of decomposition of the complexes between the enzyme and the (+)- and (−)-esters.

A further important contribution to the understanding of the mechanism of action of enzymatic reactions was made by investigations which showed that the optical selectivity of an esterase can be influenced. This may take place in various ways. Bamann[153] succeeded in changing the optical orientation of human liver esterase, a particularly suitable enzyme for studies on this problem, by simply varying the concentration of substrate. At higher concentrations of DL-mandelic acid ester the enzyme prefers the

[153] E. Bamann, *Ber.* **62**, 1538 (1929).

(+)-form, at lower concentrations the (−)-form. An intermediate concentration can therefore be found where a symmetrical hydrolysis of the racemic ester takes place. This interesting phenomenon was further studied by Ammon and Geisler[154] with homologous mandelic acid esters (from methyl- to *n*-butylmandelate) and with one and the same enzyme preparation under completely identical conditions.

The finding of Bamann that the configuration specificity of human liver esterase depends on the original substrate concentration led Fischgold and Ammon[155] to submit the problem of the mass action law as the basis of the mechanism of action of enzymes to a critical analysis. Adsorptive relationships, which were mentioned by other investigators in a general manner as a possible basis for enzymatic reactions, readily suggested themselves. In this connection, it became possible to interpret the reversal in the optical orientation of several esterases with increasing substrate concentrations on the basis of such adsorptive relationships. First of all, model experiments on the simultaneous adsorption of acetone and acetic acid on animal charcoal indicated a similar course. Making use of data of Michaelis and Menten themselves, who found a shift in the relative combination of glucose and saccharose with invertase with an increase of substrate concentration, it became possible to interpret the effect of substrate concentration on the stereochemical specificity of human liver esterase also as the distribution of two compounds, dependent on concentration, on the enzyme or adsorbent; in the instance under discussion, this involves the competition of the optical antipodes of the ester. The investigations of Schwab *et al.*,[156] which were also devoted to the problem of this influence of the substrate on the optical behavior of the enzyme, led to the following assumption of general importance: an enzyme may form with the (−)-ester two hydrolyzable complexes, $FS_{(-)}$ and $F(S_{(-)})_2$ and the decomposition of both of these complexes is strongly inhibited by the (+)-ester. The alcohol arising from the ester may also inhibit the decomposition by way of both complexes; nonhydrolyzable or slowly decomposing complexes of $FS_{(-)}$ with alcohol are formed in the process. It may thus be possible to include the influence of the substrate into the overall interpretation by way of such different effects on the catalytically active group of the enzyme (cf. below). Bamann and Laeverenz[157] have demonstrated that the ethanol formed by the hydrolysis of esters may exert an influence on the configuration specificity.

Bamann and Laeverenz[158] showed yet another way of affecting the opti-

[154] R. Ammon and W. Geisler, *Biochem. Z.* **249**, 470 (1932).
[155] H. Fischgold and R. Ammon, *Biochem. Z.* **247**, 338 (1932).
[156] G. M. Schwab, E. Bamann, and P. Laeverenz, *Z. physiol. Chem.* **215**, 121 (1933).
[157] E. Bamann and P. Laeverenz, *Ber.* **64**, 897 (1931).
[158] E. Bamann and P. Laeverenz, *Ber.* **63**, 394 (1930).

cal selectivity of the esterases. This can be done by the addition of certain substances. If the hydrolysis of mandelic acid esters by human liver esterase is allowed to proceed in the presence of strychnine, a clear-cut change in the stereochemical specificity of the enzyme takes place. This finding, which is to be explained by the formation of intermediate reaction products with new properties, could also be interpreted experimentally in this way. Ammon and Fischgold[159] determined the affinity and hydrolysis constants of the complexes of the enzyme with the (−)-and (+)-ester with and without the addition of strychnine and found that the alkaloid had no effect on the affinity of the enzyme for the antipodal forms of the ester but only accelerated the decomposition of the enzyme-(−)-ester complex, as illustrated in Fig. 6.

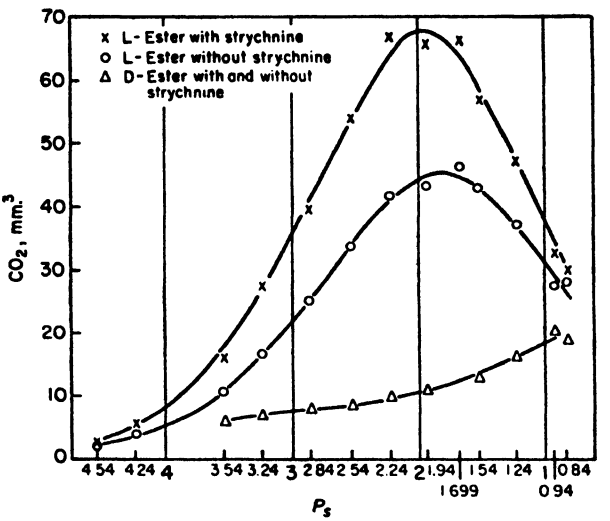


FIG. 6. Effect of strychnine on the stereochemical specificity of human liver esterase (R. Ammon and H. Fischgold, *Biochem. Z.* **234**, 54, 1931).

Another possibility of affecting the optical selectivity of an esterase, as studied by Bamann and Laeverenz[160], consists of pretreatment of the enzyme preparations (human liver esterase). Heating the enzyme powder or preliminary autolysis of the tissue brei brings about changes in the enzyme complex so that the combination of the enzyme with the mandelic acid ester isomers may lead to enzyme-substrate complexes exhibiting new properties.

On the other hand, Ammon and Geisler[161] were unable to find any

[159] R. Ammon and H. Fischgold, *Biochem. Z.* **234**, 54 (1931).
[160] E. Bamann and P. Laeverenz, *Ber.* **63**, 2939(1930).
[161] R. Ammon and W. Geisler, *Arch. path. Anat. Physiol.* (*Virchow's*) **285**, 286 (1932).

changes in the optical selectivity of liver esterase preparations from pathological human livers and also from fetal livers, although the possibility cannot be discounted that the pathological changes which take place in the liver in certain diseases may affect the behavior of the esterases toward mandelic acid esters.

A further possibility of affecting the configuration specificity of the esterases was found by Ammon and Tabor[162] in comparisons of human pancreatic esterase and human liver esterase. When both enzymes were allowed to act simultaneously on mandelic acid ethyl ester with varying substrate concentrations, it appeared as if essentially only the pancreatic enzyme were active, since the same optically specific hydrolysis took place despite variations in the substrate concentration. The effect of the liver enzyme became apparent only when an extract of pancreas powder dried at 120° was added to the ester solution. Bamann *et al.*[163] were able to confirm and extend the findings of Ammon and Tabor by exposing the racemic mandelic acid ester simultaneously to the ester-splitting principles of the pancreas and the liver. They were able to show that the pronounced decrease in the activity of the liver enzyme in the mixed experiment was due to inhibitors formed in the course of hydrolysis. This inhibition corresponds on the one hand to changes in the composition of the substrate, on the other to the appearance of the hydrolytic product alcohol; only the liver enzyme responds to these factors.

Finally, mention may be made of the investigations of Bamann and Feichtner[164] who studied the stereochemical specificity of the liver esterase prepared according to Kraut and Pantschenko-Jurewicz[165] from liver esterase pheron (apoenzyme) and pancreatic lipase agon (coenzyme). This "synthetically" prepared esterase theoretically should also exhibit the optical specificity of the natural esterase. But Bamann's experiments show that this is not so. This investigator is of the opinion "that the type of anchoring of the active group to the protein in each case is of decisive importance. Only then does it appear probable that the stereochemical preference of the synthetic esterase is different from that of the natural enzyme."

Numerous proofs for the optical selectivity of the esterases were obtained in rapid succession; in these studies it made no difference whether the asymmetric carbon atom was in the alcohol or in the acid component of the ester. The same is true for the synthetic activity of these enzymes which has been studied by Rona and Ammon since 1930 and which will be further discussed below.

[162] R. Ammon and E. Tabor, *Biochem. Z.* **267**, 26 (1933).
[163] E. Bamann, C. Feichtner, and W. Salzer, *Biochem. Z.* **288**, 310 (1936).
[164] E. Bamann and C. Feichtner, *Biochem. Z.* **288**, 70 (1936).
[165] H. Kraut and W. von Pantschenko-Jurewicz, *Biochem. Z.* **275**, 114 (1935).

Stereochemical specificity of the synthetic action. A stereochemical specificity could be shown to exist also in the synthetic action of the esterases. Rona and Ammon[166] were the first to investigate the formation of lactic acid esters by pancreas dry powder, and it could be shown that in this instance too an asymmetric synthesis took place. Table III summarizes the results obtained so far on synthesis with very different racemic substrates.

Rona *et al.*[167] studied the hydrolysis as well as the synthesis of *sec*-butylbutyrate. As indicated in Table III, the same optical isomer is also hydrolyzed more rapidly. Rona *et al.*[168,169] further attempted to synthesize esters of mandelic acid, which are in part hydrolyzed by the esterases in a

TABLE III

ROTATION OF THE MORE RAPIDLY FORMED ESTER MODIFICATION

Substrate	Origin of the esterase		
	Hog pancreas	Human liver	Hog liver
D,L-Lactic acid and isoamyl alcohol	+[a]		
D,L-*sec*-Butyl alcohol and *n*-butyric acid	−		
D,L-Methylphenylcarbinol and *n*-butyric acid	+		
D,L-Ethylphenylcarbinol and *n*-butyric acid	+		
D,L-Propylphenylcarbinol and *n*-butyric acid	+		
D,L-Butylphenylcarbinol and *n*-butyric acid	+		
D,L-Mandelic acid and *n*-butyl alcohol	±	±	±
D,L-Mandelic acid and D,L-ethylphenylcarbinol		±[b] +[c]	

[a] Amyl ester of L-(+)-lactic acid.
[b] Polarimetric result for esterification of mandelic acid.
[c] Polarimetric result for esterification of ethylphenylcarbinol.

very characteristic manner. Evidence of synthesis was indeed obtained, but the formation of mandelic acid esters was always asymmetric. Finally, if DL-mandelic acid as well as a racemic alcohol were used for the synthesis, as also shown in Table III, only the alcohol and not mandelic acid was esterified asymmetrically.

Rona and Chain[170] investigated the optical orientation in the synthesis of a series of related alcohols and acids. It was observed that the direction

[166] P. Rona and R. Ammon, *Biochem. Z.* **217**, 34 (1930).
[167] P. Rona, R. Ammon, and M. Werner, *Biochem. Z.* **221**, 381 (1930).
[168] P. Rona, R. Ammon, and H. A. Oelkers, *Biochem. Z.* **231**, 59 (1931).
[169] P. Rona, R. Ammon, and H. I. Trurnit, *Biochem. Z.* **247**, 100 (1932).
[170] P. Rona and E. Chain, *Biochem. Z.* **258**, 480 (1933).

of rotation of the more rapidly formed component tended sometimes towards the positive, sometimes towards the negative side:

α-chlorpropionic acid	(−)	vinylamylcarbinol	(−)
β-phenylpropionic acid	(−)	ethylamylcarbinol	(+)
phenylchloracetic acid	(±)	ethyl-*n*-propylcarbinol	(+)
α-hydroxybutyric acid	(−)	methylisopropylcarbinol	(−)
β-hydroxybutyric acid	(−)	propylisopropylcarbinol	(−)

This change in the direction of rotation is particularly striking in the case of the structurally similar aliphatic carbinols. It could be shown for the secondary carbinols that the enzyme has a strict configuration specificity; it always prefers the component of the same steric configuration, regardless of the direction of rotation. The optical selection by the enzyme corresponds exactly to the steric coordination which was given by Levene and Marker[171] for homologous series of secondary carbinols on theoretical grounds. It is of interest to note among the tests of acids that phenylchloracetic acid, which has a similar configuration as mandelic acid, is also esterified symmetrically although the hydrolysis proceeds asymmetrically.

cis-trans Specificity. Using suitable geometrically isomeric acids or esters, it was possible to demonstrate that esterases also possess *cis-trans* specificity.

Fabisch[172] was the first to investigate the specificity relationships in ester formation by esterases, particularly by the enzyme of hog pancreas; he used the three pairs of acids fumaric and maleic, oleic and elaidic, erucic and brassidic acids. A comparison of fumaric and maleic acids (C_4-acids) showed that the *cis*-form (maleic acid) is esterified approximately twice as rapidly (with the simple alcohols C_1 to C_6) as the *trans*-form. With oleic and elaidic acids (C_{18}-acids), only a slight difference in the rate of esterification was observed, and again the *cis*-form (oleic acid) was favored. No difference could be established among the third pair of acids (C_{22}-acids). Apparently, there is a dependence of specificity on the length of the carbon chain.

Rona *et al.*[16] attempted to characterize the formation of *n*-butyl oleic or elaidic acid ester with hog pancreas dry powder more exactly according to the concept of Michaelis and Menten. In as far as the experimentally obtained curves (which are widely different from the ideal curves of Michaelis and Menten) allow a conclusion to be made, it may be stated that the enzyme exhibits approximately the same affinity for both geometrical isomers of the acids. On the other hand, the rate of decomposition of the

[171] P. A. Levene and R. E. Marker, *J. Biol. Chem.* **97**, 379 (1932).
[172] W. Fabisch, *Biochem. Z.* **234**, 84 (1931).

enzyme-elaidic acid-alcohol complex (*trans*-form) appears to be greater than that of the corresponding oleic acid complex.

Steensholt[173] investigated the hydrolysis of the ethyl esters of fumaric and maleic acids as well as of oleic and elaidic acids by liver esterase. It was found that the rate of hydrolysis of ethyl fumarate (*trans*-form) was considerably greater than that of ethyl maleate. On the other hand, no difference in the rate of splitting could be demonstrated for the other pair of esters.

Asymmetric splitting of amino acid esters. In conclusion, mention may be made of the highly interesting observation of Brenner *et al.*[174] who encountered an unexpected esterase effect of crystallized chymotrypsin and trypsin preparations: DL-phenylalanine and DL-tryptophan methyl esters were saponified asymmetrically. If it could be shown that this effect was not caused by an esterase impurity contaminating these crystalline proteases, then a group *non*specificity with simultaneous maintenance of the stereochemical specificity could be considered to have been established.

f. Occurrence and Special Properties of the Esterases and Lipases in the Plant and Animal Kingdom

A strict separation between esterases and lipases is not possible since only relative specificity is present. The differences between the two groups of enzymes are qualitative. One enzyme behaves more like a lipase, i.e., it hydrolyzes fats better and common esters not as well; another exhibits the properties of an esterase proper, i.e., it splits common esters more easily than typical fats. For example, pancreatic esterase is a typical lipase, whereas the enzyme found in liver is an esterase. The difference between the two enzymes is clearly indicated by the experiments of Willstätter and Memmen.[45] If the esterase activities of hog liver and hog pancreas powders are compared with olive oil, tributyrin, and methylbutyrate as the substrates, then 1 g. pancreas powder corresponds to the activity of 10,600 g. liver powder in the hydrolysis of olive oil, to the activity of 100 g. liver powder in hydrolysis of tributyrin, and to the activity of 0.4 g. in hydrolysis of methylbutyrate. Although the differences are not always so pronounced, these figures clearly indicate the lipase or esterase nature of the two tissue esterases. When the synthetic actions of pancreas or liver preparations are studied, the lipase characteristics usually do not become important. However, such experiments are usually carried out under completely different conditions (cf. p. 420). Among the plant lipases, such as castor bean lipase, the lipase nature predominates; plant lipases also catalyze the saponification of common esters, but only to a very slight extent. In view of their occurrence, a subdivision of the field into plant and animal esterases appears practical.

[173] G. Steensholt, *Acta Physiol. Scand.* **5**, 103 (1943).
[174] M. Brenner, E. Sailer, and V. Kocher, *Helv. Chim. Acta* **31**, 1908 (1948).

Plant esterases, phytolipases. The occurrence in plants of enzymes that hydrolyzed fats and that played an important role in plant metabolism and especially in germination[175] was noted a long time ago. Green[176] carried out the first precise studies; one of his outstanding contributions was to call attention to the high lipase content of *castor bean seeds.* Connstein *et al.*[177] investigated the castor bean enzyme more in detail and reported the main data on its preparation and properties, including its activity in acid media and its particularly efficient hydrolytic action on fats. Willstätter and Waldschmidt-Leitz[178] carried these studies further with more modern techniques and were able to confirm many findings of the earlier investigators. The most striking property of castor bean lipase is its lack of solubility. Its preparation from seeds therefore requires different procedures than those employed to obtain the soluble esterases, such as that from the pancreas. This insoluble enzyme must be prepared as a fat- and water-containing cream which represents an emulsion of the seeds; this method was also proposed by Hoyer.[179] These preparations are difficult to purify, apparently because the colloidal system appertaining to the enzyme is changed by such treatments, and they are relatively unstable. The optimum pH of the castor bean enzyme lies between 4.7 and 5.0. However, this is true only of the enzyme originally present in the seed; during germination, the esterase acquires the capacity to split fats near the neutral pH range. It has been possible to produce this change in activity artificially by the action of pepsin, so that it may be assumed that a similar development is caused by proteolytic enzymes during germination. The new form of the enzyme was named germination- or blasto-lipase by Willstätter and Waldschmidt-Leitz, while the lipase originally present in the seed was called spermatolipase. These two enzymes differ not only in their pH optimum but also in stability; blastolipase is much more stable during drying and defatting than spermatolipase. Yang and Hsu[180] studied the effect of a whole series of salts (effect of anions and cations) on the activity of castor bean lipase. The hydrolyzability of various fats by this enzyme in comparison to pancreatic lipase was investigated by Ahmad and Bahl.[181]

Nicolai[182] also described a blastolipase in germinating *pine seeds*; according to Sandberg and Brand,[183] the lipase of raw papain also resembles a

[175] Recent reviews of phytolipases: H. E. Longenecker, *in* Anderson, Enzymes and Their Role in Wheat Technology. Interscience Publishers, New York, 1946, p. 127; B. Sullivan, *ibid.* p. 153.

[176] J. R. Green, *Proc. Roy. Soc. London* **48**, 370 (1890).

[177] W. Connstein, E. Hoyer, and H. Wartenberg, *Ber.* **35**, 3988 (1902).

[178] E. Waldschmidt-Leitz, *Z. physiol. Chem.* **134**, 161 (1924).

[179] E. Hoyer, *Z. physiol. Chem.* **50**, 414 (1906–1907).

[180] S. C. Yang and C. Hsu, *J. Biol. Chem.* **155**, 137 (1944).

[181] B. Ahmad and A. N. Bahl, *J. Sci. Ind. Research India* **5B**, 1 (1946).

[182] H. W. Nicolai, *Biochem. Z.* **174**, 373 (1926).

[183] M. Sandberg and E. Brand, *J. Biol. Chem.* **64**, 59 (1925).

blastolipase. No lipase could be found in resting *cotton seeds*. Lipolytic activity develops only during germination, as demonstrated by Olcott and Fontaine.[184] Engel[185] has reported that the aleuron cells and the seed of *wheat* and *rye kernels* are rich in esterase (hydrolysis of methylbutyrate), but no esterase could be demonstrated in the aleuron coat of barley. The esterase content of the endosperm of cereals is very low. A relationship between the number of mitochondria and esterase activity (or the activity of other enzymes) could not be established. Kinetic studies (inactivation and dissociation constants) of wheat lipase have been carried out by Singer and Hofstee.[21] Lowy[186] studied *malt lipase*. *Soybeans* also contain a lipase.[187]

Among the *molds*, most work has been done on the esterase component of takadiastase from *Aspergillus oryzae*, which is to be considered as an esterase proper on the basis of its behavior toward esters. Concerning the esterase of *Aspergillus niger*, cf. tannase (p. 441). The lipase of *Fusarium lini* Bolley is responsible for the large amounts of fat which are formed during growth of the Fusaria.[188]

Gorbach and Güntner[189] investigated the esterase activity of *yeast* which has a pH optimum of 6.6–6.8. Peters and Nelson[190] concerned themselves with the lipase of *Mycotorula lipolytica*.

In this connection, mention may be made of the lipases and esterases of *bacteria* and *viruses*. Christie and Graydon[191] demonstrated the existence in staphylococci of a lipase; there was no relation between pathogenicity and lipase levels. Concerning other bacteria, cf. Bayliss *et al.*[192] and Starr and Burkholder.[193] No esterase activity was encountered in various strains of gonococci.[194] Hoagland and collaborators,[195] who studied the lipases of the elementary bodies of vaccinia, demonstrated that this virus is able to adsorb lipase and other enzymes strongly, so that it is not certain whether this virus lipase originates in the virus itself or in the host tissue.

Zoolipases. Some different properties of the lipases and esterases of *pancreas* and *liver* have already been discussed. Therefore, a few examples of their distinct behavior toward added substances and substrates may be summarized in Table IV.

[184] H. S. Olcott and T. D. Fontaine, *J. Am. Chem. Soc.* **63**, 825 (1941).

[185] C. Engel, *Rec. trav. chim.* **64**, 318 (1945); *Biochim. et Biophys. Acta* **1**, 278 (1947); C. Engel and L. H. Bretschneider, *ibid.* **1**, 357 (1947).

[186] B. Lowy, *Western Brewing and Distributing* **53**, 8 (1945).

[187] G. Gorbach, *Fette u. Seifen* **48**, 308 (1941).

[188] R. P. Mull and F. F. Nord, *Arch. Biochem.* **5**, 283 (1944).

[189] G. Gorbach and H. Güntner, *Sitzber. Akad. Wiss. Wien. Math.-naturw. Klasse. Abt. II b* **141**, 415 (1932).

[190] I. I. Peters and F. E. Nelson, *J. Bact.* **55**, 581 (1948).

[191] R. Christie and J. J. Graydon, *Australian J. Exptl. Biol. Med. Sci.* **19**, 9 (1941).

[192] M. Bayliss, D. Glick, and R. A. Siem, *J. Bact.* **55**, 307 (1948).

[193] M. P. Starr and W. H. Burkholder, *Phytopathology* **32**, 598 (1942).

[194] I. Ciaccio, *Boll. soc. ital. biol. sper.* **19**, 224 (1944).

[195] C. L. Hoagland, S. M. Ward, J. E. Smadel, and T. M. Rivers, *J. Exptl. Med.* **76**, 163 (1942).

Virtanen and Suomalainen[196] have reported a finding which characterizes the different nature and at the same time the possible interconversion of the two enzymes. If rabbits were injected with pancreatic lipase preparations and the ester- and fat-splitting enzyme levels in the various tissues determined, the greatest increase was found in the liver. The liver esterase obtained after the injection continued to exhibit the same properties as the enzyme obtained from normal animals; it was inactivated by atoxyl, it was not affected by quinine, it hydrolyzed olive oil only slightly; the enzyme which had been injected, on the other hand, saponified olive oil well, was inhibited by quinine and was not affected by atoxyl. The Finnish investigators who were able thus to prove the interconversion of the two esterases, found themselves confirmed along different lines by Kraut and Pantschenko-Jurewicz;[55] the latter investigators stated that the active enzyme

TABLE IV

COMPARISON OF PANCREATIC LIPASE AND LIVER ESTERASE

	Behavior towards					
	Atoxyl	Quinine	Leucylglycylglycine	Tributyrin	Methylbutyrate	D,L-Methylmandelate
Pancreatic lipase	Resistant	Sensitive	Activated	Better hydrolyzed	Less well hydrolyzed	Levorotatory split product
Liver esterase	Sensitive	Resistant	Not influenced	Less well hydrolyzed	Better hydrolyzed	Dextrorotatory split product

system, the holoenzyme, is related to the active group, the coenzyme, and to the apoenzyme, on the basis of the mass action law:

$$\frac{\text{coenzyme} \times \text{apoenzyme}}{\text{holoenzyme}} = K$$

Consequently, the free components are present beside the holoenzyme, which is the active enzyme proper. And since the K of the esterases is not very small, the amounts of coenzyme and apoenzyme are not insignificant. The equation shows further that the decomposition of the holoenzyme can be prevented by an excess of the individual components. According to Kraut, an excess of free apoenzyme is present. The esterase coenzyme can be adsorbed on siliceous earth, the apoenzyme on lead phosphate. Concerning the coenzyme, it has been stated that it can be converted to a product, called anagon, which is no longer capable of form-

[196] A. I. Virtanen and P. Suomalainen, *Z. physiol. Chem.* **219**, 1 (1933); *Suomen Kemistilehti* **B5**, 1 (1932).

ing the holoenzyme. An equilibrium between coenzyme and anagon has also been postulated. The concept of Kraut and Pantschenko-Jurewicz, with the exception of this last assumption, is identical with the view inaugurated by von Euler and Myrbäck in 1923 and summarized by: apoenzyme + coenzyme $\rightleftharpoons$ holoenzyme. Kraut and collaborators were able to demonstrate further that the pancreatic and liver enzymes possess the same coenzyme and are differentiated only by the apoenzyme. This is shown also by the *in vitro* conversion which these workers carried out: if a liver esterase preparation of low activity (by removal of the coenzyme) but with a high apoenzyme content is mixed with a pancreatic lipase preparation rich in coenzyme, then a new enzyme system is formed. Concerning the stereochemical specificity of these synthetic enzymes, cf. p. 416.

As will be discussed below under blood lipase, the lipase of the pancreas, which can be given off to the blood, possesses a special significance which has been investigated recently. According to Fodor,[197] the different inhibitory effects of gum arabic on pancreatic juice indicate the presence of two lipolytic systems, a lipase and an esterase. Frazer and Sammons[198] also studied the fat- and ester-splitting action of pancreatic juice which is due not only to the pancreas (the major component), but also to the stomach, the intestine, and lipolytic bacteria. Fats are apparently hydrolyzed *in vivo* only to the mono- and diglycerides and not to glycerol. Lagerlöf[199] has made a detailed study of the dependence of the enzyme content of pancreatic juice on the function of the pancreas.

The esterase of the liver (hog) likewise seems to be composed of two isodynamic enzymes one of which is more soluble than the other, as described by Falconer and Taylor.[52] Fodor[197] also found two esterases in mammalian livers by inhibition experiments, but it has not been possible to establish that these esterases and those of Falconer and Taylor are identical. (However, hog liver was not studied by Fodor.) The esterase content of single liver cells was determined by Omachi *et al.*[200] According to this study, the microsome fraction of mouse liver contains 47% of the total esterase content of liver tissue. Experimental liver damage leads to changes in the esterase levels.[201] Normal *bile* does not contain any lipase, according to Schiller.[202]

Stomach lipase, which was discovered in 1900 by Volhard,[203] was more extensively studied by Willstätter *et al.*[204] It was found to be present in

[197] P. J. Fodor, *Exptl. Med. and Surg.* **5**, 140 (1947); *Nature* **159**, 375 (1947).

[198] A. C. Frazer, *Physiol. Revs.* **26**, 103 (1946); A. C. Frazer and H. G. Sammons, *J. Physiol. London* **103**, P 5 (1944–1945); *Biochem. J.* **39**, 122 (1945).

[199] H. O. Lagerlöf, *Acta Med. Scand.* **110**, Suppl. 128 (1942).

[200] A. Omachi, C. P. Barnum, and D. Glick, *Proc. Soc. Exptl. Biol. Med.* **67**, 133 (1948).

[201] T.-T. Chen, *Tohoku J. Exptl. Med.* **38**, 193 (1940).

[202] W. Schiller, *Surg. Gynecol. Obstet.* **72**, 70 (1941).

[203] F. Volhard, *Münch. med. Wochschr.* **47**, 141, 194 (1900).

[204] R. Willstätter, F. Haurowitz, and W. Petrou, *Z. physiol. Chem.* **144**, 68 (1925)

man and in fairly large amounts in carnivores and rodents. Lower levels of the enzyme were present in birds and fishes, while no stomach lipase at all could be shown in ruminants and pigeons. Itoh and Kamisasanuki[205] recently confirmed these results. They also showed that the lipase can be demonstrated in the human embryo in the seventh to eighth month.

The easily characterized stomach lipase is also present in human gastric juice. Schønheyder and Volqvartz[206] reported that the pH optimum of stomach lipase is dependent on the type of substrate: it lies near 5.5 for lower triglycerides and at 7.5 for higher ones. Human stomach lipase is very stable in acid medium. According to Véghelyi,[207] lipase may be increased in acute pathological states. Glick,[208] employing the micromethods developed by the school of Linderstrøm-Lang, demonstrated that an esterase is present in the stomach and also in the *duodenum* of the hog.

Bickel and Kanitz[209] found no enzyme which would hydrolyze tributyrin in their studies of the juice of the lowest portion of the human *ileum*. On the other hand, Nothmann and Wendt[210] had been able to show with depancreatized dogs that owing to the absence of the pancreas there was no or only a very slight hydrolysis of fats in the small intestine but that they were split in the large intestine, apparently by bacterial action. This hydrolyzed fat is of no importance for the organism since it is no longer resorbed. This finding explains the fatty stools which are passed in pancreas hypofunction and which contain large amounts of fatty acids together with nonsaponified fats. The latter are caused by the lack of lipase activity and the fatty acids are produced by the bacterial lipases in the lower intestine where they are no longer resorbed. According to Andersen,[211] the lipase content of duodenal juice (also the levels of trypsin and amylase) is decreased in marasmus of children.

The demonstration of hydrolysis of fats by *lung tissue* is due to Sieber.[212] Bradley[213] and Hamsik[135] showed that this tissue is enzymatically active in the direction of synthesis as well as of lipolysis. From the clinical point of view, the problem of lung lipase in tuberculosis has been of great interest. Kanócz[214] reported that the lung lipase levels of infected animals decrease, but Rordorf[215] was unable to find any differences between normal and dis-

[205] R. Itoh and K. Kamisasanuki, *J. Biochem. Japan* **33**, 269 (1941).
[206] F. Schønheyder and K. Volqvartz, *Acta Physiol. Scand.* **11**, 349 (1946).
[207] P. Véghelyi, *Ann. Paediat.* **168**, 93 (1947).
[208] D. Glick, *Z. physiol. Chem.* **223**, 252 (1934); *Medd. Carlsberg Lab.* **20**, No. 5 (1933–1934); *J. Chem. Education* **12**, 253 (1935).
[209] A. Bickel and H. R. Kanitz, *Biochem. Z.* **270**, 378 (1934).
[210] M. Nothmann and H. Wendt, *Arch. exptl. Path. Pharmakol.* **162**, 472 (1931).
[211] D. H. Andersen, *Am. J. Diseases Children* **63**, 643 (1942).
[212] N. Sieber, *Z. physiol. Chem.* **55**, 177 (1908).
[213] H. C. Bradley, *J. Biol. Chem.* **13**, 407 (1912–1913).
[214] D. Kanócz, *Z. Tuberk.* **63**, 113 (1932).
[215] G. Rordorf, *Arch. sci. biol. Italy* **20**, 442, 464, 469 (1934).

eased animals. It is very much to the point that Virtanen and Suomalainen[216] observed no decrease of lipase in the lungs of animals in experimental tuberculosis, but that the enzyme content of other tissues was lower. Bach and Lusztig[217] found significant decreases in the lipase content of the lung in human tuberculosis, but only in chronic pathological states. Concerning more recent studies on the relationship of tuberculosis to the lipolytic enzyme of blood, cf. van Oordt.[218] This investigator studied the capacity of the blood to split methylbutyrate and tributyrin. It was found that the esterase (methylbutyrate) of blood is subject to changes in tuberculosis while no effects of the disease on lipase (tributyrin) can be observed.

Takasaka[219] reported on the lipase of *brain*. This enzyme was also studied by Edlbacher *et al.*[220] The activity of the enzyme in brain appears to be slight.

Matlack and Tucker[221] found an esterase with a pronounced action on benzylbutyrate in the *muscles* of various animals (hog, mutton, beef, and fishes). This enzyme also strongly hydrolyzes the glycerides of the lower fatty acids, so that it is to be considered as an esterase. Remarkable features are its tight combination with muscle tissue and the demonstration of appreciable continued activity at temperatures below the freezing point of water.

According to Thompson and Whittaker,[222] an esterase (hydrolysis of tributyrin and methylbutyrate) as well as a cholinesterase are present in *skin*.

The lipase of the *placenta* has been extensively studied by Anselmino and Hoffmann.[223] The lipase levels are quite appreciable and rise until the middle of pregnancy is reached; such a rise was also found by Abe.[224]

Kelly[225] reported that the *mammary glands* of mammals contain lipase which, however, can be demonstrated only when the animal is pregnant. This enzyme, which can synthesize as well as hydrolyze fats, is transferred to the milk (cf. below on milk lipase).

Huggins and Moulton[226] investigated the esterase of *testicular tissue* (as well as of other tissues) under normal and pathological conditions.

[216] A. I. Virtanen and P. Suomalainen, *Nature* **133**, 532 (1934).
[217] E. Bach and L. Lusztig, *Arch. path. Anat. Physiol. (Virchow's)* **280**, 325 (1931).
[218] A. van Oordt, *Deut. med. Wochschr.* **62**, 2047 (1936).
[219] T. Takasaka, *Biochem. Z.* **184**, 390 (1927).
[220] S. Edlbacher, E. Goldschmidt, and V. Schläppi, *Z. physiol. Chem.* **227**, 118 (1934).
[221] M. B. Matlack and I. W. Tucker, *J. Biol. Chem.* **132**, 663 (1940).
[222] R. H. S. Thompson and V. P. Whittaker, *Biochem. J.* **38**, 295 (1944).
[223] K. J. Anselmino and F. Hoffmann, *Arch. Gynäkol.* **139**, 202 (1930).
[224] M. Abe, *Japan. J. Obstet. Gynecol.* **13**, 301 (1930).
[225] P. L. Kelly, *J. Dairy Sci.* **28**, 793 (1945).
[226] C. Huggins and S. H. Moulton, *J. Exptl. Med.* **88**, 169 (1948).

Brahn[227] found no lipase in *carcinoma tissue*. Gomori[228] also demonstrated that *tumors* are devoid of lipase. Greenstein,[229] who carried out very detailed and extensive studies on tissue lipases under normal and pathological conditions, found a decrease in the lipase content of tumor tissue in mice. Edlbacher and Neber[230] established that the capacity of tissues (liver, brain, blood) of tumor animals to split fats is greatly decreased in comparison to that of the corresponding tissues of normal animals (concerning blood lipase and tumors, cf. p. 426). This finding was confirmed by Troescher and Norris:[231] during the growth of an implanted adenocarcinoma in rats the blood esterase level dropped, to rise again upon removal of the tumor. According to American investigations,[232] blood esterase is about twice as high in strains of tumor-sensitive mice as in tumor-resistant animals.

On the basis of studies of Werner[233] it may be considered probable that the normalization of the blood fat level after alimentary hyperlipemia is not caused by the action of *blood lipases*. Persiel[234] and Takahashi[235] have shown that blood lipase splits tributyrin well, but it is not a true lipase since it hydrolyzes olive oil only slightly. Similar results were obtained by Goldstein and Roe.[236] It cannot yet be decided whether the enzyme in the blood comes from the individual tissues, e.g., pancreas, liver, thyroid, lung, or whether it originates in the leucocytes. The situation is different under pathological conditions: tissue lipases may then appear in the blood which can be partially characterized by their behavior toward quinine and atoxyl. A great number of studies, especially from the purely clinical point of view, have appeared on these points. Only a few of the sometimes contradictory findings can be singled out for discussion. Schmitt[237] concerned himself with the value of the estimation of atoxyl-resistant lipase in blood serum for the diagnosis of disturbances of the pancreas. He was able to show an increase in the blood level of this enzyme in all cases of acute pancreatic necrosis. Moreover, an increase was also observed in 30 cases of chronic pancreatitis. Finally, the atoxyl-resistant lipase of blood was increased in a whole series of diseases of abdominal organs and it was concluded that this increase suggested an involvement of the pancreas.

[227] B. Brahn, *Sitzber. preuss. Akad. Wiss. Physik.-math. Klasse* **1916**, 478.
[228] G. Gomori, *Arch. Path.* **41**, 121 (1946); *Proc. Soc. Exptl. Biol. Med.* **67**, 4 (1948).
[229] J. P. Greenstein, *J. Natl. Cancer Inst.* **5**, 31 (1944).
[230] S. Edlbacher and M. Neber, *Z. physiol. Chem.* **233**, 265 (1935).
[231] E. E. Troescher and E. R. Norris, *J. Biol. Chem.* **132**, 553 (1940).
[232] D. Burk and R. J. Winzler, *Ann. Rev. Biochem.* **13**, 490 (1944).
[233] M. Werner, *Z. ges. exptl. Med.* **83**, 402 (1932).
[234] H. Persiel, Doctoral thesis, München, 1925.
[235] Y. Takahashi, *Arch. ges. Physiol.* (*Pflügers*) **225**, 42 (1930).
[236] N. P. Goldstein and J. H. Roe, *J. Lab. Clin. Med.* **28**, 1368 (1943).
[237] K. Schmitt, *Arch. klin. Chir.* **174**, 512 (1933).

Similar conclusions have recently been reached by Lagerlöf.[238] This increase is of greater diagnostic value in diseases of the pancreas than that of blood diastase since the lipase can be determined more rapidly in the blood and since the higher level is maintained for a longer time than that of the amylase. On the other hand, Popper and Scholl[239] failed to attribute any great diagnostic value to the estimation of the atoxyl-resistant lipase of blood. Bernhard,[240] who found that this enzyme was also increased in the blood of patients with carcinoma, was able to show that the enzyme is given off from the tumor tissue to the blood. According to this investigator, the appearance of atoxyl-resistant lipase in carcinomatous individuals is of diagnostic value under certain conditions. After surgical removal of the tumor, this increase in serum lipase is no longer found. If the increased level persists or returns later, there is danger that a remission has taken place. Simon,[241] who at first considered the atoxyl-resistant pancreatic lipase in blood to be significant for disturbances of the pancreas and also for pernicious anemia, was able to show later[242] that the quinine-resistant serum lipase has no practical significance. On the other hand, Friesz and Hallay[243] always encountered this enzyme in the serum in acute diffuse pathological conditions of liver cells. Fiessinger and Gajdos,[244] who considered the serum lipase to be identical with the liver lipase, found that the former was decreased especially in liver cirrhosis. They therefore recommended the use of the liver enzyme in the therapy of human liver cirrhosis and apparently also had some therapeutic success. Comfort[245] stressed the diagnostic value of the estimation of serum lipase in acute pancreatitis. In such cases the olive oil-hydrolyzing effect of serum is increased.[246]

In the field of animal experiments, in pancreatectomies and ligatures of the pancreatic ducts, American investigators, e.g., Goldstein, Jacobson, Telford, and Roe[247] and Nothman, Pratt, and Benotti,[248] demonstrated that serum esterase originates from two sources, one the pancreas and the other extrapancreatic tissue. Nakagawa[249] showed that in experimental damage to the pancreas the lipase content of lung, liver, spleen, and kidneys

[238] H. O. Lagerlöf, *Nord. Med.* **24**, 1992 (1944); *Acta Med. Scand.* **120**, 407 (1945); *ibid.* **128**, 380 (1947); *ibid.* **128**, 413 (1947); *ibid.* **128**, Suppl. 196, 399 (1947).

[239] H. L. Popper and R. Scholl, *Med. Klin.* **30**, 335 (1934).

[240] F. Bernhard, *Z. Krebsforsch.* **38**, 450 (1933).

[241] H. Simon, *Klin. Wochschr.* **4**, 2295 (1925).

[242] H. Simon, *Klin. Wochschr.* **5**, 2443 (1926).

[243] J. Friesz and I. Hallay, *Z. klin. Med.* **113**, 275 (1930).

[244] N. Fiessinger and A. Gajdos, *Ann. méd.* **38**, 405 (1935).

[245] M. W. Comfort, *Am. J. Digestive Diseases Nutrition* **3**, 817 (1937).

[246] See also the review on the significance of enzymes for clinical diagnosis by R. Ammon and E. Chytrek, *Ergeb. Enzymforsch.* **8**, 91 (1939).

[247] N. P. Goldstein, M. Jacobson, I. R. Telford, and J. H. Roe, *J. Lab. Clin. Med.* **31**, 999 (1946); J. H. Roe and N. P. Goldstein, *ibid.* **28**, 1334 (1943).

[248] M. M. Nothman, T. D. Pratt, and J. Benotti, *J. Lab. Clin. Med.* **33**, 833 (1948).

[249] Y. Nakagawa, *Mitt. med. Ges. Okayama* **53**, 1039 (1941). *C.A.* **37**, 2072[4] (1943).

is also changed. The significance of serum lipase in various gynecological disturbances has been investigated by Takeda *et al.*[250] Hess and Viollier[251] reported on a sort of "protective lipase reaction": after intravenous injection of olive oil into rats, the enzyme content of plasma increases to more than twice the original value; the quinine-resistant component increases approximately twofold, the atoxyl-resistant component eightfold. In addition to quinine and atoxyl, diisopropylfluorophosphate and di-(2-chloroethyl)methylamine have recently been shown to be of use in the characterization of the esterase of human plasma.[252]

The presence of a lipase has been demonstrated in *leucocytes*, especially the eosinophils,[253] as well as in *lymphocytes*.[254] An ester-splitting enzyme could also be shown to be present in *erythrocytes*.[255]

Sussner[256] investigated the tributyrinase of human cerebrospinal fluid. The enzyme was always found but no correlations with age, sex, or the type of neurological disturbances could be established.[257] Ikebata,[258] using the stalagmometric method, found a certain lipase activity in the *aqueous humor* of the rabbit.

Cattaneo and Scoz[259] established the presence of esterase and lipase activities, among other enzymes, in *pleural effusions* and also in tubercular *pus*. Kálló[260] demonstrated the presence of a lipase in the lymph of the *ductus thoracicus*. Scheer[261] investigated the *salivary lipase* of man; this enzyme can be found in infants as early as the first month. Scheer assumed that salivary lipase acts to free the oral cavity of fatty materials. Koldajew and Pikul[262] studied the lipase of dog saliva which they obtained through fistulae placed in the parotid, submaxillary, and sublingual ducts. The enzyme obtained from the parotid gland was most active. The lipolytic activity of saliva proved to be independent of diastase. The pH optimum was 7.5–7.7.[263] Koebner[264] found that there were no deviations of the lipase titer from normal values in a series of pathological conditions.

[250] K. Takeda, K. Temma, and K. Kubo, *Japan. J. Obstet. Gynecol.* **24**, 18 (1941).
[251] W. Hess and G. Viollier, *Helv. Physiol. et Pharmacol. Acta* **6**, C 19 (1948).
[252] D. H. Adams and V. P. Whittaker, *Biochem. J.* **44**, 62 (1949).
[253] J. R. Lévy and M. Gabe, *Compt. rend. soc. biol.* **139**, 981 (1945).
[254] F. Nees, *Biochem. Z.* **124**, 156 (1921); L. Aschoff and H. Kamiya, *Deut. med. Wochschr.* **48**, 794 (1922); W. Fleischmann, *Biochem. Z.* **200**, 25 (1928); S. N. Basu, *J. Indian Chem. Soc. Ind. & News Ed.* **4**, 113 (1941).
[255] J. Broekmeyer, *Klin. Wochschr.* **3**, 1526 (1924).
[256] H. Sussner, *Klin. Wochschr.* **15**, 1490 (1936).
[257] Cf. also N. Brower and S. Tashiro, *J. Biol. Chem.* **133**, xvii (1940).
[258] T. Ikebata, *Biochem. Z.* **182**, 236 (1927).
[259] C. Cattaneo and G. Scoz, *Klin. Wochschr.* **15**, 1912 (1936); G. de Michele, *Boll. soc. ital biol. sper.* **20**, 156 (1945).
[260] A. Kálló, *Z. ges. exptl. Med.* **86**, 848 (1933).
[261] K. Scheer, *Klin. Wochschr.* **7**, 163 (1928).
[262] B. Koldajew and E. Pikul, *Biochem. Z.* **212**, 53 (1929).
[263] M. Katzenstein, *Z. ges. exptl. Med.* **69**, 179 (1930).
[264] H. Koebner, *Z. ges. exptl. Med.* **76**, 792 (1931).

As early as 1902, Moro[265] established the presence of a lipase in mother's *milk*. This finding was confirmed by Davidsohn.[266] According to Resch[267] and Behrendt,[268] mother's milk lipase is of significance for the infant, since a lipolytic action may take place in the stomach of the infant in view of the weak acidity of his gastric juice (cf. p. 400 on lipase and prolipase). The milk of animals contains an appreciably lower amount of lipase than human milk; it also possesses somewhat different properties.[269] Mattick and Kay[270] investigated the relation of cow's milk tributyrinase to the lactation cycle. According to Itoh and Kamisasanuki,[271] the enzyme, which is present in cow's and goat's milk in an inactive form and which can be activated by small amounts of NH_4OH, has a pH optimum of 8.0. More recently, milk lipase and esterase has been studied more extensively by American investigators. The esterase can be inhibited by atoxyl but not by quinine. Peterson *et al.*[272] reported further properties of the enzyme (using tributyrin as a substrate) on the basis of data obtained with their assay procedure. The inactivation of the esterase by temperature was studied by Hetrick and Tracy.[273] Hlynka and Hood[274] extensively studied the relationships between milk lipase and the lipase of cheese; it was also indicated that there is a connection between the enzyme and the appearance of rancidity in the cheese. Butter also contains the lipase derived from milk, and this enzyme is the cause of the lipolysis of butter fat.[275] According to Kay,[276] the light sensitivity of milk esterase (cow's milk) is remarkable. Irradiation of the milk with sunlight for 30 minutes leads to an 80% destruction of the enzyme. This destruction also continues in the dark following irradiation. The addition of 0.1% NaCN largely protects against this effect of irradiation. This finding may be related to the observation of Hlynka and collaborators[277] that Cu inhibits milk lipase. Even the traces of Cu present in milk may activate the dissolved oxygen so that the milk enzyme is inhibited.[278]

[265] E. Moro, *Jahrb. Kinderheilk.* **56**, 391 (1902).
[266] H. Davidsohn, *Z. Kinderheilk.* **8**, 14 (1913).
[267] A. Resch, *Jahrb. Kinderheilk.* **85**, 377 (1917).
[268] H. Behrendt, *Jahrb. Kinderheilk.* **102**, 291 (1923).
[269] A. I. Virtanen, *Z. physiol. Chem.* **137**, 1 (1924).
[270] E. C. V. Mattick and H. D. Kay, *J. Dairy Res.* **9**, 58 (1938).
[271] R. Itoh and K. Kamisasanuki, *J. Biochem. Japan* **33**, 289 (1941).
[272] M. H. Peterson, M. J. Johnson, and W. V. Price, *J. Dairy Sci.* **26**, 233 (1943).
[273] J. H. Hetrick and P. H. Tracy, *J. Dairy Sci.* **31**, 881 (1948).
[274] I. Hlynka and E. G. Hood, *J. Dairy Sci.* **25**, 111 (1942); *ibid.* **25**, 389 (1942); I. Hlynka, E. G. Hood, and C. A. Gibson, *ibid.* **26**, 1111 (1943); *ibid.* **28**, 79 (1945); *Sci. Agr.* **27**, 50 (1947); *Can. Dairy and Ice Cream J.* **20**, 26 (1941); P. B. Larsen, G. M. Trout, and I. A. Gould, *J. Dairy Sci.* **24**, 771 (1941).
[275] V. N. Krukovsky and B. L. Herrington, *J. Dairy Sci.* **25**, 231 (1942); *ibid.* **25**, 237 (1942); V. N. Krukovsky and P. F. Sharp, *ibid.* **23**, 1109 (1940).
[276] H. D. Kay, *Nature* **157**, 511 (1946).
[277] I. Hlynka, E. G. Hood, and C. A. Gibson, *J. Dairy Sci.* **25**, 923 (1942).
[278] V. N. Krukovsky and P. F. Sharp, *J. Dairy Sci.* **23**, 1119 (1940).

The ester-hydrolyzing enzyme of *urine*, which has been investigated by Bloch,[279] is quinine-resistant. The lipase content of urine is greatly increased in diffuse kidney disturbances. Yasuda[280] found an increase in cases of kidney tuberculosis. Zorn[281] observed that urinary lipase (hydrolysis of olive oil) is present in particular abundance in the urine of youths and also of rabbits. A number of relations to human pathology have been established. Ammon and Chytrek[282] also investigated the lipolytic action of urine. Stalagmometric determinations generally indicated only very slight tributyrin-splitting effects. There is no difference between the urine of children and adults in the course of the hydrolysis of tributyrin.

The tributyrin- and methylbutyrate-splitting action of *embryo extract* (chicken egg) has been studied by Ammon and Schütte.[283] In this connection, the behavior of the enzyme during incubation was also investigated. The hydrolyzability of both substrates by embryo extracts exhibits a rapid increase which is doubtless connected with the development of the embryo and the many new enzymatic accomplishments of the organism. Comparable results were obtained by Lindvall[284] with sea urchin eggs. Carlson[285] studied the lipolytic action of grasshopper eggs at various stages of development. In connection with these suggested relations of the enzymes to ontogenesis, the findings of Olcott and Fontaine with fertilized cotton seeds (cf. p. 420) may be recalled.

Arvy and Gabe[286] demonstrated a lipase in the hemolymph of various *insects*. Fodor[287] studied the olive oil- and tributyrin-splitting action of extracts of tissues and eggs of the African migratory locust. The lipolytic action of wax moth larval extracts has already been discussed (cf. p. 404). The investigations of Olivier[288] and Fodor[289] may also be mentioned. Olivier was able to show that such extracts possess antibiotic activity against tubercle bacilli; it has not yet been established to what extent a "cerase" effect is involved. Fodor found that extracts of the larvae of the mulberry silk moth can split lower as well as higher fatty acid esters and also cetylacetate. This investigator assumed that these insects carry various esterase systems.

[279] E. Bloch, *Klin. Wochschr.* **2**, 1318 (1923).
[280] R. Yasuda, *Tohoku J. Exptl. Med.* **20**, 265 (1932–1933).
[281] B. Zorn, *Fermentforschung* **15**, 397 (1938).
[282] R. Ammon and E. Chytrek, *in* Nord-Weidenhagen, Handbuch der Enzymologie. Akademische Verlagsgesellschaft, Leipzig, 1940, p. 390.
[283] R. Ammon and E. Schütte, *Biochem. Z.* **275**, 216 (1935).
[284] S. Lindvall, *Arkiv Kemi Mineral. Geol.* **26B**, N:o 9 (1948).
[285] L. D. Carlson, *Biol. Bull.* **81**, 375 (1941).
[286] L. Arvy and M. Gabe, *Compt. rend. soc. biol.* **140**, 757 (1946).
[287] P. J. Fodor, *Enzymologia* **12**, 333 (1946–1948); *ibid.* **12**, 343 (1946–1948); *ibid.* **13**, 57 (1948).
[288] H. R. Olivier, *Nature* **159**, 685 (1947).
[289] P. J. Fodor, *Enzymologia* **13**, 66 (1948).

Belgian investigators[290] studied the enzymes, including the lipases, of free-living and parasitic plathelminths. The lipase content of the free-living *worms* (Turbellariae) is of the same order of magnitude as that of mammalian tissue; the enzyme levels in the parasitic cestodes and nematodes, on the other hand, are very low. These differences in the occurrence of lipases in these worms may be explained on the basis of adaptation. Since fats constitute a poor source of energy under anaerobic conditions, the lipolytic enzymes assume a secondary role in the parasitic worms.

g. Practical Applications of Esterases

The hydrolysis of oil into acid and glycerol with the aid of castor bean lipase may be mentioned first; Hoyer[291] developed this method on a large technical scale but the procedure does not seem to have found wide acceptance.

A further example of the practical uses of esterases is provided by the interesting attempt to use the enzyme preparations obtained from the pancreas of slaughtered animals, which also contain lipases, as an organic laundry soaking agent. The principle of such a preparation ("Burnus") involves the use of a period of several hours' soaking (overnight) for the enzymatic degradation of dirt particles contained in the laundry. The diastases and proteolytic enzymes naturally aid in this endeavor.

Esterases have a certain significance in the fermentation industries. During germination of barley a degradation of the fats takes place which is of importance for the properties of the beer, since the foaming capacity of beer is lowered by fats. A direct practical use of the lipases, e.g., by artificial addition, does not seem to have been envisaged for these purposes.

Castor bean lipase appears to be useful for the preparation of vitamin A from cod liver oil: the vitamin is easily extracted following a hydrolysis of the oil by the lipase.[292]

The lipases contained in oleiferous seeds, e.g., cotton seeds, may be of significance for the storage of these seeds since the enzymes may cause a more or less pronounced fat hydrolysis. It is therefore important from a practical point of view to inhibit this enzyme action.[293] Similarly, the esterases play a role in the storage of wheat and also in baking processes.[294]

[290] E. Pennoit-de Cooman and G. van Grembergen, *Verhandel. Koninkl. Vlaam. Acad. Wetensch. Klasse Wetensch.* **4**, 7 (1942).

[291] E. Hoyer, *in* Oppenheimer, Technologie der Fermente, IV: 2. 5th ed., Georg Thieme, Leipzig, 1929, p. 1.

[292] M. Ogawa, *J. Agr. Chem. Soc. Japan* **20**, 342 (1944).

[293] A. M. Altschul, M. L. Karon, L. Kyame, and C. M. Hall, *Plant Physiol.* **21**, 573 (1946).

[294] Cf. ref. 175; B. Sullivan, *in* Anderson, Enzymes and Their Role in Wheat Technology. Interscience Publishers, New York, 1946, p. 153; H. Miyosi, *Japan J. Obstet. Gynecol.* **23**, 267 (1940).

A certain significance for milk storage[295] and cheese making[296] has likewise been attributed to the lipases.

Medicine, especially in its diagnostic and therapeutic aspects, also makes use of the esterases. Clinical interest[297] in lipase determinations centers mainly on serum lipase. Not only the increase or decrease of the absolute value is of interest but also the possibility that the addition of particular compounds may indicate the tissue of origin of the serum lipase and may thus permit conclusions to be drawn concerning the functional efficiency of the tissues in question. For example, the lipase effect of the pancreas is characterized by the fact that it can be abolished by quinine, that it is quinine-sensitive. The esterase originating from the liver, which may also get into the bloodstream, is completely inhibited by atoxyl. Serum esterase itself, the origin of which has not yet been elucidated with certainty, is sensitive to both atoxyl and quinine. In recent years, the appearance of an atoxyl-resistant esterase in serum appears to have assumed a certain significance for cancer diagnosis. There is no doubt that the determination of the lipase of duodenal juice is of practical importance, since it gives a direct indication of the function of the pancreas.

In regard to therapy, numerous preparations should be mentioned which are used to good advantage for the support of digestive processes in disturbances of the stomach and intestinal tract. However, the action involved here is not attributable solely to esterases but also to the participation of carbohydrate- and protein-splitting enzymes.

2. Acetylesterase

Among the special esterases, one enzyme in particular is worthy of note. This recently, and quite unexpectedly, has been shown to hydrolyze esters of acetic acid most efficiently and appears to be present in greater abundance in plant tissues than in the animal organism. This enzyme is therefore called acetylesterase or acylase.

Jansen *et al.*[298] have made a very extensive study of this enzyme and have investigated its occurrence in citrus fruits, its isolation, estimation, and specificity relationships; some remarkable findings have resulted from their work.

[295] N. P. Tarassuk and J. L. Henderson, *J. Dairy Sci.* **25**, 801 (1942); N. P. Tarassuk and G. A. Richardson, *Science* **93**, 310 (1941).

[296] F. J. Babel, *J. Dairy Sci.* **27**, 679 (1944); F. J. Babel and B. W. Hammer, *ibid.* **28**, 201 (1945).

[297] Cf. reviews by R. Ammon und E. Chytrek, *Ergeb. Enzymforsch.* **8**, 91 (1939); *in* Bamann-Myrbäck, Die Methoden der Fermentforschung. Georg Thieme, Leipzig, 1941, p. 2969.

[298] E. F. Jansen, M. D. F. Nutting, and A. K. Balls, *J. Biol. Chem.* **170**, 417 (1947); *ibid.* **175**, 975 (1948); E. F. Jansen, R. Jang, and L. R. MacDonnell, *Arch. Biochem.* **15**, 415 (1947).

As has been mentioned, acetylesterase hydrolyzes in a particularly pronounced manner the esters of acetic acid; the nature of the alcohol component is practically without importance. Hydrolysis was efficient with aliphatic, aromatic, and amino-alcohol acetic acid esters, such as acetals, acetyl glycols, *o*-nitrophenylacetate, and acetylcholine. If the alcohols are esterified with other acids, then the hydrolyzability by acylase decreases rapidly. Simple esters like methylethylbutyrate, ethyleneglycoldiformate, monobutyrin, tributyrin, and monopropionin are still split but the extent of hydrolysis is slight compared to that of the acetals. *N*-Acetylethanolamine, *N*-acetylglucosamine, and acetylphosphate are not hydrolyzed.

The fact that acetylcholine can be split promptly suggests an identity of acetylesterase with cholinesterase. Such is, however, not the case, since acylase cannot be inhibited by the alkaloid of the calabar bean, eserine or physostigmine, which is a good cholinesterase inhibitor. An additional piece of evidence is the fact that cholinesterase does not appear to occur in plant tissues.[299] On the other hand, both acylase and cholinesterase are inhibited by certain phosphate compounds which will be discussed below. We are thus confronted again by the remarkable phenomenon of one substrate being attacked by two different enzymes. A differentiation of acetylesterase from lipases and common esterases is provided by the fact that tributyrin is hydrolyzed only to a very slight extent and olive oil not at all, as mentioned above.

According to the American investigators, the pH optimum lies between 5.5 and 6.5 when acetals are employed as the substrates. The same authors also determined the Michaelis-Menten constants. NaCl activates the enzyme, sodium oxalate stabilizes the esterase. The fluoride ion has no influence on acylase. However, the enzyme is strongly inhibited by diisopropylfluorophosphate (DFP); the original investigators established the inhibition constants: 5×10^{-5} *M* DFP inhibits the hydrolysis of the acetic acid ester to 50% under certain conditions. Other phosphoric acid derivatives, e.g. hexaethyltetraphosphate and tetraethylpyrophosphate (TEP), are also strongly inhibitory, though less so than DFP. Acetylesterase shares this capacity of being inhibited by the phosphates mentioned with no other enzyme of plant origin, such as urease, papain, β-amylase, and pectinesterase. Dialysis does not decompose the enzyme-inhibitor complexes with DFP, in contrast to the eserine-cholinesterase complex; a small amount of decomposition is noted when the other two phosphates are the inhibitors. If, on the other hand, the enzyme is inhibited *in situ* with DFP or TEP, the enzyme in the citrus fruit reaches its original activity again after 3–4 days. The fruit apparently contains a factor which causes the regeneration of the esterase.

[299] R. Ammon and W. Dirscherl, Fermente, Hormone, Vitamine und die Beziehungen dieser Wirkstoffe zueinander. 2nd ed., Georg Thieme, Leipzig, 1948, p. 77.

Bovet Nitti[300] showed that acylase is also present in cobra venom. This indicates that it can occur in animal tissues. It has not yet been investigated to what degree acetylesterase may be identical with the morphinesterase of serum reported by Wright,[301] which can hydrolyze diacetylmorphine (heroin) and which belongs in the group of the azolesterases of Glick.[302]

3. Tropinesterases

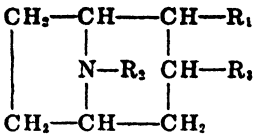
(Atropine-, Cocaine- and Tropacocainesterase)

Fleischmann[303] was the first to demonstrate that rabbit serum is capable of destroying the alkaloid atropine. It was not until 1938, however, that F. and M. Bernheim[304] presented certain proof of the enzymatically catalyzed hydrolysis of atropine into tropine and tropic acid. Later, Glick[305] and Ammon and Savelsberg[306] concerned themselves more extensively with the hydrolysis of such alkaloid esters derived from tropine or ecgonine.

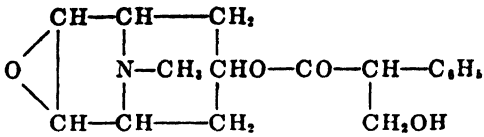
A better understanding of the specificity relationships of this special group of esterases may be aided by the schematic review (see p. 434) of the structural formulae of the substrates to be discussed.

The compounds indicated here require the existence of three enzymes, atropine-, cocaine-, and tropacocainesterase, which Glick has very appropriately grouped under the name of tropinesterases. Since cholinesterase (cf. the contribution of Augustinsson) also hydrolyzes a substrate with an *N*-alcohol and since other esterases can split a whole series of other esters with substituted amino- and nitrogen-containing heterocyclic alcohols, Glick in 1942 proposed the name of "azolesterases" for the entire group of enzymes. Included among them we find the tropinesterases, the cholinesterases (true and pseudocholinesterase), the benzoylcholinesterase of Sawyer,[307] and the esterases which hydrolyze various other *N*-alcohol esters, among them drugs with anesthetic and spasmolytic properties.[308] Of all these azolesterases, only the tropinesterases are discussed in the following section.

[300] F. Bovet Nitti, *Experientia* **3**, 283 (1947).
[301] C. I. Wright, *J. Pharmacol. Exptl. Therap.* **71**, 164 (1941).
[302] D. Glick, *J. Am. Chem. Soc.* **64**, 564 (1942).
[303] P. Fleischmann, *Arch. exptl. Path. Pharmakol.* **62**, 518 (1910).
[304] F. Bernheim and M. L. C. Bernheim, *J. Pharmacol. Exptl. Therap.* **64**, 209 (1938).
[305] D. Glick, *J. Biol. Chem.* **134**, 617 (1940); D. Glick and S. Glaubach, *J. Gen. Physiol.* **25**, 197 (1941–1942); D. Glick, S. Glaubach, and D. H. Moore, *J. Biol. Chem.* **144**, 535 (1942).
[306] R. Ammon and W. Savelsberg, *Z. physiol. Chem.* **284**, 135 (1949). (Investigations since 1942.)
[307] C. H. Sawyer, *Science* **101**, 385 (1945).
[308] See also: H. Blaschko, T. C. Chou, and I. Wajda, *Brit. J. Pharmacol.* **2**, 108 (1947); S. Ellis, *J. Pharmacol. Exptl. Therap.* **91**, 370 (1947).



Tropine skeleton



Scopolamine

	R_1	R_2	R_3
Tropine	H	CH_3	OH
Atropine	H	CH_3	O—CO—CH(CH_2OH)—C_6H_5 (Tropic acid residue)
Homatropine	H	CH_3	O—CO—CHOH—C_6H_5 (Mandelic acid residue)
Novatropine	H	$(CH_3)_2 \cdot Br$	O—CO—CHOH—C_6H_5
Tropacocaine	H	CH_3	O—CO—C_6H_5 (Benzoic acid residue)
Ecgonine	COOH	CH_3	OH
Benzoylecgonine	COOH	CH_3	O—CO—C_6H_5
Ecgonine methyl ester	CO—O—CH_3	CH_3	OH
Cocaine	CO—O—CH_3	CH_3	O—CO—C_6H_5

The most remarkable property of atropinesterase and also of cocainesterase may be mentioned first: these enzymes cannot be demonstrated in all animal species, and when they are present in a species, e.g., rabbits, they do not appear in each animal.[209] Glick and Glaubach showed that only about every fourth rabbit has an atropinolytic principle in the serum; in these animals, atropinesterase could then also be demonstrated in the tissues. No correlation between occurrence of the enzyme and the season of the year or the sex, color, age, or weight of the animals could be established. Ammon and Savelsberg concluded that the capacity to split atropine is genetically conditioned since they found that of 33 rabbits which constituted an inbred tribe started by one female and three males, 17 exhibited atropinesterase activity while this was true of only 9 of 35 animals of random origin. Sawin and Glick[210] were able to adduce further

[209] See J. Lévy and E. Michel, *Bull. soc. chim. biol.* **27**, 570 (1945).
[210] P. B. Sawin and D. Glick, *Proc. Natl. Acad. Sci. U. S.* **29**, 55 (1943).

proof for this conclusion: the capacity to split atropine is inherited in the form of a gene (As) located on the same chromosome as the gene (E) for the diffusion of the black pigment of the fur. The gene (As) is incompletely dominant since homozygotes produce the enzyme in a more active form than heterozygotes. Atropinesterase is not present at birth but appears for the first time after about a month and shows the tendency to occur in higher concentrations in females and to be demonstrable in a higher percentage of animals of that sex than of males.

Similar hereditary relationships may also hold for cocainesterase which was likewise not found in all rabbits by Ammon and Savelsberg, in contrast to the data of Glick. Of 22 animals, only one exhibited cocainesterase activity, 7 others were endowed with cocainesterase as well as atropinesterase, while 6 were able to split only atropine.

These differences in occurrence lead to the conclusion that atropin- and cocainesterase are different. Still another distinct entity is tropacocainesterase which was found by Glick to be present in all horse and rabbit sera. The following table taken from the data of Glick and Glaubach clearly shows the specificity relationships.

TABLE V

COMPARISON OF HYDROLYZABILITY OF VARIOUS TROPINE DERIVATIVES BY HORSE SERUM AND THE SERA OF TWO RABBITS

Enzyme from	Atropine hyoscyamine	Homatropine	Scopolamine	Novatropine	Tropacocaine	Cocaine	Acetylcholine
Horse	−	−	−	−	+	−	+
Rabbit I	+	+	+	+	+	+	+
Rabbit II	−	−	−	−	+	+	+

Atropinesterase thus hydrolyzes the closely related esters like hyoscyamine (as the L-isomer; atropine is the DL-form), homatropine, scopolamine, and novatropine. Tropacocaine, which represents the benzoic acid ester ψ-tropine, is hydrolyzed by all the sera, and the same is true of acetylcholine, while cocaine is split only by the rabbit sera.

Ammon and Savelsberg summarized their findings in table VI.

This summary also indicates the common features of the behavior of different sera toward atropine, hyoscyamine, and homatropine (= atropinesterase). As was mentioned above, a distinction should be made between this enzymatic entity and cocainesterase; the action of the latter consists in splitting methyl alcohol off from the carboxyl group of ecgonine without attacking the ester linkage between benzoic acid and the secondary

alcohol in ecgonine (whereas according to Glick the latter linkage can be hydrolyzed off enzymatically in pseudotropine benzoic acid ester). Cholinesterase is, of course, found in all groups.

TABLE VI

THE ACTION OF FOUR DIFFERENT RABBIT SERA ON SOME TROPINE DERIVATIVES

Rabbit serum group	Atropine hyoscyamine	Homatropine	Cocaine	Ecgonine methyl ester	Benzoylecgonine	Acetylcholine
I	−	−	−	−	−	+
II	+	+	+	+	−	+
III	+	+	−	−	−	+
IV	−	−	+	+	−	+

This enzyme may easily be distinguished from the azolesterases by means of inhibition with eserine. Atropinesterase indeed does not exhibit the high sensitivity towards physostigmine[304, 306] which characterizes cholinesterase. Glick[310] was able to show that atropinesterase is activated by KCl, NaCl, NaBr, NaI, KCNS, $MgSO_4$, and $CaCl_2$. Cyanides, fluorides, and also sulfhydryl compounds proved to be inhibitors. The pH optimum of atropinesterase is 8.1–8.4; the Michaelis constant of 6×10^{-5} indicates the unusually high affinity of the enzyme for the substrate.

The existence of atropinesterase poses the question whether certain pharmacological and clinical experiences with atropine and cocaine may be explained with the aid of the enzyme.[311] It is known, for example, that the rabbit is insensitive to high doses of atropine. But since animals devoid of atropinesterase activity also exhibit this lack of sensitivity to *Atropa belladonna*, atropinesterase does not offer any possible explanation. Likewise, the fact that patients with Parkinson's disease can stand very high doses of atropine has no connection with a possible protective enzyme effect of atropinesterase since normal human subjects and also post-encephalitic subjects treated with atropine have no atropinesterase in their sera (Ammon and Savelsberg). The same conclusion appears to hold for cocainism. Normal human serum has no effect on the hydrolysis of cocaine.

4. CHOLESTEROL ESTERASE

Cholesterol esterase, which is largely focused on the formation and hydrolysis of cholesterol esters, assumes a special position among the esterases because the conditions for the hydrolytic and synthetic actions can be obtained easily.

Some evidence for the occurrence of a cholesterol ester-splitting enzyme

[311] See also F. Bernheim, vol. II, chapter 66.

was obtained first by Kondo[312] and then by Schultz[313] in horse and beef liver. Mueller[314] and Abderhalden and Weil[315] also observed a hydrolysis of cholesterol esters by pancreas extracts and intestinal secretions. Thannhauser[316] studied this enzyme more extensively. In view of the finding that bile and duodenal juice of man contain only free cholesterol and no esterified form, a search was started for a cholesterol ester-splitting enzyme in this secretion that would explain the presence of free cholesterol. Indeed, the presence in bile and duodenal juice of an enzyme hydrolyzing cholesterol esters (cholesterol palmitate) could be demonstrated. No definite conclusions could be drawn at that time from experiments designed to show whether blood also had a similar effect. This last point is discussed below.

An indication of the presence of a cholesterol esterase, in its synthetic role, in subcutaneous tissue was given by the experiments of Basten[317]; this investigator placed a deposit of cholesterol under the skin of animals and observed microscopically that the characteristic cholesterol platelets disappeared gradually and were transformed into birefringent drops. Schönheimer and Yuasa[318] confirmed the data of Basten by chemical determinations of cholesterol esters. In eleven days about 10% of the administered free cholesterol was esterified. Schönheimer then tested the possibility that other sterols (sitosterol, stigmasterol, ergosterol, coprosterol) might also be esterified under the same conditions. This is not the case. Enzymatic esterification *in vitro* was demonstrated by Nedswedski[319] and Vercellone.[320] According to these studies, esters of cholesterol with fatty acids, e.g., oleic, palmitic, stearic, and butyric acids, are formed in long-term experiments with the aid of pancreas dry powders in the presence of glycocholate.

Klein[321] has presented proof for the assumption that there are specific cholesterol esterases. He was able to show that mammals have at least two different cholesterol ester-splitting enzymes. One acts in acid solution, at pH 5.3, and is found in liver, spleen, intestinal mucosa, kidney, etc.; the other enzyme is most effective at a neutral pH and is found only in pancreas extracts. Both cholesterol esterases act similarly to the lipases; however, they can be separated from the latter. The substrate used by Klein

[312] K. Kondo, *Biochem. Z.* **26**, 243 (1910).
[313] J. H. Schultz, *Biochem. Z.* **42**, 255 (1912).
[314] J. H. Mueller, *J. Biol. Chem.* **27**, 463 (1916).
[315] E. Abderhalden and A. Weil, *Fermentforschung* **4**, 76 (1921).
[316] S. J. Thannhauser, *Deut. Arch. klin. Med.* **141**, 290 (1923); see also S. J. Thannhauser and H. Schaber, *Klin. Wochschr.* **5**, 252 (1926); H. Wendt, *ibid.* **8**, 1215 (1929).
[317] G. Basten, *Arch. path. Anat. Physiol.* (*Virchow's*) **220**, 176 (1915).
[318] R. Schönheimer and D. Yuasa, *Z. physiol. Chem.* **180**, 19 (1929).
[319] S. W. Nedswedski, *Z. physiol. Chem.* **236**, 69 (1935).
[320] A. Vercellone, *Biochem. e terap. sper.* **25**, 207 (1938).
[321] W. Klein, *Z. physiol. Chem.* **254**, 1 (1938).

was human serum which contained the difficultly water-soluble cholesterol esters in a biological soluble form. Cholesterol esters made soluble by colloidal means, on the other hand, are split very little or not at all. Similarly, synthetic cholesterol phosphoric acid was not hydrolyzed enzymatically either in acid or neutral solution.

French investigators have been particularly interested in the cholesterol esterase of pancreatic juice. Fontaine *et al.*[322] showed that this fluid contains an enzyme which very actively catalyzes the formation of cholesterol esters in alkaline media. This cholesterol esterase may be activated by bile acid salts while monobromacetic acid and also phlorhizin have no effect; all three agents, on the other hand, inhibit the lecithinase which always accompanies the cholesterol esterase.[323] Le Breton and Pantaléon[324] discovered an interesting relationship to this lecithinase: cholesterol esterase and lecithinase are coupled with each other. Lecithinase B splits the fatty acids off from lecithin; cholesterol esterase is then needed for the formation of cholesterol esters with these fatty acids.

Pancreatic cholesterol esterase appears to be identical with that of serum which also is closely related to lecithinase.[325] The serum enzyme is responsible for the relation of free to esterified cholesterol in the blood which was first investigated by Sperry.[326] If sterile human serum or plasma is kept for a few days at 37°C., the content of total cholesterol is unchanged but the ratio of cholesterol to esterified cholesterol has shifted in the sense that the amount of the free form has decreased and that of the bound form increased. This esterifying effect is of fairly considerable magnitude. The mean per cent esterification in 30 blood samples was approximately 60%. It is of interest to note that free and esterified cholesterol apparently are not in equilibrium in flowing blood; the equilibrium is established outside the organism.[327]

The postulation by Saviano and Baccari,[328] who also studied the free and esterified cholesterol in serum, of two cholesterol esterases in blood—one with only a hydrolytic and the other with only a synthetic action—is hard to reconcile with the current concepts of the catalytic function of enzymes. It is probable that their work involved changed experimental conditions (e.g., pH changes) which sometimes permitted the hydrolytic

[322] T. Fontaine, E. Le Breton, and J. Pantaléon, *Compt. rend. soc. biol.* **137**, 611 (1943).

[323] E. Le Breton and J. Pantaléon, *Compt. rend. soc. biol.* **138**, 20 (1944).

[324] E. Le Breton and J. Pantaléon, *Compt. rend. soc. biol.* **138**, 38 (1944); *Arch. sci. physiol.* **1**, 63 (1947).

[325] E. Le Breton and J. Pantaléon, *Arch. sci. physiol.* **1**, 199 (1947); Nguyen-Van-Thoai, *Compt. rend. soc. biol.* **137**, 467 (1943).

[326] W. M. Sperry, *J. Biol. Chem.* **111**, 467 (1935); W. M. Sperry and V. A. Stoyanoff, *ibid.* **126**, 77 (1938).

[327] See also J. Pantaléon, Doctoral thesis, Paris, 1944.

[328] M. Saviano and V. Baccari, *Arch. sci. biol. Italy* **31**, 22 (1946).

effect and sometimes the synthetic function to predominate. Schramm and Wolff[329] had already described the preferentially synthetic action of pancreas dry powder and the hydrolytic action of liver dry powder (under the same conditions). Schramm and Wolff proposed an interpretation of their findings which attributes to cholesterol esterase a great significance for fat resorption. The fatty acids liberated by the intestinal fat hydrolysis are esterified by the pancreatic enzyme in the intestine at the surface of the cells of the intestinal wall, with cholesterol which is present either free in the intestinal juice or at the intestinal wall. The bile acid salts are necessary for resorption. Inside the cells, the cholesterol ester is hydrolyzed by the cholesterol esterase of the intestinal mucosa. If lecithinase, which provides the fatty acids for cholesterol esterase (as shown by French investigators), is taken into account, cholesterol esterase appears to have a particular significance for the introduction of fatty acids into the organism.

The specificity of cholesterol esterase depends on the type of sterol as well as on the other component, the acid.

While Schönheimer[330] had at first reported that stigmasterol, ergosterol, and coprosterol are not esterified, Schramm and Wolff[329] were able to show, under their conditions, a clear-cut esterification of these three sterols by the enzyme, though at a much lower rate than with cholesterol. These investigators found that dihydrocholesterol and dehydroandrosterone were esterified well. This observation provides an explanation for the clinical finding that dehydroandrosterone is active when given *per os:* like cholesterol, it is easily esterified and resorbed. Although sitosterol, stigmasterol, and ergosterol can be esterified to a small extent, the corresponding esters do not get a chance to be formed and resorbed because of the ever-present excess of cholesterol.

The specificity relationships for the acid component have already been briefly touched upon a few times: the best conditions for esterification are provided by the higher fatty acids, e.g., palmitic, stearic, and oleic acids.[331] Butyric acid is also esterified but phosphoric acid is not. Imaizumi[332] found a bacterial cholesterol esterase in *Bact. prodigiosum, proteus,* and *typhi* and in *Staphylococcus aureus,* which hydrolyzes monocholesterol esters of phthalic acid. In this connection, we may mention the occurrence of cholesterol esterase in the larvae of the large wax moth (*Galleria mellonella*), which according to Clement and Frisch[333] transforms cholesterol into its fatty acid esters at pH 7.2.

[329] G. Schramm and A. Wolff, *Z. physiol. Chem.* **263**, 61 (1940); *ibid.* **263**, 73 (1940); see also G. Quagliariello, F. Cedrangolo, and C. Senise, *Boll. soc. ital. biol. sper.* **16**, 447 (1941).

[330] R. Schönheimer, H. von Behring and R. Hummel, *Z. physiol. Chem.* **192**, 117 (1930).

[331] R. G. Sinclair and L. Chipman, *J. Biol. Chem.* **167**, 773 (1947).

[332] M. Imaizumi, *J. Biochem. Japan* **27**, 227 (1938).

[333] G. Clement and A. M. Frisch, *Compt. rend. soc. biol.* **140**, 472 (1946).

5. Chlorophyllase

Chlorophyllase, which was discovered in 1910 by Willstätter and Stoll[334] and shown to accompany chlorophyll, is not an esterase proper since it also catalyzes an alcoholysis very easily. It splits chlorophyll into chlorophyllide and phytol (R indicates the chlorophyll residue) with an uptake of alcohol:

$$RCOOC_{20}H_{39} + C_2H_5OH = RCOOC_2H_5 + C_{20}H_{39}OH$$

The enzymatically catalyzed alcoholysis is possible even in more than 90% alcohol or in the presence of acetone.[335]

In moist ether this enzyme, which was later shown to have particular interest in botany, can also cause a hydrolysis which justifies its classification among the hydrolases, especially since the free acid is formed and not the ethyl ester as in alcoholysis.

The synthetic action of chlorophyllase was also proved by Willstätter and Stoll. The ether solution of the chlorophyll which was formed exhibited the properties of the natural pigment.

According to the investigations of Mayer,[336] the pH optimum of chlorophyllase was at 5.9 when an enzyme dry preparation was used which was obtained by the method of Noack[337] from the leaves of *Heracleum sphondylium* and was chlorophyll-free. Both leaf pigments, chlorophylls *a* and *b*, are attacked by chlorophyllase. However, the *a* component is more rapidly hydrolyzed. The specificity of this enzyme also extends to the magnesium-free plant pigments, phaeophytins *a* and *b*, and here too *a* is hydrolyzed more rapidly than *b*.

Chlorophyllase has the remarkable property of being firmly bound to the cell structure; it may be considered to be the best example of a desmoenzyme among the esterases. A crystallizable chlorophyllase preparation may be obtained from *Scrophularia californica*.[338]

The enzyme is very widely distributed in the plant world; it is localized in the chloroplasts.[338] In agreement with Willstätter, Mayer found that it occurred in all species of plants which were studied. The differences in concentration, however, are very great. All monocotyledons and all tropical plants studied by Mayer had low levels of the enzyme although chlorophyll was usually present in abundance. On the other hand, high levels of the enzyme were generally present in Umbelliferae, Labiatae, and Solanaceae. The enzyme occurs not only in the leaves but could be demonstrated also in other green parts, such as stalks and leaf stems, and in roots.

[334] R. Willstätter and A. Stoll, *Ann.* **378**, 18 (1911); *ibid.* **380**, 148 (1911); Untersuchungen über Chlorophyll. J. Springer, Berlin, 1913.
[335] C. A. Weast and G. Mackinney, *J. Biol. Chem.* **133**, 551 (1940).
[336] H. Mayer, *Planta* **11**, 294 (1930).
[337] K. Noack, *Biochem. Z.* **183**, 135 (1927).
[338] G Krossing, *Biochem. Z.* **305**, 359 (1940).

The enzyme content of the plants is highest in May and September; this is probably related to the formation and degradation of chlorophyll. In the phase of the Fall color changes, a decrease of enzymatic activity is also connected with the disappearance of chlorophyll. Plants grown entirely in the dark also contain chlorophyllase, but much lower levels than plants grown normally in the light. In various mosaic virus diseases of tobacco, the chlorophyllase activity of the tobacco leaves may be changed.[339]

Chlorophyllase can also be found in bacteria if they contain bacteriochlorophyll, like the purple bacteria.[340]

6. Tannase

This enzyme, whose action was already discovered by Scheele in 1786, acts on the depside linkages of the tannins. According to Emil Fischer, depsides are ester-like derivatives of the phenol-carbonic acids. The simplest example of a didepside is the anhydride of *p*-hydroxybenzoic acid in which the carboxyl group of one molecule has combined with the hydroxyl group of another:

$$HO \cdot C_6H_4 \cdot CO \text{——} O \cdot C_6H_4 \cdot COOH.$$

Tannase, which occurs mainly in extracts of molds, e.g., *Aspergillus niger*, hydrolyzes optimally in the acid pH range of 3.1–4.1. Dyckerhoff and Armbruster[341] found the pH optimum of purer tannase preparations to be 5–6. None of the usual complex formers, as NaF, KCN, oxalate, pyrophosphate, etc., inhibit the enzyme. The dialyzed enzyme is not activated by alkaline earth metals or by Mn.[342] A synthetic action of the enzyme—the formation of *m*-digallic acid from gallic acid—has also been observed.[343]

Tannase not only decomposes gallotannin into gallic acid and sugar but also chlorogenic acid into cinchona and caffeic acid. It hydrolyzes chebulinic acid, a constituent of oak tannins, into ellagic and quercetic acids. Freudenberg reported that gallic acid methyl ester, $C_6H_2(OH_3) \cdot COOH$, also was split by tannase. The method of estimation of tannase is based on the hydrolysis of this ester.[344]

Dyckerhoff and Armbruster were able to separate the concomitant mold esterase in mold extracts from tannase by the use of phenylacetate as the

[339] P. D. Petersen and H. H. McKinney, *Phytopathology* **28**, 329 (1938); C. H. Hills and H. H. McKinney, *ibid.* **32**, 857 (1942).

[340] H. Fischer, R. Lambrecht, and H. Mittenzwei, *Z. physiol. Chem.* **253**, 1 (1938); H. Fischer and R. Lambrecht, *ibid.* **253**, 253 (1938).

[341] H. Dyckerhoff and R. Armbruster, *Z. physiol. Chem.* **219**, 38 (1933).

[342] L. Massart and R. Dufait, *Verhandel. Koninkl. Vlaam. Acad. Wetenschap. Belg. Klasse Wetenschap.* **3**, 3 (1941).

[343] M. Nierenstein, *Biochem. J.* **26**, 1093 (1932).

[344] O.Th. Schmidt. *in* Bamann-Myrbäck, Die Methoden der Fermentforschung. Georg Thieme, Leipzig, 1941, p. 1590.

substrate; they reported that the acid component of an ester must contain at least two phenolic hydroxyl groups in order to be split off by tannase. Further, the esterified carboxyl group must be directly linked to the oxidized benzene ring and may not be in the ortho-position to a phenolic hydroxyl group. Chromatographic adsorption, as applied to tannase preparations by Tóth and Bársony,[345] also permits the separation of tannase from other enzymes such as the esterase and β-glucosidase. The interesting fact was observed that glucogallin can be split by the β-glucosidase as well as by tannase. It also appears probable that the hydrolysis of the simple gallic acid ester and of the depsides is not carried out by the same tannase.

Freudenberg and Vollbrecht[346] described suitable culture methods for *Aspergillus niger* and the isolation of tannase preparations from the mold mycelium. Dyckerhoff and Armbruster were unable to find any tannin-splitting activity in animal extract, e.g., glycerol extracts of stomach, intestine, kidneys, spleen, pancreas, and bile of hogs and calves, so that tannase appeared to be restricted to the plant kingdom. Sieburg and Mordhorst,[347] on the other hand, have claimed that tannase occurs in some animal tissues. Their investigations were based on the old observation of Wöhler in 1824 that the urine of dogs contains gallic acid after the administration of tannin, and on the work of Mörner in 1892 who demonstrated tannic acid in the urine after the administration of fairly large amounts of tannin to dogs and human subjects. In following the fate of orally administered tannin, Sieburg and Mordhorst found that tannin hydrolysis starts in the small and large intestine. Tannin is also split in liver extracts and in blood serum.

Little can be said at this time on the significance of tannase. There is no doubt that certain lower organisms, e.g., Aspergillus and Penicillium, can use tannin as a source of carbon since they are able to split it. No other molds and no bacteria grow on nutrient media with tannin as the only source of carbon. The great importance of tannase in the study of the constitution of the natural tannins[348] may be alluded to in closing.

[345] G. Tóth and G. Bársony, *Enzymologia* **11**, 19 (1943–1945).
[346] K. Freudenberg and E. Vollbrecht, *Z. physiol. Chem.* **116**, 277 (1921).
[347] E. Sieburg and G. Mordhorst, *Biochem. Z.* **100**, 204 (1919).
[348] K. Freudenberg, Die Chemie der natürlichen Gerbstoffe. Julius Springer, Berlin, 1920.

CHAPTER 10

Acetylcholine Esterase and Cholinesterase

BY KLAS-BERTIL AUGUSTINSSON

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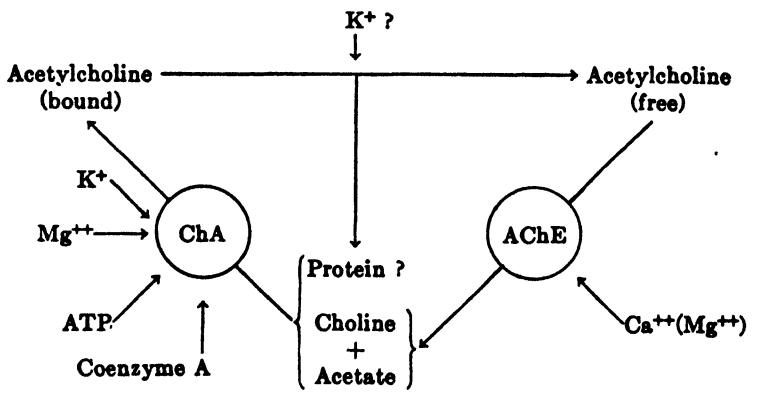
I. Introduction[1]

Acetylcholine (ACh) has been assigned by many workers the important physiological role of chemical mediator of parasympathetic postganglionic and autonomic preganglionic (synaptic) impulses. Evidence has been presented that it might mediate the nerve impulses from motor nerves to striated muscles. In addition, many neurophysiologists have been inclined to believe that the same mechanism predominates in the central nervous system for synaptic transmission.

The transmission of the nerve impulses along the axon and across the synapse has been claimed to be an electrical phenomenon by some investigators and a chemical one by others. A more modern view, based on studies of the enzymes connected with the formation and hydrolysis of ACh, postulates that the impulse transmission is both chemical and electrical in nature, the chemical action being the primary event. The release and removal of ACh are said to be essential factors in conduction, the transmitting agent being the flow of current. The precise function of the ester, however, is still unknown[2] (*cf.* Sect. IX).

There are two enzymes concerned in the ACh metabolism. *Acetylcholine esterase* (AChE) is capable of hydrolyzing ACh into acetic acid and choline, which are much less active than the parent substance. The decrease of free energy in this process is great and the reaction is essentially irreversible.[3] The resynthesis of ACh *in vivo* is most probably not catalyzed by AChE; in this reaction *choline acetylase* (ChA) takes part. The following scheme is a tentative model of ACh metabolism:



[1] It seems undesirable to obscure the text by too many detailed references to the more than 1300 papers on this subject. References, therefore, have been made to more detailed reviews of particular aspects, to key papers from which other references may be traced, and to the recent papers published since 1943.

[2] The role of acetylcholine in nerve action is discussed in a series of papers in *Ann. N. Y. Acad. Sci.* **47**, 375–602 (1946), *Federation Proc.* **7**, 435–463 (1948), and *Bull. Johns Hopkins Hosp.* **83**, 463–603 (1948). See also M. C. Sanz, *Arch. ges. Physiol.*

Besides AChE, the physiological function of which is to split ACh, there are esterases which split ACh and other choline esters faster than ordinary esters; they are not, however, materially concerned in nerve function. Some of these esterases behave like AChE; others are distinct enzymes and are in the following referred to as *cholinesterases* (ChE). The physiological functions of these enzymes are more obscure. Both types of choline ester-splitting enzymes have properties which definitely separate them from ordinary esterases (aliesterases).

AChE and ChE belong to a group of esterases called "azolesterases,"[4] which hydrolyze nitrogen–alcohol esters. Other examples of enzymes belonging to this group are morphine esterase, the tropine esterases, and procaine esterase. They are present in blood serum, and, most probably, they are not identical with serum ChE.

II. Existence of Specific Esterases for Acetylcholine and other Choline Esters

As early as 1914, Dale[5] suggested that an enzyme is present in the blood which brings about the destruction (hydrolysis) of ACh. Such an enzyme was shown to exist in nearly all animal tissues;[6] it has not yet been found in the plant kingdom. In 1932, Stedman, Stedman, and Easson[7] prepared from horse serum an enzyme which was considered to be a specific esterase for ACh and other choline esters; this they called *choline-esterase* (the single word cholinesterase has lately gained in popularity). During the following years, a great many papers appeared dealing with the ACh-splitting activity of various animals, vertebrates as well as invertebrates. The literature on this subject is very extensive.[8] One of the problems involved has

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(*Pflügers*) **246**, 597 (1943), **247**, 317 (1943); W. Feldberg, *Physiol. Revs.* **25**, 596 (1945); O. Loewi, *J. Mt. Sinai Hosp. N. Y.* **12**, 803, 851 (1945); A. von Muralt, Die Signalvermittlung in Nerven, Birkhäuser, Basle, 1946; D. Nachmansohn, *in* J. F. Fulton, Physiology of the Nervous System, 3rd ed., Oxford Univ. Press, New York, 1950; D. Nachmansohn, *in* G. Pincus and K. V. Thimann, The Hormones, Vol. II, Academic Press, New York, 1950, p. 515.

[3] Recent studies, however, have shown that an acid shift of pH displaces the equilibrium in the direction of synthesis. This has been demonstrated with the purified AChE of the electric tissue of *Electrophorus* in the case of ACh and propionylcholine; see S. Hestrin, *Biochem. et Biophys. Acta* **4**, 310 (1950).

[4] D. Glick, *J. Am. Chem. Soc.* **64**, 564 (1942).

[5] H. H. Dale, *J. Pharmacol. Exptl. Therap.* **6**, 147 (1914).

[6] F. Plattner and H. Hintner, *Arch. ges. Physiol.* (*Pflügers*) **225**, 19 (1930).

[7] E. Stedman, E. Stedman, and L. H. Easson, *Biochem. J.* **26**, 2056 (1932).

[8] Reviews: R. Ammon, *Ergeb. Enzymforsch.* **4**, 102 (1935), *in* Nord-Weidenhagen, Handbuch der Enzymologie, Akadem. Verlagsgesellschaft, Leipzig, 1940, p. 394; W. Hanske, *Angew. Chem.* **54**, 357 (1941); E. Werle, *Fermentforschung* **17**, 230 (1943); O. Bodansky, *Ann. N. Y. Acad. Sci.* **47**, 521 (1946); J. Lévy, *J. physiol. et path. gén.* **39**, 413 (1947); K.-B. Augustinsson, *Acta Physiol. Scand.* **15**, Suppl. 52 (1948); R. Balbi and L. Saitta, *Acta Neurol.* **3**, 73 (1948).

been the question of the existence of an esterase specific for ACh, a question of considerable interest in view of the physiological function of this ester.

Stedman *et al.*[7] and Simonart[9] thought that the enzyme from blood serum is specific for choline esters, although not for ACh since it splits butyrylcholine and propionylcholine at a higher rate than ACh. Later experiments, however, have indicated that the esterases present in some sera are not specific for choline esters although they split them faster than noncholine esters. Vahlquist[10] definitely pointed to this fact, working with human blood plasma, and Glick[11] made a systematic study of the specificity of horse serum ChE. Later on, the esterase of red blood cells was demonstrated to differ markedly from serum esterase. Thus Alles and Hawes[12] found that the former enzyme is inhibited by high concentration of ACh in contrast to the latter; the former hydrolyzes acetyl-β-methylcholine, which is not split by serum esterase.[11] Richter and Croft,[13] Mendel and Rudney,[14] Zeller and Bissegger,[15] and Schaefer[16] confirmed and extended these observations. It was found also that a "specific cholinesterase," acting exclusively on choline esters, is present in the erythrocytes of some animals and in the brain, and that the "cholinesterase" present in blood serum and pancreas is a "nonspecific" one. Erythrocyte and brain esterases do not split benzoylcholine, which is hydrolyzed by serum and pancreas esterases.[17] An esterase capable of splitting benzoylcholine, but not engaged in the hydrolysis of ACh, was demonstrated by Sawyer[18] in the liver of some animals. Langemann[19] studied the esterase types in various human tissues, and Augustinsson[20] showed that a true chemical, established difference exists between the ACh-hydrolyzing enzymes in horse serum and erythrocytes. Nachmansohn and Rothenberg[21] demonstrated that the esterases in nervous and muscular tissues and in erythrocytes, in contrast to serum esterase, split propionylcholine at the same or at a lower rate than ACh, whereas butyrylcholine and noncholine esters are split at a very low rate or not at all. Some of these observations were confirmed by Casier and Delaunois.[22]

[9] A. Simonart, *Rev. belge sci. méd.* **3**, 757 (1931); **5**, 73 (1933).
[10] B. Vahlquist, *Skand. Arch. Physiol.* **72**, 133 (1935).
[11] D. Glick, *J. Biol. Chem.* **125**, 729 (1938); **130**, 527 (1939); **137**, 357 (1941).
[12] G. A. Alles and R. C. Hawes, *J. Biol. Chem.* **133**, 375 (1940); *J. Lab. Clin. Med.* **26**, 845 (1941).
[13] D. Richter and P. G. Croft, *Biochem. J.* **36**, 746 (1942).
[14] B. Mendel and H. Rudney, *Biochem. J.* **37**, 59 (1943).
[15] E. A. Zeller and A. Bissegger, *Helv. Chim. Acta* **26**, 1619 (1943).
[16] H. Schaefer, *Arch. ges. Physiol.* (*Pflügers*) **249**, 405 (1947).
[17] B. Mendel, D. B. Mundell, and H. Rudney, *Biochem. J.* **37**, 473 (1943).
[18] C. H. Sawyer, *Science* **101**, 385 (1945).
[19] H. Langemann, *Helv. Physiol. Pharmacol. Acta* **2**, C17 (1944); *cf. Helv. Chim. Acta* **25**, 464 (1942).
[20] K.-B. Augustinsson, *Arkiv Kemi Mineral. Geol.* **18A**, No. 24 (1944).
[21] D. Nachmansohn and M. A. Rothenberg, *J. Biol. Chem.* **158**, 653 (1945).
[22] H. Casier and A. L. Delaunois, *Experientia* **2**, 180 (1946).

These results suggested the existence of two types of choline ester-splitting enzymes for which various names have been proposed; the esterases in erythrocytes and nervous and muscular tissues have been called "specific ChE," [23] "true ChE," [14] "e-type," [15] or simply "cholinesterase"; [21] the second type in certain sera, pancreas, and other tissues was called "nonspecific ChE," [23] "pseudo-ChE," [14] "s-type," [15] or regarded as an unspecified esterase.[21] None of these names has been generally excepted.

Recent investigations have shown that it is inappropriate to refer to a "specific (true) ChE." Thus Bodansky[24] observed that brain and erythrocyte esterases split triacetin, that of human brain splitting this substrate even more rapidly than ACh. Furthermore, these esterases have been shown to catalyze the hydrolysis of a large number of noncholine esters, especially acetates. According to Augustinsson,[25] acetylsalicylic acid and the acetyl–ester linkage in acetylsalicylcholine are split by brain and erythrocyte esterases. Adams and Whittaker[26] demonstrated that the more closely the alcohol group simulates the choline configuration, the more rapidly is the ester hydrolyzed. The carbon analog of ACh, 3,3-dimethylbutyl acetate, is the most rapidly split, next to ACh itself. The observation of Zeller, that ethyl chloroacetate, β-chloroethyl acetate,[27] desoxycorticosterone acetate, and others[27a] are attacked by erythrocyte cholinesterase, also indicated that the ability to hydrolyze noncholine esters can no longer be used as a criterion to distinguish the two types of esterases. The investigations on the esterase of *Helix* blood[28] and other invertebrate tissues,[29] and those on the esterase of snake venoms[30] showed, moreover, that the principles of nomenclature previously used are not adequate. Zeller[27a] has recently considered his "e-type" to be an "acetylesterase" rather than a cholinesterase.

Augustinsson[25] emphasized the importance of a careful consideration of the substrate concentration in following the enzymatic hydrolysis of various esters; the relationship between activity and substrate concentration may differ for a given enzyme from substrate to substrate. For the so-called "specific ChE" this difference is such that at high substrate concentration

[23] See D. Glick, *Science* **102**, 100 (1945).
[24] O. Bodansky, *Ann. N. Y. Acad. Sci.* **47**, 521 (1946).
[25] K.-B. Augustinsson, *Acta Physiol. Scand.* **15**, Suppl. 52 (1948).
[26] D. H. Adams and V. P. Whittaker, *Biochem. J.* **43**, P14 (1948). D. H. Adams, *Biochim. et Biophys. Acta* **3**, 1 (1949); V. P. Whittaker, *Biochem. J.* **44**, P43 (1949).
[27] E. A. Zeller, G. A. Fleisher, and R. A. McNaughton, *Federation Proc.* **8**, 238 (1949). R. A. McNaughton and E. A. Zeller, *Proc. Soc. Exptl. Biol. Med.* **70**, 165 (1949).
[27a] E. A. Zeller, G. A. Fleisher, R. A. McNaughton, and J. S. Schweppe, *Proc. Soc. Exptl. Biol. Med.* **71**, 526 (1949).
[28] K.-B. Augustinsson, *Biochem. J.* **40**, 343 (1946).
[29] K.-B. Augustinsson, *Acta Physiol. Scand.* **11**, 141 (1946).
[30] E. A. Zeller, *Advances in Enzymol.* **8**, 459 (1948).

the rate of hydrolysis with, for instance, triacetin is even higher than with ACh.[31]

In view of the fact that there exists a type of esterases with well defined properties, described in detail below, and with an affinity for its physiological substrate, *i.e.*, ACh,[31a] higher than for any other ester so far tested, Augustinsson and Nachmansohn[32] suggested the use of the term *acetylcholine esterase* (AChE). This type of esterase occurs in nervous and muscular tissues and in erythrocytes. Esterases with similar characteristics occur in *Helix* blood and in the venom of certain snakes. Other esterases (*e.g.*, in some sera, pancreas, certain salivary glands, and presumably other sites) which split choline esters at a higher rate than noncholine esters, but which differ from the acetylcholine esterases in activity–substrate concentration relationship and the increasing rate of hydrolysis with increasing length of the acyl group (from acetyl to *n*-butyryl), are called *cholinesterases* (ChE), as was originally proposed by Stedman *et al.* Their physiological substrate is at present unknown. This terminology and classification are used in the following treatise.

III. Measurement of Activity

The activity of choline ester-splitting enzymes is determined quantitatively by measuring the rate of hydrolysis of ACh. Formerly biological methods were chiefly used, but today chemical techniques are more in favor and should always be used when accurate data are wanted. Some methods are described in detail by Ammon.[33] In the following, the various methods used are briefly summarized.

1. Biological Methods

The evanescence of ACh is followed pharmacologically by measuring its action on isolated intestine, heart, frog rectus abdominis, leech muscle, or by measuring the blood pressure. These methods are much less accurate than the chemical techniques.

2. Chemical and Physicochemical Methods

In estimating the activity chemically, the acetic acid liberated by the hydrolysis of ACh is usually determined. The chemical estimation of ACh itself is the principle of some modern methods.

a. Titration Methods

The liberated acetic acid is titrated with an alkaline solution of known concentration. The difficulties in these methods are the dilution effect and the pH changes due to the liberation of acid.

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[31] K.-B. Augustinsson, *Arch. Biochem.* **23**, 111 (1949).

[31a] According to Zeller *et al.*[27a] however, "no enzymologic clues are available at present to show that acetylcholine is the physiological substrate of the e-ChE."

[32] K.-B. Augustinsson and D. Nachmansohn, *Science* **110**, 98 (1948).

[33] R. Ammon, *in* Bamann-Myrbäck, Die Methoden der Fermentforschung, Thieme, Leipzig, 1941; Academic Press, New York, 1945, p. 1585.

[34] G. Scoz and C. Cattaneo, *Enzymologia* **4**, 157 (1937). H. G. Barbour and V. C. Dickerson, *J. Pharmacol. Exptl. Therap.* **65**, 281 (1939).

Indicator Methods. Various indicators have been recommended, *e.g.*, phenolphthalein, phenol red, cresol red, or bromthymol blue. In using dyes as indicators, it is often very difficult to observe a clearly defined color change. In addition, many other troublesome precautions must be observed (*e.g.*, possible influence of the indicator on the enzyme).

Electrometric Methods. A more convenient method than using indicators is to titrate electrometrically, using a quinhydrone electrode,[34] glass electrode,[35] or antimony electrode.[36]

b. Manometric Methods

Warburg Method. The Warburg manometric method is the most convenient for following the hydrolysis of ACh, and was used for the first time by Ammon.[37] This method is most suitable for comparative and serial experiments and is now used almost exclusively. One estimates manometrically the volume of carbon dioxide evolved from a bicarbonate-containing system, buffered against carbon dioxide by the acetic acid formed in the hydrolysis of ACh.

Other Manometric Methods. The Barcroft differential method[38] and the Van Slyke method[39] have also been employed. The principle of the Cartesian diver has been successfully used in the estimation of esterase activity.[40,41]

c. Other Methods

A step photometric method, based on the ferric chloride reaction of acetic acid, has been described.[42] Another photometric method, using *m*-nitrophenol,[43] and an opalescence method[44] have been evolved. A method for ACh determination utilizes the fact that ACh, and certain other esters, are rapidly converted at alkaline pH by hydroxylamine into the corresponding hydroxamic acids, which can be determined colorimetrically.[45]

A histochemical method has been developed.[45a] The histological distribution of ChE in the gastric mucosa has been discussed.[46]

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[34] D. Glick, *J. Gen. Physiol.* **21**, 289 (1938); *Compt. rend. trav. lab. Carlsberg Sér chim.* **21**, 263 (1938). M. C. Sanz, *Helv. Physiol. Pharmacol. Acta* **2**, C29 (1944). N. Schümmelfeder, *Arch. exptl. Path. Pharmacol.* **204**, 454 (1947); H. O. Michel, *Lab. Clin. Med.* **34**, 1564 (1949).

[35] A. L. Delaunois and H. Casier, *Experientia* **2**, 67 (1946); *Arch. intern. pharmacodynamie* **75**, 371 (1948).

[36] R. Ammon, *Arch. ges. Physiol.* (*Pflügers*) **233**, 486 (1933).

[37] E. Stedman and E. Stedman, *Biochem. J.* **29**, 2107 (1935).

[38] M. Rinkel and M. Pijoan, *J. Pharmacol. Exptl. Therap.* **64**, 228 (1938). D. G. Friend and O. Krayer, *ibid.* **71**, 246 (1941).

[39] K. Linderstrøm-Lang and D. Glick, *Compt. rend. trav. lab. Carlsberg, Sér. chim.* **22**, 300 (1938).

[40] E. J. Boell and S. C. Chen, *J. Exptl. Zoöl.* **97**, 21 (1944).

[41] N.-O. Abdon and B. Uvnäs, *Skand. Arch. Physiol.* **76**, 1 (1937).

[42] H. Croxatto, R. Croxatto, and F. Huidobro, *Anales acad. Biol., Univ. católica Chile* **3**, 55 (1939); *Compt. rend. soc. biol.* **130**, 236 (1939). A similar method is described by C. Huggins and J. Lapides, *J. Biol. Chem.* **170**, 467 (1947).

[43] L. Gal, *Med. Klin.* (*Munich*) **36**, 385 (1940).

[44] S. Hestrin, *J. Biol. Chem.* **180**, 249 (1949).

[45] G. B. Koelle and J. S. Friedenwald, *Proc. Soc. Exptl. Biol. Med.* **70**, 617 (1949). A histochemical demonstration has been described by G. Gomori (*Proc. Soc. Exptl. Biol. Med.* **68**, 354, 1948), but it is highly questionable whether AChE or ChE is responsible for this test.

[46] D. Glick, *J. Gen. Physiol.* **21**, 297 (1938).

3. Methods for Distinguishing between Acetylcholine Esterases and Cholinesterases

After evidence had been presented that various types of choline ester-splitting enzymes exist, methods were proposed for distinguishing them. One method is based on the observation that acetyl-β-methylcholine is split only by AChE and not by ChE, whereas benzoylcholine is hydrolyzed only by ChE.[17] A more valuable tool is the test of hydrolysis rates of propionylcholine and butyrylcholine.[21,31] AChE splits propionylcholine at the same or at a lower rate than ACh, butyrylcholine at a low rate or not at all. The rate of hydrolysis by ChE, on the other hand, increases with increasing length of the acyl chain.[11,26] AChE splits β-chloroethyl acetate more rapidly than ChE.[27] If the AChE activity is plotted against the log of the molar ACh concentration a bell-shaped curve is obtained, *i.e.*, inhibition by excess of substrate.[12] The ChE is not inhibited by high concentrations of ACh. The substrate concentration must be carefully considered in following the enzymatic hydrolysis of various esters, as the activity substrate concentration relationship may differ for a given enzyme from substrate to substrate.[25]

The ability of certain compounds to inhibit selectively either AChE or ChE can also be used for distinguishing the two types. Several attempts in that direction have been made. In many cases, however, the data obtained are based only on preliminary experiments with no tests of the action against specific substrates and at various substrate concentrations. Examples of selective inhibitors are given in Table I (Sect. IX).

A biological method has been proposed which employs frog muscle and various choline esters.[47]

4. Units Used in Expressing Enzyme Activity

Esterase activity is frequently expressed in Q values or milligrams ACh (chloride) hydrolyzed in 60 minutes by 100 mg. (or 1.0 g.) tissue.[48] For purified preparations the unit A_p has been proposed, *i.e.*, milligrams ACh hydrolyzed in 60 minutes by 1 mg. protein.[49] *Cf* signifies the quantity of ACh chloride, in micromoles, destroyed in 60 minutes by 1 mg. dry substance.[20]

The activity measured by the manometric method is simply expressed as the volume of carbon dioxide, in micromoles, evolved in 20 to 60 minutes at STP. The initial hydrolysis rate should always be used, as this factor is independent of the decrease in substrate concentration and directly proportional to the enzyme concentration.[25,50]

In titration methods, using 0.01 M sodium hydroxide, the unit is the amount of

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[47] A. Denys and J. Lévy, *Compt. rend. soc. biol.* **141**, 650, 731 (1947).

[48] D. Nachmansohn and E. Lederer, *Compt. rend. soc. biol.* **130**, 321 (1939); *Bull. soc. chim. biol.* **21**, 797 (1939).

[49] M. A. Rothenberg and D. Nachmansohn, *J. Biol. Chem.* **168**, 223 (1947).

[50] G. Arragon and E. Sala, *Bull. soc. chim. biol.* **30**, 44 (1948).

enzyme necessary to liberate 1.00 ml. of 0.01 *M* acetic acid in 10 minutes at pH 8.0. The activity must also in this case be defined in terms of initial hydrolysis rate.[51]

IV. Occurrence of Acetylcholine Esterase and Cholinesterase

The distribution of choline ester-splitting enzymes has been studied in considerable detail in connection with the physiological significance of ACh.[8] The enzymes have been found in almost all multicellular animals; they were present in all vertebrates and in most invertebrates investigated. The earlier data concerning the esterase activity of various tissues (except those discussed in Sect. II) furnish no information as to the type of enzyme present. The first accounts dealing with the specificity of ACh-splitting esterases in various tissues of a single species are those on *Helix pomatia*[28] and on the rat.[52] In the following, only the most prominent features concerning the distribution of the esterases can be mentioned.

1. Tissues Containing Predominantly Acetylcholine Esterase

Nervous Tissues. It is now well established that the esterase in all nervous tissue, vertebrate as well as invertebrate, is predominantly AChE and present in high concentration.[14,15,19,21,52] The brains of all species investigated contain this enzyme. The values vary considerably in different species and in the different parts of the brain.[53] AChE is much more abundant in the gray matter than in the white matter. The smaller the brain, the higher generally the enzyme concentration.[25,54] There seems, however, to be no increase in the concentration with the phylogenetic order of the species. The highest AChE value was obtained with the head ganglion of the squid.[55]

The peripheral nervous system has also a very high concentration of AChE. The sympathetic ganglia have a higher concentration than most parts of the central nervous system.[53,56] A mixture of AChE and ChE has been reported to exist in these ganglia.[57,58] The concentration of AChE is high in all nerve fibers, but rises still higher in the synaptic region. High concentration of AChE usually corresponds to high ACh concentration.[59] Cholinergic nerves have higher activity than sensory ones.[60]

[51] G. E. Hall and C. C. Lucas, *J. Pharmacol. Exptl. Therap.* **59**, 34 (1937).
[52] C. H. Sawyer and J. W. Everett, *Am. J. Physiol.* **148**, 675 (1947).
[53] G. Pighini, *Biochim. e terap. sper.* **25**, 347 (1938), **26**, 157 (1939); *Boll. soc. ital. biol. sper.* **15**, 237 (1940); D. Nachmansohn, *Bull. soc. chim. biol.* **21**, 761 (1939). E. Egaña, *Pubs. lab. med. exptl. clin. med. E. Prado-Tagle, Univ. Chile* **1**, 99, 117 (1946).
[54] E. A. Zeller, *Helv. Chim. Acta* **32**, 448 (1949).
[55] E. J. Boell and D. Nachmansohn, *Science* **92**, 513 (1940).
[56] D. Glick, *Nature* **140**, 426 (1937); *J. Gen. Physiol.* **21**, 431 (1938).
[57] B. Mendel and H. Rudney, *Science* **100**, 499 (1944).
[58] C. H. Sawyer and W. H. Hollinshead, *J. Neurophysiol.* **8**, 137 (1945).
[59] H. F. Hellauer, *Arch. ges. Physiol.* (*Pflügers*) **242**, 382 (1939).
[60] K. Umrath and H. F. Hellauer, *Arch. ges. Physiol.* (*Pflügers*) **250**, 737 (1948).

In experiments with the giant axon of squid, AChE has been found to be located exclusively at the neuronal surface; no enzyme is found in the axoplasm.[55] *Tubularia*, a hydrozoan coelenterate and one of the lowest animals to possess a nervous system, contains AChE.[61] The esterase activity of insect nervous system has been studied in detail, especially in connection with the action of the recently discovered insecticides (diisopropyl fluorophosphate, tetraethyl pyrophosphate).[62] This enzyme is said to differ in certain respects from vertebrate nerve AChE.

The cerebrospinal fluid (CSF) also splits ACh.[63] The esterase activity of human CSF is about 1% of the activity of human serum. CSF contains predominantly AChE, but also ChE.[64]

Muscular Tissues. Muscles have a relatively low esterase activity. The enzyme in striated muscles is predominantly AChE;[19,21] heart muscle does not contain only AChE but also other esterases. The enzyme is not evenly distributed, as was first demonstrated in experiments on the frog sartorius muscle;[65] it is present in high concentration at the motor end plates. There it is apparently localized in the postsynaptic membrane, an exclusively muscular element.[66]

Electric Organs. There are three known species with powerful electric organs (*Electrophorus electricus*, *Malapterurus electricus*, and *Torpedo marmorata*) and several others with weak electric organs. These organs, except those of *Malapterurus* which have relatively low esterase activity, are phylogenetically evolved from striated muscle. They have the highest concentration of ACh-splitting enzymes found in any tissue.[67] One g. tissue splits 1 to 5 g. ACh per hour. The activity is ascribed to AChE[21] and detailed studies of the enzyme concentration in relation to the electromotive force have been carried out with *Electrophorus* (see Sect. IX—1).

Erythrocytes. In most species, the AChE of blood is located in the erythrocytes; the plasma contains smaller amounts. The erythrocyte AChE is bound to the cell membrane.[68] Scarcity or lack of AChE in the erythro-

[61] T. H. Bullock, H. Grundfest, D. Nachmansohn, and M. A. Rothenberg, *J. Neurophysiol.* **10**, 11 (1947).

[62] S. J. Mihalonis and R. H. Brown, *J. Cellular Comp. Physiol.* **18**, 401 (1941). O. W. Means, Jr., *ibid.* **20**, 319 (1942). A. G. Richards, Jr., and L. K. Cutkomp, *ibid.* **26**, 57 (1945). J. M. Tobias, J. J. Kollros, and J. Savit, *ibid.* **28**, 159 (1946).

[63] H. Altenburger, *Klin. Wochschr.* **16**, 398 (1937). M. Reiss and R. E. Hemphill, *Nature* **161**, 18 (1948).

[64] W. Ferrari, *Boll. soc. med. clin. Modena* **46**, 2 pp. (1946), quoted from *Chem. Abstracts* **42**, 3446 (1948). B. Glasson and S. Mutrux, *Helv. Physiol. Pharmacol. Acta* **4**, C12 (1946). D. B. Tower and D. McEachern, *Rev. can. biol.* **7**, 198 (1948).

[65] A. Marnay and D. Nachmonsohn, *Compt. rend. soc. biol.* **124**, 942 (1937), **125**, 41 (1937); *J. Physiol.* **92**, 37 (1938).

[66] R. Couteaux and D. Nachmansohn, *Proc. Soc. Exptl. Biol. Med.* **43**, 177 (1940). R. Couteaux, *Bull. biol. France Belg.* **76**, 14 (1942); *Rev. can. biol.* **6**, 563 (1947).

[67] A. Marnay, *Compt. rend. soc. biol.* **126**, 573 (1937). D. Nachmansohn, *Yale J. Biol. Med.* **12**, 565 (1940).

[68] P. G. Croft and D. Richter, *J. Physiol.* **102**, 155 (1943). R. W. Brauer and M. A. Root, *Federation Proc.* **4**, 113 (1945).

cytes of birds and fish is compensated for by the presence of a comparatively larger amount of AChE in the plasma.[17,25] The erythrocyte AChE activity decreases according to the following series: man, cow, guinea pig, horse, dog, sheep, rabbit, and cat. The activity varies from individual to individual, but tends to remain fairly constant in any one individual. In addition to AChE, human erythrocytes usually contain a small quantity of an ordinary esterase (aliesterase).[25,26,69]

The leucocytes do not split ACh.[70]

Other Sources. AChE is said to be predominant in thymus,[19] spleen, red bone marrow, lymph nodes, and adrenal cortex,[62] but closer analysis of the type of esterase present has not been published. In addition to an aliesterase, *Paracentrotus* (a sea urchin) larvae contain AChE[25,71] *Sepia* liver seems to contain predominantly AChE.[25]

Closer investigations of the type of esterases have been carried out with *Helix* blood and snake venom. The blood of *Helix pomatia* hydrolyzes ACh at a very high rate.[26] The enzyme is of the AChE type, but has a few features which distinguish it from the esterase in nervous and muscular tissues and in erythrocytes.[31] The high rate of the hydrolysis of acetylaneurine is especially noticeable.[25]

The ACh-splitting activity of cobra venom was first observed by Iyengar *et al.*[72] The venoms of the species of the Colubridae possess marked activity, of the same magnitude as the electric organs, while the venoms of the species of the Viperidae show no such activity.[73] In addition to ACh, other esters containing acetyl groups are hydrolyzed by cobra venom.[74] The same esterase splits ACh and triacetin[31,75] and halogen acetic acid esters.[27,76] The enzyme behaves like an AChE.[31,77]

Bee venom[25] and scorpion venom[78] do not split ACh.

2. Tissues Containing Predominantly Cholinesterase

Blood Serum (Plasma). The literature on the ChE activity of blood serum

[69] B. Glasson, *Pharm. Acta Helv.* **19**, 279 (1944); *Schweiz. med. Wochschr.* **75**, 1011 (1945).

[70] J. Fegler, H. Kowarzyk, and J. Szpunar, *Bull. intern. acad. polon. sci. Classe méd.* **7/10**, 517 (1937).

[71] K.-B. Augustinsson and T. Gustafson, *J. Cellular Comp. Physiol.* **34**, 311 (1949).

[72] N. K. Iyengar, K. B. Sehra, B. Mukerji, and R. N. Chopra, *Current Sci.* **7**, 51 (1938). The properties of snake venom esterases have been extensively reviewed by Zeller.[80]

[73] E. A. Zeller, *Experientia* **3**, 375 (1947).

[74] F. Bovet-Nitti, *Experientia* **3**, 283 (1947).

[75] P. Holton, *Biochem. J.* **43**, P13 (1948); H. Blaschko and P. Holton, *Brit. J. Pharmacol.* **4**, 181 (1949).

[76] E. A. Zeller and D. C. Utz, *Helv. Chim. Acta* **32**, 338 (1949).

[77] E. A. Zeller and A. Maritz, *Helv. Physiol. Pharmacol. Acta* **3**, C19 (1945). E. A. Zeller, *ibid.* **6**, C36 (1948); *Helv. Chim. Acta* **32**, 94 (1949). Zeller has regarded snake venom esterase as a new type of choline ester-splitting enzyme ("c-type"), but in later papers he includes this enzyme in the group of AChE.

[78] E. C. del Pozo, *Brit. J. Pharmacol.* **3**, 219 (1948).

is very extensive and has been reviewed previously.[8] The blood ChE is located in the plasma of most species, but the esterase is absent or present in very low concentration in the blood of certain animals (ruminants, birds, fish).[17,25,79] The high ChE activity of horse serum in contrast to that of ox serum has been used to differentiate the two sera from each other.[80] Serum and plasma of the same species have the same activity. The activity in normal human beings varies markedly. The serum of most species contains, in varying proportions, a mixture of ChE and AChE.[17,81] Human and horse serum contain predominantly ChE, whereas rabbit serum contains mainly AChE. In addition, some sera seem to contain ordinary esterases;[13,75] such an aliesterase of human plasma accounts for 5 to 20% (depending on the substrate) of the aliphatic esterase activity of the plasma.[82]

Other Sources. ChE is the predominant esterase in pancreas,[14] and it occurs presumably in other tissues, *e.g.*, human ovary,[19] Harderian glands, brown fat, uterus, liver,[52] and testis[83] of rat. The enzyme seems to be absent on the whole from the tissues of ruminants.[79] ChE is present in the parotid glands of pig and guinea pig; in dog and cat parotids AChE is present as well, and in the parotids of rabbit and cow AChE is present alone.

3. Tissues Containing Esterases of Unknown Type

In most other cases definite conclusion cannot yet be drawn regarding the specificity. In many cases it is not even shown whether it is justified to speak about AChE or ChE or an aliesterase. The earlier investigations have been concentrated on the degree of esterase activity rather than on the type of enzyme present. Almost all tissues possess ACh-splitting activity.[8] Only in very few cases such esterases are absent, *e.g.*, saliva, gastric juice, gall bladder, bile, urine, and milk.

In the liver of guinea pigs and rats there is an esterase capable of splitting benzoylcholine, but not of hydrolyzing ACh.[18,84] A similar esterase is present in the kidney of guinea pig and cow.[25] There is, however, no reason for suggesting a specific "benzoylcholine esterase" as these enzymes do split noncholine esters at a higher rate.

The esterase content of invertebrate tissues has been extensively reviewed.[29,85] Generally, AChE is present in nervous and muscular tissues. Squid ganglion,[55] the dorsal longitudinal muscle of the flatworm *Cerebratulus lacteus*,[86] and the dart sac of *Helix*[28] have very high activity. In the protozoans neither esterase nor ACh are present. As a rule coelen-

[79] J. M. Gunter, *Nature* **157**, 369 (1946).
[80] D. Vincent and J. Broca, *Ann. pharm. franç.* **4**, 187 (1946).
[81] R. D. Hawkins and B. Mendel, *Brit. J. Pharmacol.* **2**, 173 (1947).
[82] D. H. Adams and V. P. Whittaker, *Biochem. J.* **44**, 62 (1949).
[83] C. Huggins and S. H. Moulton, *J. Exptl. Med.* **88**, 169 (1948).
[84] H. Blaschko, T. C. Chou, and I. Wajda, *Brit. J. Pharmacol.* **2**, 108, 116 (1947).
[85] C. L. Prosser, *Physiol. Revs.* **26**, 337 (1946).
[86] C. C. Smith, B. Jackson, and C. L. Prosser, *Biol. Bull.* **79**, 377 (1940).

terates do not show esterase activity. In worms and crustaceans considerable quantities of ACh-hydrolyzing enzyme are often present; the esterase in *Lumbricus* muscle is of the AChE type.[31] The blood of these animals has no or very low activity. This is also the case with spiders and insects. The blood of mollusks has high esterase activity. The enzyme is lacking in the purple cyst of *Murex*; from this animal Erspamer[87] has isolated a new choline derivative (murexine) which has marked ACh-like action but is not acted upon by any esterases. The enzyme is present in the blood of echinoderms, but absent in that of the tunicates.

Bacteria in some cases have been found to have a low ACh-splitting activity.[88]

V. Enzyme Preparations

1. Acetylcholine Esterase

The AChE of red blood cells may be obtained by adsorbing the enzyme on infusorial earth,[14] or by extracting[89] it with ammonia solution of pH 8.3. By adding hydrochloric acid to pH 6 to a hemolyzate,[90] AChE is precipitated together with the stroma and can then be eluted with lysolecithin.[25] Other methods have been described.[91]

A method for the purification of AChE from the electric organ by fractional ammonium sulfate precipitation is described.[48,49] In the solutions obtained, 1 mg. of protein splits 20,000 to 21,000 mg. of ACh per hour. The AChE activity of brain has been separated into two fractions.[92] The greatest difficulty in the purification of the enzyme from brain is to obtain it in solution.

The AChE from snake venom has also been purified.[93]

2. Cholinesterase

Purified preparations of serum ChE can be obtained by fractional precipitation with ammonium sulfate.[94] Serum ChE is then precipitated in the albumin fraction. More careful separation of the plasma proteins into fractions[95] has shown that the esterase is associated with the α- and β-globulin fractions,[96] which are close to the

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[87] V. Erspamer, *Experientia* **4**, 226 (1948).

[88] K. Schaller, *Z. physiol. Chem.* **276**, 271 (1942). D. Vincent and J. de Prat, *Compt. rend. soc. biol.* **139**, 1148 (1945).

[89] J. Mentha, H. Sprinz, and R. Barnard, *J. Biol. Chem.* **167**, 623 (1947).

[90] S. Paléus, *Arch. Biochem.* **12**, 153 (1947).

[91] H. Scheiner, *Compt. rend. soc. biol.* **142**, 36 (1948). G. Arragon and E. Sala, *Bull. soc. chim. biol.* **30**, 51 (1948).

[92] J. M. Little, *Am. J. Physiol.* **153**, 436 (1948); **155**, 60 (1948).

[93] D. K. Chowdhury, *Science and Culture* **8**, 238 (1942), quoted from *Chem. Abstracts* **37**, 1458 (1943); *Ann. Biochem. and Exptl. Med. (India)* **4**, 77 (1944).

[94] E. Stedman and E. Stedman, *Biochem. J.* **29**, 2563 (1935). T. L. McMeekin, *J. Biol. Chem.* **128**, P66 (1939). M. Faber, *Acta Med. Scand.* **114**, 72 (1943). K.-B. Augustinsson, *Arkiv Kemi Mineral. Geol.* **18A**, No. 24 (1944). F. Strelitz, *Biochem. J.* **38**, 86 (1944).

[95] Review: J. T. Edsall, *Advances in Protein Chem.* **3**, 384 (1947). The separation of ChE from other plasma proteins has recently been described by D. M. Surgenor, L. E. Strong, H. L. Taylor, R. S. Gordon, Jr., and D. M. Gibson, *J. Am. Chem. Soc.* **71**, 1223 (1949).

[96] D. Glick, S. Glaubach, and D. H. Moore, *J. Biol. Chem.* **144**, 525 (1942).

serum albumin. A crystalline serum mucoprotein with high ChE activity is described,[97] as well as a preparation from dog pancreas.[98]

VI. Activators and Nature of the Active Groups

Although much chemical work has been carried out with choline ester-splitting enzymes, little is known about the nature of the active groups of AChE and ChE. The enzymes are inactivated by dialysis and then reactivated by adding the dialyzate[48] or certain bivalent metallic ions.[99–101] The activating effects of Ca^{++}, Mg^{++}, and Mn^{++} are well established, but there are different opinions regarding the amplitude of these effects. Other bivalent ions (Sr^{++}, Ba^{++}, Cd^{++}) activate weakly or not at all. Regarding the actions of monovalent ions (K^+ and Na^+) the disagreement has been considerable; they are resolved, however, at least partly if we note that both AChE and ChE from various sources have been studied under various conditions. These ions seem to activate the AChE of *Torpedo* electric organ,[101] rat serum,[102] and erythrocytes.[12,25] Serum ChE is said[25,102] to be very little influenced by K^+. The observed shift of optimum activity of AChE to higher ACh concentration with increasing salt concentration[103] has not been confirmed.[25] Activity determination must be carried out under optimum conditions with respect to salt concentration. A medium giving optimum activity is the following: 0.15 *M* sodium chloride, 0.04 *M* magnesium chloride, and 0.025 *M* sodium bicarbonate.

Some organic substances have been reported to activate the esterase activity. Reduced glutathione activates both serum ChE[104] and the AChE of the electric organ.[48] Certain amino acids (*e.g.*, arginine, lysine, histidine) potentiate the activity of serum ChE.[105] The observed activation *in vivo* by vitamin C is still under discussion.[106] In female rats the activities of brain AChE and serum ChE seem to be reduced in E-avitaminosis;[107] the activity may be regained by adding DL-α-tocopherol acetate to the

[97] R. Bader, F. Schütz, and M. Stacey, *Nature* **154**, 183 (1944); **155**, 239 (1945).

[98] B. Mendel and D. B. Mundell, *Biochem. J.* **37**, 64 (1943).

[99] L. Massart and R. Dufait, *Enzymologia* **6**, 282 (1939); *Bull. soc. chim. biol.* **21**, 1039 (1939); *Nature* **145**, 822 (1940). G. Scoz and G. de Michele, *Boll. soc. ital. biol. sper.* **19**, 24 (1944).

[100] B. Mendel, D. Mundell, and F. Strelitz, *Nature* **144**, 479 (1939); **145**, 822 (1940).

[101] D. Nachmansohn, *Nature* **145**, 513 (1940).

[102] D. Glick, *Nature* **148**, 662 (1941).

[103] B. Mendel and H. Rudney, *Science* **102**, 616 (1945).

[104] E. Keeser, *Klin. Wochschr.* **17**, 1811 (1938).

[105] E. Aron, A. D. Herschberg, and E. Frommel, *Helv. Physiol. Pharmacol. Acta* **2**, 495 (1944).

[106] O. Granzner, *Folia Haematol.* **63**, 217 (1939). A. Rubino, *Ormoni* **2**, 595 (1940). E. Frommel, A. D. Herschberg, and J. Piquet, *Helv. Physiol. Pharmacol. Acta* **1**, 229 (1943); **2**, 507 (1944). A. D. Herschberg and E. Frommel, *Compt. rend. soc. phys. hist. nat. Genève* **61**, 33 (1944). E. Frommel and J. Piquet, *ibid.* **63**, 113 (1946).

[107] H. Bloch, *Helv. Chim. Acta* **25**, 793 (1942). W. Hess and G. Viollier, *ibid.* **31**, 381 (1948).

vitamin E-free diet of controls. A substance in incubated rat heart augmenting the esterase activity of rat heart muscle has been described.[108]

The fact that the AChE of electric tissue, the molecular weight of which is about 3 million, is inhibited by those substances (*e.g.*, maleic acid, oxidized glutathione) which are capable of transforming sulfhydryl groups to disulfide groups caused Nachmansohn and Lederer[48] to assume that the enzyme molecule contains sulfhydryl groups. Muscle AChE is also inhibited by cystine.[109] This view is supported by the inhibitory effect on brain AChE of certain organic arsenicals which are said to be specific inhibitors of sulfhydryl enzymes.[110,111]

Serum ChE also is supposed to contain active sulfhydryl groups. This enzyme, however, seems to be very resistant to oxidizing agents;[112] cystine

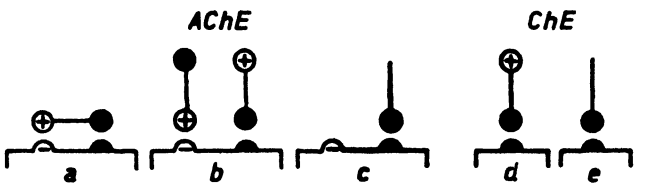


FIG. 1. Models of acetylcholine esterase (AChE) and cholinesterase (ChE) (modified from Zeller and Bissegger[15,30]). Black circles, ester group; black half-circles, ester-combining group of the enzyme; white circles, positively charged group of ACh; white half-circles, negatively charged group of AChE. (*a*) Normal linkage of AChE with ACh. (*b*) Form of linkage produced by excessive ACh concentration. (*c*) Linkage of noncholine esters (*e.g.*, triacetin, β-chloroethyl acetate) with AChE. (*d*) Linkage of ChE with ACh. (*e*) Linkage of noncholine esters (*e.g.*, methyl butyrate) with ChE. *Cf.* Sect. VII—2*a*.

has practically no effect.[25,113] The activity of *Helix* blood AChE is also uninfluenced by cystine.[25]

A possible heme nature of serum ChE has been assumed,[114] but no real support for this hypothesis has been revealed. Although the existence of a coenzyme for AChE or ChE has not yet been established, Kraupp and Werner[115] have made the interesting observation that horse serum ChE can be partially separated into two components, one having an active acid group with pK' of about 6.0, the other having an isoelectric point between

[108] M. Wright and B. Mendel, *J. Biol. Chem.* **165**, 389 (1946).
[109] W. Riechert and E. Schmid, *Arch. exptl. Path. Pharmakol.* **199**, 66 (1942).
[110] E. S. G. Barron and T. B. Singer, *Science* **97**, 356 (1943).
[111] J. J. Gordon and J. H. Quastel, *Nature* **159**, 97 (1947); *Biochem. J.* **42**, 337 (1948).
[112] W. C. Stadie, B. C. Riggs, and N. Haugaard, *J. Biol. Chem.* **161**, 175 (1945).
[113] P. Wels and K. Repke, *Arch. exptl. Path. Pharmakol.* **204**, 323 (1947).
[114] R. D. Barnard, *Proc. Soc. Exptl. Biol. Med.* **54**, 254 (1943); *Science* **104**, 331 (1946).
[115] O. Kraupp and G. Werner, *Arch. intern. pharmacodynamie* **75**, 288 (1947); **76**, 1, 13 (1948).

pH 3.0 and 5.0. A partly purified enzyme preparation[20] from horse serum had the isoelectric point at pH 4.4. Data for the isoelectric point of AChE have been reported for erythrocyte (4.7)[20] and snake venom esterases (5.55 and 5.9).[116]

Unlike ChE, AChE activity is depressed by excess of ACh. The Murray-Haldane interpretation of such a mechanism is very well fitted to the reactions of AChE.[15,24,25] It has been used by Zeller and Bissegger for tentative models of the two enzymes (Fig. 1).

VII. Kinetics

1. Effect of Concentration of Enzyme

Direct proportionality between reaction rate and esterase concentration is usually found, provided the substrate concentration is continually in excess. Several investigations have dealt with this relationship for serum ChE,[20,42] nerve AChE,[41] and a series of other choline ester-splitting enzymes.[25] Large dilutions partially inactivate serum ChE.[117]

The turnover number of serum ChE has been reported[118] to be 1500 molecules of ACh and 3500 molecules of butyrylcholine per second at 30°. A very high value for the AChE of electric organ (300,000 molecules of ACh per second at 25°) has been proposed.[49] The molar concentration of active ChE centers in 4.54% dog serum has been estimated[119] to be less than 1.8×10^{-8}.

2. Effect of Concentration of Substrate

The study of the relationship between esterase activity and concentration of the substrate has been of great interest in the development of our knowledge of these enzymes. This relationship, discussed in detail in some reviews,[16,24,25] was one of the first clues in the separation of AChE and ChE (Sect. II). High concentration of ACh inhibits AChE activity; this is not the case with ChE.

a. Inhibition of Acetylcholine Esterases by Excess of Acetylcholine

The inhibition of erythrocyte AChE by excess of ACh was first observed by Alles and Hawes.[12] This finding was confirmed and extended to the esterases of brain and other tissues (Sect. II). It may be accounted for in terms of the theory brought forward by Murray and Haldane:[120] a complex of the enzyme with two molecules of the substrate is formed at high substrate concentrations; this complex is incapable of yielding acid and alcohol

[116] D. K. Chowdhury, *Ann. Biochem. Exptl. Med.* (*India*) **6**, 91 (1946).
[117] O. Kraupp, *Z. Vitamin-, Hormon- u. Fermentforsch.* **2**, 179 (1949).
[118] L. H. Easson and E. Stedman, *Proc. Roy. Soc. London* **B121**, 142 (1936).
[119] A. Goldstein, *J. Gen. Physiol.* **27**, 529 (1944).
[120] D. R. P. Murray, *Biochem. J.* **24**, 1890 (1930). J. B. S. Haldane, Enzymes, Longmans, Green, London, 1930.

(see Fig. 1). A symmetric bell-shaped curve is obtained when the hydrolysis rate is plotted against the logarithm of the ACh concentration, pS.

These are the experimental results for the esterases of nervous tissue, electric organs, erythrocytes, the blood of *Helix*, and snake venom. Fig. 2 shows the pattern of the AChE of electric organs, typical for all esterases

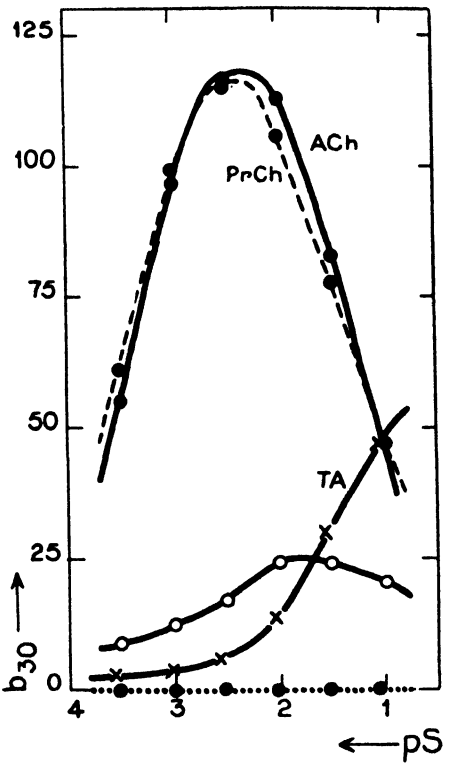


FIG. 2. Activity-pS curves for the enzymatic hydrolysis of various esters by the AChE of *Electrophorus* electric organ.[31] *Cf.* Fig. 3. ●——● Acetylcholine (ACh); ●- - -● propionylcholine (PrCh); ●····● butyrylcholine; O——O acetyl-β-methylcholine; ×——× triacetin (TA); b_{30}, μl. carbon dioxide evolved in 30 min. (extrapolated values).

of the AChE type (propionylcholine is split at a lower rate or at the same rate at ACh, and butyrylcholine is not split at all). The relationship between activity and substrate concentration is the same for ACh and propionylcholine; it may, however, differ for a given enzyme when other substrates are used.[25] Erythrocyte AChE splits acetyl-β-methylcholine at a lower rate than ACh at low substrate concentration, but at a higher rate when the substrate concentrations are high. This shows that, although both esters inhibit the enzyme in high concentration, the optimum sub-

strate concentration is not the same for the two esters. Acetylsalicylcholine[25] and triacetin,[31] which are also split by this enzyme, do not inhibit the enzyme activity at high concentration. In the case of triacetin,[26,75] this has been demonstrated with AChE from various sources. At high substrate concentration the rate of hydrolysis of triacetin may be even higher than that of ACh.[31] Therefore, estimating the hydrolysis rate at *one* arbitrarily chosen substrate concentration, a distorted picture may be obtained. In all cases the affinity of AChE is much higher for ACh than for noncholine esters.

The AChE activity has an optimum at 3×10^{-3} *M* ACh and the dissociation constant (K_S) of the AChE–ACh complex is 5×10^{-4}. In pernicious anemia the K_S value is increased about threefold.[121]

b. No Inhibition of Cholinesterases by Excess of Acetylcholine

Serum ChE, acting upon ACh, gives a familiar dissociation curve, $K_S = 2 \times 10^{-3}$, and ACh of high concentration does not inhibit.[25,119,122,123] This is characteristic of ChE, which splits propionylcholine at a higher rate than ACh, and butyrylcholine at a still higher rate. All these esters give the same value of *K*. The activity–pS curves are not the same for various substrates (Fig. 3). Benzoylcholine and acetylsalicylcholine in high concentration depress the activity.[25] The affinities for noncholine esters are definitely lower than for the choline esters.

3. Effect of Concentration of Hydrogen Ions

Serum ChE is completely destroyed at pH 2; on the alkaline side the enzyme activity starts to decline at pH 11.[20,124] Erythrocyte AChE is completely destroyed at pH 4.5 and its stability at alkaline reactions seems also to be less than for serum ChE.[20] Brain AChE behaves like erythrocyte AChE in this respect.[125]

The optimum pH[118,126] of serum ChE is 8.0 to 8.5. The value for erythrocyte AChE has been reported[12] to be somewhat lower, pH 7.5 to 8.0.

4. Effect of Temperature

The esterases are fairly stable in tissue extracts. Purified preparations in high dilution are very unstable and must be stabilized with gelatin. Hemolyzed blood collected aseptically and preserved for many years in the dark at room temperature loses only a few per cent of its original ac-

[121] J. C. Sabine, *Federation Proc.* **8**, 136 (1949).
[122] D. Glick, *Biochem. J.* **31**, 521 (1937).
[123] G. S. Eadie, *J. Biol. Chem.* **146**, 85 (1942).
[124] F. Strelitz, *Biochem. J.* **38**, 86 (1944). V. V. Mihailesco, *Bull. acad. méd. Roumanie* **18**, 51 (1946).
[125] K.-B. Augustinsson, *Nature* **156**, 303 (1945).
[126] E. Werle and H. Uebelmann, *Arch. exptl. Path. Pharmakol.* **189**, 421 (1938).

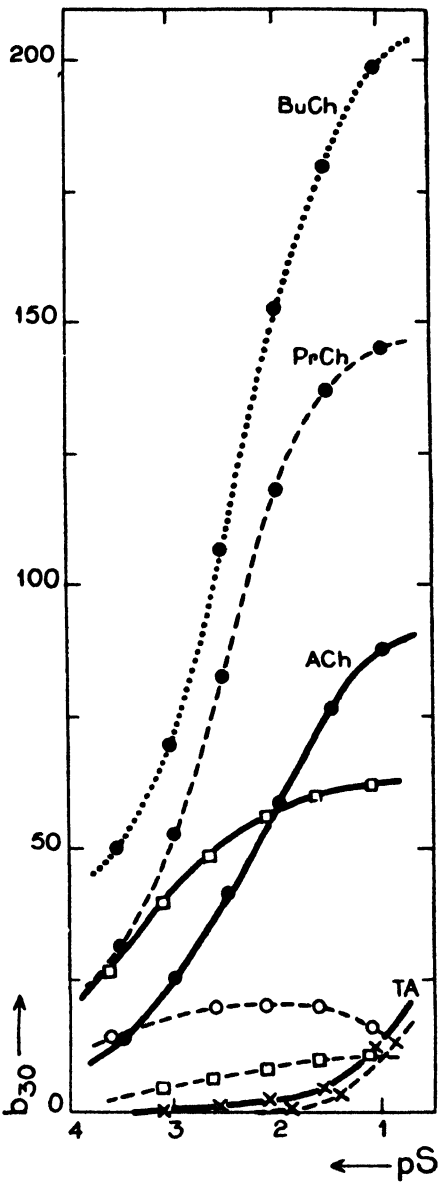


FIG. 3. Activity–pS curves for the enzymatic hydrolysis of various esters by a purified esterase preparation from human plasma.[31] *Cf.* Fig. 2. O- - -O Benzoylcholine; X- - -X methyl butyrate; other symbols as in Fig. 2.

tivity.[127] At about 56° the enzymes begin to be destroyed; at 70° the activity is quite lost. Evaporation of blood and tissue extracts gives active dry

[127] D. Keilin and Y. L. Wang. *Biochem. J.* 41. 491 (1947)

powders;[128] drying with acetone, however, destroys the enzymes.[48] The freezing-drying process does not affect the activity.[25,41,129]

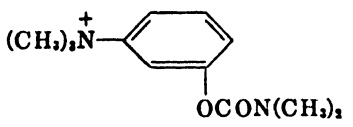
The temperature coefficient (K_{25-35}) of ACh hydrolysis by serum[10,42,130] is 1.36. The optimum temperature of serum ChE and nerve AChE is 37 to 40°.

5. Effect of Light and Other Radiations

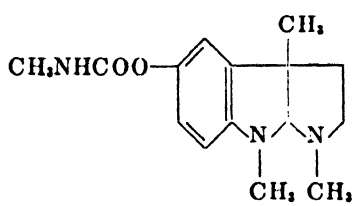
Visible light causes a reversible increase in serum ChE activity.[117] This enzyme is unaffected by ultraviolet and fluorescent light.[131] No definite effect of X-rays on serum ChE has been reported.[132]

VIII. Inhibitors

A great variety of compounds are known to inhibit the enzymatic hydrolysis of ACh. A comprehensive list of such "anticholinesterases"[133] has been published elsewhere.[134] The inhibitory action of many compounds, however, is observed in fairly high concentration. The most active inhibitors of AChE and ChE can be classified in four groups: (*1*) quaternary ammonium bases (prostigmine, methylene blue); (*2*) physostigmine and other urethans; (*3*) alkyl fluorophosphates (DFP); (*4*) alkyl polyphosphates



Prostigmine



Physostigmine (Eserine)

[128] F. Bernheim and M. L. C. Bernheim, *J. Pharmacol. Exptl. Therap.* **57**, 427 (1936).

[129] C. B. Anfinsen, O. H. Lowry, and A. B. Hastings, *J. Cellular Comp. Physiol.* **20**, 231 (1942).

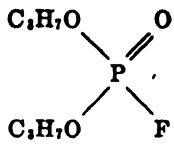
[130] D. Glick, *Proc. Soc. Exptl. Biol. Med.* **40**, 140 (1939).

[131] E. Engelhart and O. Loewi, *Arch. exptl. Path. Pharmakol.* **150**, 1 (1930).

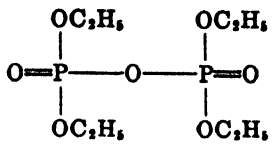
[132] H. Kwiatkowski, *Fermentforschung* **15**, 138 (1936). T. Baglioni and M. Piemonte, *Boll. soc. ital. biol. sper.* **23**, 732 (1947).

[133] The word "anticholinesterase" is frequently used, especially in pharmacological literature, for designating a drug which is supposed to exert its pharmacological activity by inhibiting the enzymatic hydrolysis of ACh. The existence of a true anticholinesterase, that is, an immune body (antibody) produced by animals as the result of parenteral injections of the esterase, has not yet been demonstrated.

[134] R. Ammon, *Ergeb. Enzymforsch.* **9**, 35 (1943). K.-B. Augustinsson, *Acta Physiol. Scand.* **15**, Suppl. 52, 28 (1948). In these reviews detailed references are found; only references to the recent data are given in the following. G. B. Koelle and A. Gilman */Pharmacol. Revs.* in the *J. Pharmacol. Exptl. Therap.* **95**, April, Part II, 166 (1949)/ have recently reviewed the pharmacological aspects of the "anticholinesterase" drugs.



Diisopropyl fluorophosphate (DFP)



Tetraethyl pyrophosphate (TEPP)

TABLE I

INHIBITORS USED IN DETERMINING TYPES OF ACETYLCHOLINE-SPLITTING ENZYMES

Compound	Ref. No.
Selective Inhibition of AChE	
Methylhydroxy purines	15, 135
Priscol	136
Di-(2-chloroethyl)-methylamine	137
Neurine	25
N-*p*-Chlorophenyl-*N*-methylcarbamate of *m*-hydroxyphenyl-trimethylammonium bromide (Nu-1250)	137*a*
Selective Inhibition of ChE	
Pyrazolone derivatives	15, 138
Percaine, procaine	15, 139
Privine	136
Sulfonamides	15, 140
Tri-*o*-cresyl phosphate	57
Quinine, quinidine	135, 141
Paludrine	84
Curare (intocostrin)	142
Eucupine	143
Phenanthrene amino alcohols	144
Dimethylcarbamate of 2-hydroxyphenylbenzyltrimethylammonium bromide (Nu-683)	145
DFP	81
Amidone	146
N-Diethylaminoethylphenothiazine hydrochloride	147

[135] D. Nachmansohn and H. Schneemann, *J. Biol. Chem.* **159**, 239 (1945).
[136] B. Schär-Wüthrich, *Helv. Chim. Acta* **26**, 1836 (1943).
[137] D. H. Adams and R. H. S. Thompson, *Biochem. J.* **42**, 170 (1948).
[137a] R. D. Hawkins and B. Mendel, *Biochem. J.* **44**, 260 (1949).
[138] E. A. Zeller, *Helv. Chim. Acta* **25**, 1099 (1942).
[139] A. Denys and J. Lévy, *Compt. rend. soc. biol.* **141**, 653, 735 (1947).
[140] E. A. Zeller, *Helv. Chim. Acta* **25**, 216 (1942).
[142] M. M. Harris and R. S. Harris, *Proc. Soc. Exptl. Biol. Med.* **56**, 223 (1944).
[143] P. Zamboni and W. Ferrari, *Boll. soc. med. chir. Modena* **45**, 435 (1945).
[144] C. I. Wright, *J. Pharmacol. Exptl. Therap.* **87**, 109 (1946).
[145] R. D. Hawkins and J. M. Gunter, *Biochem. J.* **40**, 192 (1946). H. Blaschko, E. Bülbring, and T. C. Chou, *Brit. J. Pharmacol.* **4**, 29 (1949).
[146] M. E. Greig and R. S. Howell, *Proc. Soc. Exptl. Biol. Med.* **68**, 352 (1948).
[147] J. J. Gordon, *Nature* **162**, 146 (1948).

(TEPP). Some other compounds, the pharmacological actions of which have been discussed in connection with their inhibitory effect on these enzymes, will be considered as a fifth group.

The actions of certain inhibitors of choline ester-splitting enzymes have been used to differentiate AChE and ChE. Table I lists such compounds. The problem of the relationship between specific structural configuration and inhibitory capability can only be obtained if the effect of an inhibitor is studied under various experimental conditions. In the system enzyme–substrate–inhibitor the concentrations of all these components must be varied one after the other. Detailed studies of that kind, however, with the compounds listed in Table I, have in many cases not yet been performed. Recent studies on the kinetics of the inhibition of ChE[25,148] and AChE,[25,141,149,150] respectively, have given us valuable information of the mechanism of this action and cleared up many previous contradictions.

1. Quaternary Ammonium Bases

All quaternary ammonium bases (except betaine) are strong inhibitors of AChE and ChE. They are said to be more active on brain AChE than on serum ChE, while tertiary amines seem to inhibit the serum ChE more powerfully.[151]

a. Choline and its derivatives inhibit the blood serum ChE reversibly and competitively. Contradictory results have been obtained with the AChE from various other sources. This probably is due to the fact that choline causes a shift of optimum ACh concentration to higher concentrations.[25] The action on *Helix* blood AChE is such that, at high substrate concentration, choline may even have a weak activating effect on the enzyme activity.

Muscarine, chemically closely related to choline, is a strong inhibitor of serum ChE.

b. Methylene blue and other basic dyes are strong inhibitors of serum ChE[152] and the AChE of brain and erythrocytes.[25] This action is due to the presence of the quaternary ammonium ion, for the leuco form has no effect.

c. Prostigmine is a strong inhibitor of both AChE and ChE. This action

[148] O. H. Straus and A. Goldstein, *J. Gen. Physiol.* **26**, 559 (1943). A. Goldstein, *ibid.* **27**, 529 (1944); *Federation Proc.* **7**, 223 (1948).

[149] C. I. Wright and J. C. Sabine, *J. Pharmacol. Exptl. Therap.* **93**, 230 (1948).

[150] D. Nachmansohn, M. A. Rothenberg, and E. A. Feld, *J. Biol. Chem.* **174**, 247 (1948).

[150] K.-B. Augustinsson and D. Nachmansohn, *J. Biol. Chem.* **179**, 543 (1949). Most of the results in this paper has recently been confirmed by A. S. V. Burgen, *Brit. J. Pharmacol.* **4**, 219 (1949), see also J. A. Bain, *Proc. Soc. Exptl. Biol. Med.* **72**, 9 (1949).

[151] M. C. Sanz, *Helv. Physiol. Pharmacol. Acta* **3**, C14 (1945).

[152] L. Massart and R. P. Dufait, *Enzymologia* **9**, 364 (1941). P. Klein, *Biochem. Z.* **317**, 210 (1944). K.-B. Augustinsson, *Acta Chem. Scand.* **4**, 536 (1950).

is due to its being a urethan derivative *and* quaternary ammonium base. The action of prostigmine is a reversible and competitive one. The optimum ACh concentration for AChE is changed to higher concentrations in the presence of prostigmine[150] (Fig. 4). Consequently, prostigmine at high ACh concentration (10^{-2} to 10^{-1} M) lowers the activity of AChE much less than that of serum ChE. The dissociation constant (K_I) for the AChE–prostigmine complex is 1.6×10^{-7}.

It is not clear whether the beneficial results obtained with prostigmine in myasthenia gravis can be ascribed only to its inhibitory action on the enzymatic hydrolysis of ACh. More recent investigations on the pharma-

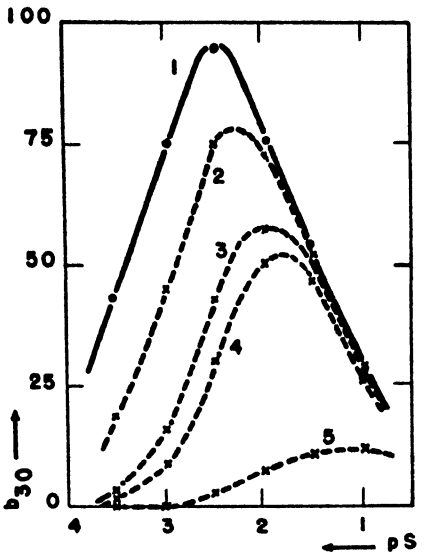


FIG. 4. Activity–pS curves for the enzymatic hydrolysis of ACh by AChE (*Electrophorus* electric organ) in the presence of prostigmine bromide.[150] Prostigmine concentration $\times 10^7$ M: (1) 0, (2) 4.0, (3) 10.0, (4) 20.0, (5) 100.0.

cology of prostigmine suggest that some of the effects of this substance are not related to the action on AChE (or ChE).[153]

A great number of analogs of prostigmine have been prepared[154] and their activities as esterase inhibitors determined. The dimethyl carbamate of 2-hydroxy-5-phenylbenzyltrimethylammonium bromide (Nu-683) is said to inhibit serum ChE selectively.[145] Recent observations on the actions

[153] C. Heymans, *Experientia* **2**, 260 (1946). C. Heymans, R. Pannier, and R. Verbeke, *Arch. intern. pharmacodynamie* **72**, 405 (1946). W. F. Riker, Jr., and W. C. Wescoe, *J. Pharmacol. Exptl. Therap.* **88**, 58 (1946). See also M. Guggenheim, *Schweiz. med. Wochschr.* **77**, 657 (1947).

[154] J. A. Aeschlimann and A. Stempel, *in* Emil Christoph Barell Jubilee Volume, Hoffmann-La Roche, Basle, 1946, p. 306.

of these substances on end plate potential have thrown light on the detailed mechanisms involved in neuromuscular transmission.[155]

d. Curare, recently shown to contain quaternary ammonium bases, is a powerful inhibitor of ACh-hydrolyzing esterases. Pure curare preparations (*e.g.*, D-tubocurarine), however, do not inhibit dog serum ChE and the AChE of insect nerves.[156] The curarelike action of polymethylene bis-quaternary ammonium salts are said to be related to their inhibitory action on AChE.[157]

2. Physostigmine and Other Urethans

a. Physostigmine (eserine), the most important alkaloid derived from the Calabar bean, protects ACh from enzymatic hydrolysis in 10^{-6} *M* concentration and its pharmacological action is probably accounted for by this. The drug combines reversibly with the enzyme and this reaction is a slow one. Detailed studies on the mechanism of action of physostigmine on serum ChE[148] have shown that one molecule of the drug combines with one center of ChE; K_I is 3.11×10^{-8}. Similar results have been obtained with AChE (electric tissue[149]; for this enzyme,[150] K_I is 6.1×10^{-8}. Physostigmine changes the activity - ACh concentration relationship only slightly at high inhibitor concentration. At high ACh concentrations, therefore, this drug has a much stronger inhibitory effect than prostigmine. Physostigmine probably affects two different active groups of AChE.[158]

The inhibition of horse serum ChE has been proposed[159] as a method for the estimation of physostigmine at low concentration (10^{-8} to 10^{-6} *M*).

b. Other Urethans. The action of physostigmine on the enzymatic hydrolysis of ACh has been suggested to be due to the urethan group. Other urethans (*e.g.*, miotine; prostigmine also is a urethan) have an effect similar to that of physostigmine. Urethan itself, however, has very little activity as an inhibitor and phenol bases containing no urethan group an action like physostigmine.[160]

3. Alkyl Fluorophosphates

These substances act as powerful inhibitors of AChE and ChE both *in vivo* and *in vitro*. The dimethyl ester was the first to be investigated.[161]

[155] J. C. Eccles and W. V. MacFarlane, *J. Neurophysiol.* **12**, 59 (1949).

[156] A. R. McIntyre and R. E. King, *Science* **97**, 69 (1943). A. G. Richards, Jr., and L. K. Cutkomp, *J. Cellular Comp. Physiol.* **26**, 57 (1945).

[157] R. B. Barlow and H. R. Ing, *Brit. J. Pharmacol.* **3**, 298 (1948).

[158] J. A. Cohen, F. Kalsbeek, and M. G. P. J. Warringa, *Biochim. et Biophys. Acta* **2**, 549 (1948).

[159] S. Ellis, F. L. Plachte, and O. H. Straus, *J. Pharmacol. Exptl. Therap.* **79**, 295 (1943).

[160] H. Bloch, *Arch. exptl. Path. Pharmakol.* **193**, 292 (1939).

[161] See E. D. Adrian, W. Feldberg, and B. A. Kilby, *Brit. J. Pharmacol.* **2**, 56 (1947).

The most active one is diisopropyl fluorophosphate (DFP),[162] which is one of the most powerful and specific enzyme inhibitors known.[163] In contrast to the inhibitory action of prostigmine and physostigmine, the action of DFP is progressive and irreversible.[164] The irreversible inactivation, however, is a relatively slow process.[149] ACh protects AChE against the action of DFP, which suggests that DFP acts on the same active center of the enzyme molecule as ACh.[150,165] Prostigmine effectively protects the enzyme against the action of DFP,[150] which has also been demonstrated in *in vivo* experiments.[166] The protective action of physostigmine is less pronounced.[167] The affinity of DFP for the enzyme is of a similar order of magnitude to that of prostigmine. DFP has a stronger inhibitory action on serum ChE than on AChE.[81] P^{32} from radioactive DFP binds to protein in amounts directly proportional to the degree of inhibition of AChE.[168] The action on other enzymes, including other esterases, is much less pronounced.[169]

Extensive pharmacological and biochemical studies have been carried out with DFP during recent years.[170] Aside from its inhibitory effect on ACh-splitting enzymes, DFP has no action that has been conclusively demonstrated.[171]

4. Alkyl Polyphosphates

The most potent inhibitors belonging to this group are tetraethyl pyrophosphate (TEPP) and hexaethyl tetraphosphate (HETP),[172] introduced as insecticides during the last war by the Germans. A great number of alkylated phosphorus compounds have been studied with respect to their inhibitory action on AChE (erythrocytes) and ChE (serum).[173] TEPP is the most powerful inhibitor known. It inactivates the AChE and ChE irreversibly; this reaction is very fast compared with that of DFP.[150] ACh protects AChE against the action of TEPP.

The inhibiting activity of these compounds on AChE seems to be re-

[162] H. McCombie and B. C. Saunders, *Nature* **157**, 287, 776 (1946).
[163] D. Nachmansohn and E. A. Feld, *J. Biol. Chem.* **171**, 715 (1947).
[164] A. Mazur and O. Bodansky, *J. Biol. Chem.* **163**, 261 (1946).
[165] *Cf.* E. C. Webb, *Biochem. J.* **42**, P27 (1948).
[166] T. Koppanyi, *Federation Proc.* **8**, 309 (1949).
[167] G. B. Koelle, *J. Pharmacol. Exptl. Therap.* **88**, 232 (1946).
[168] H. O. Michel and S. Krop. *Federation Proc.* **8**, 320 (1949). See, however, B. J. Jandorf and P. D. McNamara, *ibid.* **8**, 210, 223 (1949).
[169] E. C. Webb, *Biochem. J.* **42**, 96 (1948). S. Ellis. *Proc. Soc. Exptl. Biol. Med.* **69**, 363 (1948).
[170] References and a comprehensive report: R. Verbeke, *Arch. intern. pharmacodynamie* **79**, 1 (1949).
[171] See, however, C. Heymans *et al.*, *Arch. intern. pharmacodynamie* **74**, 233 (1947); **75**, 415 (1948); **77**, 64 (1948).
[172] K. P. Dubois and G. H. Mangun, *Proc. Soc. Exptl. Biol. Med.* **64**, 137 (1947). L. E. Chadwick and D. L. Hill, *J. Neurophysiol.* **10**, 235 (1947).
[173] R. W. Brauer, *J. Pharmacol. Exptl. Therap.* **92**, 162 (1948).

sponsible for most of their pharmacological effects. The toxicity appears to be a function of their potency as AChE inhibitors.[174]

5. Other Compounds

The great variety of compounds tested for their inhibitory action on AChE and especially on serum ChE has been summarized elsewhere.[134] The most important features, in addition to some recent observations, will be briefly mentioned in the following.

Bufotenine, the structure of which is similar to that of physostigmine, is a very active inhibitor. Atebrine inhibits both AChE and ChE strongly, but the new antimalarial drug paludrine has little effect. No relationship has been found between the relative inhibiting action on serum ChE and erythrocyte AChE, respectively, and the antimalarial activity or chemical constitution.[141] In the group of local anesthetics, which generally inhibit serum ChE more strongly than AChE (nervous system, erythrocytes), no connection exists between chemical constitution and inhibiting action on serum ChE, but a certain correlation is to be found between this action and pharmacological action.[175]

Prolonged administration of barbiturates causes serum ChE to reach low values. This action is said to be due to a lowering of the enzyme concentration rather than to an inhibition.[176] The sulfonamides and *p*-aminobenzoic acid inhibit serum ChE, the latter being a stronger inhibitor.[140]

Adrenaline increases the response of the muscle and the nervous system to ACh, but this increase is probably due to a different mechanism than an inhibition of AChE.[177]

AChE is strongly inhibited by certain chemical vesicants (chloroalkyl amines) and some of their pathological effects may be due, in part, to this inhibition.[178] The British antilewisite (BAL) has a weak inhibiting effect.[179]

A great variety of alkaloids has been investigated.[180] The more potent inhibitors are morphine, papaverine, strychnine, sparteine, eucupine, the ergot alkaloids, and others. A very strong inhibitor of serum ChE is the alkaloid from *Tabernanthe iboga*, ibogaine, the action of which is said to be of the same order as that of physostigmine.[181]

Tricresyl phosphate inhibits serum ChE strongly; among the three possible isomers, the ortho derivative is the only one which has this action both *in vivo* and *in vitro*.[182]

Thiamine (vitamin B_1) inhibits both ChE and AChE, but whether this action is physiologically important is still unsettled.[183] Inconsistent results have been reported for the action of vitamin C.

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[174] C. Dayrit, C. H. Manry, and M. H. Seevers, *J. Pharmacol. Exptl. Therap.* **92**, 173 (1948). H. W. Jones, Jr., B. J. Meyer, and L. Karel, *ibid.* **94**, 215 (1948).

[175] K. Bullock, *Quart. J. Pharm. Pharmacol.* **21**, 266 (1948). G. Dastugue and P. Dupuis, *Compt. rend. soc. biol.* **142**, 598, 601 (1948).

[176] F. Schütz, *J. Physiol.* **102**, 259 269 (1943); *Quart. J. Exptl. Physiol.* **33**, 35 (1944). E. Frommel, M. Favre, and F. Vallette, *Helv. Med. Acta* **15**, 314 (1948).

[177] J. H. Burn, *Physiol. Revs.* **25**, 377 (1945).

[178] R. H. S. Thompson, *J. Physiol.* **105**, 370 (1947).

[179] E. S. G. Barron, Z. B. Miller, and J. Meyer, *Biochem. J.* **41**, 78 (1947).

[180] A comprehensive study has been performed by P. Beaujard, Recherches sur les alcaloïdes inhibiteurs de la cholinestérase (applications toxicologiques). Douladoure, Toulouse, 1944.

[181] D. Vincent and I. Sero, *Bull. soc. biol. chim.* **24**, 1352 (1942).

[182] H. Bloch, *Helv. Med. Acta* **8**, Suppl. 7, 15 (1941); *Helv. Chim. Acta* **26**, 733 (1943).

[183] G. Stüttgen, *Klin. Wochschr.* **26**, 136 (1948).

The only hormone which has a significant effect on serum ChE is vagotonine, a preparation from pancreas which increases vagal tone and slows the heart. It is said to combine with the same group of the enzyme molecule as does physostigmine.[184]

In general, it is very uncertain whether the pharmacological and therapeutic effects of all these and other drugs are connected with the cholinergic impulses, *i.e.*, with the liberation and stabilization of ACh. Anyhow, the effect on serum ChE cannot have any connection with these impulses and the effects of the drugs are intimately connected with the localization and fixation of the drugs on specific receptors, a condition connected with the physicochemical properties of the drugs.

IX. Physiological Significance of Acetylcholine-Hydrolyzing Enzymes

1. Acetylcholine Esterase

The presence of AChE is necessary for any function of ACh. What this function is has not yet been conclusively demonstrated. According to the hypothesis of chemical transmission,[2] AChE is said to destroy ACh immediately after transmission has occurred. ACh is removed so rapidly from the autonomic synapses or from the junctional zones between parasympathetic postganglionic fibers and the muscles or glands which they supply that activity in response to a single nerve impulse immediately ceases. A nerve impulse is then able to exert a separate effect. The significantly high concentration of AChE at the precise place where it can play an essential role in such a chemical transmission can hardly be purely incidental and without physiological significance. The hydrolysis of ACh may be prevented or greatly slowed by injection of AChE inhibitors (*e.g.*, physostigmine, prostigmine, etc.); ACh then accumulates, and a single nerve impulse may set up a prolonged response in muscle or gland.

Especially due to Nachmansohn and coworkers,[2] the question of the role of ACh in the mechanism of nerve activity has been approached by the study of the enzyme systems involved in the formation (by choline acetylase) and hydrolysis (by AChE) of the ester. The activity of AChE has been correlated in different ways with events in the living cell recorded by physical methods. The following features, emerging from the study on AChE, are of physiological importance: (*1*) AChE is present in nervous and muscular tissues throughout the animal kingdom; (*2*) AChE is located in the neuronal surface; (*3*) ACh is hydrolyzed by AChE at an extremely high speed (high turnover number); (*4*) AChE is relatively specific for ACh; (*5*) direct proportionality is found between the relative AChE concentration and the voltage developed in the unitary elements of the electric organ; (*6*) if AChE is reversibly inhibited, the abolition of the nerve action potential is reversible, and irreversible inhibition of AChE abolishes the action potential irreversibly; (*7*) absence of AChE in the brain coincides with death.

[184] M. Polonovski, D. Santenoise, and A. Pelou, *Compt. rend. soc. biol.* **137**, 115 (1943).

These facts, together with those obtained with the choline acetylase system, form the basis of the hypothesis of Nachmansohn that AChE is necessary for conduction both in nerves and muscles; the release and removal of ACh is an intracellular process, occurring at points along the neuronal surface and directly associated with the nerve action potential.

All these observations with AChE are of great interest, though the validity of some of them has not yet been generally accepted.[2] The attention is centered on the problem of removal of ACh after it has performed its function. Therefore, it would be more strict to say that AChE is not essential for conduction but rather for repolarizing the nerve fiber following the action of free ACh. The hypothesis relates the electric effect not with the presumed electrogenic agent but with its inactivator. It does not give us information as to the precise point at which ACh enters the cycle of activity. Among other things, it would be desirable to show whether "the time relations of the electrical activity can be modified predictably by experimental changes in the acetylcholine system."[185] It is a possibility, as has been claimed by Nachmansohn, that the change in the nerve membrane resistance, *i.e.*, the increased permeability for ions, during the passage of the impulse, is due to the release of ACh and its effect on the proteins or lipoproteins of the active membrane. The AChE in the membrane of the erythrocytes may then probably be associated with permeability. It is of interest that Greig and Holland[185a] have observed that changes in the permeability of the erythrocytes may be effected by inhibitors of AChE. Their suggestion that the ACh-AChE system may have a widespread function in maintaining the normal permeability of the living cell, is of great importance. Recent studies by Rothenberg[185b] on the permeability of the surface membranes of the giant axon of Squid to ions have given promising results in that direction. The AChE inhibitors, physostigmine and DFP, seem to produce an increase in membrane permeability; the rate of potassium penetration is decreased, that of sodium increased.

The function of AChE in snake venom and the blood of *Helix* is more difficult to understand. Regarding the snake venom esterase, it is a possibility that the venom is a natural solution of the "membrane" AChE. The hemolytic effect of the venom is well known and it has been demonstrated that lysolecithin dissolves the esterase out of the "ghosts."[25] Therefore, the venom in the hosts also may dissolve the esterase from the erythrocytes, or the glands in which it is produced. These glands are phylogenetically electric organs.

Very few studies have been performed to correlate the AChE activity

[185] H. Grundfest, *Ann. Rev. Physiol.* **9**, 477 (1947).

[185a] M. E. Greig and W. C. Holland, *Arch. Biochem.* **23**, 370 (1949); *Science* **110**, 237 (1949).

[185b] M. A. Rothenberg, *Biochem. et Biophys. Acta* **4**, 96 (1950). For discussion, see D. Nachmansohn, *ibid.* **4**, 78 (1950).

with various pathological conditions of the nervous system. Such studies, however, have been carried out in great number with serum ChE, but it must be stressed that abnormal functioning of the ACh system in the nervous system is not necessarily indicated by an alteration of serum ChE activity.[16,137a,183,187]

The investigations of AChE activity at different stages of animal development have added much to our knowledge of the significance of the system ACh–AChE. It has been demonstrated in many ways that a relation exists between AChE concentration and function during embryonic development. Thus, for instance, the functional maturation of the neuromuscular apparatus of *Amblystoma* coincides with the development of AChE to a quantitatively high level.[41,188]

2. Blood Serum Cholinesterase

Because of the complete lack of evidence regarding the physiological substrate(s) of serum ChE, the physiological function of this enzyme is still unknown. It may be that this "transport ChE"[189] is merely a barrier responsible for the destruction of any ACh that leaks out into the blood stream and that might escape hydrolysis by AChE. It is, however, difficult to understand why some animals (*e.g.*, the ruminants) have no ChE in their plasma. ACh injected directly into the blood vessels is rapidly destroyed by the ChE which undoubtedly is concerned with the vasodepressor effects of ACh. Pharmacologists are particularly interested in ChE inhibitors, because they find that the effects of injected ACh are prolonged, potentiated, or otherwise modified by these inhibitors.

The ChE concentration in the blood of man has been studied in order to correlate if possible the enzyme activity with various diseases. The results are not easy to interpret because of the marked variation in the ChE activity of normal human beings.[190] Normal physiological processes have no influence on ChE. It is suggested, however, that the ChE level of human plasma (and of the liver) is influenced by certain sex hormones.[191] An

[186] B. Mendel, R. D. Hawkins, and M. Nishikawara, *Am. J. Physiol.* **154**, 495 (1948).

[187] O. Bodansky, *Ann. N. Y. Acad. Sci.* **47**, 521 (1946).

[188] C. H. Sawyer, *J. Exptl. Zool.* **92**, 1 (1943), **94**, 1 (1943). Problems of that kind are also discussed by: D. Nachmansohn, *J. Neurophysiol.* **3**, 396 (1940); K. A. Youngstrom, *ibid.* **4**, 473 (1941); B. F. Lindeman, *Am. J. Physiol.* **148**, 40 (1947); E. J. Boell, *Ann. N. Y. Acad. Sci.* **49**, 773 (1948); K.-B. Augustinsson and T. Gustafson, *J. Cellular Comp. Physiol.* **34**, 311 (1949).

[189] T. Koppanyi, *Bull. Johns Hopkins Hosp.* **83**, 532 (1948).

[190] A. Sawitsky, H. M. Fitch, and L. M. Mayer, *J. Lab. Clin. Med.* **33**, 203 (1948).

[191] E. A. Zeller, H. Birkhäuser, H. von Wattenwyl, and R. Wenner, *Helv. Chim. Acta* **24**, 962, 1465 (1941); **26**, 2063 (1943). D. B. Mundell, *Nature* **153**, 557 (1944). C. H. Sawyer and J. W. Everett, *Endocrinology* **39**, 307, 323 (1946). M. G. Levine and R. E. Hoyt, *Proc. Soc. Exptl. Biol. Med.* **70**, 50 (1949).

association between thyroid activity and plasma ChE activity has been proposed.[192]

Investigations carried out with a great number of patients with various diseases have been described.[193] Reduced ChE values have been obtained in cases of liver diseases; the liver is supposed to be the main site of ChE formation.[191,193,194] Any increase in plasma ChE activity in patients treated for pernicious anemia, however, is said to be the resultant of recovery and not the cause of it.[195] Special interest has been directed toward myasthenia gravis and myotonia congenita, supposed to be associated with an unbalance between the rate of liberation of ACh and the rate of its destruction. However, evidence indicating that such a state of affairs actually exists is still lacking.[196] Serum ChE activity has been studied also in certain mental diseases, but the results obtained have not permitted any conclusions as to the relation between enzyme activity and the disease investigated.[197] In general, the data obtained do not seem to have any diagnostic value; they may probably help in the search for a general factor governing the distribution of ChE.

[192] W. Antopol, L. Tuchman, and A. Schifrin, *Proc. Soc. Exptl. Biol. Med.* **36**, 46 (1937), R. D. Hawkins, M. Nishikawara, and B. Mendel, *Endocrinology* **43**, 167 (1948).

[193] See, among others: M. McGeorge, *Lancet* **232**, 69 (1937). A. T. Milhorat, *J. Clin. Invest.* **17**, 649 (1938). M. Faber, Studier over serumcholinesterasens variationer, Nyt Nordisk Forlag, Copenhagen, 1941; *Acta Med. Scand.* **114**, 59 (1943). H. R. Butt, M. W. Comfort, T. J. Dry, and A. E. Osterberg, *J. Lab. Clin. Med.* **27**, 649 (1942). J. de Prat, La cholinestérase du sérum (application clinique), Imprimerie Moderne, Toulouse, 1945. E. Aron and A. D. Herschberg, *Presse méd.* **54**, 107 (1946). P. Cristol, P. Passouant, C. Benezech, and J. Dutarte, *ibid.* **54**, 557 (1946). H. Sträter, Over het specifieke- en pseudo cholinesterasegehalte bij enigen ziekten. N. V. Erven B. van der Kamp, Groningen, 1948.

[194] B. McArdle, *Quart. J. Med.* **9**, 107 (1940). W. C. Wescoe, C. C. Hunt, W. F. Riker, and I. C. Litt, *Am. J. Physiol.* **149**, 549 (1947). J. E. Davis, *Am. J. Digestive Diseases* **15**, 52 (1948); *Federation Proc.* **8**, 285 (1949). L. M. Meyer, A. Sawitsky, N. D. Ritz, and H. M. Fitch, *J. Lab. Clin. Med.* **33**, 189 (1948). A. Sawitsky, M. Rowen, and L. M. Meyer, *ibid.* **34**, 178 (1949).

[195] A. M. Kunkel, S. Krop, and W. C. Wescoe, *Am. J. Physiol.* **152**, 309 (1948).

[196] Review: C. L. Hoagland, *Advances in Enzymol.* **6**, 193 (1946).

[197] M. S. Jones and H. Tod, *J. Mental Sci.* **83**, 202 (1937). D. Richter and M. Lee, *ibid.* **88**, 428, 435 (1942). S. Mutrux and B. Glasson, *Monatsschr. Psychiat. Neurol.* **114**, 20 (1947). S. Platania and P. Pappalardo, *Acta neurol.* **2**, 714 (1947); **3**, 51 (1948).

CHAPTER 11

Phosphatases*

BY JEAN ROCHE

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I. Historical Introduction

Evidence for phosphatase activity, that is to say, the enzymatic liberation or fixation of orthophosphoric acid, was obtained by several biochemists from 1907 to 1913; it did not create real interest until 1923, when Robison[1] discovered the "bone enzyme"; the role of this enzyme in bone calcification was immediately shown to be important. The study of the phosphatases covers a large number of very diverse problems and has been the subject of several thousand publications; therefore, this chapter can provide no more than an incomplete summary and discussion of the results obtained up to the present time.

The preliminary work of Suzuki, Yoshima, and Takahishi,[2] MacCollum and Hart,[3] Neuberg and Karczag,[4] Levene and Medigreceanu,[5] and von Euler and Funke[6] established that very diverse substrates liberate orthophosphoric acid in the presence of many animal and plant tissues. Later on, the demonstration of the role played by the phosphate radical in glucose metabolism and the advances in the biochemistry of ossification led numerous investigators to a study of the phosphatases. The biochemistry and the physiological chemistry of these enzymes evolved simultaneously step by step. The individual phosphatases were separated and classified and the phosphatase systems of the various tissues were described. This portion of research on the phosphatases is about completed; on the other hand, the preparation of the enzymes in the pure state and the recognition of their active group and structure are going to require renewed efforts. The physiological and pathological chemistry of the phosphatases, initially approached by Kay, Robison, Roberts in England and Canada, by Demuth in Germany and by Bodansky in the United States, has undergone a rapid and extensive development, owing to its medical applications. Until now, only the role of the enzyme present in bone may be considered to have been elucidated; on the other hand, it is not yet possible to define with certainty the role of the phosphatases of the intestinal mucosa, kidney and prostate, tissues rich in these enzymes. Histochemistry has taken a more and more important part in this field, as shown by a large number of recent investigations, while the pathological chemistry of the phosphatases concentrates on the study of the variations of phosphoesterase activity of serum. These subdivisions of the study of the phosphatases are far from exhausted, as shown by the results obtained during the last few years on the hormonal control of the level of these enzymes in cer-

[1] R. Robison, *Biochem. J.* **17**, 286 (1923).
[2] U. Suzuki, Y. Yoshima, and M. Takahishi, *J. Coll. Agr. Tokyo Imp. Univ. Bull.* **7**, 503 (1907).
[3] E. V. McCollum and E. B. Hart, *J. Biol. Chem.* **4**, 497 (1908).
[4] C. Neuberg and L. Karczag, *Biochem. Z.* **36**, 60 (1911).
[5] P. A. Levene and F. Medigreceanu, *J. Biol. Chem.* **9**, 65 (1911).
[6] H. von Euler and Y. Funke, *Z. physiol. Chem.* **77**, 488 (1912).

tain tissues and on the modifications of phosphatasemia during the development and treatment of cancer of the prostate.

This brief introduction explains the selection of our plan and gives the reasons for the lack of precision in certain paragraphs, since our discussion includes not only the data that can be logically coordinated but also others whose significance is still vague. In our present state of knowledge, the general picture of the study of the phosphatases is well defined, but the study itself in almost all its aspects is still in the process of being developed.

II. General Biochemistry of the Phosphatases

1. Substrates and Measurement of Phosphatase Activity

Biological fluids and cellular extracts liberate orthophosphoric acid from compounds of different types. Since the phosphatases selectively hydrolyze some of them, it is necessary to define their nature exactly in order to study the specificity of these enzymes in a later paragraph.

The esters of phosphoric acid, its amides, pyrophosphoric acid and its higher polyphosphoric homologues, and metaphosphoric acid are the substrates of the principal phosphatases. Among the first, only the monoesters of alcohols and phenols $(R—O)O:P(OH)_2$, and the diesters, $(R—O)_2O:P(OH)$, are hydrolyzed by phosphatases, and the nature of the R radical may, in certain instances, be a factor affecting specificity. The substrates of the most common of these types are the α- and β-glycerophosphoric, hexosephosphoric, mono- and diphenylphosphoric acids and the nucleotides. The orthophosphoric triesters, which are easily hydrolyzed by acids, bases, or even boiling water, are not hydrolyzed enzymatically. Certain anhydrides of organic acids (acetic among others) and of orthophosphoric acid are hydrolyzed by a specific enzyme. The same is true of pyrophosphoric and triphosphoric acid and of their esters (adenosinetriphosphoric acid among others).

Various natural or synthetic compounds endowed with these structures are commonly employed as substrates in the study of the phosphatases. Some are chosen by reason of their great sensitivity to the action of the enzymes (easily hydrolyzable substrates such as the glycerophosphoric acids) or of their constitution, which corresponds to a specific enzymatic activity (pyrophosphates, adenosinetriphosphoric acid). Others are employed because of particular properties, useful for analytical purposes, of the products of hydrolysis (color in the case of phenolphthaleinphosphate, fluorescence in that of the phosphoric esters of eosin and fluorescein, the capacity of phenol to give more intense color reactions than the phosphates in the case of phenylphosphate).

The study of the phosphatases is based upon the measurement of their activity under various conditions. The principle of this analytical operation is the determination of one of the products of hydrolysis of the substrate, liberated under carefully standardized conditions (pH of a buffer solution free of inhibitors, constant tempera-

ture, suitable substrate concentration). In investigations of pure enzymology, it is customary to measure the initial rate of the reaction by determining the products to which it gives rise at various successive times. In orientation experiments and in investigations of physiological or medical chemistry, it is sufficient to make a single determination, at a time chosen in such a way that a hydrolysis of the substrate not surpassing 10% of the latter is achieved. Beginning with a certain degree of purity, it is desirable to refer the activity to milligrams of weight or of protein nitrogen acting during the time unit on the substrate under defined experimental conditions. This provides the only way for a comparison of the results obtained during purification operations or by different authors. As for the other enzymes, the absolute activity Q_P may be expressed in mm.^{3}, considering that one mole of orthophosphoric acid in the gaseous state occupies a volume of 22,400 ml. Q_P is the number of cubic millimeters of H_3PO_4 liberated per milligram of preparation in 1 hour under the chosen conditions of pH, temperature, and substrate and activator concentrations. The activity of tissue extracts or blood serum is expressed only in relative units. These can be defined only empirically and it is not possible to compare one with the other and, *a fortiori*, to convert one into the other, since they correspond to amounts of phosphoric acid, or of a radical bound to it, liberated from different substrates under nonidentical conditions. The units defined by Bodansky (amount of enzyme liberating 1 mg. P in 1 hour from 0.5% sodium-β-glycerophosphate, at pH 8.6 and at 37°, per 100 ml. of serum)[7], by Gutmann and Gutmann[8] and by King and Armstrong[9] (amount of enzyme liberating 1 mg. of phenol in a given time from 0.005 *M* sodium monophenylphosphate at the optimum pH of the acid or alkaline phosphatase and at 37°) are the most commonly employed.

2. Substrate Specificity and General Classification of the Phosphatases

Since the beginning of research on these enzymes, it has been noted that the tissue extracts hydrolyze phosphate derivatives of very varied structures; therefore, much work has been devoted to the identification and separation of specific phosphatases. The definition of phosphatase activity given above includes the catalysis of many reactions: hydrolysis and synthesis of monoesters and diesters, pyrophosphates, phosphoamides, anhydrides of organic acids and of orthophosphoric acid, hydration of metaphosphoric acid. In addition, the cellular reactions in which phosphate radicals participate do not proceed autonomously but are coupled to others, so that their evolution takes place in cycles of reactions. For these two reasons, a great number of phosphatase activities have been described, while only certain of them offer a real specificity.

The principal criterion employed in separating and classifying the phosphatases is the specificity towards the substrate.[10,11,12] It makes it possible to characterize, on the one hand, the phosphatases *acting on a type of bond*

[7] A. Bodansky, *J. Biol. Chem.* **101**, 93 (1933).
[8] E. B. Gutmann and A. B. Gutmann, *J. Biol. Chem.* **136**, 201 (1940).
[9] E. J. King and A. R. Armstrong, *Canad. Med. Assoc. J.* **31**, 376 (1934).
[10] H. D. Kay, *Biochem. J.* **22**, 1446 (1928).
[11] S. J. Folley and H. D. Kay, *Ergebn. Enzymforsch.* **5**, 159 (1936).
[12] Nguyen-van-Thoai, Thèse Doct. Sci. phys., Marseille, 1946, 177 pp.

independently of the nature of the radical combined with the orthophosphoric acid, and, on the other hand, those *specific for a single substrate or a small number of substrates of closely related structure.* Table I lists the best-known phosphatases and their principal sources.

The corresponding enzymatic actions take place with substrates that have a constitution generally related to the name of these phosphatases

TABLE I

SUBSTRATE SPECIFICITY AND CHIEF SOURCES OF THE PHOSPHATASES

Enzyme type	Substrates	Chief sources
A. PHOSPHATASES SPECIFIC FOR ONE BOND		
Phosphomonoesterases	Orthophosphoric monoesters	Bone, intestinal mucosa, kidney, fungi, seeds, molds, yeasts
Phosphodiesterases	Orthophosphoric diesters	Liver, kidney, yeasts, snake venoms
Pyrophosphatases	Pyrophosphoric acid and its esters	Intestinal mucosa, kidney, yeasts, fungi, molds
Phosphoamidases	Orthophosphoric amides	Kidney, cereals
Phosphoacylases	Orthophosphoric anhydrides of organic acids	Muscle, liver
B. PHOSPHATASES SPECIFIC FOR ONE OR SEVERAL SUBSTRATES		
Adenosinetriphosphatases:	Adenosinetriphosphate	
1. Adenylpyrophosphatase		Muscle, intestinal mucosa, potato
2. Apyrase		Muscle, potato
Phytase	Phytic acid	Cereals, seeds
Polyphosphatase	Triphosphoric acid and higher homologues	Intestinal mucosa, molds, yeasts
Cholinephosphatase	Cholineglycerophosphate	Intestinal mucosa, snake venoms
Hexosediphosphatase	1,6-Fructosediphosphate	Liver, kidney, yeasts
Polynucleotidases	Nucleic acids and polynucleotides	Intestinal mucosa, pancreas, liver
5-Nucleotidase	5-Nucleotides	Testis, snake venoms
Metaphosphatases	Metaphosphoric acid	Kidney, molds, yeasts

(Group A: monoesters and diesters of phosphoric acid, pyrophosphates, phosphoric amides, anhydrides of organic acids and of orthophosphoric acid; Group B: adenosinetriphosphoric acid, cholineglycerophosphate, fructosediphosphate, 5-nucleotides, metaphosphates). It has been possible to separate almost all of them from each other. However, this is not absolute proof of their individuality, since it may suffice to destroy or block a functional group of the apoenzyme which preferentially fixes a substrate

in order to abolish the capacity of an enzyme to hydrolyze that substrate; this can be done without bringing about the elimination of a specific phosphatase and the separation of another. This reservation does not apply to the phosphatases of group A, at least with regard to the first three, which are the only ones that have been extensively studied. On the other hand, it is of great importance with regard to the enzymes of group B; on the list of the latter, only those appear whose characterization is certain beyond doubt. In this regard, mention should be made of the fact that the strict specificity of the adenosinetriphosphatases and of metaphosphatase is assured in the sense that it has been possible to obtain preparations of these enzymes active on a single substrate under satisfactory conditions of fractionation; this is not true of the others. The purest preparations of several enzymes of group B are not rigorously without action on substrates other than the one corresponding to their name. For example, it is possible to obtain phosphomonoesterases without phytase activity, but the latter cannot be isolated. In other instances, the capacity to hydrolyze a substrate is specifically increased without complete elimination of the other phosphatase actions. The specificities of the enzymes in group B must be rechecked with purified preparations in order to prevent confusion. Natural factors may inhibit the breakdown of one substrate more than that of another and thus apparently modify the specificity. Facts of this type undoubtedly explain the discussion[13] of the existence of an α-glycerophosphatase and likewise of a hypothetical amylophosphatase.[14] Table I is not complete with regard to the enzymes of group B described in the literature, because of a lack of characterization of certain ones among them. Various authors, studying the hydrolysis of a single substrate, have stated without proof that this reaction is carried out by a specific phosphatase (glycerophosphatase, glucosemonophosphatase, saccharosephosphatase, adenylase). Some phosphatase actions originally considered to be specific, *e.g.*, that of a nucleotidase hydrolyzing all the mononucleotides,[15] were later attributed to the enzymes of group A. The study of hexosediphosphatase[15a] and cholinephosphatase (see p. 000) merits completion like those of proteinphosphatase[16] and diphosphopyridinenucleotidase,[17] which have recently been described.

3. Isodynamics of the Phosphatases

The demonstration of the presence in cells or body fluids of phosphatase activities of the same specificity but of a different pH optimum was ac-

[13] A. Schäffner and E. Bauer, *Z. physiol. Chem.* **232**, 66 (1935).
[14] E. Waldschmidt-Leitz and K. Mayer, *Z. physiol. Chem.* **236**, 168 (1935).
[15] P. A. Levene and R. T. Dillon, *J. Biol. Chem.* **88**, 753 (1930).
[15a] G. Gomori, *J. Biol. Chem.* **148**, 139 (1943).
[16] R. M. Feinstein and M. E. Volk, *J. Biol. Chem.* **177**, 339 (1949).
[17] A. Kornberg and O. Lindberg, *J. Biol. Chem.* **176**, 665 (1948).

complished for the first time by Akamatsu,[18] Bamann and Diederichs,[19] Bamann and Riedel,[20] and Belfanti, *et al.*[21] Owing to the identity of the reactions that they catalyze, these enzymes are called isodynamic[19] and it has been possible to base the classification of the phosphatases of group A on the concept of isodynamics.[12,21,22,23] Four phosphomonoesterases of different pH optima, three pyrophosphatases, and, less precisely, three phosphodiesterases have been identified. The study of the first has progressed the farthest.

The presence of several isodynamic enzymes in a medium cannot always be established by a simple study of its activity at different pH levels. The existence of natural inhibitors and the unequal stability of related enzymes complicates the observations. For example, the aqueous extract of many fungi hydrolyzes pyrophosphates at a pH optimum of 3.8–4.0 and apparently contains a single pyrophosphatase. After adsorption on kaolin at pH 6.0, the same sample exhibits two pH optima (3.8 and 6.0) of phosphatase action,[24] owing to the elimination of a natural inhibitor which masks one of the pyrophosphatases. The interference of another factor with the description of natural mixtures of isodynamic phosphatases is illustrated by the following facts. Mammalian red blood cells contain a phosphomonoesterase of pH optimum = 3.8 and two other isodynamic enzymes endowed with much greater stability at pH = 6.5–7.5. Unless the hemolyzates are very fresh the first of these is lacking. On the other hand, it becomes noticeable in the product obtained once the blood leaves the blood vessels and its activity is then added, at a slightly acid pH, to that of another phosphomonoesterase present in red blood cells together with small quantities of alkaline phosphatase. For this reason, the pH optimum of the red blood cell phosphatase acting in slightly acid solution is apparently 5.0–5.2 in fresh hemolyzates and approaches 6.2 in proportion as the spontaneous destruction of the enzyme acting at the pH optimum of 3.8 proceeds.[25] The consequence of such facts is that the characterization of a phosphatase by its pH optimum cannot be carried out with certainty except on purified preparations.

The same remark applies to other criteria adopted for the identification of isodynamic enzymes, the most important being sensitivity towards modifying factors. It is necessary to add other elements of characterization to the pH optimum, and Fig. 1 [26] indicates how the activation of alkaline phosphatase by Mg^{++} [27] differentiates this enzyme from the isodynamic phosphomonoesterase of pH optimum = 5.2. But in this field too the results obtained with crude extracts are not always satisfactory. For example, the highly purified yeast pyrophosphatase of pH optimum = 7.0 is very strongly activated by Mg^{++}, while the efficacy of the same ion on this enzyme in crude

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[18] S. Akamatsu, *Biochem. Z.* **142**, 184 (1923).
[19] E. Bamann and K. Diederichs, *Ber.* **67**, 2019 (1934); **68**, 6 (1935).
[20] E. Bamann and E. Riedel, *Z. physiol. Chem.* **229**, 125 (1934).
[21] S. Belfanti, A. Contardi, and A. Ercoli, *Biochem. J.* **29**, 517 (1935).
[22] S. Munemura, *J. Biochem.* (*Japan*) **17**, 343 (1933).
[23] J. Roche and J. Courtois, *Exposés Ann. Biochim. Med.* **4**, 219 (1943); J. Roche, *Helv. Chim. Acta* **29**, 1253 (1946).
[24] Nguyen-van-Thoai, *Bull. soc. chim. biol.* **23**, 1277 (1941).
[25] J. Roche, Nguyen-van-Thoai, and J. Baudoin, *Bull. soc. chim. biol.* **24**, 1247 (1942).
[26] J. Roche and E. Bullinger, *Enzymologia* **7**, 278 (1939).
[27] H. D. Jenner and H. D. Kay, *J. Biol. Chem.* **98**, 733 (1931).

extracts is so negligible that it is considered nonexistent by many authors.[28] Many contradictory results are undoubtedly due to the study of preparations that have different origins and degrees of purity.

A provisional classification of the isodynamic phosphomonoesterases[11,22,23] and of the pyrophosphatases[29] has been elaborated on the basis of the pH optimum, modifying factors, and stability of these enzymes. It is summarized in Table II, compiled on the basis of the publications of Folley and Kay[11] and Roche and Courtois[23] on the phosphomonoesterases; the cor-

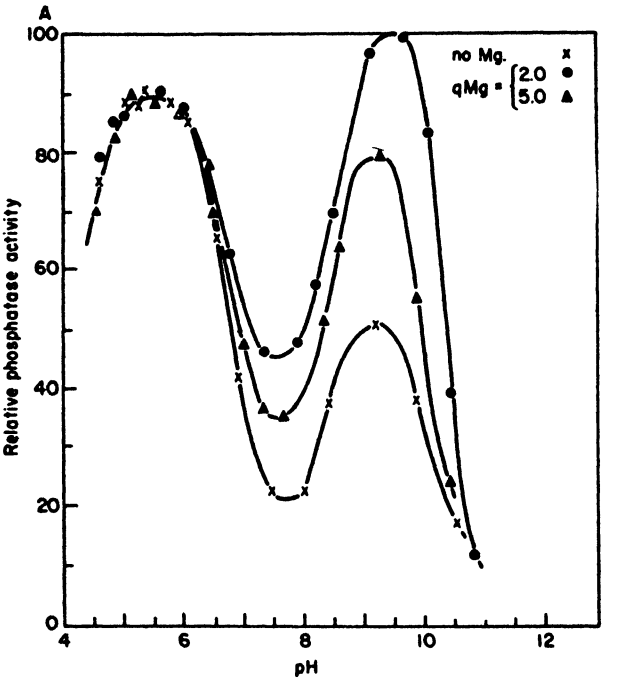

FIG. 1. Influence of Mg^{++} ions on the phosphomonoesterases of an aqueous extract of pig liver (x: no addition of $MgSO_4$; ● and ▲: addition of $MgSO_4$ at concentrations of $-\log [MgSO_4] = q$ Mg 2 and 5) on sodium β-glycerophosphate at various pH's. (Cf. Ref. 26.)

responding table on the pyrophosphatases is too incomplete in certain regards to be presented here.

Table II represents only an attempt at classification in a preliminary manner, since it has been set up on data obtained with preparations of unequal purity. Nevertheless, it has rendered undeniable service in the descriptive study of the natural phosphatase systems. These are generally

[28] K. Bailey and E. C. Webb, *Biochem. J.* **38**, 394 (1944).

[29] J. Roche, Nguyen-van-Thoai and J. Durand, *Bull. soc. chim. biol.* **25**, 1166 (1943).

made up of mixtures of isodynamic enzymes, with the exception of that of the prostate, which contains a high level of phosphomonoesterase II and only traces of enzyme I, and of that of growing bone, which is very rich in the first and poor in the second. Most animal cells contain mainly phosphoesterases and pyrophosphatases that are active in an alkaline environment, while plant cells largely contain those with an acid pH optimum. This difference is not absolute; green leaves, yeasts, and bacteria contain small quantities of phosphatases with an alkaline pH optimum. Owing to the presence of similar mixtures in the cells and body fluids, the descriptive study of the phosphatase systems necessarily must be undertaken

TABLE II

CLASSIFICATION OF ISODYNAMIC PHOSPHOMONOESTERASES

Type	Optimal pH	Chief sources	Chief characteristics in cell or tissue extracts
I	8.6–9.4	Bone, kidney, intestinal mucosa, mammary gland	Activation by Mg^{++}; inhibition by —SH; more active on β- than on α-glycerophosphate; optimal stability at pH = 7.5–8.5.
II	5.0–5.5	Liver, seeds, fungi, prostate	No action of Mg^{++}; inhibition by F^-; more active on β- than on α-glycerophosphate; optimal stability at pH = 5.0–6.0.
III	3.4–4.2	Liver, top yeasts	Inhibition by Mg^{++}; more active on β- than on α-glycerophosphate; optimal stability at pH = 4.5–5.5.
IV	5.0–6.0	Red blood cells, bottom yeasts	Activation by Mg^{++}; more active on α- than on β-glycerophosphate; optimal stability at pH = 6.5–7.5.

before the separation and purification of their constituents is begun. Twenty years ago, this study was still one of the principal objects of research on the phosphatases; at the present time it has been about completed and would appear to have been the initial phase of the biochemistry of these enzymes.

4. HYDROLYTIC AND SYNTHETIC ACTIVITY

Enzymatic synthesis has long been studied with the aim of demonstrating the reversibility of the biocatalytic action of enzymes. Investigations of this type are of no more than historical interest; they opened the way to more constructive endeavors. The synthesis of various esters, including

derivatives of starch and glycogen[30] by alkaline or acid phosphatases[31,32,33] has been shown to proceed according to the law of mass action. It is generally so regular that the synthesis of glycerophosphates from glycerol and phosphates by the kidney enzyme has been chosen in several manuals as an example for the demonstration of the synthetic activity of the enzymes in laboratory courses. Pyrophosphates have been synthesized *in vitro* from phosphates by intestinal pyrophosphatase.[34]

The synthesis of glycerophosphoric acids in the presence of a constant concentration of phosphate ions is governed by the concentration of glycerol in the solution. The rate may nevertheless be reduced by the inhibitory action of phosphate ions if they are present in too high a concentration. The reaction gives rise to a mixture of the two isomeric glycerophosphates, generally richer in α- (up to 80%) regardless of whether the synthesis is carried out by a phosphatase of acid or alkaline optimum.[32,33]

Factors affecting hydrolytic activity are in the same way activators or inhibitors of the enzymatic syntheses, but their action is not always easy to establish in the presence of the natural impurities of the crude tissue extracts usually employed. Purification of the latter greatly reduces their capacity to catalyze the syntheses owing to the elimination of modifying factors of a particular type. Purified alkaline or acid phosphatases retain their ability to cause hydrolysis of esters, but their synthesizing activity is greatly diminished in the sense that synthesis proceeds only at an extremely slow rate. A natural activator that increases the rate of synthesis, but does not appreciably increase the rate of hydrolysis of the glycerophosphates has been isolated from crude extracts of many animal and plant tissues;[35] it is destroyed by autolysis and its sensitivity to this process explains why the storage of crude enzyme preparations causes them to lose their synthesizing capacity. The addition of this activator to purified enzyme preparations restores their capacity for esterifying the alcohols; it can be replaced, at least partially, by amino acids (alanine and others) associated with divalent cations (Mg^{++} or Zn^{++}) and added in optimum concentrations.[35] Phosphocreatine and substances containing an energy-rich phosphate bond increase the velocity of ester synthesis[35a]. A natural activator of the enzymatic synthesis of pyrophosphates, distinct from the one mentioned above, has been prepared; it cannot be replaced by other agents.

[30] J. Roche, Nguyen-van-Thoai, E. Danzas, and M. Silhol-Bernère, *Arch. sci. physiol.* **1**, 81 (1947).
[31] H. D. Kay, *Biochem. J.* **22**, 855 (1928).
[32] H. D. Kay, *Physiol. Revs.* **12**, 384 (1932).
[33] J. Courtois, Thèse Doct. Sci. phys., Paris, 1938, 205 pp.
[34] J. Roche, Nguyen-van-Thoai, and E. Danzas, *Bull. soc. chim. biol.* **27**, 599 (1945).
[35] J. Roche, Nguyen-van-Thoai and E. Danzas, *Bull. soc. chim. biol.* **26**, 1138 (1944); **27**, 401 (1945).
[35a] O. Meyerhof and H. Green, *J. Biol. Chem.* **178**, 655 (1949).

5. Kinetics of Phosphatase Action

The kinetics of the action of phosphomonoesterases has been intensively studied, but only the data obtained under rigorously comparable conditions can be discussed since the value of the affinity constant K_M is greatly modified by the presence of impurities and is very sensitive to the pH. At the pH optimum and at 37°, the phosphomonoesterases I (alkaline) exhibit very different affinities for sodium β-glycerophosphate (K_M = 880 for the bone enzyme, 550 for that of kidney and liver and 80 for that of intestine under certain conditions).[36]

A study of the kinetics of phosphatase action at different substrate concentrations has led to an explanation of the behavior of the various enzymes on the substrates and the action of certain modifying factors.[33] The preferential action of the phosphomonoesterase III of taka-diastase on β-glycerophosphate is due to the greater affinity, measured by the value of K_M, of the enzyme for this isomer. On the other hand, the phosphatase of sweet almonds, which is equally active on the two glycerophosphates, exhibits the same affinity for both. As stated by Albers[37] (p. 455), "certain compounds by combining with the enzyme render it unable to fix the substrate while others form with it complexes having a smaller catalytic activity." The first are the competitive inhibitors, the second the absolute inhibitors. Competition between a substrate and an inhibitor does not affect the maximum rate of hydrolysis of the substrate but reduces the affinity of the enzyme for the latter. On the other hand, absolute inhibition decreases the maximum rate of the reaction but not the affinity, which may actually be increased in the presence of the modifying factor. Finally, the same compound may behave as a competitive and an absolute inhibitor for different phosphatases. For example, sodium fluoride is a competitive inhibitor of the phosphatase of the prostate[38] and an absolute inhibitor of the enzyme of the same type (II) of seeds.[32] In the latter instance, it reduces the maximum rate of hydrolysis of the esters but increases the affinity of the enzyme for them. At low concentrations of substrate (glycerophosphates), where the role of affinity is the more important, F^- has a small action, but at high substrate levels, where the role of the maximum rate predominates, the same ion inhibits strongly. Thus, part of the mechanism of action of phosphatase inhibitors on these enzymes may be interpreted within the framework of the classical hypothesis of Michaelis and Menten.

[36] J. Roche and H. Sarles, *Compt. rend. soc. biol.* **142**, 917 (1948).
[37] H. Albers *in* Handbuch der Enzymologie. Akademische Verlagsgesellschaft, Leipzig, 1940, p. 672.
[38] W. Kutscher and W. Wüst, *Biochem. Z.* **310**, 292 (1941).

III. Biochemistry of the Various Types of Phosphatases

1. Phosphomonoesterases

These enzymes, classified as shown in Table II, act on very many alcoholic or phenolic monoesters; the nature of the radical attached to the orthophosphoric acid has a limited influence on the rate of hydrolysis of the substrate. They are generally associated in nature with pyrophosphatases possessing a similar pH optimum. Adsorption, though providing preparations of low activity, permits a separation of the two types[39,40,41,42]; the same is true of thermal inactivation[43] and inactivation by maintenance at different pH levels peculiar to each enzyme.[19,42,44] The four types of phosphomonoesterases and their properties must be described before their constitution and specific sources are studied.

a. Different Types of Phosphomonoesterases. Modifying Factors

(1) *Phosphomonoesterase I or alkaline phosphatase.* This enzyme is very widely distributed in nature. It is found in all animal cells with the exception of the hyaline cartilage and the tunics of the vessels[45]; but only certain tissues contain an elevated level: zones of growth of the bones of young animals, intestinal mucosa, kidney cortex, lactating mammary gland, and, to a smaller extent, liver and brain. White blood cells and lymphoid tissues are rich in this enzyme; plant cells contain little (yeasts, bacteria) or, more commonly, none (green plants, higher fungi). Its purification has been accomplished by several authors[46,47,48,49]; the starting material of choice is the intestinal mucosa of the dog or hog, either the fresh or spray-dried tissue or the residue excreted in dog feces. This purification is based on fractionations with salts, acetone and alcohol and on the elimination of inactive proteins by adsorption or proteolysis. The most active preparations have a Q_P = 210,000 (37°, pH 9.2, $M/50$ β-glycerophosphate).[50*]

[39] K. Asakawa, *J. Biochem.* (*Japan*) **10**, 157 (1928); **11**, 143 (1929).

[40] S. Uzawa, *J. Biochem.* (*Japan*) **15**, 19 (1932).

[41] H. Kobayashi, *J. Biochem.* (*Japan*) **8**, 205 (1927); **10**, 147 (1928); **11**, 173 (1929).

[42] E. Bamann and W. Salzer, *Ber.* **70**, 1263 (1937).

[43] E. Jacobsen, *Biochem. Z.* **242**, 292 (1931).

[44] E. Bamann and M. Meisenheimer *in* Bamann-Myrbäck, Die Methoden der Fermentforschung. Thieme, Leipzig, 1941; Academic Press, New York, 1945, p. 1621.

[45] M. G. MacFarlane, L. M. B. Patterson, and R. Robison, *Biochem. J.* **28**, 720 (1934).

[46] H. Albers and E. Albers, *Z. physiol. Chem.* **232**, 165 (1935); **232**, 189 (1935).

[47] A. R. Armstrong, *Biochem. J.* **29**, 2020 (1935).

[48] R. Caputto and A. Marsal, *Rev. soc. argentina biol.* **17**, 1 (1941).

[49] G. Schmidt and S. J. Tannhauser, *J. Biol. Chem.* **149**, 369 (1943).

[50] Nguyen-van-Thoai, J. Roche, and M. Roger, *Biochim. et Biophys. Acta* **1**, 61 (1947).

* The crystallization of this enzyme has been reported,[51] but it is probable that the described product is an adsorbate of phosphatase on mineral salts (King, Abdul-Fadl, Nguyen-van-Thoai, and Roche, *Biochem. J.* **44**, 428, 1949).

[51] J. Roche, Nguyen-van-Thoai, and L. Sartori, *Compt. rend. soc. biol.* **138**, 47 (1944).

The principal properties of alkaline phosphatase are its pH optimum and its sensitivity toward certain modifying factors. The former exhibits small variations depending on the substrate being studied; it lies between 9.2 and 9.6 for the purified enzymes, but occasionally it is slightly below 9.0 in crude preparations. It can be determined rigorously only during a very short hydrolysis (30 minutes at most) owing to the rapid inactivation of the enzyme in alkaline solution. The action of modifying factors has given rise to an important series of investigations, some of which present contradictory results.

Without taking up here the significance of the differences observed between preparations of different origin, it is appropriate to discuss the meaning of other differences. It has been admitted that the lessened sensitivity toward modifying factors of the fraction of tissue enzymes which is not extractable by aqueous solvents (desmophosphatases) represents a profound difference from the soluble fraction (lyophosphatases).[52] As a matter of fact, this difference disappears upon prolonged dialysis of the first fraction owing to the elimination of natural modifying factors,[53] and there is no reason for assuming that the lyo- and desmophosphatases exhibit important structural differences. In addition, as we have already indicated, sensitivity towards modifying factors is regular only with purified enzymes, for various reasons. On the one hand, crude tissue extracts may contain some of them in amounts sufficient to saturate the enzyme. On the other hand, many modifying factors exhibit an optimal concentration beyond which their action is decreased and may even be reversed. Hence the addition of a modifying factor is without effect in the first instance and variable in the second, and this explains the numerous contradictions. Thus it has been possible to report that bone phosphatase is insensitive to the Mg^{++} ion which activates it strongly after purification.[54] In the same way, the Mn^{++} ion, regularly an activator of the purified enzymes regardless of their origin,[55] is considered inactive for some of them.[56] Finally, the interpretation of results obtained in this field on crude preparations is sometimes difficult owing to phenomena of anti-inhibition. Various substances may appear to activate the phosphatase because they block or eliminate a natural inhibitor. For example, alkaline cyanides at low concentrations (1×10^{-5} to 1×10^{-4} M) activate certain tissue extracts (liver, intestine) that are rich in inhibitory metals by forming complexes with the latter; on the other hand, they are inhibitors of purified alkaline phosphatase at the same levels and even of the crude enzyme at a higher concentration. All these facts explain that one should not consider an activation or inhibition to be significant unless it occurs regularly and reproducibly in all instances.

The principal activators of alkaline phosphatase are the divalent cations, among them Mg^{++}. Insufficiently purified preparations exhibit a progressive activation by Mg^{++} in proportion to its concentration up to an optimum level ($M/200$ in many instances); beyond that point, this effect is less pronounced.[57] Starting with a certain degree of purification, maximum activa-

[52] E. Bamann, E. Riedel, and K. Diederichs, *Z. physiol. Chem.* **230**, 175 (1934).
[53] Nguyen-van-Thoai and J. Raymond, *Compt. rend. soc. biol.* **139**, 814 (1945).
[54] M. Martland and R. Robison, *Biochem. J.* **23**, 237 (1929).
[55] E. Bamann, *Naturwissenschaften*, **28**, 142 (1940); E. Bamann and E. Heumüller, *Ibid.*, **28**, 539 (1940).
[56] D. Albers, *Z. physiol. Chem.* **265**, 129 (1940).
[57] H. Erdtmann, *Z. physiol. Chem.* **172**, 182 (1927); **177**, 211 (1928); **177**, 231 (1928).

tion is almost always observed at more and more elevated concentrations of Mg^{++} above a certain threshold. Amino acids (alanine and higher homologues among others) increase the effect of the cations to a significant degree,[58,59] according to the circumstances to be discussed below. Mg^{++} is not the only activating cation; it may be replaced by Mn^{++}, Zn^{++}, Co^{++} [55,60,61] but at optimum concentrations characteristic of each of them and much lower for some (M/10,000 to M /50,000 for zinc salts). Fe^{++}, Ni^{++}, and under certain conditions Ca^{++} are less effective activators. Above an optimum level, amino acids become inhibitory.[59,61]

The most important inhibitors are phosphate and arsenate ions[62] and various metal-complex-forming compounds. Among these, the cyanides have already been mentioned. Sulfhydryl compounds, particularly cysteine, are powerful inhibitors[63,64] and the same is true of hydrogen sulfide, while the fluorides and pyrophosphates inhibit to a much lesser degree and under special conditions.[50] Oxalates inhibit slightly but regularly.[65] The oxidation-reduction potential of the solution is without effect on phosphatase activity, except in strongly oxidizing (+400 mV) solutions where the enzyme is less active.[66] Finally, alkaline phosphatase is more stable in neutral or slightly acid solution than at alkaline pH levels and hydrolyzes β-glycerophosphate more rapidly than the α-isomer. Purification may modify the proportion of hydrolysis of the two isomers.

(2) *Phosphomonoesterase II or acid phosphatase.* This phosphomonoesterase is the most widely distributed in nature after the preceding one but its distribution is not the same. It is abundant in higher plants and fungi; the best sources are oleagenous seeds, basidiomycetes, and potato tubers. The human prostate is very rich in this enzyme and the spleen and liver contain appreciable quantities, while most of the tissues endowed with an intense alkaline phosphatase activity are practically devoid of acid phosphatase.[32,67,68,69] It is present in many bacteria.[70] The most suitable

[58] E. Hove, C. A. Elvehjem, and E. B. Hart, *J. Biol. Chem.* **134**, 425 (1940).

[59] J. Roche, Nguyen-van-Thoai, and M. Roger, *Bull. soc. chim. biol.* **26**, 1047 (1944).

[60] L. Massart and L. Vandendriessche, *Naturwissenschaften* **28**, 143 (1940).

[61] O. Bodansky, *J. Biol. Chem.* **165**, 605 (1946); **174**, 465 (1948); H. Aebi and I. Abelin, *Helv. Chim. Acta* **31**, 1943 (1948).

[62] J. Roche and Nguyen-van-Thoai, *Bull. soc. chim. biol.* **25**, 1365 (1943).

[63] M. Martland and R. Robison, *Biochem. J.* **21**, 665 (1927).

[64] E. Waldschmidt-Leitz, A. Scharikova, and A. Schäffner, *Z. physiol. Chem.* **214**, 75 (1933); S. Edlbacher and W. Kutscher, *Ibid.* **207**, 1 (1932).

[65] S. Belfanti, A. Contardi, and A. Ercoli, *Biochem. J.* **29**, 834 (1935); *Ibid.* **29**, 1491 (1935).

[66] I. W. Sizer, *J. Biol. Chem.* **145**, 405 (1942); J. Roche, Nguyen-van-Thoai, and E. Danzas, *Bull. soc. chim. biol.* **26**, 1143 (1944).

[67] F. Cedrangolo and A. Ruffo, *Arch. sci. biol. Italy* **24**, 59 (1938).

[68] D. R. Davies, *Biochem. J.* **28**, 529 (1934).

[69] Nguyen-van-Thoai, *Bull. soc. chim. biol.* **23**, 1183 (1941).

[70] L. B. Pett and A. M. Wynne, *Biochem. J.* **27**, 1660 (1933); **28**, 365 (1934); **32**, 563 (1938); M. Paget and M. Vittu, *Compt. rend. soc. biol.* **138**, 415 (1944).

starting materials for its preparation are the human prostate, the liver of mammals, basidiomycetes, sweet almonds, and potatoes. Purification of extracts of plant material is less satisfactory[70,71,72] than that of extracts of human prostate[73] when fractionations by electrodialysis, adsorption, and precipitation by neutral salts are employed. However, since the levels of the enzyme are low in mammalian prostates with the exception of that of man and the higher apes,[73] it is desirable that this phosphatase be obtainable from plant material in order to proceed easily with investigations on it. Its isolation from kidney has been achieved but has no more than theoretical interest.[74]

The pH optimum of this enzyme varies from 5.0 to 6.0 depending on its origin; it is generally near 5.2–5.4.[33,69] The most characteristic modifying factors are inhibitors. Besides phosphate and arsenate ions, which are effective against all phosphoesterases, certain ions inhibit acid phosphatase quite regularly, even at low concentrations, while they are ineffective or much less active against the alkaline enzyme. F^- is strongly inhibitory at a concentration of $M/1000$-$M/5000$[33,66,75] and its action in this respect is characteristic since it indicates a much greater sensitivity of this enzyme towards this ion than that of the alkaline phosphomonoesterase, which is only slightly inhibited by F^- after purification. Molybdate ion likewise is an inhibitor of enzyme II but not of enzyme I.[76] Conversely, Mg^{++} and the divalent cations, sulfhydryl compounds, amino acids, and alkaline cyanides have no effect on acid phosphatase.[19,33] Oxalates are generally strong inhibitors.[65] Phosphomonoesterase II of animal origin regularly hydrolyzes β- more than α-glycerophosphate and the same is true of most of the preparations of plant origin (fungi, almonds, mustard). However, depending on the conditions of measurement (pH and substrate concentration), the affinity for the two isomers and their rate of hydrolysis may be reversed.[33] It is clear from this that the problem of the possible existence of an α- and a β-glycerophosphatase is hard to attack.

(3) *Phosphomonoesterase III.* This enzyme, less studied than the preceding ones, is present in relatively slight amounts in many animal tissues, among them liver and spleen[24,39,42,68,77,78] where it is very unstable since it is very rapidly inactivated spontaneously in neutral solution. For this

[71] E. Pfankuch, *Z. physiol. Chem.* **241**, 34 (1936).
[72] B. Helferich and H. Stetter, *Ann.* **558**, 234 (1947); S. Bouchilloux, Y. Derrien, J. Roche, and M. Roger, *Bull. soc. chim. biol.* **30**, 417 (1948).
[73] W. Kutscher, *Z. physiol. Chem.* **235**, 62 (1935); W. Kutscher and J. Pany, *Ibid.* **255**, 169 (1938).
[74] J. Courtois and P. Denis, *Enzymologia* **5**, 288 (1938); **6**, 325 (1939); W. Kutscher and H. Wüst, *Biochem. Z.* **310**, 292 (1941).
[75] G. E. Perlmann and R. M. Ferry, *J. Biol. Chem.* **142**, 513 (1942).
[76] J. Courtois and M. Bossard, *Bull. soc. chim. biol.* **27**, 464 (1944).
[77] K. Inouye, *J. Biochem.* (*Japan*) **10**, 133 (1928).
[78] C. Neuberg and J. Wagner, *Biochem. Z.* **171**, 485 (1926).

reason, correct curves of the activity of extracts of these tissues in relation to pH can be obtained only in the presence of very fresh extracts. Phosphatase III is present in molds (takadiastase), higher yeasts, and rice bran; until the present time, only preparations derived from a fractionation of takadiastase have been purified to a high level. Its pH optimum lies between 3.4 and 4.2, depending on its origin. Activation and inhibition data are still not very precise, in the sense that they differ greatly in preparations derived from animal or plant tissues.[79] Magnesium salts inhibit the enzyme of basidiomycetes much more than that of takadiastase or liver extracts. Similarly, inhibition by fluorides is erratic, as is activation by Ca^{++}.[79] A systematic investigation of this enzyme in a suitably purified state is required in order to be able to interpret with assurance the many observed facts. Phosphatases II and III of animal tissues are very easily distinguished but this is not true with plant tissues, where the two enzymes generally have very similar pH optima.

(4) *Phosphomonoesterase IV*. Only erythrocytes, bottom yeasts, and perhaps certain propionic bacteria contain this enzyme.[26,80,81,82] Its pH optimum lies between 5.2 and 6.2 for the reasons discussed above; it is even higher in certain yeasts. In contrast to phosphatase II, which has a similar pH optimum, it is strongly activated by Mg^{++} and Mn^{++}; the differences in sensitivity of animal and plant preparations towards these activators are attenuated after adsorption on and elution from kaolin. Sulfhydryl compounds inhibit the yeast enzyme but activate the nonpurified erythrocyte enzyme; this also holds true for ascorbic acid.[25,82,83] Finally, phosphatase IV is notably more active on α- than on β-glycerophosphate, so much so that some investigators have thought to use the enzyme of bottom yeasts for the preparation of a specific α-glycerophosphatase.[13] A study of the purified enzyme should be undertaken.

b. Constitution. Role of Metals

Only phosphomonoesterases I and II can be examined along these lines; the absence of a test which would make it possible to determine the purity of the most active preparations further complicates research in this field.

To begin with, the question has been raised whether the various phosphomonoesterases have a similar constitution, and this question remains open. Dialysis at different pH levels of enzymes I, II, and III permits a separation of proteins from the diffusible products; each fraction is inactive but a mixture of the two has phosphatase activity.[84,85,86,87,88] The pH opti-

[79] Nguyen-van-Thoai, *Bull. soc. chim. biol.* **24**, 1077 (1942).
[80] A. Schäffner and E. Bauer, *Z. physiol. Chem.* **225**, 245 (1934); **232**, 64 (1935).
[81] J. Roche, *Biochem. J.* **25**, 1724 (1931); *Bull. soc. chim. biol.* **13**, 841 (1931).
[82] B. Naganna and V. K. Narayana Menon, *J. Biol. Chem.* **174**, 501 (1948).
[83] E. J. King, E. J. Wood and G. E. Delory, *Biochem. J.* **39**, xxiv (1945).
[84] E. Bauer, A. Schäffner and F. Krumey, *Z. physiol. Chem.* **237**, 191 (1935).

mum of this regenerated enzymatic action is identical with that of the enzyme from which the protein is derived, regardless of the origin of the dialyzate. It has been possible to reconstitute phosphomonoesterase I (kidney) dissociated by dialysis at pH 5.5 by mixing with its inactive protein the inactive dialysate of phosphomonoesterase II (yeast) dissociated at pH 6.5. In the same way, phosphatases II and III have been regenerated from their apoenzyme and the dialyzate of enzymes I, II, or III. The ash of these dialyzates is inactive. These results have been interpreted to indicate removal of a coenzyme by dialysis; each phosphatase is assumed to contain a particular protein apoenzyme combined to a coenzyme which is the same in all instances.[89,90] Actually, this interpretation is entirely hypothetical since it is not possible to determine to what extent the observations which have been made indicate a specific coenzyme effect or an activation caused by a modifying agent. No information on the nature of the coenzyme is available; the enzyme does not exhibit a characteristic ultraviolet spectrum that would distinguish it from nonenzymatic proteins[87,88,91] and alkaline phosphatase can be completely reactivated by the addition to the protein of various cations in association with amino acids.[50,59] It is evident that the problem of the existence of a coenzyme common to all phosphatases has not yet been resolved.

Investigations of several types have led to the concept that alkaline phosphatase is an enzyme with a dissociable metal and that the natural metallic constituent can be replaced by various divalent cations. Numerous complex-forming compounds (cyanides, sodium azide, sodium diethyldithiocarbamate, sulfhydryl compounds) inhibit the enzyme. The activation by divalent metals, magnesium among others, at suitable concentrations may be interpreted as a saturation of the enzyme by these activators. Likewise, the inhibition by phosphates, fluorides, pyrophosphates may indicate the formation of combinations of these anions with a metal present in the enzyme, as in the case of enolase. Experiments on reactivation of alkaline phosphatase after the enzyme has been totally inactivated by dialysis against complex-forming compounds or double-distilled water are significant in this respect.[50] The theory of Cloetens,[91] according to which two metals are required to reactivate the enzyme, is no longer tenable. This theory is based mainly on attempts at reactivation by zinc and magnesium after dialysis against alkaline cyanides. The results would seem to be re-

[86] D. Albers, *Z. physiol. Chem.* **261**, 43 (1939); **261**, 269 (1939).
[87] J. Roche, Nguyen-van-Thoai and O. Michel-Lila, *Compt. rend.* **218**, 249 (1943).
[88] H. von Euler and A. Fono, *Arkiv Kemi Mineral. Geol.* **25A**, No. 15, 1 (1947); B. Ek, H. von Euler, and L. Hahn, *Ibid.* **26A**, No. 9 (1948) and *Arkiv Kemi* **1**, 117 (1949).
[89] A. Ruffo, *Boll. soc. ital. biol. sper.* **19**, 9 (1944).
[90] H. Albers, E. Beyer, A. Bohnenkamp, and G. Müller, *Ber.* **71**, 1913 (1938).
[91] D. Albers, *Biochem. Z.* **306**, 143 (1940).
[92] R. Cloetens, *Biochem. Z.* **307**, 352 (1941); **308**, 37 (1941); **310**, 42 (1941).

lated to an incomplete elimination of the cyanides and their blocking (anti-inhibition) by metals such as zinc or mercury for which the CN radical exhibits a strong affinity. Alkaline phosphatase completely inactivated by a dialysis against water for several weeks is partially reactivated by incubation for 30 minutes at 37° and at pH 9.2 in the presence of various cations (Mg^{++}, Mn^{++}, Zn^{++}, Co^{++}, Fe^{++}) at optimum concentration. A much more pronounced reactivation is achieved if the solution contains, in addition, a suitable concentration of an amino acid (alanine); it is then possible to obtain enzymatic activities of much greater magnitude than that of the initial preparation.[50] Under these conditions, reactivation does not require two metals; it reaches a maximum in the presence of Mg^{++} (intestine).

Two important facts must be stressed and commented upon. On the one hand, the divalent cations mentioned above are interchangeable as activators or reactivators, but each one exhibits its optimum effectiveness at a different concentration (1×10^{-2} *M* for magnesium salts, 5×10^{-4} approximately for zinc salts).[50] On the other hand, the coactivating effect of amino acids is very pronounced and regular in the reactivation of the enzyme after prolonged dialysis. Finally, the analysis of purified preparations of alkaline phosphatase has demonstrated that it contains magnesium together with minimal amounts of zinc, a significant portion of which is dialyzable.[92] Similar data have been obtained with yeast carboxylase, the constitution of which is better defined. Reasoning by analogy, it may be assumed that alkaline phosphatase is a protein with an activity related to a metal complex (probably of magnesium in general) of certain primary amine groups of the apoenzyme and to a dissociable peptide. The observations during reactivation show that neither the metal nor the peptide are strictly specific. The participation of the primary amine groups of the apoenzyme in enzymatic activity is supported by the fact that the latter is destroyed if these groups are blocked.[93,94] The existence of a metal other than magnesium in the alkaline phosphatases is discussed below.

It is still difficult to define the nature of the acid phosphomonoesterases precisely. Enzyme II is dissociable by dialysis only under very special circumstances; divalent metals do not activate it and complex-forming compounds are without effect. However, fluorides inhibit the enzyme reversibly in all instances and this fact could indicate the existence of magnesium in its molecule;[95] but this metal has not been found in the ash of purified preparations. Primary amine groups of the apoenzyme are indispensable to the activity of phosphatase II.[94,96]

c. Tissue Specificity and Constitution

In view of the unequal sensitivity of phosphatases, depending on their origin, toward modifying factors, the assumption has been made that each

[92] S. Bouchilloux, M. Roger, et J. Roche, *Compt. rend. soc. biol.* **142**, 1144 (1948).
[93] B. S. Gould, *J. Biol. Chem.* **156**, 365 (1944).
[94] J. Roche and M. Abul-Fadl, *Bull. soc. chim. biol.* **30**, 427 (1948).
[95] L. Massart and R. Dufait, *Z. physiol. Chem.* **272**, 157 (1942).
[96] V. Baccari and G. Auricchio, *Boll. soc. ital. biol. sper.* **22**, 1 (1946).

of the various tissues contains a particular enzyme. Bile acids do not inhibit the alkaline phosphatase of intestine but have a slight effect on that of other tissues.[97,98] Inhibition by CN^- takes place under circumstances which have been thought to indicate the existence of two alkaline phosphatases in animal tissues.[99] In addition, the inhibitory effects of the methyl esters of the two tautomeric forms of hydrogen cyanide are not the same on the purified alkaline phosphatases of various tissues.[100] Finally, the fact that magnesium, manganese, cobalt and zinc are preferential activators of the enzyme in particular tissue extracts has suggested the hypothesis that the latter contain magnesium, manganese, cobalt, and zinc phosphatases. There would then exist a tissue specificity of the alkaline enzyme based on differences in the metal constituent.[91,95,100–104] However, the significant differences in the value of the substrate affinity constant K_M of this enzyme depending on the origin of the preparations indicates that the apoenzymes are not identical,[36] while the interchangeability of the reactivating metals suggests that the tissue specificity is probably not related to the metal constituent.

The concept of a difference in the nature of phosphomonoesterase II depending on origin was connected not with a metal but rather with a difference of the apoenzymes. These are in effect unequally sensitive towards inactivation by alcohol and formol.[105,104] The acid phosphatase of the prostate is much more easily inactivated by alcohol and less so by formaldehyde than that of other animal tissues.

It is not possible to accept without reservation the conclusions formulated in this field by most authors, since the sensitivity of the enzymes towards activators and inhibitors cannot be considered to be constant in such insufficiently purified media as tissue extracts or biological fluids. Nevertheless, a tissue specificity of the phosphatases is very probable. It is undoubtedly based upon differences in the constitution of the protein apoenzymes, which explain the differences in the behavior of the enzymes in regard to various inactivating factors (pH, temperature, alcohol). We have discussed how hypothetical the differences in the metals present in the alkaline phosphatases of varying origin are. The question can be resolved only by a study of highly purified enzymes since crude preparations always contain small amounts of different metals, only a fraction of which is related

[97] O. Bodansky, *J. Biol. Chem.* **118**, 341 (1937).
[98] J. Roche, S. de Laromiguière, and A. Laurens, *Bull. soc. chim. biol.* **25**, 1157 (1943).
[99] R. Cloetens, *Enzymologia* **6**, 46 (1939).
[100] J. Monche, J. Jimenes-Vargas, and A. Sols, *Rev. espan. fisiol.* **3**, 289 (1947).
[101] H. D. Kay, *J. Biol. Chem.* **89**, 235 (1930).
[102] L. Massart and L. Vandendriessche, *Enzymologia* **11**, 261 (1944).
[103] L. Massart, R. Dufait, and L. Vandendriessche, *Naturwissenschaften* **27**, 806 (1939); **28**, 143 (1940).
[104] R. Cloetens, *Arch. intern. Pharmacodynamie* **68**, 419 (1942); **69**, 389 (1944).
[105] F. K. Herbert, *Biochem. J.* **38**, xxiii (1944); *Quart. J. Med.* **15**, 221 (1946).
[106] M. Abul-Fadl and E. J. King, *J. Clin. Pathol.* **1**, 80 (1948).

to their activity even in the case of magnesium in alkaline phosphatase.[92,102] For these various reasons, we have not deemed it proper to discuss separately the investigations of the enzymes derived from specific tissues and whose absolute characteristics are not known. Likewise, the study of animal or plant phosphatases deserved to be distinguished while it was purely descriptive, but there is no longer any profit in maintaining this distinction. It is time, on the contrary, to consider the biochemistry of the various types of phosphatases under a more general plan.

2. Phosphodiesterases

The separate entity of this type of phosphatases was based initially on the variability of the ratios of hydrolysis of the mono- and diesters, on the differences between their pH optimum and that of the phosphomonoesterases, and, finally, on the separation by adsorption on alumina of products active on monoesters and not on diesters.[39,40,107] These criteria of specificity are not absolute and the existence of diesterases was definitely established when the venom of a snake, *Trimesurus flavoviridis* (a Japanese poison called Habu), was found to contain an enzyme that liberated a molecule of phenol and no phosphate ion from diphenylphosphate;[40,108,109] this fact was confirmed by later studies of the venoms of the rattlesnake, of the water moccasin (*Ancistrodon piscivorus*, *A. mokassin*) and of a Colubrida (*Bungarus fasciatus*).[110]

The phosphodiesterases are generally associated with the phosphomonoesterases in the cells and body fluids.[39,108,109,111] Two isodynamic enzymes of this group have been described and characterized by their pH optimum (pH = 8.5–9.0 in venoms and serum, pH = 5.5 in rice bran, takadiastase, liver), and it is possible that a third enzyme active at a more acid pH also exists.[109] Certain organs, such as the prostate[112] and the citrus fruit,[113] are devoid of diesterase activity.

The problem of the specificity of cholinephosphatase and of the polynucleotidases is related to the question of the nature of the diesterases. According to Contardi and Ercoli,[114] the glycerophosphoric ester of choline which arises by the action of lecithinase B on lecithin is subject to the action of a cholinephosphatase which liberates glycerophosphoric acid and choline. This enzyme, which has a pH optimum of 4.0, is inhibited by physostigmine as is cholinesterase; its action is apparent in extracts of kidney, intestine,

[107] R. Hotta, *J. Biochem.* (*Japan*) **20**, 343 (1934).
[108] U. Udagawa, *J. Biochem.* (*Japan*) **22**, 323 (1935).
[109] H. Takahashi, *J. Biochem.* (*Japan*) **16**, 447 (1932); **16**, 463 (1932).
[110] J. M. Gulland and E. M. Jackson, *Biochem. J.* **32**, 590 (1938); *J. Chem. Soc. London* **1938**, 1492; **1939**, 1842.
[111] J. Roche and M. Latreille, *Enzymologia* **3**, 75 (1937).
[112] J. Courtois and P. Biget, *Bull. soc. chim. biol.* **25**, 103 (1943).
[113] B. Axelrod, *J. Biol. Chem.* **167**, 57 (1947).
[114] A. Contardi and A. Ercoli, *Biochem. Z.* **261**, 275 (1933).

and pancreas. It is different from the phosphodiesterase of rice bran or *Aspergillus oryzae*[109,115] which gives rise to glycerol and phosphorylcholine from the same substrate; phosphorylcholine is hydrolyzed by the phosphomonoesterases.[116,117] At the present state of our knowledge, further investigations are necessary before the existence of a specific cholinephosphatase can be admitted without reservations.

The existence of polynucleotidases, diesterases hydrolyzing only the phosphoric ester linkages that hold the nucleotides together, has been considered;[118,119] it is to be discussed in another chapter together with the study of ribonuclease and thymonucleodepolymerase.

3. Pyrophosphatases

The separate entity of the pyrophosphatases rests upon their separation from the phosphoesterases which almost always accompany them. This operation can be accomplished by the selective inactivation of the latter.[22,120–122] Ammonia extracts of hog liver maintained for 24 hours at pH 9.0 and at 37° retain their pyrophosphatase activity and lose their esterase activity. Specific adsorption and elution have yielded the same results. Adsorption of the phosphomonoesterase of extracts of Basidiomycetes on alumina leaves the accompanying acid pyrophosphatase in solution, while the reverse situation prevails in hemolyzates. Finally, dialysis of aqueous extracts of Basidiomycetes and of bottom yeasts of pH 9.0 and of acetic acid extracts of hog liver at pH 2.5 inactivates the phosphoesterases more rapidly than the pyrophosphatases. Bailey and Webb[28] prepared from baker's yeast a very active pyrophosphatase devoid of phosphoesterase activity by adsorption of autolyzates on tricalcium phosphate gel, elution in the presence of ammonium sulfate, and fractional precipitation with this salt. Their preparation is undoubtedly the purest obtained to date.

As in the case of the phosphoesterases, there exist isodynamic pyrophosphatases characterized above all by their pH optimum and also by modifying factors peculiar to each. Three enzymes are distinguished. One of them (type 1) abounds in animal tissues,[10,81,120,122] among others the intestine, kidney, and white blood cells, and its pH optimum lies between 7.2 and 8.2; it is present also in yeasts where its pH optimum is 7.0–7.2. Very sensitive to slight acidity, it is destroyed when kept at pH 4.5 and at 30° for 60 minutes. It is strongly activated by Mg^{++} [27] and by various

[115] A. Contardi and C. Ravazzoni, *Rend. reale ist. lombardo sci.* **67**, 503 (1934).
[116] J. Roche and S. Bouchilloux, *Compt. rend. soc. biol.* **141**, 1068 (1947); **141**, 1249 (1947).
[117] V. Baccari, *Arch. sci. biol. Italy* **32**, No. 6, 1 (1948).
[118] R. J. Dubos and R. H. S. Thompson, *J. Biol. Chem.* **124**, 501 (1928).
[119] S. J. Tannhauser, *Z. physiol. Chem.* **91**, 329 (1914); **100**, 121 (1917).
[120] J. Roche and J. Baudoin, *Compt. rend. soc. biol.* **137**, 245 (1943).
[121] E. Bauer, *Z. physiol. Chem.* **239**, 195 (1936).
[122] E. Bamann and H. Gall, *Biochem. Z.* **293**, 1 (1937).

other divalent cations (Zn^{++}, Mn^{++}, Co^{++}, Fe^{++}).[59,62] The effect and interchangeability of these ions is particularly apparent after prolonged dialysis against double-distilled water, or even better against solutions of complex-forming compounds (α,α'-dipyridyl, diethyldithiocarbamate);[123] however, the activation by metals is never as pronounced as in the case of the phosphomonoesterases and the presence of amino acids has no coactivating effect. These are differences in intensity of activation or inhibition of the enzyme by different modifying factors depending on the origin of the pyrophosphatase preparations, so that the question of tissue specificity of this enzyme arises. This question can be resolved only by a study of purified enzymes, as was discussed for the phosphomonoesterases.

Another pyrophosphatase (type II) is distinguished from the preceding one by a pH optimum situated between 5.0 and 5.5, by its lack of sensitivity towards divalent cations, and by its very strong inhibition by F^-.[40,121,124] It is found in plants (fungi, seeds) and in almost all animal tissues containing phosphomonoesterase I, among others the kidney and the liver; the latter organ is a very rich source.[120] This enzyme is frequently inhibited in tissue extracts by metals, so that the addition of complex-forming compounds (sulfhydryl compounds, ascorbic acid) then activates it irregularly by an anti-inhibitory effect.[120] The pair cysteine-Fe^{++} is a direct activator of the enzyme of liver.[125] Type III of the pyrophosphatases, with a pH optimum of 3.2–4.0, exhibits a distribution parallel to that of phosphomonoesterase III. Like the enzyme discussed above, it has not been prepared in a high stage of purity and its modifying factors are still ill-defined.[22,24,124,126,127] Takadiastase, yeasts, and liver are the best sources.

These few facts permit us to state that the study of the pyrophosphatases is still relatively little advanced and that, in addition, these enzymes exhibit analogies with the phosphomonoesterases. Moreover, they, like the latter, may be partially inactivated by dialysis and reactivated by means of their dialyzate,[85] but not of the ash of the dialyzate. No precise data have been obtained on their constitution; pyrophosphatase I is probably an enzyme with a dissociable metal and containing magnesium. It has been assumed that either the phosphomonoesterases and pyrophosphatases of the same pH optimum are made up of the same apoenzyme associated with different coenzymes[124] or that their specificity is related to an active group particular to each in the same enzyme protein.

[123] R. Falconer, J. M. Gulland, G. I. Hobday, and E. M. Jackson, *J. Chem. Soc. London* **1939**, 907.

[124] P. Fleury and J. Courtois, *Enzymologia* **1**, 377 (1937); **5**, 254 (1938).

[125] J. Roche, Nguyen-van-Thoai and O. Milhau, *Bull. soc. chim. biol.* **25**, 1217 (1943).

[126] A. Schäffner and F. Krumey, *Z. physiol. Chem.* **243**, 149 (1936).

[127] K. Kurata, *J. Biochem. (Japan)* **14**, 25 (1931).

4. Phosphoamidases

These enzymes are hydrolases that act on the amide linkage which unites orthophosphoric acid to a primary amine group, as in phosphocreatine and phosphoarginine. They have been separated from the phosphomonoesterases of a similar pH optimum by selective adsorption of the latter on kaolin at pH 4.5.[128] Two isodynamic phosphoamidases have been characterized. One, present in kidney[129] and certain snake venoms,[120] has a pH optimum of 9.0; the other, in rice bran extracts, acts at a pH optimum of 5.2.[130] It is probable that a third phosphoamidase, in kidney, acts at a pH optimum of 3.0. The study of these enzymes is very incomplete and should be taken up again, particularly with the aid of artificial substrates having greater stability than the natural phosphoamides (phosphoric anilides).

5. Phosphoacylases

Animal tissues contain an enzyme, particularly abundant in muscle and liver, which hydrolyzes acetylphosphate and its higher homologues (propionyl-, butyryl-, and succinylphosphate). It is generally called acetylphosphatase.[131] It is easily extracted from tissues by water and traces of it are contained in the culture media of various bacteria. It is destroyed by pepsin but exhibits exceptional resistance to certain denaturing agents. It remains unaltered in trichloracetic acid solution and after prolonged boiling at pH 3–4; this thermostability is analogous to that of ribonuclease. It is constituted by a basic protein, and this fact has been called upon to explain its inhibition by many anions. It is inhibited strongly by phosphates, pyrophosphates, hexosephosphates, nucleic acids, and hyaluronic acid, less strongly by sulfates, citrates, and oxalates, and only slightly by fluorides. It is certain that the phosphoacylase extracted from muscle is different from the phosphoesterases; these enzymes have been separated and their modifying factors are different.[113,131,131a] Moreover, their distribution in the tissues is not the same.[132]

Since the substrates of acetylphosphatase contain a high-energy phosphate bond, the study of the metabolic role of this enzyme, inaugurated by Lipmann, has stimulated many investigations. It is not yet possible to state whether or not isodynamic or strictly specific phosphoacylases exist.

6. Phosphatases Specific for a Single Substrate or a Small Number of Structurally Related Substrates

The uncertainty which reigns over any attempt to individualize the numerous apparently specific phosphatase effects limits this paragraph to the discussion of

[128] M. Ichihara, *J. Biochem.* (*Japan*) **18**, 87 (1933).
[129] E. Waldschmidt-Leitz and F. Köhler, *Biochem. Z.* **258**, 360 (1933).
[130] H. Bredereck and E. Geyer, *Z. physiol. Chem.* **254**, 223 (1938).
[131] F. Lipmann, *Advances in Enzymol.* **6**, 231 (1946).
[131a] J. F. Danielli, *J. Exptl. Biol.* **22**, 110 (1946).
[132] S. Shapiro and E. Wertheimer, *Nature*, **156**, 690 (1945).

those enzymes whose autonomy is certain beyond doubt. In this regard, all definite conclusions in the field of the phosphomonoesterases are difficult to formulate, although it is possible to obtain preparations which act preferentially, but not exclusively, on one substrate or a limited group of them, such as fructose-1,6-diphosphate, or the 5-nucleotides. We shall confine ourselves to summarizing here, as an example of the difficulties which confront research in this field, some data obtained on phytase. The study of the enzymes which hydrolyze the pyro- and polyphosphate derivatives is more advanced; this subject, and the study of metaphosphatase, will be discussed below.

a. Phytase

Phytic acid, the hexaphosphate ester of meso-inositol, is one of the important organic compounds of plants, particularly of cereals, where it is found as the calcium and magnesium salt associated with proteins. It is hydrolyzed by enzymes whose activity provided the first occasion for demonstrating the existence of a phosphatase.[2] Phytase action is evident in many seeds, rye and wheat being richest in the enzyme; it occurs also in molds.[133–136] Phytase is less widely distributed and less active than the phosphoesterases in animal tissues; among these, the nucleated erythrocytes of birds and of fish and the intestinal mucosa of the rat constitute the best sources.[137,138] There are several isodynamic phytase activities. Of the two that have been definitely identified, one, with a pH optimum of 7.8, is activated by Mg^{++} and occurs in rat intestine;[137] the other, present in seeds of cereals, mustard, and soybeans is strongly inhibited by fluorides and molybdates at a pH optimum of 5.5, and it is not activated by Mg^{++}.[135]

The specificity of the phytases is as yet ill-defined. In effect, all enzymatic preparations that hydrolyze phytic acid also split numerous other esters, while other preparations contain phosphomonoesterases of very wide specificity but without action on phytic acid. Finally, the ratios of the rates of hydrolysis of the mono-, di-, tri-, tetra-, penta-, and hexaphosphate derivatives of inositol vary from one preparation to the next. It is, after all, rather improbable that there are phosphatases specific for one or several phosphoric esters of inositol. According to Fleury and Courtois,[136] phytase activity belongs to certain phosphomonoesterases that have a very wide specificity extending to phytic acid, while this is not true of phosphomonoesterases I, II, III, and IV, which correspond to the classical types.

b. Phosphatases Specific for the Pyrophosphate Type of Linkage

It has been demonstrated that the hydrolysis of mineral pyrophosphates and that of their esters, liberating orthophosphoric esters, are accomplished

[133] R. H. A. Plimmer, *Biochem. J.* **7**, 43 (1913).
[134] R. A. MacCance and E. M. Widowson, *Nature* **153**, 650 (1944).
[135] J. Courtois, *Bull. soc. chim. biol.* **27**, 411 (1945).
[136] P. Fleury and J. Courtois, *Helv. Chim. Acta* **29**, 1297 (1946).
[137] S. Rapoport, E. Leva, and G. M. Guest, *J. Biol. Chem.* **139**, 621 (1941).
[138] V. N. Patwardhan, *Biochem. J.* **31**, 560 (1937).

by the same enzymes[24,110,126] except in a certain number of particular instances. The condensation of orthophosphoric acid to the pyrophosphate radical or to its esters causes a modification of manifest specificity. The mineral tri- and tetraphosphates are split by a polyphosphatase of pH optimum 6.3–7.7 which is present in molds, yeasts, and various animal tissues.[139] This enzyme is inactivated without alteration of pyrophosphatase, by treatment of the preparations with ethyl acetate. Adenosinetriphosphoric acid is the substrate the hydrolysis of which exhibits the greatest peculiarity in this field, since it is hydrolyzed by two specific enzymes with different modes of action: adenylpyrophosphatase[140] and apyrase.[141,142]

The former removes a single phosphate group from the substrate at the expense of which adenosinediphosphoric acid is formed in addition.[143,144,145] This fact demarcates the field of the specificity of adenylpyrophosphatase, which acts solely on adenosinetriphosphoric acid and its isologue inosinic acid. Apyrase hydrolyzes adenosinetriphosphoric acid with the liberation of adenylic acid and two phosphate radicals. The two enzymes are present in muscle and in potatoes. The specificity of the former may be altered by the coexistence of other enzymes or of myosin on which adenylpyrophosphatase is fixed in muscle. The purified enzyme is inactive on pyrophosphates and on adenosinediphosphate, while the latter is hydrolyzed by the enzyme in certain myosin preparations that contain a soluble protein constituent.[146,147]

Adenylpyrophosphatase is endowed with properties similar to those of the pyrophosphatases.[148,149] Depending on the degree of purity and the modifying factors present, the enzymatic activity has one or two pH optima. That of the enzyme adsorbed on crystallized myosin is 9.4; that of actomyosin in the presence of veronal buffer is 7.6; that of the enzyme completely free of myosin is 7.5 in the presence of histidine. In media containing Ca^{++} ions, myosin with adenylpyrophosphatase activity has two pH optima at 9.0 and 6.3. Likewise, the sensitivity of the enzyme towards modifying factors is a function of its degree of purity and of its union with myosin. In the presence of the latter, it is activated by Ca^{++} and inhibited by Mg^{++}, while the reverse situation prevails after removal of the muscle

[139] C. Neuberg and H. A. Fischer, *Enzymologia*, **2**, 191 (1937); **2**, 241 (1937); **2**, 360 (1937).
[140] K. Lohmann, *Biochem. Z.* **282**, 109 (1935).
[141] V. A. Engelhardt, *Advances in Enzymol.* **6**, 147 (1946).
[142] P. S. Krishnan, *Arch. Biochem.* **20**, 261 (1949); **20**, 272 (1949).
[143] O. Meyerhof, *J. Biol. Chem.* **157**, 105 (1945).
[144] H. M. Kalckar, *J. Biol. Chem.* **153**, 355 (1944).
[145] A. Szent-György, Chemistry of Muscle Contraction. Academic Press, New York, 1946.
[146] K. Bailey, *Advances in Protein Chem.* **1**, 289 (1944).
[147] I. Banga and G. Josepovits, *Hung. Acta Physiol. Biol.* **1**, 67 (1947).
[148] K. Bailey, *Biochem. J.* **36**, 121 (1942).
[149] V. A. Engelhardt and M. N. Lyubimova, *Biokhimya* **7**, 205 (1942).

protein, a procedure which renders the phosphatase very labile. Amino acids activate this enzyme, but this finding may simply indicate a blocking of inhibitors. The study of adenylpyrophosphatase is discussed in detail with that of apyrase in another chapter.

c. Metaphosphatase

The hydration of metaphosphates to orthophosphates is accomplished by an enzyme of pH optimum 7.0–8.0 which is present first of all in yeasts[150] and also in liver, kidney, and *Aspergillus niger*.[139,150,151] This enzyme is different from pyrophosphatase I; in the presence of the latter it is specifically inactivated by heating for 10 minutes in a neutral solution.

IV. Physiological Chemistry of the Phosphatases

1. Distribution in the Tissues and Histochemistry of the Phosphatases

The principal sources of the phosphatases and their localization have been indicated above, beginning with data of a chemical nature. The determination of phosphatases in the tissues cannot be rigorous since the extraction of the enzymes is always incomplete when carried out on maceration juices and, in addition, the diffusion of the substrate is equally incomplete when it is done on tissue fragments. The chemical study of the distribution of the phosphatases has, therefore, been of a preliminary character in certain respects; nevertheless, it has made it possible to locate the tissues and organs which are exceptionally rich in phosphatases and, for that very reason, it has posed the problem of the physiological roles of the enzymes. The only data available on the distribution of phosphatases other than the phosphomonoesterases and adenylpyrophosphatase at the present time are of a chemical nature. They have been supplemented, as regards alkaline (I) and acid (II) phosphomonoesterase, by a large number of data of a histochemical nature. In contrast to the former, these are qualitative, but they have had the merit of introducing the chemistry of the phosphatases in the fields of the cell and of tissue organization.

The histochemical characterization of the phosphatases is based on the demonstration of the formation of phosphates at the locus where the hydrolysis of an ester proceeds in the presence of ions capable of giving rise to insoluble phosphates under the selected pH conditions (tricalcium phosphate at pH = 9.0 and lead phosphate at pH = 5.5). It is evident that this principle could be applied to the histochemical detection of phosphatases other than the esterases, on the condition that other substrates be employed. It is desirable that a comparative histochemical study of the various types of phosphatases be undertaken. The principle of the histochemical methods confers a very great sensitivity upon them; they can well distinguish strong from slight enzymatic activities, but with a much lower precision than that of an assay. It follows that the quantitative interpretation of data obtained with these

[150] T. Kitasato, *Biochem. Z.* **197**, 257 (1928); **201**, 206 (1928).
[151] T. Mann, *Biochem. J.* **38**, 339 (1944).

methods is uncertain, especially in the case of slight activities which are sometimes overestimated. Moreover, histochemistry does not make it possible to define the mechanism of the reaction which liberates the phosphates, so that it may cause the activity of a complex enzymatic system to be attributed to a phosphatase when the activity is slight. Also, the interpretation of the liberation of minimal amounts of phosphates from tissue slices immersed in a solution of a phosphate ester should often be held in abeyance while that of abundant quantities of phosphates entails no ambiguity.

The most commonly employed method for the histochemical detection of the phosphatases[152,153,154] is based upon the conversion of the freed phosphates into cobalt phosphate followed by the conversion of the latter into cobalt sulfide; this black precipitate saturates the region endowed with phosphatase activity. The enrichment of the region in phosphates, recovered as precipitated calcium and lead salts, by a preliminary hydrolysis of the substrate suffices to make it appear more or less deeply black. Demonstrative results have also been obtained by fixing as tricalcium phosphate the phosphate ions which are formed and then coloring the calcium deposit on the slice with acridine red,[155] or by precipitating an azo dye obtained from the organic radical of the substrate of enzyme action (barium β-naphthylphosphate).[156] Special methods have been worked out for the study of calcified tissues.[157,158] The results of histochemical studies of the phosphatases have been of importance for the understanding of special physiological processes, such as ossification; but they have also demonstrated the general role of these enzymes and called attention to their participation in biological phenomena in which they had not been considered.

Phosphatase (acid or alkaline) activity is generally very uniformly distributed in protoplasm, but the content of the nucleus varies in the different cellular types.[156,157,159–162] For example, the acidophil cells of the anterior lobe of the hypophysis are rich in these enzymes while the basophil cells are poor; this fact may be connected with the synthesis of protein hormones (lactogenic and growth hormones) by the former.[163] The alkaline phosphatase content of nuclei seems to be in correlation with the rate of turnover of the phosphorus of thymonucleic acid; moreover, a portion of the cytoplasmic phosphatases are included in the ribonucleic acid granules.[160,164,165]

Various physiological processes are related to the phosphatase activity of the cells. On the whole, all protein formation goes on a par with it. It has been possible to demonstrate its appearance and evolution in the course of cellular differentiation in the embryonic development of the

[152] G. Gomori, *Proc. Soc. Exptl. Biol. Med.* **42**, 23 (1939).
[153] H. Takamatsu, *Trans. Soc. Path. Japon.* **29**, 492 (1939).
[154] J. F. Danielli, *J. Exptl. Biol.* **22**, 110 (1946).
[155] G. Gomori, *Am. J. Path.* **19**, 197 (1943).
[156] M. L. Menten, J. Junge, and M. H. Green, *J. Biol. Chem.* **153**, 471 (1944).
[157] G. Bourne, *Quart. J. Exptl. Path.* **32**, 1 (1943).
[158] J. Lorch, *Quart. J. Microscop. Sci.* **88**, 159 (1947).
[159] J. Brachet, *Experientia* **2**, 143 (1946).
[160] J. Brachet and R. Jeener, *Biochimica et Biophysica Acta* **2**, 423 (1948).
[161] J. F. Danielli and D. G. Catcheside, *Nature* **156**, 234 (1945).
[162] G. Montalenti and M. de Nicola, *Experientia* **4**, 315 (1948).
[163] L. Abolin3, *Nature* **161**, 556 (1948).
[164] R. Jeener, *Biochimica et Biophysica Acta* **2**, 439 (1948).
[165] R. Jeener, *Experientia* **2**, 458 (1946).

chick[166,167] and of mammals,[168,169] in the repair of wounds and cutaneous burns[170] and of bone fractures,[171] in the formation of secretions of protein nature, such as silk,[172] and in cell proliferations stimulated by sex hormones.[173] In the chick embryo, acid and alkaline phosphatase are present from the fourth day of development on, in the mesoderm tissue, which later gives rise to the posterior membrane; the osteoblasts do not appear until the seventh to eighth day of development. The calcification of bone, which takes place later, is preceded briefly by a great increase in alkaline phosphatase activity. These facts speak for the existence of a relation between protein formation and the presence of phosphatases in the cells; this may be an indirect relation by way of nucleoproteins and nucleic acids or a direct participation in a synthetic reaction. The metabolism of cellular nucleic acids necessarily involves the action of several phosphatases; at least one aspect is demonstrated histochemically. A characteristic example is provided by the study of the evolution of the nucleoproteins that constitute Nissl's granules in nerve fibers.[174,175] Chromatolysis of these granules after section is accompanied by a great increase in the acid phosphatase activity at the level of these granulations; the same is true of their regeneration.

The participation of phosphatases in less general processes has been studied histochemically with success in the fields of ossification, kidney secretion, and digestive resorption, as will be seen below.

2. Bone Phosphatase and Calcification

Robison,[1] studying the effect of sections of bone of growing animals on solutions of calcium hexosemonophosphates, observed in 1923 that the enzymatic hydrolysis of these esters is followed by the precipitation of tricalcium phosphate in the junction cartilage. The role of a phosphatase in bone calcification has had to be taken into account since that time. A very important group of chemical[176–181] and histochemical[156,157,168,169,171]

[166] F. Moog, *Proc. Natl. Acad. Sci. U. S.* **29**, 176 (1943).
[167] F. Moog, *Biol. Bull.* **86**, 51 (1944).
[168] M. B. Engel and W. Furuta, *Proc. Soc. Exptl. Biol. Med.* **50**, 5 (1942).
[169] E. A. Kabat and J. Furth, *Am. J. Path.* **17**, 303 (1941).
[170] J. F. Danielli, H. B. Fell and E. Kodicek, *Brit. J. Exptl. Path.* **24**, 196 (1943); **26**, 367 (1945).
[171] G. H. Bourne, *J. Physiol.* **102**, 319 (1943).
[172] R. G. Bradfield, *Nature* **157**, 876 (1946).
[173] R. Jeener, *Nature* **159**, 578 (1947).
[174] D. Bodian and R. C. Mellors, *Proc. Soc. Exptl. Biol. Med.* **55**, 243 (1947).
[175] D. Bodian, *in* Nucleic Acids. Cambridge Univ. Press, 1947, p. 163.
[176] A. Policard, M. Péhu, J. Roche, and J. Boucaumont, *Bull. histol. appl. physiol. et path. et tech. microscop.* **13**, 645 (1931).
[177] M. Martland and R. Robison, *Biochem. J.* **18**, 1354 (1924).
[178] O. Bodansky, R. M. Bakwin and H. Bakwin, *J. Biol. Chem.* **94**, 551 (1931).
[179] J. Roche and E. Bullinger, *Bull. soc. chim. biol.* **21**, 166 (1939).
[180] C. B. Huggins, *Biochem. J.* **25**, 728 (1931).
[181] J. Roche and R. Martin-Poggi, *Bull. soc. chim. biol.* **23**, 1534 (1941).

observations has demonstrated that those regions of growing bone and teeth and of fracture calluses where a depot of salts is being organized are rich in alkaline phosphatase. This is the case in the zones of ossification of the junction cartilage, while the overlying serous cartilage is very poor in or entirely devoid of the enzyme, depending on the animal species. The same is true of the regions of dental pulp underlying the odontoblasts at the level of which the dentine arises in embryonic teeth.[168] The abundance of alkaline phosphatase in the teeth of Selachians, where the cartilagenous skeleton is devoid of the enzyme, is also significant.[178,179] Intramuscular grafts of fragments of bladder usually give rise to heterotopic osseous tissue, and only the grafts which are calcified are rich in the enzyme.[180] Finally, the osseous calluses do not contain a large amount of the enzyme until a short time before their consolidation.[171,181] The distribution of alkaline phosphatase in the skeleton therefore offers a first argument in favor of its role in calcification.

This may also be said of the study of the variations in its level in the long bones. Robison and Fell and their students[182,183,184] have demonstrated that the cultures of embryonic chick or rabbit bones exhibit an intense phosphatase activity starting at or a little before the stage of their evolution where they begin to be calcified. The same is true in the course of extra-embryonic development; thus in man, the kneecap is not ossified until the second year of life and it begins to contain the enzyme only then. In bony fish, the seasonal spurts of growth go hand in hand with a very great increase in the enzymatic activity of the bones, especially in the scales—membranous bones of very simple structure—which have an intensive vernal proliferation.[185] All these facts, chosen from among many others, suggest that the enzyme participates in the formation of the "bone salt." Its mechanism of action in this process is almost completely known. Robison and his students have given it a first representation on the basis of a qualitative study of the calcification of sections of epiphyses of young rachitic rats, immersed in solutions containing either PO_4^{--} and Ca^{++} ions or a phosphoric ester and Ca^{++} ions. At equal phosphorus and calcium concentrations, calcification is much more intense in the second instance than in the first. Its quantitative study[187] demonstrated that it then occurs at the expense of the phosphates liberated by the enzymatic hydrolysis of the ester. These phosphates participate in the formation of tricalcium phosphate as soon as the solubility product $[PO_4^{--}]^2 \times [Ca^{++}]^3 = S$ is reached.[186–189] A quantitative study of this phenomenon has been

[182] H. B. Fell and R. Robison, *Biochem. J.* **23**, 767 (1929).
[183] H. B. Fell and R. Robison, *Biochem. J.* **24**, 1905 (1930).
[184] H. B. Fell and R. Robison, *Biochem. J.* **28**, 2243 (1934).
[185] J. Roche and J. Collet, *Bull. soc. chim. biol.* **22**, 245 (1940).
[186] J. S. F. Niven and R. Robison, *Biochem. J.* **28**, 2237 (1934).
[187] R. Robison, M. Macleod and A. H. Rosenheim, *Biochem. J.* **24**, 1927 (1930).
[188] R. Robison and A. H. Rosenheim, *Biochem. J.* **28**, 684 (1934).
[189] R. Robison and K. M. Soames, *Biochem. J.* **24**, 1922 (1930).

undertaken by Roche and Deltour[190] and the results are illustrated in Fig. 2. It indicates that the presence of a phosphoric ester in the medium into which the section of bone is immersed and the hydrolysis of this ester not only permit the formation *in situ* of phosphate ions in abundance,

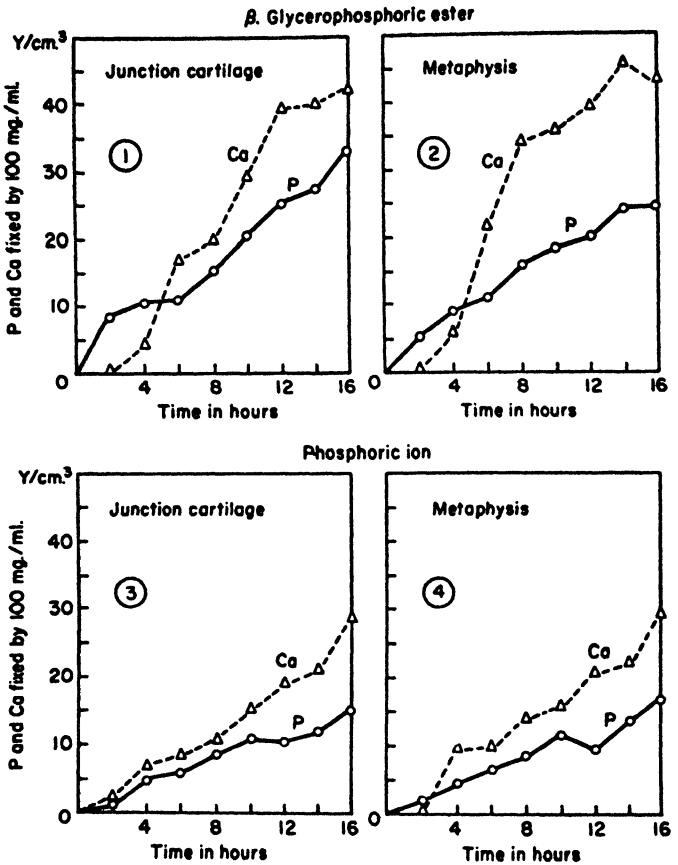

FIG. 2. Fixation of PO_4^{---} and Ca^{++} *in vitro* by junction cartilage and metaphysis (slices) of long bones of sheep embryo (35–45 cm. long): *1* and *2* from solutions containing Ca^{++} (10 mg./100 ml.) and β-glycerophosphate (sodium salt, 5 mg. P/100 ml.); *3* and *4* from solutions containing Ca^{++} (10 mg./100 ml.) and disodium orthophosphate (5 mg. P/100 ml.) at pH = 7.2 and at 37°. (Cf. Ref. 190.)

but also cause a draining of Ca^{++} ions present in the immersion fluid towards the section of bone. This occurs physiologically, where the formation of PO_4^{---} ions in the bone stimulates the movement of Ca^{++} ions from the blood toward the bone.

The important biological problem which remains to be resolved is the

[190] J. Roche and G. H. Deltour, *Bull. soc. chim. biol.* **25**, 1261 (1943).

supply of phosphoric esters to the osseous tissue. Plasma contains only traces (0.1–0.2 mg. esterified P per 100 ml.) and, in addition, circulation through the bones is of low intensity, so that the existence of another source of esters besides those carried in by the blood must be looked for. Histochemical observations have shown that all osseous regions during intensive calcification contain glycogen[168,169,191,192] together with a phosphorylase. This enzyme accomplishes the phosphorylating glycogenolysis by fixing the phosphate radicals from the blood, and glucose-1-phosphate is synthesized by this enzyme in the tissue itself where it serves as a substrate

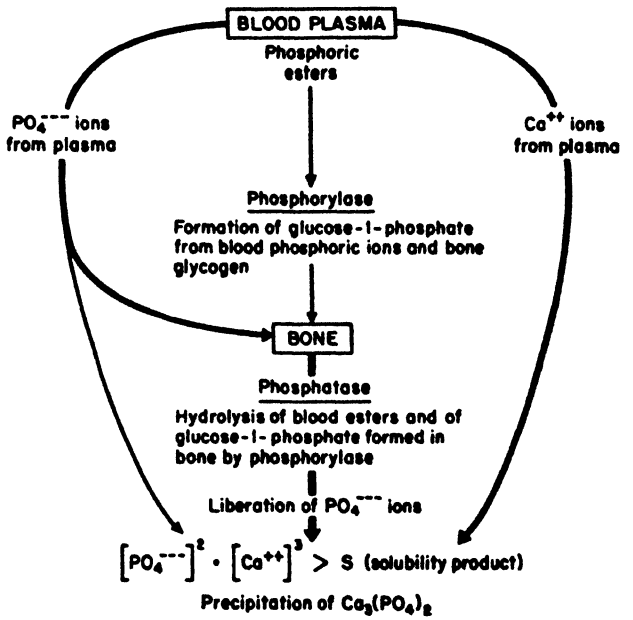

FIG. 3. General scheme of enzymatic processes involved in calcification of the bones. (Cf. Ref. 193.)

for the phosphatase. The latter acts physiologically together with the phosphorylase according to this scheme:[193]

Thus, the blood phosphates are fixed as esters in the bones, where the phosphatase can then liberate them at a high concentration by means of the hydrolysis of these esters. This permits the rapid and abundant formation of tricalcium phosphate, the renewal of Ca^{++} ions in the osseous fluids being assured by an actual draining of blood calcium. Bone phosphatase is thus one of the most important elements in the mineralisation

[191] G. E. Glock, *J. Physiol.* **98**, 1 (1940).
[192] N. H. Horowitz, *J. Dental Research* **21**, 519 (1942).
[193] J. Roche, *Ann. nutrition alimentation* **1**, 3 (1947).

of bones and teeth, especially in periods of growth. It is indispensable for all rapid calcification. Nevertheless, its activity plays an important role only in one of the phases of ossification, since the fixation of tricalcium phosphate, formed according to the mechanism outlined above, to the proteins of the bone is a phenomenon independent of its precipitation. This is especially clear in experimental rickets, at the start of which the junction cartilage of the long bones of the rat is richer in phosphatase but exhibits a diminution of the ability to fix the salts.[194]

3. Other Specialized Roles of the Phosphatases and Quantitative Variations of Phosphatase Activities in the Tissues

The localization of phosphatases at high concentrations in various tissues besides the bones is necessarily related to physiological processes which are not all understood at the present time.

Alkaline phosphatase participates in the transport of calcium as the phosphate in vertebrates and in those invertebrates in which rapid changes of calcium take place, such as the molting of the crustaceans. The mammary gland, which in the cow secretes 1.75 g. tricalcium phosphate per liter of milk, probably concentrates the blood phosphates by a mechanism analogous to the formation of bone salts;[195] the phosphorylation of casein is independent of this process. Anyway, alkaline phosphatase intervenes actively in a group of processes which involve the formation of tricalcium phosphate as an intermediate or end product.

The function of this enzyme in resorption phenomena has been shown in kidney and intestinal mucosa. The kidney cortex is one of the animal tissues richest in this enzyme and Lundsgaard suggested in 1933 that the activity of the latter is connected with the resorption of glucose in the convoluted tubules; but this hypothesis has taken on weight only after the development of the histochemistry of the phosphatases. The cycle of reactions that keeps blood glucose from being eliminated in the urine after its diffusion into the glomerular liquid takes place at the level of the convoluted tubules. It involves a phosphorylation at the level of the cells of the latter and a dephosphorylation at their distal end. The cells of the convoluted tubules are particularly rich in the enzyme and the latter is especially concentrated at the level of their brush borders,[171,196] site of glucose resorption, while the glomeruli, organs of filtration, and the distal tubules do not contain the enzyme in most animal species. In the toadfish, *Opsanus tau*, an aglomerular animal which can neither secrete nor excrete sugar, there is no tubular phosphatase.[197] The observations made

[194] J. Roche and M. T. Simonot, *Enzymologia* **10**, 239 (1942).
[195] E. Lundsgaard, *Biochem. Z.* **264**, 221 (1933).
[196] G. Gomori, *J. Cellular Comp. Physiol.* **17**, 71 (1941).
[197] H. A. Wilmer, *Arch. Path.* **37**, 227 (1944).

during experimental uranium nephritis[198] or pathological nephritis require a more cautious interpretation. On the other hand, the blocking of kidney phosphatase simultaneously with the glycosuria of alloxan diabetes is significant.[199,200] Although it is not actually possible to state precisely the mechanism of action of alkaline phosphatase in kidney secretion, its abundance and its localization at least suggest that the enzyme takes part in the tubular resorption of glucose, probably in the dephosphorylating phase. Acid phosphatase II is present, less abundantly, in a diffuse state in the tubular cells; it is not concentrated in the brush borders.

Intestinal mucosa is richer in alkaline phosphatase than any other tissue and many authors consider that it may participate in the resorption of glucose or fatty acids. It is localized mostly in the superficial layers of the epithelium, where it occupies at the same time the striated circular border of the cells and an intermediary zone between the nucleus and the luminal border.[171,197,201] The duodenum is an especially rich source. Intestinal desquamation leads to a pronounced enrichment of the feces with this enzyme, beyond its secretion proper which has an important digestive function, and beyond its probable participation in the absorption of hexoses and fatty acids. The secretion of alkaline phosphatase by the intestine takes place in response to a very poorly differentiated alimentary stimulus; it is more or less intensive in the presence of hexoses, fatty acids, amino acids, phosphoric esters, nil in the presence of mineral solutions. An isolated intestinal loop into which a solution of glucose is injected can secrete in 1 hour about a twentieth of the phosphatase it contains.[202,203]

The functions of the acid phosphatases are much less well defined, except perhaps in the case where they act outside the secreting tissue. The human prostate eliminates phosphomonoesterase II, which is found in the seminal fluid. This enzyme may participate in the sperm in the dephosphorylation of glucose esters to liberate fructose, which serves as food for the spermatozoa, but it is also undoubtedly responsible for the formation of choline from the phosphorylcholine of sperm.[204]

Quantitative variations of the phosphatase activity of tissues have been found; the most characteristic are those of hormonal origin. The phosphoesterase activity of intestinal mucosa is increased by the injection of adrenocortical hormones and diminished by adrenalectomy or injection of alloxan;[197,198,205] this fact is often interpreted as an indication that the en-

[198] C. Breedis, C. M. Flory and J. Furth, *Arch. Path.* **36**, 402 (1943).
[199] M. L. Menten and M. Janouch, *Proc. Soc. Exptl. Biol. Med.* **63**, 33 (1946).
[200] A. Soulairac, *Compt. rend. soc. biol.* **142**, 643 (1948); P. Desclaux and F. Katz, *Ibid.* **142**, 311 (1948).
[201] V. M. Emmel, *Anat. Record* **91**, 39 (1945).
[202] A. Sols and F. Pons, *Rev. espan. fisiol.* **2**, 283 (1946).
[203] J. Lopez-Navarro, *Rev. espan. fisiol.* **2**, 211 (1946).
[204] F. Lundquist, *Acta Physiol. Scand.* **13**, 322 (1947).
[205] W. Kutscher and H. Wüst, *Z. physiol. Chem.* **273**, 235 (1942).

zyme takes part in glucose or lipide metabolism. Alloxan diabetes goes hand in hand with a very pronounced regression of the phosphatase activity of kidney,[199,200] and this appears to be proof for the role of the enzyme in the resorption of glucose after its glomerular filtration. The significant effect of male sex hormones on kidney phosphatases, on the other hand, is hard to explain. Testosterone, androsterone, and various derivatives substituted in position (α), or 3(α or β), 17(α), when administered to castrated mice, causes kidney hypertrophy and, at the same time, a diminution of alkaline phosphatase activity and a great increase in acid phosphatase activity; the enzymes of the liver and intestine are not affected by these hormones.[206] Neither these facts nor the simultaneous increase in kidney arginase activity can as yet be interpreted simply, while the progressive enrichment of the mammary gland in alkaline phosphatase from the onset of lactation hardly requires comment.[207]

4. Physiological and Chemical Roles of the Phosphatases in the General Metabolism of the Cell

The participation of the phosphatases in the metabolic processes that take place in the cells cannot be precisely indicated. The universality of their distribution suggests that they have a general direct or indirect role. The metabolic importance of the phosphate bond is developed elsewhere but it may be proper to consider here to what extent the phosphatases are associated with it.

The phosphorylating enzyme systems are independent of a synthesizing action of the phosphatases; they include other enzymes (phosphorylase, hexokinase) and phosphate radical donors other than mineral phosphates. Phosphatase synthesis of esters appears to have little physiological importance, or at least to be rare. The phosphatases take part in metabolism as far as the hydrolase activity in liberating phosphates is concerned, but their action is then included in reaction cycles and depends on enzymes of very strict specificity, or at least acting preferentially on certain esters (hexosediphosphatase, nucleotidases, etc.). This explains the fact that enzymes of wide specificity are abundant above all in cells where their presence responds to a specialized function, such as bone calcification or kidney secretion. Within the framework of general metabolism, the phosphatases are of less importance than the enzymes that transfer phosphates and those which carry out the synthesis of esters by more complex processes than the reaction inverting hydrolysis. An important but still obscure point is the extent to which these enzymes participate in metabolic reaction cycles. The findings are clear for adenylpyrophosphatase, but far from unequivocal for the phosphoesterases and pyrophosphatases.

V. Pathological Chemistry of the Phosphatases

As with a few other enzymes, pathological chemistry has attempted to put to good use the knowledge obtained in the field of physiological chem-

[206] C. D. Kochakian, *Recent Progress in Hormone Res.* **1**, 177 (1947).
[207] S. J. Folley and A. L. Greenbaum, *Biochem. J.* **41**, 261 (1947).

istry of the phosphatases. Only quantitative data have any significance in the field of pathology: Attempts have been made to obtain such data on serum, white and red blood cells, and the various tissues. We shall examine the findings which have been made on serum and tumor tissue.

1. Alkaline and Acid Phosphatases of Serum

Blood serum contains a mixture of small quantities of phosphatases, mainly phosphomonoesterases, phosphodiesterases, and pyrophosphatases.[81,208,209,210] Up to the present time, only the first have been studied systematically; hyperpyrophosphatasemia seldom occurs.[211] No exact significance has been ascertained until now for the variations of the level of this enzyme in the formed elements of the blood.[81,212] Alkaline phosphatase (phosphomonoesterase I) is accompanied in normal serum and plasma by minimal amounts of the isodynamic enzyme of acid pH optimum (type II) and, occasionally, of phosphomonoesterase III. Normally these enzymes are derived by diffusion from various tissues, first of all, without doubt, the liver. In various pathological conditions, a particular tissue may pour high levels of one or the other enzyme into the blood; the existence of a hyperphosphatasemia may, therefore, direct or confirm the diagnosis of a disease and give useful information on its course and on the efficacy of treatment.

In adult man, plasma contains 2.5 to 5 Bodansky units (B.u. = γ P liberated per hour at pH = 8.6 and at 37° by 100 ml. plasma acting on 0.01 *M* sodium β-glycerophosphate); the growing infant has appreciably higher levels.[213] Pathological or experimental variations are significant only in hyperphosphatasemia when they reach or surpass a level twice the average normal values, since hypophosphatasemia is hard to demonstrate. The most important of the former have been observed in osseous or hepatic disturbances. In osteitis deformans (Paget's disease), phosphatasemia may reach and surpass 100 B.u.; it is less elevated in osteitis fibrocystica (Recklinghausen's disease) and even less so in other osteites.[214,215] It is increased to a moderate degree (10–15 B.u.) in the acute stages of human or experimental[213] rickets, and it is brought back to normal by vitamin therapy of this osseous dystrophy. But the main conditions in which the study of phosphatasemia has been of use to bone pathology are the osteites and certain cancer metastases, as will be seen below. In liver pathology, determina-

[208] F. Demuth, *Biochem. Z.* **159**, 415 (1925).
[209] E. Lundsteen and M. Vermehren, *Enzymologia* **6**, 27 (1939).
[210] J. Roche, Nguyen-van-Thoai, and J. Marcelet, *Compt. rend. soc. biol.* **138**, 517 (1944).
[211] G. Desruisseaux, *Compt. rend. soc. biol.* **140**, 644 (1946); **140**, 646 (1946).
[212] L. Chevillard, *Compt. rend. soc. biol.* **139**, 249 (1945).
[213] D. J. Barnes and B. Munks, *Proc. Soc. Exptl. Biol. Med.* **44**, 327 (1940).
[214] H. D. Kay, *Brit. J. Exptl. Path.* **10**, 253 (1929).
[215] H. L. Jaffe and A. Bodansky, *Bull. N. Y. Acad. Med.* **19**, 831 (1943).

tions of serum phosphatase are of service in jaundice. Like experimental biliary obstruction,[215,216] the obstructions caused by various diseases (calculi, tumors) lead to alkaline hyperphosphatasemia which rarely surpasses 25 B.u. Icterus of different origin never or rarely involves such a manifestation.[217,218]

Acid phosphatase (type II) shows significant increases only in cancer of the prostate, especially when it involves bone metastases.[218,219,220] Values as high as 1000 King-Armstrong units (K.A.u. = γ phenol liberated by 100 ml. of serum acting on 0.005 *M* sodium phenylphosphate at pH =

TABLE III

SERUM ACID AND ALKALINE PHOSPHATASE IN DISEASES

Disease	Phosphatase	
	Alkaline	Acid
Prostatic cancer:		
a. with bone metastases	Normal or slightly raised	Usually markedly raised
b. without bone metastases	Normal	Most frequently normal
Other diseases of prostate	Normal	Normal
Osteitis deformans	Markedly raised	Normal or slightly raised
Osteitis fibrosa cystica	Markedly raised	Normal or slightly raised
Osteogenic sarcoma	Raised	Usually normal
Myelomatosis	Normal	Usually normal
Other bone tumors	Normal or raised	Usually normal
Cancer or organs (other than prostate) with bone metastases	Normal or raised	Usually normal
Rickets	Raised	Usually normal
Osteomalacia	Raised	Usually normal
Senile osteoporosis	Normal	Usually normal
Jaundice	Markedly raised in obstructive, less so in toxic, normal in hemolytic jaundice	May be slightly raised.

5.0 for 1 hour at 37°) have been found in this condition. However, only 50% of the cases surpass 10 units; the normal average is 3.3 units. Table III summarizes the principal modifications of phosphatasemia observed

[216] S. Freemann and Y. P. Chen, *J. Biol. Chem.* **123**, 239 (1938).
[217] T. Meranze, D. R. Meranze, and M. M. Rathman, *Rev. gastro-enterol. Mex.* **6**, 254 (1939).
[218] A. B. Gutmann, K. B. Olson, E. B. Gutmann, and C. A. Flood, *J. Clin. Invest.* **19**, 129 (1940).
[219] A. B. Gutmann and E. B. Gutmann, *J. Clin. Invest.* **17**, 273 (1938).
[220] C. Huggins and C. V. Hodges, *Cancer Research* **1**, 293 (1941).

in pathological conditions, on the basis of findings established or assembled by Sullivan *et al.*,[221] King and Delory,[222] and Sundermann.[223]

The mechanism of pathological hyperphosphatasemias has been discussed from two points of view: the identification of their cellular origin and the part which the activation of the enzymes or the actual increase in their level play in their manifestation. With regard to the first, experiments on specific inhibition by alcohol and formol[105,106] showed that serum contains a mixture of enzymes of different origin. In addition, hyperphosphatasemias more frequently reflect an actual enrichment in the enzyme than an activation.[224]

2. Phosphatases and Cancer

The acid or alkaline phosphatase activity of pathological or experimental tumors is very variable.[225] In rat hepatoma, phosphatase activity remains high after three transplantations, then decreases considerably. Besides, different results are obtained with different carcinogenic agents which appear equivalent. Very systematic results have been obtained only in bone tumors and in cancer of the prostate.[226] Osteogenic sarcomas are very rich in alkaline phosphatase, which participates in the formation of osteoid tissue.[227] The same is true of all osteogenic tumors while certain metastases in which the processes of bone destruction are more important than the tendency for repair have low enzyme concentrations. The origin of the metastases plays an undeniable role in determining their enzyme content; for example, those of prostatic cancer are generally rich in acid phosphatases while those of other osseous neoplasms are not.

The principal attempts at demonstrating an increase in alkaline phosphatase in connection with tumors have been carried out on serum. Enzymatic activity increases to very different degrees depending on whether there are a neoplastic proliferation, a formation of osseoid tissue or repair processes simultaneous with or consecutive to a neoplastic destruction of the bones. The existence of hyperphosphatasemias of certain osseous tumors is connected with an osteoblastic activity but is not involved in the purely destructive lesions of myelomatosis.

Acid hyperphosphatasemia of cancers of the prostate has stimulated a great number of investigations and its correlation with the existence of tumors is certain. Orchidectomy and stilbestrol treatment cause at the

[221] T. J. Sullivan, E. B. Gutmann and A. B. Gutmann, *J. Urol.* **48**, 426 (1942).
[222] E. J. King and G. E. Delory, *Postgrad. Med. J.* (June number) 1 (1948).
[223] F. W. Sundermann, *Am. J. Clin. Path.* **12**, 404 (1942).
[224] G. E. Delory and E. J. King, *Biochem. J.* **38**, 50 (1944).
[225] J. P. Greenstein, Biochemistry of Cancer. Academic Press, New York, 1947.
[226] J. P. Greenstein, *J. Natl. Cancer Inst.* **2**, 511 (1942).
[227] H. Q. Woodard, *Cancer Research* **2**, 497 (1942); *Am. J. Roentgenol.* **47**, 227 (1942).

same time a regression in the volume of the tumors and of the level of serum acid phosphatase,[220] while the repair of osseous lesions due to metastases of the parent tumor causes an increase of alkaline phosphatasemia.[221] This hyperphosphatasemia is, however, less pronounced than that caused by Paget's disease; the latter, moreover, has no effect on the serum level of acid phosphomonoesterase II. At the present state of our knowledge, the interest in and study of the phosphatases in tumors and in the blood of the tumor-bearing host are therefore limited and it is not possible to connect the histological forms of the tumors with their different enzymatic activities.

VI. General Conclusions

The knowledge accumulated on the phosphatases is still fragmentary on very many points and the study of these enzymes has posed more problems than it has resolved. Even the individuality of the multiple phosphatases remains uncertain. The constitution of none of these enzymes is known and, *a fortiori*, we are ignorant of the structural factors responsible for their specificity and the fundamental reasons of isodynamics. It is urgent that progress be made in the preparation of the various phosphatases at a high degree of purity so that these problems can be studied. In the field of physiological chemistry and of its applications, only the role of bone phosphatase has been elucidated and histochemistry has again raised many questions that can now be properly approached. This means that the chemistry of the phosphatases offers very vast possibilities for development and that the importance of this field increases as a function of progress in it.

This field abounds in problems of great importance for general enzymology, so that it merits the attention of investigators not only from the point of view of extending our knowledge about the phosphatases themselves, but also from that of resolving these problems. The biochemistry of enzymes having a dissociable metal, that of intermediary metabolism, that of the mode of action of certain hormones, and that of the renal and intestinal mechanisms of glucose resorption are directly related to the biochemistry of the phosphatases.

CHAPTER 12

Metaphosphate and its Enzymatic Breakdown

BY BJÖRN INGELMAN

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I. Introduction

Metaphosphatases are enzymes able to hydrolyze salts of metaphosphoric acid, *e.g.*, the sodium salts $(NaPO_3)_n$. Metaphosphates of low molecular weight as well as colloidal metaphosphates are known. Thus the number n in the formula $(NaPO_3)_n$ can vary within a wide range for different metaphosphate preparations. The trimetaphosphate $(NaPO_3)_3$ is considered to have a cyclic structure. Another metaphosphate of comparatively low molecular weight is the so-called hexametaphosphate, $(NaPO_3)_6$. It is, however, very unlikely that the generally used commercial preparations called hexametaphosphate are monodisperse substances with molecular weights corresponding to this formula. Very high molecular metaphosphates can be prepared by heating KH_2PO_4. The molecular weights of such preparations often are of the order of 1,000,000, as has been found by Malmgren and Lamm.[1,2] These substances are sometimes called polymetaphosphates, not to be confused with the notation polyphosphate, which often refers to tripolyphosphate or triphosphate, $Na_5P_3O_{10}$. The structure of these colloidal metaphosphates is not yet definitely established. Probably they are built up of long chains of the following type:

—O—PO(ONa)—O—PO(ONa)—O—PO(ONa)—O—PO(ONa)—O—

Regarding earlier chemical investigations on metaphosphates reference is made to a review by Karbe and Jander.[3]

II. Enzymatic Breakdown of Metaphosphate

Kitasato[4] in 1928 demonstrated that metaphosphate can be converted into orthophosphate by means of enzyme extracts from molds, yeast, and

[1] H. Malmgren and O. Lamm, *Z. anorg. Chem.* **232**, 236 (1944).
[2] H. Malmgren, *Acta Chem. Scand.* **2**, 147 (1948).
[3] K. Karbe and G. Jander, *Kolloid-Beihefte* **54**, 1 (1942).
[4] T. Kitasato, *Biochem. Z.* **197**, 257 (1928); *ibid.* **201**, 206 (1928).

rabbit organs. As substrate in these investigations he used hexametaphosphate. The orthophosphate liberated was determined colorimetrically. Later this observation was confirmed by Neuberg and Fischer[5] and other investigators. Schäffner and Krumey[6] found that a metaphosphatase in a yeast extract had its optimum activity at pH 7–8 and that the enzyme was activated by magnesium ions. They could show that the enzyme was not identical with yeast pyrophosphatase. The metaphosphatase activity disappeared after heating at 50°C. for some minutes. This treatment decreased the pyrophosphatase activity of the extract only to a small extent. Bamann and Heumüller[7] studied the enzymatic breakdown of trimetaphosphate into orthophosphate by liver extracts. They found that several divalent metallic ions activated this enzyme.

Investigating phosphorus metabolism in molds, Mann[8] in 1944 observed powerful metaphosphatase activity in water extracts from *Aspergillus niger*. A purified enzyme preparation (60 γ) incubated with a solution of sodium metaphosphate or pyrophosphate (0.3 mg. P) for 2 hr. at pH 5 and 30° converted both compounds quantitatively to orthophosphate.

It might also be mentioned that the transformation of sodium triphosphate, $Na_5P_3O_{10}$, to orthophosphate by enzyme extracts from microorganisms and organs of animals has been studied by several investigators.[5–7,9–11] The metaphosphate preparations used as substrate in many earlier investigations were often incompletely described. However, the molecular weights of the substances seem to have been comparatively low. (The preparations used were described only as metaphosphate, trimetaphosphate, and hexametaphosphate.)

In 1947 Ingelman and Malmgren[12] published investigations which gave evidence that metaphosphates of very high molecular weight are broken down by enzymes from microorganisms such as *Aspergillus niger* and *Penicillium expansum*. The potassium metaphosphate preparations used had molecular weights larger than one million. Thus in these experiments a purely inorganic colloid was broken down enzymatically. By means of viscosity measurements, ultracentrifugations, dialysis experiments, and colorimetric orthophosphate determinations indications were obtained that the breakdown proceeds through a—probably random—scission of —P—

[5] C. Neuberg and A. H. Fischer, *Enzymologia* **2**, 191, 241, 360 (1937–1938).
[6] A. Schäffner and F. Krumey, *Z. physiol. Chem.* **255**, 145 (1938).
[7] E. Bamann and E. Heumüller, *Naturwissenschaften* **28**, 535 (1940).
[8] T. Mann, *Biochem. J.* **38**, 339 (1944).
[9] C. Neuberg and A. H. Fischer, *Compt. rend. trav. lab. Carlsberg, Sér. chim.* **22**, 366 (1938).
[10] C. Neuberg, L. Frankenthal, and I. S. Roberts, *Exptl. Med. and Surg.* **1**, 386 (1943).
[11] M. Dainty, A. Kleinzeller, A. S. C. Laurence, M. Miall, J. Needham, D. M. Needham, and Shih-Chang Shen, *J. Gen. Physiol.* **27**, 355 (1944).
[12] B. Ingelman and H. Malmgren, *Acta Chem. Scand.* **1**, 422 (1947).

O—P— links in the metaphosphate chains. Therefore, to begin with comparatively large fragments are formed. Thus in the first stage of the breakdown only very small amounts of orthophosphate are liberated. The hydrolysis does not occur as a splitting off of one or a few phosphoric acid residues at a time from the ends of the chains.

In the continuation of these investigations[13] a convenient method, based on viscosity measurements, was worked out for the determination of relative enzyme activities. The high-molecular potassium metaphosphates are soluble in sodium salt solutions of suitable concentration. These solutions have a high viscosity. Hence it is easy to follow the first phase of the breakdown by viscosity measurements. The viscosity, however, depends not only upon the degree of polymerization and the concentration of the colloidal metaphosphate but also upon the low-molecular salts added to the solution. Therefore, in activity determinations of this kind allowance must

TABLE I

pH OPTIMA OF ENZYMES FROM SOME MICROORGANISMS ACTING ON HIGH-MOLECULAR METAPHOSPHATE

Organism	pH optimum
Penicillium expansum	4.5
Penicillium chrysogenum	4.8
Penicillium funiculosum	4.5
Aspergillus niger	5.7
Saccharomyces cerevisiae	7.2
Proteus vulgaris	4.7

be made for this influence. Using this method and an enzyme from *Aspergillus niger* Ingelman and Malmgren could show that the breakdown velocity is proportional to the enzyme concentration. This is valid at concentration levels of metaphosphate and enzyme at which the measurements could conveniently be performed.

Some physicochemical properties of the enzyme from *Aspergillus niger* which degrades metaphosphate of high molecular weight were also studied in the same investigation.[13] As the enzyme was not pure and the concentration very low, ordinary methods of observation could not be used. Therefore, the course of the experiments was followed by means of the above-mentioned activity determination method. The electrophoretic mobility was studied in the Tiselius apparatus. The isoelectric point of the enzyme was found to be about pH 3.1. The sedimentation constant of the enzyme was determined in the Svedberg ultracentrifuge using a separation cell. The sedimentation constant, s_{20}, was found to be 3.2 *S*. The diffusion

[13] B. Ingelman and H. Malmgren, *Acta Chem. Scand.* **2**, 365 (1948).

constant of the enzyme was determined according to "the porous-disk method" and D_{20} thus obtained was 8.8×10^{-7} cm.²/sec. The molecular weight of this enzyme from *Aspergillus niger* was estimated to be about 33,000.

Investigations into the further occurrence of enzymes degrading colloidal metaphosphates hitherto have shown that such enzymes are found in some fungi belonging to the group of *Ascomycetes* and in the bacterium *Proteus vulgaris*.[14] The pH optima of these enzymes were determined with the viscosity method. The values obtained are given in Table I.

Preliminary experiments with enzyme extracts from liver of rabbit and cow[14] gave no evidence of a breakdown of high-molecular metaphosphates. However, metaphosphates of comparatively low molecular weights, *e.g.*, sodium trimetaphosphate $(NaPO_3)_3$, can be broken down by extracts from animal organs.

Regarding the specificity of metaphosphatases very little is known. Very little has been done on the purification of these enzymes.

III. Occurrence of Metaphosphate in Microorganisms

During the last century investigations were published which indicated the occurrence of metaphosphate in yeast. The first publications on this subject seem to be from 1888 and 1890, in which Liebermann[15,16] says that metaphosphoric acid occurs in yeast. In 1893 Kossel[17] isolated from yeast a substance which he gave the name "*Plasminsäure*" and which was supposed to contain metaphosphoric acid. Six years later Ascoli[18] described further experiments on this "*Plasminsäure*." He also concluded that the substance contained metaphosphoric acid. These early investigations seem to have been forgotten for a long time. In 1936 MacFarlane[19] obtained from yeast a nucleic acid preparation of high phosphorus content. On extracting this substance with hydrochloric acid, inorganic metaphosphate was obtained and identified. Later also Wiame[20] isolated, from yeast, such preparations containing 17% phosphorus. In 1944 Mann[21] isolated metaphosphate from *Aspergillus niger* by extracting the mycelia with trichloroacetic acid. Metaphosphate has also been found in different mutants of *Neurospora*.[22] During recent years the occurrence of metaphosphate

[14] B. Inge'man and H. Malmgren, *Acta Chem. Scand.* **3**, 157 (1949).
[15] L. Liebermann, *Arch. ges. Physiol.* (Pflügers) **43**, 97 (1888); *ibid.* **47**, 155 (1890).
[16] L. Liebermann, *Ber.* **21**, 598 (1888).
[17] A. Kossel, *Arch. Anat. u. Physiol., Physiol. Abt.* p. 160 (1893).
[18] A. Ascoli, *Z. physiol. Chem.* **28**, 426 (1899).
[19] M. G. MacFarlane, *Biochem. J.* **30**, 1369 (1936).
[20] J. M. Wiame, *Biochim. et Biophys. Acta* **1**, 234 (1947).
[21] T. Mann, *Biochem. J.* **38**, 345 (1944).
[22] M. Houlahan and H. Mitchell, *Arch. Biochem.* **19**, 257 (1948).

in yeast has been further confirmed.[23–26] Some of the investigations indicate the possible occurrence of two different kinds of metaphosphate in yeast: an acid-soluble and an acid-insoluble form differing in their metabolic and physiological functions.[24,25]

In the earlier literature on the naturally occurring metaphosphate no molecular weight determinations are described. Investigating the enzymatic breakdown of very high molecular metaphosphates, Ingelman and Malmgren[12] raised the question whether at least a fraction of the metaphosphate found in microorganisms, *e.g.*, *Aspergillus niger*, could be of colloidal nature. However, the isolation of undegraded metaphosphate of high molecular weight in a soluble form is difficult. First, the cells contain a powerful enzyme which breaks down the substance and, second, the high-molecular metaphosphate is a labile substance. Both at low and high pH values, the rate of spontaneous breakdown is high. Therefore, it is important to find a suitable method to remove the enzyme and other organic substances in such a procedure. Applying the following technique, Ingelman[27,28] succeeded in isolating a high-molecular, nondialyzable substance of high phosphorus content. *Aspergillus niger* was extracted at a slightly alkaline pH. The extract was then filtered through large amounts of active carbon in order to remove the enzyme and other organic material. Finally the solution was dialyzed in cellophane bags and dried *in vacuo* in the frozen state. One of the preparations contained 25% phosphorus and 15% sodium. The characteristic absorption of nucleic acids in ultraviolet light could not be observed. The ash content was about 85%. The sedimentation constant found in the ultracentrifuge was 2.3 *S*. Another preparation obtained contained 29.3% phosphorus. The ash content was more than 90% and the sedimentation constant 2.3 *S*. This investigation indicated that almost all the substance isolated must consist of sodium metaphosphate, $(NaPO_3)n$, of high molecular weight, *i.e.*, an inorganic colloid. From the sedimentation constants obtained a molecular weight of about 8000 can be estimated for this substance.

Wiame's[26,29,30] investigations suggest that the substance in yeast cells called "volutin," which can be detected by a special staining technique, contains metaphosphate. The role of volutin in yeast has been investigated by Lindegren.[31] Due to the energy-rich phosphate bonds in meta-

[23] G. Schmidt, L. Hecht, and S. J. Tannhauser, *J. Biol. Chem.* **166**, 775 (1946).
[24] E. Juni, M. Kamen, J. Reiner, and S. Spiegelman, *Arch. Biochem.* **18**, 387 (1948).
[25] J. M. Wiame, *Rev. fermentations et inds. aliment.* **3**, 83 (1948).
[26] J. M. Wiame, *Federation Proc.* **6**, 302 (1947).
[27] B. Ingelman, *Acta Chem. Scand.* **1**, 776 (1947).
[28] B. Ingelman, *Svensk Kem. Tid.* **60**, 222 (1948).
[29] J. M. Wiame, *Bull. soc. chim. biol.* **28**, 552 (1946).
[30] J. M. Wiame, *J. Am. Chem. Soc.* **69**, 3146 (1947).
[31] C. C. Lindegren, *Proc. Natl. Acad. Sci. U. S.* **34**, 187 (1948).

phosphate this substance probably supplies energy to important processes in bacteria and fungi. Lindegren's studies suggest that in yeast this volutin is essential for chromosome division. It is not yet definitely established whether the metaphosphate itself or perhaps an organic complex containing metaphosphate represents the compound originally present in the cell.

As far as the author knows metaphosphate has not yet been found in higher animals. It is rather unlikely that metaphosphate of high molecular weight should occur in animals. A preliminary study[14] showed that only low-molecular metaphosphates are broken down by organ extracts. Whether or not substances of low molecular weight with a metaphosphate-like structure occur in animals is a question which cannot yet be answered. A more thorough study of the occurrence of metaphosphate and its enzymatic degradation has just begun; consequently it is yet very difficult to make definite statements about the biological significance of these substances. However, it seems very probable that metaphosphate and the enzymes which break down this substance are of great significance for important physiological functions in at least certain species of microorganisms.

References added in proof:

[32] H. Malmgren, *Acta Chem. Scand.* **3**, 1331 (1949).
[33] G. Schmidt, L. Hecht, and S. J. Tannhauser, *J. Biol. Chem.* **178**, 733 (1949).
[34] J. M. Wiame, *J. Biol. Chem.* **178**, 919 (1949).
[35] B. Ingelman and H. Malmgren, *Acta Chem. Scand.* **4**, 478 (1950).

Chapter 13

Sulfatases*

By CLAUDE FROMAGEOT

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I. Introduction

The sulfatases are enzymes which hydrolyze the numerous sulfuric esters, according to the general reaction:

$$ROSO_3K + H_2O \rightarrow ROH + KHSO_4$$

* Translated by Erich Hirschberg, McArdle Memorial Laboratory, The University of Wisconsin, Madison, Wisconsin.

These enzymes have been the subject of a number of reviews[1-4]; reference should be made to these for the details which cannot be covered here.

Several types of sulfatases may be distinguished according to the nature of the sulfuric esters which they hydrolyze:

Type of sulfatase	Esters hydrolyzed
Phenolsulfatase	Esters with an aromatic radical e.g., monophenylsulfuric ester)
Glucosulfatase	Esters with a carbohydrate radical (e.g., glucose-6-sulfuric ester)
Chondrosulfatase	Chondroitinsulfuric acid
Myrosulfatase	Sinigrin

This classification results from the combination of the specificities of action of the different preparations of sulfatases obtained up to the present time. For example, the sulfatase of taka-diastase hydrolyzes only sulfuric esters with an aromatic radical, without attacking sinigrin[5] or the esters with an aliphatic radical, or, in a general manner, any other sulfuric ester; on the other hand, the enzyme preparations obtained from animal tissues are capable of hydrolyzing the sulfuric esters with an aromatic radical as well as potassium myronate (sinigrin)[6]; the enzyme preparations obtained from *Pseudomonas eisenbergii* can hydrolyze chondroitinsulfuric acid as well as glucose—and saccharose—sulfuric acids and potassium myronate[7]; the sulfatase contained in myrosinase (or sinigrinase) liberates sulfuric acid from sinigrin, but has no effect on phenolsulfuric ester[1] or chondroitinsulfuric acid.[7]

II. Phenolsulfatase

In 1911, Derrien[8] demonstrated the existence in *Murex* of an enzyme, which he named purpurase, which hydrolyzes potassium indoxyl sulfate to free indoxyl and potassium acid sulfate. In 1924, Neuberg and Kurono[9] found in taka-diastase a similar enzyme capable of hydrolyzing potassium phenyl sulfate. This is the enzyme to which Neuberg gave the name sulfatase, later changed to the more precise one of phenolsulfatase.

[1] C. Neuberg and E. Simon, *Ergeb. Physiol.* **34**, 896 (1932).
[2] C. Oppenheimer, Die Fermente und ihre Wirkungen. Suppl. I, Junk, The Hague, 1936, p. 171.
[3] C. Neuberg and J. Wagner, *in* Oppenheimer, Methodik der Fermente. Thieme, Leipzig, 1929, p. 760.
[4] C. Fromageot, *Ergeb. Enzymforsch.* **7**, 50 (1933).
[5] C. Neuberg and J. Wagner, *Biochem. Z.* **174**, 457 (1923).
[6] C. Neuberg and J. Wagner, *Z. ges. exptl. Med.* **53**, 331 (1927).
[7] C. Neuberg and E. Hofmann, *Biochem. Z.* **231**, 345 (1931).
[8] M. Derrien, *Bull. soc. chim. France* (IV) **9**, 110 (1911).
[9] C. Neuberg and K. Kurono, *Biochem. Z.* **140**, 235 (1923).

1. METHODS OF INVESTIGATION

Qualitatively, the action of phenolsulfatase may be demonstrated by using potassium indoxyl sulfate as the substrate: in the course of enzymatic hydrolysis, indoxyl is liberated; the latter is easily oxidized to indigo, and the solution under test therefore assumes a green color. The dye promptly precipitates in blue flakes; on the other hand, it dissolves partly in toluene, which then takes on a red coloration.[10]

Most of the quantitative studies on the action of phenolsulfatase have been carried out in the following manner[11]:

To a flask is added a solution of the potassium salt of the sulfuric ester, at about 1% concentration, and then the enzyme preparation to be studied; calcium carbonate is added to maintain neutrality of the mixture during the course of the experiment (this neutrality would be speedily destroyed by the liberation of potassium acid sulfate by hydrolysis of the sulfuric ester). The sterility of the mixture is assured by the addition of a little toluene. After a certain period of time, the sulfuric acid which has remained combined with the organic radical is assayed.

The manometric Warburg apparatus may also be employed; use is made of the action on sodium bicarbonate of the potassium acid sulfate produced.

Huggins and Smith[12] recently reported an excellent method for the study of phenolsulfatase, with *p*-nitrophenyl sulfate as the substrate. *p*-Nitrophenol liberated by the enzyme gives a yellow color in alkaline medium which lends itself perfectly to quantitative measurement; the unit of phenolsulfatase is defined as the quantity of enzyme which produces a color intensity equivalent to that of 10 γ of *p*-nitrophenol in 10 hours at 37°, when placed into 10 ml. 0.5*N* acetate buffer at pH 5.8 with 0.005*M* substrate.

2. DISTRIBUTION

Phenolsulfatase is present in lower plants (*Aspergillus oryzae*[10]), in lower animals (molluscs[13]), and in higher animals (in most of the organs of the rat, guinea pig, dog, pig, calf, and man). The livers, adrenals, and kidneys are generally the most abundant sources of the enzyme.[14-16] According to Huggins and Smith,[12] phenolsulfatase is found in rat neoplasms in significantly higher amounts than in normal tissues. Thus, a pleomorphic transmissible rat sarcoma contains 0.8 to 1.7 units of phenolsulfatase per mg., while the striated muscles and connective tissues, considered to be the tissues of origin for this type of tumor, contain, respectively, only 0.2 and 0.02 units of the enzyme.

[10] C. Neuberg and J. Wagner, *Biochem. Z.* **161**, 492 (1925).
[11] C. Neuberg and E. Hofmann, *Naturwissenschaften* **19**, 484 (1931).
[12] C. Huggins and D. R. Smith, *J. Biol. Chem.* **170**, 391 (1947).
[13] T. Soda, *J. Fac. Sciences Tokyo* (I) **3**, 149 (1936).
[14] C. Neuberg and E. Simon, *Biochem. Z.* **156**, 365 (1925).
[15] C. Hommerberg, *Z. physiol. chem.* **200**, 69 (1931).
[16] L. Rosenfeld, *Biochem. Z.* **157**, 434 (1925).

3. Methods of Preparation

Phenolsulfatase, of plant as well as of animal origin, is not tightly bound to the solid portions of the cell. This fact makes it possible to obtain enzyme preparations which, in relation to their total mass, are richer in sulfatase than simply ground tissues or even taka-diastase. Concerning the latter preparation, Neuberg and Wagner[10] showed that the maceration juice obtained by leaving taka-diastase in aqueous solution for some time, then clarifying by filtration, possessed the same hydrolytic activity as a corresponding amount of taka-diastase itself. Concerning the sulfatase of animal organs, Neuberg and Simon[14] established that it likewise is not dependent on the presence of intact cells of these organs; these authors indeed obtained active enzyme preparations either as dry powders derived from ground tissues, treated with ethanol and diethyl ether or with acetone, or as solutions prepared by extraction of the various tissues with water containing some toluene, followed by centrifugation of the aqueous suspension of the tissues. Later, Hommerberg[15] studied the purification of phenolsulfatase by various procedures. But up to the present time no extended purification of the enzyme has really been achieved.

4. Specificity

Chemical Specificity. Fromageot[4] has brought together the whole series of the various sulfuric esters which have been studied from the point of view of their hydrolyzability by phenolsulfatase. The results demonstrate that sulfuric esters, in order to be hydrolyzed by phenolsulfatase, not only must possess an aromatic nucleus, but it is also necessary that the esterification from which they result be between the sulfuric acid and a hydroxyl group directly attached to the nucleus (phenolic hydroxyl group).

Optical Specificity. Weinmann[17] was the first investigator to attempt to obtain evidence of stereochemical specificity of phenolsulfatase by allowing the enzyme to act on DL-*m*-methylcyclohexyl-*p*-phenolsulfuric ester; this investigation did not reveal any difference in the rates of hydrolysis of the D- and L-forms. On the other hand, the stereochemical specificity of the action of phenolsulfatase is clearly apparent during the hydrolysis of DL-*p*-*sec*-butylphenolsulfuric ester, carried out by Fromageot[18]; the phenol liberated by this hydrolysis is indeed dextrorotatory, and this is true of the action of plant sulfatase (taka-diastase) as well as of animal sulfatase (ground pig liver).

5. Kinetics

Influence of pH. The effect of pH on the rate of hydrolysis of potassium *p*-nitrophenyl sulfate by the phenolsulfatase of taka-diastase has been

[17] F. Weinmann, *Biochem. Z.* **205**, 214 (1929).
[18] C. Fromageot, *Biochem. Z.* **208**, 482 (1929).

studied by Huggins and Smith[12] in acetate (pH 4 to 6.6) and phosphate (pH 6 to 8.12) buffers. Enzyme activity is greater in acetate buffer, pH being equal; it reaches a maximum at pH 6.12, when the buffer system is made up of a mixture of 0.5*N* sodium acetate–acetic acid.

Influence of Substrate Concentration. The rate of enzyme action as a function of substrate concentration follows the law of Michaelis and Menten. The maximum rate is attained at a substrate concentration of 0.01*M*. The Michaelis constant (at 37° and pH 5.8) is $0.7 \times 10^{-4}M$.

III. Glucosulfatase

Glucosulfatase was discovered by Soda and Hattori[19,20] in snails belonging to the genus *Eulota*. This enzyme hydrolyzes glucose-6-sulfuric ester according to the reaction:

$$C_6H_{11}O_5\text{—}O\text{—}SO_2K + H_2O \rightarrow C_6H_{12}O_6 + KHSO_4$$

which has been established by determination of the glucose and sulfuric acid produced.[21] In addition to a series of publications by Soda and collaborators, glucosulfatase was the subject of a general work[13] which brought together the results of each of the separate publications.

1. Methods of Investigation

The typical substrate for the study of glucosulfatase is glucosesulfuric ester, which is used as the potassium, sodium, or barium salt; it is conveniently obtained by the method indicated by Soda.[22] To study its enzymatic hydrolysis, a solution of the ester is mixed with the enzyme preparation, calcium carbonate to maintain the neutrality of the medium, and 1 to 2% chloroform; the mixture is maintained at a temperature of 35°. The course of the reaction is followed by determining either the sulfuric acid liberated into the medium or the sulfuric acid which remains esterified; the latter is separated by hydrolysis with hydrochloric acid. The following indirect method[23] may also be used: since the pH optimum of glucosulfatase is close to 5, the enzyme is allowed to act on the barium salt of glucosesulfuric ester in a buffer solution made up of one part 2*N* acetic acid and 5 parts 2*N* neutral barium acetate (pH 5.2). The reaction:

$$(C_6H_{11}O_6SO_3)_2Ba + 2\,H_2O + (CH_3COO)_2Ba \rightarrow 2\,C_6H_{12}O_6 + 2\,BaSO_4 + 2\,CH_3COOH$$

liberates acetic acid, which is easily titrated. It is evident that the Warburg manometric method may be employed here to good advantage.

[19] T. Soda and C. Hattori, *Proc. Imp. Acad. Tokyo* **7**, 269 (1931).
[20] T. Soda and C. Hattori, *Bull. Chem. Soc. Japan* **6**, 258 (1931).
[21] T. Soda and F. Egami, *Bull. Chem. Soc. Japan* **8**, 148 (1933).
[22] T. Soda, *Bull. Chem. Soc. Japan* **8**, 37 (1933).
[23] T. Soda and F. Egami, *J. Chem. Soc. Japan* **55**, 256 (1934).

2. Distribution

Glucosulfatase is found in bacteria belonging to the group of *Pseudomonas eisenbergii*.[24] Among the lower animals, it is present in many molluscs, arthropods, and echinoderms.[25-27] A general article by Fromageot[4] contains a list of these animals with their relative contents of enzymes.

3. Method of Preparation

The best preparations of glucosulfatase have been obtained from the liver of *Charonia lampas*.[13] Soda and Egami[21,28] described a method of purification of the enzyme which yielded a product 20 times more active than the initial preparation. This method, which takes into account the fact that phosphates are inhibitory to glucosulfatase, results among other things in the elimination of phosphates, which are always present, to a greater or lesser degree, in the enzyme solution. To 40 ml. of autolyzate prepared from 20 g. acetone-dried liver and 100 ml. water are added 2 ml. 2*N* ammonia, 4 ml. 12% sodium sulfate, and 8 ml. 10% barium chloride; the pH of the solution rises to 9.5–10.0. The precipitate which forms is eliminated by centrifugation; the clear liquid is neutralized with acetic acid. This solution is then treated with 4 ml. aluminum hydroxide gel and with 2 g. activated charcoal.

4. Specificity

Besides glucosesulfuric ester, the glucosulfatase of animal origin is capable of hydrolyzing various sulfuric esters of carbohydrates, mono-, di-, or even tetrasulfuric esters of galactose, saccharose, maltose, etc.[4,13,28] Soda, Hattori, and Terasaki[29] isolated from molluscs (*Charonia*) a white, fat-free powder consisting of a very impure natural sulfuric ester which could be hydrolyzed, at least in part, by glucosulfatase. On the other hand, the hydrolysis of chondroitinsulfuric acid is essentially nil under the same conditions which bring about almost 80% hydrolysis of glucose sulfate. Glucosulfatase and chondrosulfatase are, therefore, different enzymes.

On the other hand, the glucosulfatase of bacterial origin is capable of hydrolyzing the sulfuric esters of glucose and saccharose as well as chondroitinsulfuric acid.[24]

5. Kinetics

Influence of pH. With purified preparations obtained from *Charonia* liver, and using acetic acid – barium acetate buffer solutions, Soda[13,27] found that the pH optimum of the action of glucosulfatase is close to 5.

[21] B. Tanko, *Biochem. Z.* **247**, 486 (1932).
[24] T. Soda and C. Hattori, *J. Chem. Soc. Japan* **54**, 59 (1933).
[26] T. Soda and C. Hattori, *Bull. Chem. Soc. Japan* **8**, 65 (1933).
[27] T. Soda and S. Koyama, *J. Chem. Soc. Japan* **56**, 1338 (1935).
[28] T. Soda and F. Egami, *J. Chem. Soc. Japan* **54**, 1069 (1933).
[29] T. Soda, C. Hattori, and H. Terasaki, *J. Chem. Soc. Japan* **57**, 981 (1936).

Influence of Various Substances. The action of glucosulfatase is inhibited by fluorides, phosphates, borates, and sulfates.[13,30] The inhibition by fluorides is of particular importance; that by sulfates is the weakest. A 0.5*M* concentration of borates or phosphates is sufficient to stop hydrolysis entirely.

Soda[13,31] furthermore compared the effect of a series of sugars on the rate of hydrolysis by glucosulfatase.[4] Among the hexoses, glucose causes the strongest inhibition, but among all the sugars, maltose and cellobiose have an inhibitory effect of distinctly greater magnitude than glucose.

6. Glucosulfatase and Phenolsulfatase

The distinction between glucosulfatase and phenolsulfatase is based on the following observations: (*1*) Glucosulfatase is labile in alkaline medium; it loses most of its activity if it is maintained for some hours near pH 8; under these conditions, phenolsulfatase is entirely stable. (*2*) The pH optimum of phenolsulfatase is 6.1 while that of glucosulfatase is near 5. (*3*) The two enzymes are adsorbed in a different manner on activated charcoal and, under suitable conditions, the adsorption of phenolsulfatase may be considered selective compared to that of glucosulfatase. (*4*) The activity of glucosulfatase is considerably decreased in a 0.5*M* glucose solution while that of phenolsulfatase is either unchanged or significantly increased under the same conditions.[13] (*5*) Soda and Kawamatsu[32] showed that although the concentrations of glucosulfatase and phenolsulfatase are of the same order of magnitude in a series of invertebrates, there are certain molluscs which contain a sizable amount of glucosulfatase but practically none of phenolsulfatase.

IV. Chondrosulfatase

The existence of a chondrosulfatase has been probable since 1914 when Neuberg and Rubin[33] established the formation, among other products, of inorganic sulfate from chondroitinsulfuric acid by the action of putrefactive bacteria. It was not until 1931, however, that Neuberg and Hofmann[7,11] demonstrated the existence of chondrosulfatase by obtaining a preparation from an organism related to *Pseudomonas eisenbergii*; this preparation was capable of separating quantitatively the sulfate radical of chondroitinsulfuric acid, according to the reaction: potassium chondroitinsulfate $\rightarrow$ 2 $KHSO_4$ + degradation products of the chondroitin radical (acetic acid, reducing substances, etc.).

1. Methods of Investigation

Since chondrosulfatase acts on chondroitin- or mucoitinsulfuric acid, the investigation of this enzyme requires the preparation of one or the

[30] T. Soda and F. Egami, *J. Chem. Soc. Japan* **55**, 1164 (1934).
[31] T. Soda, *J. Chem. Soc. Japan* **57**, 599 (1936).
[32] T. Soda and S. Kawamatsu, *J. Chem. Soc. Japan*, **57**, 597 (1936).
[33] C. Neuberg and O. Rubin, *Biochem. Z.* **67**, 82 (1914).

other of these acids. The preparation of chondroitinsulfuric acid is carried out by combining the methods described by Levene[34] and Sawjalow.[35] Mucoitinsulfuric acid is obtained either by following the technique reported by Neuberg and Cahill[36] starting with umbilical cords (100 cords give about 5 g. of a product containing neither inorganic sulfate nor reducing substances) or according to the method of Levene and Lopez-Suarez[37] starting with beef eyes (using the corneas and lenses).

As has been indicated for the other sulfatases, the action of chondrosulfatase may be followed by determining hydrolyzed sulfuric acid; however, in contrast to the preceding instances, this determination must be made directly, by assay of the liberated sulfuric acid, and cannot be carried out indirectly, by assay of the sulfuric acid which remains attached to the organic radical. The reason for this restriction is the fact that chondroitinsulfuric or mucoitinsulfuric acids, which serve as substrate for the enzyme, are precipitated by the reagents used to eliminate sulfate and phosphate ions and proteins from the medium, as required in the indirect assay.

2. Distribution

Chondrosulfatase has been shown to be present in several species of bacteria: *Proteus vulgaris*, *Pseudomonas aeruginosa*, and an organism isolated by Neuberg and Hofmann[11] and related to *Pseudomonas eisenbergii*. Chondrosulfatase is not present in the molds of the type *Aspergillus* or in higher plants. It does not appear to be present either in the higher animals.

3. Methods of Preparation

Bacteria containing chondrosulfatase are grown under suitable conditions,[11] collected and washed with distilled water; when treated with acetone–ether, they yield a powder from which the enzyme may be extracted by maceration for 3 days at 25° with 20 times by weight of water in the presence of toluene. Centrifugation yields a clear liquid, active on chondroitinsulfuric acid but still containing several other enzymes.

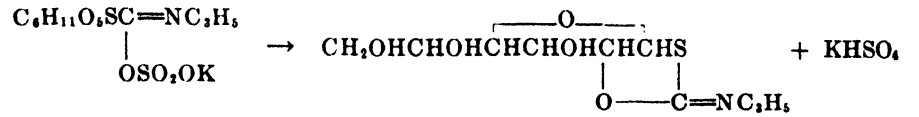
4. Specificity

Preparations of chondrosulfatase attack chondroitinsulfuric acid and mucoitinsulfuric acid; they also hydrolyze glucosesulfuric acid, but the reasons which establish the fact that glucosulfatase and chondrosulfatase are two different enzymes have been indicated (Sect. III-4). On the other hand, chondrosulfatase does not attack phenolsulfuric acid or sinigrin.

[34] P. A. Levene, Monographs of the Rockefeller Institute for Medical Research, No. 18, 1922.
[35] W. Sawjalow, *Z. physiol. Chem.* **126**, 219 (1923).
[36] C. Neuberg and W. L. Cahill, *Enzymologia* **1**, 22 (1936).
[37] P. A. Levene and J. López-Suárez, *J. Biol. Chem.* **36**, 105 (1918).

V. Myrosulfatase

Neuberg and Wagner[6] found an enzyme in animal tissues which detached the sulfate radical of sinigrin (potassium myronate) without causing the slightest hydrolysis of the glucosidic linkage:



Sinigrin Merosinigrin

This is the enzyme which Neuberg and Wagner called myrosulfatase.

1. Methods of Investigation

The typical substrate of myrosulfatase is sinigrin; this substance is prepared to the method of Schneider and Wrede[38] and is obtained entirely pure after recrystallization from alcohol according to the technique described by Neuberg and Wagner.[5]

The action of myrosulfatase is followed by determining the sulfuric radical which remains attached to the organic radical of the substrate, as with phenolsulfatase and glucosulfatase; the elimination of released sulfate, phosphates, etc. from the medium must be carried out always by precipitation with a solution of barium acetate rather than with an alkaline solution of barium chloride, since the latter significantly decomposes potassium myronate even in the cold. The remainder of the procedure follows the details given for phenolsulfatase.

2. Distribution

Myrosulfatase is not present in molds of the type *Aspergillus*[5]; it has not yet been encountered in bacteria. In higher plants, it is present in the Cruciferae, where together with thioglucosidase it constitutes myrosinase.[39] In higher animals, it is found in the liver, muscle, and kidney of the horse and the rabbit.[6]

3. Methods of Preparation

The myrosulfatase preparations obtained up to the present time from animal tissues consist simply of these tissues ground and used fresh; the maceration liquid obtained from the same tissues does not contain any myrosulfatase.

In order to obtain the enzyme from the Cruciferae it is necessary to

[38] W. Schneider and F. Wrede, *Ber.* **47**, 2225 (1914).
[39] C. Neuberg and O. von Schönebeck, *Biochem. Z.* **265**, 223 (1933).

separate it from the thioglucosidase. First of all, an aqueous solution of myrosinase is prepared according to the technique described by Neuberg and Wagner,[5] by aqueous extraction of white mustard seeds followed by precipitation of the enzyme with 90% ethanol and washing of the precipitate with 70% ethanol; the washing is repeated several times, and the activity of the precipitate is significantly increased by this step. Finally, the washed residue is redissolved in water. In order to separate myrosulfatase and thioglucosidase in this solution, Neuberg and Schönebeck[33] make use of adsorption phenomena, employing successively kaolin, iron hydroxide, different varieties of alumina and "osmosil." These authors also make use of the fact that myrosulfatase may be precipitated by mercury acetate and lead phosphate, and that it is easily eluted with secondary sodium phosphate. Neuberg and Schönebeck have thus been able to obtain from the crude myrosinase an enzyme which in 24 hours detaches 80% of the sulfuric radical or sinigrin without attacking the thioglucosidic linkage.

4. Specificity

It is probable that myrosulfatase is capable of detaching the sulfate radical not only of sinigrin but also of analogous substances such as sinalbin and glucocheiroline.[4]

CHAPTER 14

Invertase

BY CARL NEUBERG AND INES MANDL

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I. Historical

Invertase has been known the longest of all the carbohydrases. Its history goes back to 1828, when Dumas and Boullay[1] showed that sucrose

[1] J. Dumas and P. Boullay, *Ann. chim. et phys.* **37**, 45 (1828).

fermentation by yeast takes place only when one molecule of water is taken up. The inverting action of yeast was discovered by Persoz in 1833. In 1860 Berthelot[2] isolated the enzyme by alcohol precipitation and gave it the name "*ferment inversif*," whence the present terms "invertase" and "invertin" are derived. Other synonyms are "sucrase" and "saccharase."

Two types of invertase can be distinguished according to whether the fructose or glucose end of the molecule is attacked: fructosidase (fructofuranosidase, β-*h*-fructosidase) and glucosidase (α-*n*-glucosidoinvertase, α-glucopyranosidase) (see pages 542–546).

II. Occurrence

1. Yeasts

Both types are contained in ordinary yeast,[3,4] which is the main source of invertase. It is present in *Saccharomyces carlsbergensis* and *S. cerevisiae*, and occurs in most baker's, brewer's, and distiller's, and even in lactose-fermenting yeasts; however, several *Saccharomyces* varieties (*S. albicans*, *S. octosporus*, *S. apiculatus*, and some *Torula* varieties) are said to be invertase free. The large excess present in yeast cells may be a symptom of physiological hypertrophy and does not seem to have any function. The hydrolysis of raffinose by the enzyme of *Schizosaccharomyces octosporus*, which does not attack saccharose but was claimed to be saccharase by Lindner,[5] may be due to an α-galactosidase. Organisms (*S. exiguus*, *S. ludwigii*, *Zygosaccharomyces marxianus*) which contain invertase but lack maltase will not ferment maltose, which shows that invertase and maltase could hardly be identical (see also p. 543).

2. Other Cryptogams

Invertase has been detected in several other cryptogams.[6–9] (See also p. 1322 of Bamann-Myrbäck[10]). According to von Euler *et al.*[11] *Penicillium glaucum* contains glucoinvertase while *Aspergilli* show fructoinvertase activity. The presence of fructosidase in *Aspergillus oryzae* has been proved,[11–14] but different conditions of culture may lead to adaptive enzyme formation.

[2] M. Berthelot, *Compt. rend.* **50**, 980 (1860).
[3] H. von Euler and S. Asarnoj, *Fermentforschung* **3**, 318 (1920).
[4] H. von Euler, *Fermentforschung* **4**, 242 (1920).
[5] P. Lindner, *Wochschr. Brau.* **17**, 173 (1900).
[6] A. Béchamp, *Compt. rend.* **46**, 44 (1358).
[7] U. Gayon, *Compt. rend.* **86**, 52 (1878).
[8] C. Kosmann, *Bull. soc. chim.* (N.S.) **27**, 251 (1877).
[9] E. Bourquelot, *J. pharm. chim.* (6) **16**, 578 (1902).
[10] E. Bamann and K. Myrbäck, Die Methoden der Fermentforschung. Thieme, Leipzig, 1941; Academic Press, New York, 1945.
[11] H. von Euler, K. Josephson, and B. Soederling, *Z. physiol. Chem.* **139**, 1 (1924).
[12] H. Amelung, *Z. physiol. Chem.* **187**, 171 (1930).
[13] E. Hofmann, *Biochem. Z.* **273**, 198 (1934).
[14] O. Guagnini and D. Jacovkis, *Industria y quimica Buenos Aires* **9**, 207 (1947).

Pringsheim *et al.*[15] state that *Aspergillus wentii* sometimes contains fructoinvertase, sometimes glucoinvertase. *A. niger*[13] forms fructosidase exclusively when grown on raffinose, but melibiase as well in Raulin's medium and no invertase at all unless the culture medium contains sucrose or raffinose.[16–18] Most invertase is produced if the culture medium contains sucrose and inulin; abnormally high quantities are found when potassium or magnesium is lacking.[19] Generally the invertase content of molds is lower than that of yeasts. In molds as in yeasts invertase is dissolved within the cell but not secreted by it, *i.e.*, an endoenzyme.[20] According to Wasserzug,[21] however, fusaria secrete invertase after the development of the conidia. In wine[22] and in beer[23] autolysis of the dying yeast cells is responsible for the presence of invertase. In must invertase is derived from both yeast and grapes. Similarly cell damage caused by prolonged autolysis results in the presence of invertase in older culture fluids.[24] Invertase dissolved in wine is stable for prolonged periods.[25]

3. Bacteria and Other Microorganisms

Green algae[26] and many bacteria contain invertase, *i.e.*, acetic acid and lactic acid bacteria, though not all strains. The amounts vary in *Escherichia coli* but are rather uniform in *Thermobacterium mobile* and sulfur bacteria.[27] The lower the nitrogen content of the *E. coli* the lower the saccharase activity.[28] Invertase has also been demonstrated in several pathogenic bacteria (*Vibrio cholerae*, streptococci, and pneumococci) (see also p. 1213 of Bamann-Myrbäck[10]).

4. Plants

Blossoms,[29, 30] leaves, and fruits[31–33] of most higher plants contain in-

[15] H. Pringsheim, H. Borchardt, and F. Loew, *Z. physiol. Chem.* **202**, 23 (1931)
[16] Z. I. Kertész, *Fermentforschung* **9**, 300 (1928).
[17] Z. I. Kertész, *Plant Physiol.* **6**, 249 (1931).
[18] Z. I. Kertész, *Chem. Centr.* **1932, I**, 686.
[19] G. von Doby and Z. I. Kertész, *Z. physiol. Chem.* **189**, 177 (1930).
[20] R. K. Saksena and S. K. Bose, *J. Indian Botan. Soc.* **23**, 108 (1944); *Chem. Abstracts* **42**, 4206 (1948).
[21] E. Wasserzug, *Ann. Inst. Pasteur* **1**, 525 (1886); *Chem. Centr.* **1888, II**, 974.
[22] H. Müller-Thurgau, *Landw. Jahrb.* **14**, 909 (1885).
[23] A. Bau, *Wochschr. Brau.* **19**, 41 (1902).
[24] A. Fernbach, *in* Oppenheimer, Die Fermente. 5th ed., Vol. I, G. Thieme, Leipzig, 1925, p. 550.
[25] C. von der Heide, *Z. Untersuch. Lebensm.* **57**, 13 (1929).
[26] K. Sjoeberg, *Fermentforschung* **4**, 97 (1920).
[27] S. Forssman, *Biochem. Z.* **264**, 231 (1933). E. Hofmann, *Biochem. Z.* **273**, 198 (1934).
[28] A. I. Virtanen and J. Ley, *Arch. Biochem.* **16**, 169 (1948).
[29] A. Béchamp, *Compt. rend.* **59**, 496 (1864).
[30] V. Plouvier and A. Sosa, *Bull. soc. chim. biol.* **30**, 273 (1948); V. Plouvier, *Ann. sci. nat. Botan. et biol. végétale* **3**, 204 (1942).
[31] L. Rosenthaler, *Fermentforschung* **8**, 282 (1925).
[32] A. Sosa-Bourdouil, *Compt. rend.* **224**, 1651 (1947).
[33] J. S. Trifu, *Polytechnica Bucuresti Facultatea Agronom.* **20**, 3 (1945); *Chem. Abstracts* **42**, 2399 (1948).

vertase, which is reported to be an endoenzyme also in plastids.[34] A significant amount is present in Gramineae[35] and it increases in the barley and tomato plant during ripening.[36–38] In sugar beet invertase occurs in stem and leaves. For distribution in the roots, see Wanner and Leopold.[39] The mineral nutrients exert a definite influence on the invertase content of plants[10, 40] (*cf.* page 529).

5. Invertebrates

In invertebrates extracts,[41, 42] blood, eggs,[43] saliva, intestines, and particularly the honey bladder of bees[44, 45] were found to contain invertase. Primitive animals such as the jelly fish[46] also contain the enzyme. Honey invertase may not be identical with yeast invertase.[47–50] It has no effect on raffinose,[49, 50] which points to a glucoinvertase.

6. Vertebrates

The same is true for the invertase of higher animals which does not cleave raffinose, is inhibited by α-glucose, and appears to be glucoinvertase exclusively. In contrast to invertebrates, neither gastric juice[51] nor saliva[52] of vertebrates contain saccharase (but, see Jona[53] and Vandevelde[54] for contrary claims). In adults invertase is not a metabolic enzyme, since sucrose injected parenterally is eliminated unchanged. Its presence in the intestines

[34] N. M. Sisakyan and A. M. Kobyakova, *Biokhimiya* **13**, 83 (1948); *Chem. Abstracts* **42**, 7841 (1948).
[35] J. O'Sullivan, *Proc. Chem. Soc.* **16**, 61 (1900).
[36] J. Kjeldahl, *in* von Lippmann, Die Chemie der Zuckerarten. Fr. Vieweg u. Sohn, Braunschweig, 1904, p. 1304. M. Gonnermann, *ibid.*, p. 1044.
[37] H. K. Archbold, *Biochem. J.* **34**, 749 (1940).
[38] L. F. Bailey and J. S. McHargue, *Am. J. Botany* **30**, 763 (1943).
[39] H. Wanner and U. Leopold, *Ber. schweiz. botan. Ges.* **57**, 153 (1947); *Chem. Abstracts*, **42**, 6390 (1948).
[40] L. F. Bailey and J. S. McHargue, *Plant Physiol.* **19**, 105 (1944).
[41] E. Bourquelot, *Compt. rend.* **126**, 1015 (1898).
[42] H. Bierry, *Compt. rend.* **148**, 949 (1909); **152**, 465, 904 (1911).
[43] R. Kobert, *Arch. ges. Physiol.* (*Pflüger's*) **92**, 116 (1903); *Biol. Zentr.* **2**, 37 (1904).
[44] Erlenmeyer, *Münch. Akademie der Wissenschaften* **1874**, 205.
[45] Erlenmeyer, *Malys Jahresber. Tierchemie* **1875**, 270.
[46] F. Haurowitz, and H. Waelsch, *Z. physiol. Chem.* **161**, 300 (1926).
[47] J. M. Nelson *et al.*, *J. Biol. Chem.* **61**, 193 (1924).
[48] J. M. Nelson, *et al.*, *J. Biol. Chem.* **62**, 139 (1924).
[49] P. E. Papadakis, *J. Biol. Chem.* **83**, 581 (1929).
[50] P. E. Papadakis, Dissertation, Columbia University, New York, 1929.
[51] G. Lusk, *Proc. Am. J. Physiol.* **10**, xxi–xxii (1903–1904).
[52] H. T. Brown and J. Heron, *Ann.* **204**, 223 (1880).
[53] J. L. Jona, *Proc. J. Physiol.* **40**, xxi–xxii (1910).
[54] A. J. J. Vandevelde, *Biochem. Z.* **23**, 324 (1909).

of the fetus[58] and the stillborn[58–60] show that it is not due to ingested food. As early as 1878[61, 62] it had been described in intestinal extracts where its rough localization was determined some 40 years later.[63–66] Apparently none is present in the small intestine of cattle.[67] Invertase found in certain cysts of the ovaries in humans[68] also points to an origin in the intestines since these are pathological structures derived from fetal endoderm.

Human invertase is much more thermolabile than yeast invertase.[69] The mucosa of intestines of pigeons[70] and the gizzard of chickens also contain invertase. Its presence in leucocytes[71] is still contested.[72] It is said to occur occasionally in the blood of young animals[73, 74] after sucrose injections, but this effect may be due[63–65] to gradual mobilization of the invertase of the intestine rather than to a new formation.

III. Separation

Numerous methods of separating and purifying invertase have been published, but as yet no pure or crystalline invertase has been obtained. The purest has recently been prepared by Sumner and O'Kane.[75]

1. Yeast Invertase

a. Plasmolysis and Autolysis

Essentially all methods are based on Salkowski's discovery of autolysis in 1889,[76–79] *i.e.*, the aseptic self-digestion of which all cells are capable. Any

[58] K. Miura, *Z. Biol.* **32**, 266 (1895); *Chem. Centr.* **1895**, II, 231.
[59] M. F. L., Keene, and E. E. Hewer, *Chem. Centr.* **1929**, I, 3109.
[60] T. Tachibana, *Japan. J. Obstet. Gynecol.* **12**, 21 (1929).
[61] T. Tachibana, *Japan. J. Obstet. Gynecol.* **12**, 40 (1929).
[62] T. Tachibana, *Ber. ges. Physiol. u. exptl. Pharmakol.* **53**, 115 (1930).
[63] T. Tachibana, *Ber. ges. Physiol. u. exptl. Pharmakol.* **54**, 756 (1930).
[64] V. Paschutin, *in* Gillespie, The Natural History of Digestion. W. Scott, Ltd. London, 1898, p. 16.
[65] C. Bernard, Leçons sur le Diabète et la Glycogenèse animale, Baillière., Paris, 1877, p. 259.
[66] F. Röhmann, *Biochem. Z.* **61**, 464 (1914).
[67] F. Röhmann, *Biochem. Z.* **72**, 26 (1916).
[68] F. Röhmann, *Biochem. Z.* **84**, 382 (1917).
[69] H. von Euler and O. Svanberg, *Z. physiol. Chem.* **115**, 43 (1921).
[70] E. Fischer and J. Niebel, *Sitzbe . preuss. Akad. Wiss. physik. math. Klasse.* 1896, 30; *Chem. Centr.* **1896**, I, 499.
[71] P. Lecène and H. Bierry, *Compt. rend.* **177**, 222 (1923).
[72] H. von Euler and K. Myrbäck, *Z. physiol. Chem.* **115**, 68 (1921).
[73] A. Bernardi and M. A. Schwarz, *Biochem. Z.* **256**, 406 (1932).
[74] C. H. Boissevain, *Chem. Abstracts* **12**, 2356 (1918).
[75] R. S. Morris and T. R. Boggs, *Arch. Internal Med.* **8**, 806 (1911).
[76] E. Weinland, *Z. Biol.* **47**, 279 (1906).
[77] E. Abderhalden *et al.*, *Z. physiol. Chem.* **69**, 23, 1910; **90**, 419 (1914).
[78] J. B. Sumner and D. J. O'Kane, *Enzymologia* **13**, 251 (1948).
[79] E. Salkowski, *Arch. path. Anat. Physiol. (Virchow's)* **70**, 158 (1877).
[77] E. Salkowski, *Z. physiol. Chem.* **13**, 506 (1889).
[78] E. Salkowski, *Z. klin. Med.* **17**, 77 (1890).
[79] E. Salkowski, *Malys Jahresber. Tierchem.* **1890**, 454.

decrease of surface tension of the surrounding medium may cause plasmolysis and leakage of invertase from the yeast cells,[80] but the release of an enzyme is not merely a matter of mechanical opening of the cell walls and liquefaction of the cell contents. Simultaneous degradation of the substances loosely combined with the invertase sets free the enzyme but causes contamination.

Besides the plasmolyzing agents introduced by Hoppe-Seyler[81] and Salkowski,[76–79, 82, 83] *i.e.*, diethyl ether and chloroform, toluene and ethyl acetate are most frequently used, but benzene, xylene, carbon disulfide, carbon tetrachloride, isosulfocyanates, amyl alcohol, and benzyl alcohol were also applied.[84] Decreasing the surface tension of water to half results in plasmolysis and the agent need not be a fat solvent. Glycerol[85–91] and even sucrose [92, 93] and soluble polysaccharides such as gum arabic have been used.

The first fundamental progress can be ascribed to the method of slow autolysis devised by O'Sullivan and Tompson,[94] while a substantial simplification was introduced by Hudson[95, 96] through his rapid liquefaction by the use of cell poisons such as toluene. Autolyzates of this type contain considerable amounts of native proteins, nucleoproteins, their degradation products, and polysaccharides, especially gum from the yeast.

Generally slow autolysis methods are obsolete today and have been replaced by rapid autolysis of ordinary or stimulated yeasts. Preparatory treatment with sucrose may increase the enzyme content threefold.[97, 98] Any zymohexose acts as stimulating agent; mannose is said to be superior to other monosaccharides.[99, 100] Probably general anabolic activity increases invertase as the cell substance. Much higher accumulation of invertase is, however, brought about by stimulation of the yeast with small

[80] B. Kisch, *Biochem. Z.* **40**, 152 (1912).
[81] F. Hoppe-Seyler, *Ber.* **4**, 810 (1871).
[82] E. Salkowski, *Z. physiol. Chem.* **31**, 305 (1900–1901).
[83] E. Salkowski, *Z. physiol. Chem.* **61**, 124 (1909).
[84] C. Neuberg and H. Lustig, *Arch. Biochem.* **1**, 211 (1942).
[85] C. Naegeli and O. Löw, *Sitzber. math. naturw. Kl., Bayer. Akad. Wiss.* München, **1878**, 177.
[86] v. Wittich, *Arch. ges. Physiol.* (*Pflüger's*) **2**, 193 (1869).
[87] v. Wittich, *Arch. ges. Physiol.* (*Pflüger's*) **3**, 339 (1870).
[88] v. Wittich, *Arch. ges. Physiol.* (*Pflüger's*) **5**, 435 (1872).
[89] G. Hüfner, *J. prakt. Chem.* (N. F.) **5**, 372 (1872).
[90] I. W. Gunning, *Ber.* **5**, 821 (1872).
[91] C. Nägeli and O. Löw, *Ann.* **193**, 339 (1878).
[92] C. J. Lintner, *Chem. Centr.* **1900**, I, 54.
[93] W. Issaew, *Z. ges. Brauw.* **23**, 796 (1900).
[94] C. O'Sullivan and F. W. Tompson, *J. Chem. Soc.* **57**, 834 (1890).
[95] C. S. Hudson, *J. Am. Chem. Soc.* **30**, 1564 (1908).
[96] C. S. Hudson, *J. Am. Chem. Soc.* **36**, 1566 (1914).
[97] H. von Euler and H. Cramér, *Biochem. Z.* **58**, 467 (1914).
[98] H. von Euler and H. Cramér, *Z. physiol. Chem.* **89**, 272 (1914).
[99] J. Meisenheimer, S. Gamberjan, and L. Semper, *Biochem. Z.* **54**, 122 (1913).
[100] J. Meisenheimer, S. Gamberjan, and L. Semper, *Biochem. Z.* **67**, 364 (1914).

quantities of sucrose during fermentation[101,102] rather than pretreatment with large quantities. Relatively purer solutions of invertase are obtained by fractional autolysis at neutral reaction. In all cases pH control by addition of ammonia or diammonium phosphate is advantageous.

b. Purification

Once an invertase solution containing a minimum of impurities is obtained, the enzyme must be isolated by adsorption and eluted from the adsorbate. Both procedures are complicated and must be adapted to the purpose. Adsorption of fructosidase on aluminum hydroxide C[103] either from the untreated autolyzate or from a dialyzed autolyzate which has previously been freed of protein may yield preparations of considerable purity. The precipitable protein can be removed by fractional precipitation with ethanol, addition of dilute acetic or sulfuric acid, or by lead acetate,[104] though normal lead acetate is said partially to precipitate invertase from yeast extracts,[105] which may be due to absorption by the lead phosphate formed. Monsel's salt (basic ferric sulfate) or aluminum hydroxide,[106–110] which may suitably be replaced by fuller's earth or bentonite may also be used. In strong ethanol or acetone, kaolin and various modifications of aluminum hydroxide adsorb little or no invertase. Ferric hydroxide[111] and its colloidal form[112–114] as well as hematite, the natural ferric hydroxide, are equally suitable. Precipitation[115,116] of the fructosidase from rapid autolyzates by strontium hydroxide at about pH 8, or ethanol under carefully controlled conditions[117] have been used. Uranyl acetate has also been applied[118–122] for purification of the enzymes. An enzymatic digestion

[101] R. Willstätter, C. D. Lowry, Jr., and K. Schneider, *Z. physiol. Chem.* **146**, 158 (1925).
[102] R. Willstätter and C. D. Lowry, Jr., *Z. physiol. Chem.* **150**, 287 (1925).
[103] H. Albers and I. Meyer *Z. physiol. Chem.* **228**, 122 (1934).
[104] F. Hofmeister, *Z. physiol. Ch m.* **2**, 288 (1878–1879).
[105] M. Hahn, *in* Buchner, Zymasegärung. R. Oldenbourg. Munich and Berlin, 1903, p. 325.
[106] L. Michaelis and M. Ehrenreich, *Biochem. Z.* **57**, 70 (1913).
[107] L. Michaelis, *Biochem. Z.* **7**, 488 (1908).
[108] L. Michaelis, *Biochem. Z.* **10**, 283 (1908).
[109] L. Michaelis and P. Rona, *Biochem. Z.* **25**, 359 (1910).
[110] L. Michaelis and P. Rona, *Biochem. Z.* **115**, 269 (1921).
[111] P. Cornette, *Chem. Ztg.* **22**, Rept. 79 (1898).
[112] L. Michaelis and P. Rona, *Biochem. Z.* **7**, 329 (1907).
[113] L. Michaelis and P. Rona, *Biochem. Z.* **8**, 356 (1908).
[114] L. Michaelis and P. Rona, *Biochem. Z.* **16**, 60 (1909).
[115] R. Weidenhagen, *Z. Ver. deut. Zuckerind.* **86**, 473 (1936).
[116] R. Weidenhagen and L. Nenninger, *Z. Ver. deut. Zuckerind.* **89**, 149 (1939).
[117] H. von Euler and K. Josephson, *Ber.* **56**, 446 (1923).
[118] M. Jacoby, *Z. physiol. Chem.* **30**, 135 (1900).
[119] K. Glässner, *Hofmeisters Beitr. chem. physiol. Pathol.* **1**, 1 (1902).
[120] H. Rosell, Thesis, Strassburg, 1901.
[121] R. Willstätter, Untersuchungen über Enzyme, Springer, Berlin, 1928, p. 589.
[122] E. I. Kritschewskaja, *Biochem. Z.* **272**, 348 (1934).

method[123] is more complicated and gives less pure invertase. Yet another preliminary purification is carried out by ethanol, lead acetate, or tannic acid.[124] When the contaminating protein has been removed, optimal selective adsorption of the invertase on aluminum hydroxide under slightly acid conditions[125] in a solution containing acetone or on kaolin or lead phosphate[126] and optimum conditions for elution by sucrose, dilute ammonia, or salts such as sodium carbonate or disodium phosphate must be established. Adsorption methods on bentonite[126] or zinc sulfide[127] have been worked out by Hudson and coworkers (fermenting yeast strongly adsorbs added invertase[127a]). Generally adsorption is fairly rapid but elution, which also shows greater dependence on temperature, takes a measurable length of time.

Numerous methods have been evolved for the purification of invertase and the reader is referred to recent reviews[10,128,129] for greater details.

The complete elimination of the accompanying compounds formed by cellular enzymes during autolysis presents a problem of preparative enzyme chemistry which as yet has not been completely solved. Elimination of glycogen presents no difficulties since it is hydrolyzed during autolysis, but considerable quantities of yeast gum, a glucomannan, passes into the autolysate. By rapid extraction of fresh yeast with chloroform water Salkowski[82,83] succeeded in obtaining an invertase preparation free of gum and at the same time refuted the theory that invertase was nothing but a polysaccharide. Willstätter[130–136] effected the separation by an adsorption technique. Adsorption also causes partial preferential elimination of inactive enzyme from purified and concentrated invertase solutions.[124] Most recently Sumner[75] found that yeast gum could be washed out with water from a tricalcium phosphate adsorbate thus causing a 1500–2500-fold purification or more on an ash-free basis and 3000-fold purification by a second adsorption, giving the purest invertase preparation obtained so far. Concavalin A, a crystalline jackbean globulin known to precipitate

[123] W. Grassmann and T. Peters, *Z. physiol. Chem.* **204**, 135 (1932).
[124] R. Willstätter, K. Schneider, and E. Wenzel, *Z. physiol. Chem.* **151**, 1 (1926).
[125] H. Kraut and E. Wenzel, *Z. physiol. Chem.* **142**, 71 (1925).
[126] M. Adams and C. S. Hudson, *J. Am. Chem. Soc.* **60**, 982 (1938).
[127] N. K. Richtmyer and C. S. Hudson, *J. Am. Chem. Soc.* **60**, 983 (1937).
[127a] A. I. Oparin, *Chem. Abstracts* **43**, 8003 (1949).
[128] R. Willstätter, Untersuchungen über Enzyme, Vols. I, II, Springer, Berlin, 1928. K. Schneider, *in* Oppenheimer, Die Fermente und ihre Wirkungen; Vol. III, pp. 756–854, 1929.
[129] C. Neuberg and I. S. Roberts, Invertase. Sugar Research Foundation Scientific Rept. No. 4, New York, 1946.
[130] R. Willstätter and K. Schneider, *Z. physiol. Chem.* **133**, 193 (1924).
[131] R. Willstätter and K. Schneider, *Z. physiol. Chem.* **142**, 257 (1925).
[132] R. Willstätter, K. Schneider, and E. Bamann, *Z. physiol. Chem.* **147**, 248 (1925).
[133] R. Willstätter, *Ber.* **59**, 1591 (1926).
[134] R. Willstätter, J. Graser, and R. Kuhn, *Z. physiol. Chem.* **123**, 1 (1922).
[135] R. Willstätter and F. Racke, *Ann.* **425**, 1 (1921).
[136] R. Willstätter and F. Racke, *Ann.* **427**, 11 (1922).

glycogen, precipitates the yeast gum and also saccharase itself, whence Sumner concludes that saccharase is most likely a protein–carbohydrate.

Loss of protective colloids, poisoning of the adsorbent surface, storage, other enzymes, as well as dialysis, electrodialysis, concentration, and light may account for this partial destruction of invertase. At best purified invertase cannot be stored for more than 6 weeks without loss of activity, but crude enzyme solutions retain their activity for decades (19 years).[137,138] While sensitivity increases with dilution, concentration or preparation of dry material also causes considerable loss. Pure enzyme cannot be preserved even in sealed glass tubes in the dark at 0°.

2. Glucoinvertase

Separation of glucoinvertase has been investigated to a much lesser extent. Inert material may be precipitated with lead acetate and superficial purification accomplished by dialysis and low temperature concentration.[139] Dialysis of aqueous solutions of taka-diastase against ethanol or acetone[140] has been reported but some kinds seem to be sensitive toward these solvents.[139]

IV. Properties

By determining its rate of diffusion the molecular weight of invertase was found to be greater than 20,000. Moelwyn-Hughes[141] gives it as 60,000. More recently Dieu[142] by electrophoretic studies and diffusion reports that at least two components are involved, an active substance of an approximate molecular weight of 3500 and a much larger enzymatically inactive moiety of a molecular weight of approximately 100,000. Separation is said to be difficult on account of the instability of the dilute solution. The isoelectric point lies at pH 5.

Analyses show variable nitrogen content between 6.6 and 12.7% and only part of it in the form of amino nitrogen, which led Willstätter and coworkers[130–133] to doubt the protein nature of the enzyme. Ash of from 0.1 to 0.25% may be due to minerals forming part of the molecule or to impurities. Traces of phosphorus are also present. Some preparations are said[143] not to give the biuret reaction. Polypeptide character has not been disputed, however; both peptides and amino acids increase the stability of invertase solutions.[134] Invertase practically free of tryptophan or tyrosine has been prepared by both Willstätter *et al.*[130–133] and Albers[103] although

[137] C. Neuberg, *Biochem. Z.* **56**, 495 (1913).
[138] C. Neuberg, *Biochem. Z.* **238**, 251 (1931).
[139] N. Takatomi, *Chem. Centr.* **1935**, I, 532.
[140] J. Feigenbaum, *Nature* **150**, 318 (1942).
[141] E. A. Moelwyn-Hughes, *Ergeb. Enzymforsch.* **2**, 1 (1933).
[142] H. Dieu, *Bull. soc. chim. biol.* **28**, 543 (1946); *Bull. soc. chim. Belg.* **55**, 327 (1947).
[143] B. Hafner, *Z. physiol. Chem.* **42**, 1 (1904).

von Euler and Josephson[144-147] considered the tryptophan content characteristic of the enzyme; at one time, by an obviously mistaken deduction,[148] tryptophan was even stipulated as the enzyme carrier. The importance of the tyrosyl group appears to follow from the partial inactivation of saccharase by iodine and by tyrosinase (see page 538 and 539).

V. Stability

As mentioned in Sect. III-1-*b*, invertase is surprisingly stable when protected by the accompanying substances present in yeast,[137,138,148a] probably colloids of the yeast juice which remain unchanged on autolysis, although the possibility of protective carbohydrates must also be considered. The lability of invertase will as a rule increase with purification. Part of the enzyme may be irreversibly inactivated during adsorption on kaolin.

1. pH Dependence

In alkaline solution purified preparations rapidly lose activity, but if invertase is not separated from the cells sucrose may be completely split at pH values above 8 by fermentation in the presence of sodium sulfite, magnesium oxide, ammonium hydroxide, disodium phosphate, and sodium carbonate to yield glycerol and acetaldehyde.[149-152] Yeast invertase is most active at slightly acid pH[94,153] and most stable at pH 4–5. Careful studies of the optimum conditions[154,155] led to the generally accepted value of pH 4.5, but the divergence at pH 3.5 or 5.5 is small, *i.e.*, the optimum range is rather wide. Invertases from *Aspergillus oryzae* or intestinal tissue have a higher optimum pH of 6–8. Invertases of different origin show optimum values[156] ranging from 4.2 to 7 and shifts have frequently been observed.[157]

2. Temperature Dependence

The optimum temperature for formation of invertase in yeast is[99,100,158] approximately the same as for cell growth and proliferation, 16–30°. When

[144] H. von Euler and K. Josephson, *Ber.* **56**, 1097 (1923).
[145] H. von Euler and K. Josephson, *Ber.* **57**, 299 (1924).
[146] H. von Euler and K. Josephson, *Ber.* **57**, 859 (1924).
[147] K. Josephson, *Z. physiol. Chem.* **136**, 224 (1924).
[148] G. Gorbach and K. Lerch, *Biochem. Z.* **219**, 122 (1930).
[148a] C. Henberg and I. S. Roberts, *Antonie van Leeuwenhoek J. Microbiol. Serol.* **12**, 78 (1947).
[149] C. Neuberg and E. Reinfurth, *Biochem. Z.* **89**, 365 (1918).
[150] C. Neuberg and J. Hirsch, *Biochem. Z.* **96**, 175 (1919).
[151] C. Neuberg and J. Hirsch, *Biochem. Z.* **100**, 304 (1919).
[152] C. Neuberg and J. Hirsch, *Biochem. Z.* **105**, 307 (1920).
[153] A. Fernbach, *Ann. inst. Pasteur* **4**, 1 (1890).
[154] A. Kanitz, *Arch. ges. Physiol.* (*Pflüger's*) **100**, 547 (1903).
[155] F. Steward, *Biochem. J.* **6**, 131 (1911).
[156] E. Hofmann, *Biochem. Z.* **275**, 320, 1934/5.
[157] L. Frankenthal, *Exptl. Med. and Surg.* **2**, 229 (1944).
[158] H. von Euler, *Biochem. Z.* **85**, 406 (1918).

dry, invertase is extremely resistant to heat. According to Salkowski[82,83] it may be heated to 105° or even to 145–160°; heating *in vacuo*[159] to 140–150° will not destroy all the invertase. In solution, heating to 60° leads to destruction of about half the activity. According to von Euler and Laurin[160] the energy for heat inactivation of saccharase at pH 4 is 101,000 cal./mole. A compilation of energy and entropy of inactivation values can be found elsewhere.[161,162] Reversible heat denaturation at 50° in acid solution has been reported by Chase *et al.*[163] Apparently the "raffinase" component of the enzyme is the more sensitive.[164] Von Euler[165] maintains that a 40% increase in activity may occasionally though not always be effected by heating dialyzed yeast invertase. Crude invertase precipitated by ethanol was found to withstand[166] a 45-minute exposure at −191°. The rate of hydrolysis increases with temperature[167] and is 3.5 times greater at 45° than at 35°.

Effects of temperature and pressure on invertase kinetics ($E_{act.}$) are discussed in Section IX.

3. Irradiation Effects

The damaging action of ultraviolet light on invertase was recognized at an early date by Downes and Blunt[168] and has been the subject of many later investigations.[169] Agulhon[170,171] in his attempt to classify enzymes by their behavior under irradiation states that saccharase is not attacked by visible light in vacuum, but that ultraviolet light below 3022 Å attacks the enzyme, although less than in the presence of oxygen. Inactivation under nitrogen[172] and at different pH values[172,173] has been reported. Short activations which were said to occur at the start of irradiation of crude solutions[172] or by intermittent irradiation[174–176] may be due to destruction of inhibitors. On the other hand postirradiation effects are attributed to the

[159] E. Buchner, *Ber.* **30**, 1110 (1897).
[160] H. von Euler and I. Laurin, *Z. physiol. Chem.* **110**, 55 (1920).
[161] H. Eyring and A. E. Stearn, *Chem. Revs.* **24**, 253 (1939).
[162] I. W. Sizer, *Advances in Enzymol.* **3**, 35 (1943).
[163] A. M. Chase, E. H. Reppert, and R. M. Ruch, *J. Cellular Comp. Physiol.*, **23** V, (1944).
[164] J. M. Nelson and P. E. Papadakis, *J. Biol. Chem.* **80**, 163 (1928).
[165] H. von Euler and J. Lindstål, *Svensk Kem. Tid.* **37**, 18 (1925); *Chem. Centr.* **1925**, **I**, 1793.
[166] E. Pozerski, *Compt. rend. soc. biol.* **52**, 714 (1900).
[167] A. N. Kapanna and I. G. Shrikhanda, *Chem. Centr.* **1932**, **II**, 1005.
[168] A. Downes and T. B. Blunt, *Proc. Roy. Soc. London* **28**, 199 (1879).
[169] A. Fernbach, *Ann. inst. Pasteur* **3**, 473 (1889); A. Chauchard and B. Mazoué, *Compt. rend.* **152**, 1709 (1911;) J. Giaja, *Compt. rend. soc. biol.* **72**, 2 (1912).
[170] H. Agulhon, *Compt. rend.* **152**, 398 (1911).
[171] H. Agulhon, *Compt. rend.* **153**, 979 (1911).
[172] O. Svanberg, *Arkiv. Kemi Mineral. Geol.* **8**, 1 (1921).
[173] G. Gorbach and H. Pick, *Monatshefte* **61**, 29 (1932).
[174] G. Gorbach *et al.*, *Biochem. Z.* **219**, 122 (1930).
[175] G. Gorbach *et al.*, *Biochem. Z.* **235**, 259 (1931).
[176] G. Gorbach *et al.*, *Biochem. Z.* **271**, 338 (1934).

accompanying impurities, *e.g.*, yeast gum, and tryptophan.[177,178] Yeast- and taka-invertase behave differently in this respect.

α- and β-radiations of radium have a damaging action on invertase,[179,180] but X-rays were reported by Emmerling[181] to be ineffective.

4. Inorganic Inhibitors

The loss of activity in oxidizing solutions has not been fully explained as yet. Sizer[182] considers it due to high oxidation potential, not a toxic effect on substrate, and finds inhibition of the enzyme irreversible and independent of purity. Reducing agents such as hydrogen sulfide and sodium thiosulfate are said to cause a slight increase in activity[183]; generally, invertase is stable over a wide range of oxidation-reduction potentials.

Inactivation by metal salts has been studied extensively.[184] Silver and mercury salts cause reversible inactivation,[185,186] the activity being re-established by dialysis or addition of substances which combine with silver or mercury, thus bringing about a spontaneous self-regeneration through displacement of the impure enzyme bound to the metal. (This phenomenon is similar to the well known Danysc effect.) In the absence of impurities this effect can no longer be observed. In the presence of saccharose the metal ion apparently does not reach the active group of the invertase molecule and is therefore protected to a considerable extent. Silver is believed to form the normal salt, and mercury a complex with the basic groups of the enzyme. The effect of nitrous acid was interpreted similarly as interaction with the amino group.[187–189] The amino group is attacked when not combined and thus protected by carbohydrate, and the enzyme–substrate complex cannot be formed. Zinc nitrate[190] and copper salts inactivate equally. Fluorides which inhibit fermentation by yeast are said not to affect invertase.[101,102,190v,191] Iodine inhibits by a different mechanism, but even large quantities will not inactivate more than 50% at room temperature. A new fairly stable substance, iodoinvertase, with an activity about 60% of the original enzyme, is believed to be formed, presumably by iodination of tyrosine and other cyclic compounds. In this connection

[177] G. Goldhaber and J. Leibowitz, *Nature* **152**, 274 (1943).
[178] G. Gorbach and D. Kimovec, *Monatshefte* **61**, 39 (1932).
[179] R. G. Hussey and W. R. Thompson, *J. Gen. Physiol.* **9**, 211 (1925).
[180] G. A. Bonino and V. Mazzucchetti, *Chem. Centr.* **1927**, I, 2084.
[181] O. Emmerling, *Ber.* **34**, 3811 (1901).
[182] I. W. Sizer, *J. Gen. Physiol.* **25**, 399 (1942).
[183] R. Itoh and F. Obo, *Brit. Chem. Physiol. Abstracts* **AIII**, 686 (1940).
[184] H. von Euler and O. Svanberg, *Fermentforschung* **3**, 330 (1920).
[185] M. Jacoby, *Biochem. Z.* **181**, 194 (1927).
[186] M. Jacoby, *Fermentforschung* **10**, 1 (1928).
[187] K. Myrbäck, *Z. physiol. Chem.* **158**, 160 (1926).
[188] K. Myrbäck, *Z. physiol. Chem.* **159**, 1 (1926).
[189] J. B. Sumner and K. Myrbäck, *Z. physiol. Chem.* **189**, 218 (1930).
[190] E. Boeri, *Enzymologia* **12**, 114 (1947).
[191] J. Courtois, *Enzymologia* **11**, 313 (1945).

interest attaches to the recent findings[192] that active tyrosinase (probably by oxidation of essential tyrosyl groups) inactivates invertase by 10–43%.

5. Organic Inhibitors

a. Aliphatic

Among organic inhibitors acetone, glycerol, and alcohol are of particular importance in view of their preparative application. Acetone yields poisonous diacetoneamine with the ammonium ions present in the enzyme solution[193]; glycerol inactivates only in high concentration,[194] possibly by inhibiting the decomposition of the enzyme–substrate complex. In dilute solutions glycerol actually acts as a stabilizer.[86–88] Ethanol may completely inactivate invertase.[82,83,94,195,196] Since it slows down all forms of sucrose hydrolysis[197] an effect on the substrate for the system must be involved. Maximum inactivation takes place at 50%, while absolute ethanol is rather inactive. Sucrose protects the enzyme and here pH and temperature play a part. No destruction occurs at −20° at a slightly acid pH maintained, *e.g.*, by acetic acid.[130,131] At the isoelectric point inactivation is at a maximum.[198,199] Strict observance of temperature and pH is especially important for preliminary purification of fructosidase by ethanol precipitation.[117] Precipitation at +37° yields an enzyme preparation which has less than 1% of the activity of a material prepared at +7°.

b. Aromatic

Other organic inhibitors include amines like aniline, *o*-, *m*-, and *p*-toluidine, the three chloro and bromoanilines, aminobenzoic acids, and sulfanilic acid,[187–189,200,201] which are believed to react with a carbonyl group,[187–189] similarly to phenylhydrazine and aminoguanidine, which equally have an inhibiting effect. (Semicarbazide reacts sluggishly; the behavior of hydroxylamine, hydrogen cyanide, potassium cyanide, and sodium sulfite[187–189,202] also agrees with a carbohydrate character, as postulated for invertase by Myrbäck.[187–189]) Decomposition of the substrate—enzyme complex rather than its formation appears to be inhibited by combination of the amine with the complex with an affinity equal to that between amine and enzyme.

[192] I. W. Sizer, *Science*, **108**, 335 (1948).

[193] R. Willstätter, Untersuchungen über Enzyme. Springer, Berlin, 1923, pp. 572–573.

[194] E. Bourquelot, *Compt. rend.* **165**, 557 (1917).

[195] A. Mayer, Die Lehre von den chemischen Fermenten oder Enzymologie. Winter's Verlag, Heidelberg, 1882, p. 79.

[196] J. Sestri *et al.*, *Indian Inst. Sci.* **II(A)**, Part I, 1 (1923).

[197] C. S. Hudson and A. S. Paine, *J. Am. Chem. Soc.* **32**, 1350 (1910).

[198] J. M. Nelson and E. T. Palmer, *J. Biol. Chem.* **93**, Lxxviii (1931).

[199] J. M. Nelson, E. T. Palmer, and B. G. Wilks, *J. Gen. Physiol.* **15**, 401 (1932).

[200] H. von Euler, *Z. physiol. Chem.* **125**, 207 (1922–1923).

[201] H. von Euler, L. Ahlström, and B. Högberg, *Arkiv Kemi, Mineral. Geol.* **17A**, No. 20 (1944); *Chem. Abstracts* **39**, 3311 (1945).

[202] E. Rona, *Biochem. Z.* **109**, 279 (1920).

Thiamine has also been claimed as an inhibitor.[203] Yeast invertase is blocked by atropine, cocaine, and pilocarpine.[203a] Inhibition by adrenaline is prevented by tyrosinase, which oxidizes adrenaline to a quinone derivative.[203b]

c. Dyes

Dilute solutions of dyes (Congo red, fuchsin, safranine)[204–206] seem to combine loosely with the enzyme, but are displaced on addition of sucrose. The damaging action of photodynamic dyes,[207–209] reversible inactivation by both basic and acid dyes, and also a competition of different sugars with dyestuffs for invertase has been reported.[206] The action of picric acid and also aniline is discussed in detail by Bamann and Myrbäck.[10] *m*-Nitrophenol also inhibits.[190]

d. Sugars

The most interesting inhibitors are the cleavage products of sucrose itself.[210–212] Both α- and β-fructose, but not α-glucose, inhibit yeast invertase. β-glucose inhibits fructosidase; α-glucose, but not fructose, inhibits taka-invertase. Carbohydrates other than those obtained by cleavage of sucrose also inhibit, *e.g.*, α- and β-galactose, α- and β-arabinose, α- and β-methyl glucosides,[213,214] and β-rhamnose.[215–217] The α-forms are more effective than the β-forms but the differentiation is neither clear cut nor regular. The explanation originally advanced—*i.e.*, that inactivation of fructoinvertase by fructose and glucoinvertase by glucose is due to preferential combination of the enzyme with the monosaccharide rather than the original substrate—is undoubtedly an oversimplification. The inhibitions may be competitive inhibitions of affinity or nonspecific (noncompetitive).

Attempts have been made to gain some insight into the structure of invertase from a consideration of the mechanism of inhibition.

[203] A. A. Titaev, *Biokhimiya* **13**, 197 (1948); *Chem. Abstracts* **42**, 8303 (1948).
[203a] P. Karrer, *Chemistry and Industry* **1949**, 634.
[203b] I. W. Sizer, *Am. Brewer*, **82**, 24 (July, 1949).
[204] S. S. Mereshkowsky, *Chem. Ztg.* **27**, Rept. 271 (1903).
[205] S. S. Mereshkowsky, *Z. Bact.* (2) **11**, 33 (1903).
[206] J. H. Quastel and E. D. Yates, *Enzymologia* **1**, 60 (1936–1937).
[207] H. von Tappeiner, *Ber.* **36**, 3035 (1903).
[208] H. von Tappeiner, *Ergeb. Physiol.* 8, 713 (1909).
[209] A. Jodlbauer, *Biochem. Z.* **3**, 488 (1907).
[210] V. Henri, *Compt. rend.* **135**, 916 (1902).
[211] H. E. Armstrong and E. F. Armstrong, *Proc. Roy. Soc. London* **79B**, 360 (1907).
[212] L. Michaelis and H. Pechstein, *Biochem. Z.* **60**, 79 (1914).
[213] R. Kuhn and H. Münch, *Z. physiol. Chem.* **150**, 220 (1925).
[214] R. Kuhn and H. Münch, *Z. physiol. Chem.* **163**, 1 (1927).
[215] H. von Euler and K. Josephson, *Z. physiol. Chem.* **132**, 301 (1924).
[216] H. von Euler and K. Josephson, *Z. physiol. Chem.* **152**, 31 (1926).
[217] K. Josephson, *Z. physiol. Chem.* **136**, 62 (1924).

VI. Configuration

From investigations of the pH dependence of activity[146,218] it appears that invertase is a weak acid,[219,220] with a carboxyl and a basic amino group. Possibly the latter is involved in the formation, the former in the hydrolysis of the enzyme–substrate complex. Furthermore the presence of a carbonyl group has been suspected.[187–189] Enzyme poisons react with these groupings which may form part of the pheron or agon (see below). Where there is no apparent affinity a retardation of the decomposition of the enzyme–substrate complex has been postulated.[212]

The concept of an agon—the active group of the enzyme—and a pheron or carrier, both being integral parts of the system, is related to the theory of the protein or polypeptide nature of invertase.[221] The agon and the pheron may combine to form a specific protein, or the agon may merely be adsorbed by the pheron. The pheron is a protein or at least a nitrogen-containing ampholyte. Carbohydrate character has been ascribed to the agon.[187–189] Accompanying substances may be unessential or may be part of the system in the form of excess pheron. If the agon can combine to form active complexes with various pherons, the differences in the behavior of various invertases may lose much of their significance. The same concept could also form the basis of an explanation of the existence of different invertases. Fructo- and glucoinvertase may contain the same agon but different pherons or at least different accompanying substances. There would be no reason that both types of invertase should not coexist in the same kind of cell. Transitional forms attacking both substrates at an atypical pH can likewise be accounted for, since the proteinlike pheron can combine with a large variety of substances, while pheronlike compounds may act as colloidal stabilizers protecting the enzyme from inactivation. Replacement of the typical carrier by an atypical one formed the basis of the earlier theory of Fodor and Epstein,[222] who distinguished a zymohaptic, i.e., functional group, and a carrier and explained the lesser stability of highly purified invertase solutions by a decrease in stability caused by such replacement. The fact that neutral autolysis yielded preparations containing proteins and carbohydrates, while invertase of the same activity obtained by weakly acid autolysis at pH 5 showed negative or indefinite protein reactions though positive tests for their degradation products, points to the existence of mixed carrier complexes.[223] Nelson and Saul[223a] found that proteins, both natural and denatured, accelerate the activity

[218] H. von Euler, *Ber.* **59**, 1129 (1926).
[219] L. Michaelis and M. L. Menten, *Biochem. Z.* **49**, 333 (1913).
[220] L. Michaelis, *Biochem. Z.* **115**, 269 (1921).
[221] H. Kraut and W. von Patschenko-Jurewicz, *Biochem. Z.* **275**, 114 (1934).
[222] A. Fodor and C. Epstein, *Z. physiol. Chem.* **167**, 1 (1927).
[223] J. G. Lutz and J. M. Nelson, *J. Biol. Chem.* **107**, 169 (1934).
[223a] J. M. Nelson and E. L. Saul, *J. Am. Chem. Soc.* **56**, 1994 (1934); *J. Biol. Chem.* **111**, 95 (1935).

of purified yeast invertase at pH 3, but accelerate to a lesser extent or not at all at its optimum pH 4.5. Herriott[223b] had shown that the enzyme, when inactivated by acid, may be reactivated on standing at pH 6–7. Wagreich *et al.*[224] studied the effect of a number of proteins and amino acids on the reactivated invertase and the process of reactivation. Reactivation was inhibited by trypsin, pepsin, chymotrypsin and to a lesser degree by some, though not all, foreign proteins. The activity of purified reactivated invertase was accelerated like that of native one. Amino acids not containing —SH or —S.S— groups had no effect; cysteine accelerated, cystine inhibited reactivation. The increase in activity of very pure invertase preparations on addition of native protein may also be due to pheron addition. The decrease in solubility in water as inactivation of the enzyme progresses may be interpreted as separation of the agon from the carrier, which becomes insoluble due to denaturation. Invertase is characterized as a polysaccharide–protein by Sumner and O'Kane,[75] while Gortner and Dieu[225] simply assume a microprotein, and Adams and Hudson[226] a carbohydrate–protein. It is suggested by Quastel and Yates (206) that invertase acts as a zwitterion whose oppositely charged groups are bridged by saccharose, the glucose moiety being attached to the anion, the fructose moiety to the cation.

VII. Specificity

There is no doubt that two isodynamic invertases exist; both hydrolyze sucrose, but β-*h*-fructosidase, or yeast invertase, attacks the fructose part, while α-*n*-glucosidoinvertase or taka-invertase attacks the glucose end of the molecule. Whereas in sucrose both ends are open to attack, the glucose end is blocked by the linkage with galactose in raffinose (fructosylglucosylgalactoside = saccharose-α-galactoside).[227,228] That is why raffinose resists taka-invertase but is hydrolyzed into fructose and melibiose (galactosylglucose) by yeast invertase. Similarly α-*n*-glucoinvertase does not attack gentianose (sucrose-β-glucopyranoside), in which the functional glucose is linked by a β-glucosidic bond while melezitose (turanose-α-glucopyranoside) is hydrolyzed by an α-D-glucosidase into glucose and turanose[229,230] but resists the β-fructosidase of yeast.[231,232] The effect of saccharase on the

[223b] R. M. Herriott, Thesis, Columbia Univ., 1932.
[224] H. Wagreich, L. I. Schwartz, and H. Kamin, *J. Gen. Physiol.* **25**, 207 (1941); H. Wagreich, W. Halpert, and A. Hirschman, *J. Gen. Physiol.* **26**, 479 (1943).
[225] E. Gortner and H. Dieu, *Ned. Akad. Wetenschapen* **50**, 325 (1947); *Chem. Abstracts* **42**, 939 (1948).
[226] M. Adams and C. S. Hudson, *J. Am. Chem. Soc.* **65**, 1359 (1943).
[227] C. Neuberg, *Biochem. Z.* **3**, 519 (1907).
[228] C. Neuberg and F. Marx, *Biochem. Z.* **3**, 535 (1907).
[229] C. S. Hudson, *J. Org. Chem.* **9**, 117: 470 (1944).
[230] H. S. Isbell, *J. Research Natl. Bur. Standards* **26**, 35 (1941).
[231] R. Kuhn and G. E. von Grundherr, *Ber.* **59**, 1655 (1926).
[232] C. S. Hudson, *Advances in Carbohydrate Chem.* **2**, 2 (1946).

pentasaccharide verbascose (trigalactosido-glucosido-fructose) yielding fructose[232a] has been reported.

This differentiation of two forms of invertase is due to Kuhn[232–236] but has been contested by Weidenhagen,[237–239] who maintains that the α-glucopyranosidase is identical with maltase. His main argument is the inverting power of maltase at pH 7 after its purification to a degree which leaves it inactive toward sucrose at the optimum pH for β-fructoinvertase, i.e., pH 4.5. Glucoinvertases are most active[240] at pH 6–8 with the exception of that of barley malt, which, like β-*h*-fructosidases, is most effective at pH 4.5 to 5. Separations of the invertase and maltase from yeast extracts by adsorption of the maltase on kaolin[241] or meta-aluminum hydroxide[242] have been described, but Weidenhagen asserts[237–239] that these methods bring about separation of fructosidase and maltase only, not of invertase and maltase. Weidenhagen's view has not been generally accepted, however. Several authors[13,243–251] have reported experiments which contradict his theory, in particular Hofmann,[13,243] who successfully separated the two enzymes from *Schizosaccharomyces octosporus Beijerinck* and isolated a maltase which did not attack sucrose at any pH although it completely hydrolyzed maltose. Neutral autolysis leaves invertase in the cell debris. Recently Hofmann and Scheck[243a] also obtained enzyme preparations (autolysis juices and acetone dried powders) from animal organs, such as liver, kidneys and spleen; they readily attack maltose, but do not cleave saccharose. Enzyme preparations derived from haploid hybrid Saccharomyces strains of genetically defined constitution according to Lindegren,

[232a] S. Murakami, *Chem. Abstracts* **34**, 3694 (1940).
[233] R. Kuhn, *Z. physiol. Chem.* **125**, 23 (1923).
[234] R. Kuhn, *Z. physiol. Chem.* **127**, 234 (1923).
[235] R. Kuhn, *Z. physiol. Chem.* **129**, 57 (1923).
[236] R. Kuhn, *Naturwissenschaften* **11**, 732 (1923).
[237] R. Weidenhagen, *Ergeb. Enzymforsch.* **1**, 163 (1932).
[238] R. Weidenhagen, *Ergeb. Enzymforsch.* **2**, 90 (1933).
[239] R. Weidenhagen, *Handbuch der biochemischen Arbeitsmethoden*, Abt. IV, **2**, 2051 (1933).
[240] G. Bertrand and Rosenblatt, *Compt. rend.* **156**, 261 (1913).
[241] L. Michaelis and P. Rona, *Biochem. Z.* **57**, 70 (1913).
[242] R. Willstätter and E. Bamann, *Z. physiol. Chem.* **151**, 273 (1926).
[243] E. Hofmann, *Biochem. Z.* **272**, 417 (1934).
[243a] E. Hofmann and H. Scheck, *Biochem. Z.* **320**, 34 (1949). See also E. Hofmann and E. Latzko, *Biochem. Z.* **320**, 269 (1950).
[244] N. N. Iwanoff, E. W. Dodonowa, and W. J. Tschastuchin, *Fermentforschung* **11**, 433 (1930).
[245] L. Zechmeister, W. Grassmann, *et al.*, *Ann.* **502**, 20 (1933).
[246] W. Grassmann, L. Zeichmeister, *et al.*, *Ann.* **503**, 167 (1933).
[247] J. M. Nelson and M. P. Schubert, *J. Am. Chem. Soc.* **50**, 2188 (1925).
[248] M. Hotchkiss, *J. Bact.* **29**, 391 (1935).
[249] H. Tauber and I. S. Kleiner, *J. Gen. Physiol.* **16**, 763 (1933).
[250] H. Tauber and I. S. Kleiner, *J. Biol. Chem.* **99**, 241 (1933).
[251] H. Tauber and I. S. Kleiner, *J. Biol. Chem.* **105**, 679 (1934).

Lindegren, and Hestrin[252] have provided a specific invertase which acts on saccharose but not on raffinose or on maltose. Feigenbaum[253,254] distinguished maltase and taka-invertase as two different enzymes by means of $Na_2S_2O_4$ which destroys taka-invertase but does not attack the maltase. Other workers (Karström, Myrbäck, and Virtanen) who found maltases of different origin without effect on saccharose and invertase without effect on maltose or α-glucosides are quoted by Hofmann[243]. Maltases from saliva, pancreatic extract, or other animal tissues do not split sucrose or raffinose at any pH. The digestive tract of *Helix pomatia* does, however, contain an enzyme which hydrolyzes both sucrose and raffinose. Gentianose is split into gentiobiose and fructose by the digestive juice of invertebrates.[41]

Extension of Weidenhagen's concept to tri- and polysaccharides has also been disputed. While he maintains that turanose and melezitose are hydrolyzed by the same α-glucosidase and raffinose, gentianose, and inulin by the same fructosidase, trehalose, an α-glucoside, resists the action of α-glucosidase and all invertases. (The cleavage of trehalose is attributed to a special enzyme, trehalase.)

According to Leibowitz and Mechlinski[255] invertase should act on raffinose only after it has been enzymatically split into galactose and sucrose. In the case of taka-diastase lack of melibiase is alleged to prevent hydrolysis, but Weidenhagen[237-239] demonstrated that melibiase-free taka-invertase directly attacks raffinose. Kuhn[233-236] denies this for the general case in which only fructoinvertase is effective, but occasionally he too observed hydrolysis. Since commercial taka-diastase preparations are by no means uniform conclusive results can as a rule not be expected unless the material is characterized by the investigator.

In general the question whether hydrolysis of tri- and polysaccharides requires special enzymes basically different from invertase has not been solved. Quantitative changes might be expected according to whether the third component is a sugar, as in raffinose, or an aglucon (*e.g.*, a phosphate radical), as in sucrose phosphates, or has a methyl group replacing glucose, as in the case of methyl-β-fructopyranoside. Sucrose phosphate is split by fructoinvertase free of phosphatase, yielding free hexose and phosphorylated hexose, but hydrolysis by taka-invertase has also been noted.[256] Probably the substrate is not entirely uniform, part of the phosphoric acid being linked to the fructose end. (See also Courtois.[191]) Carefully purified[257] saccharose monophosphate is hardly attacked by glucoinvertase. Fructose-6-phosphate (Neuberg ester) inhibits hydrolysis by yeast invertase like unsubstituted fructose. Fruc-

[252] C. C. Lindegren, G. Lindegren and S. Hestrin, *Proc. U. S. Natl. Acad. Sci. U. S.* **35**, 23 (1949); S. Hestrin and C. C. Lindegren, *Nature* **165**, 158 (1950).
[253] J. Feigenbaum, *Science*, **96**, 521 (1942).
[254] J. Feigenbaum, *Biochem. J.* **36**, 768 (1942).
[255] J. Leibowitz and P. Mechlinski, *Z. physiol. Chem.* **154**, 64 (1926).
[256] S. Ohmiya, *Japan. J. Biol. Chem.* **18**, 125 (1933).
[257] C. Neuberg and O. Dalmer, *Biochem. Z.* **131**, 188 (1922).

tose-1,6-diphosphate (Harden-Young ester) is active, but to a lesser degree, whereas the Robison ester (glucose-6-phosphate) has no inhibiting effect.

General differentiation of the two invertases by inhibition is unsatisfactory, however,[215–217] since glucose, especially β-glucose, also inhibits fructosidase and differences are often only a matter of degree. Inhibition by glucose but not by fructose is affected by galactose, probably because galactose and glucose have greater affinity for the enzyme than saccharose. Both sucrose monophosphate[213,214,256,258–261] and sucrose monosulfate[262,263] are split by invertase free from phosphatase (resp-sulfatase) and also are fermented by yeast.[262–266] Simple fructosides which may be regarded as sucrose in which the glucose component is replaced by an aglucon, *e.g.*, a methyl group, should be split as well, but in the case of the simple α- or β-methyl fructosides the pyranoid structure does not allow attack by invertase. γ-Methyl fructoside[267, 268, 268a] is a mixture containing some methyl-β-furanoside and is partly split by invertase from yeast and hog liver.[269, 270] The question whether pure methyl fructofuranoside had α[271–274] or β[275] configuration was solved by a determination of the action of yeast invertase on the two forms prepared by complete dephosphorylation of the 1,6-phosphorylated fructosides. The β form was hydrolyzed as its diphosphate ester,[276–278] showing an absolute ring specificity as well as α,β specificity of the enzyme comparable to that found for other carbohydrases. Although pure α-methyl fructoside resists yeast invertase,[276–278] taka-diastase is said to hydrolyze it[275]; however, different preparations behave differently. Since α- and β-methyl fructofuranosides are the simplest substrates for invertase they deserve special attention. The analogous β-benzyl D-fructofuranoside is equally cleavable.[279]

[258] C. Neuberg and H. Pollak, *Biochem. Z.* **23**, 515 (1910).
[259] C. Neuberg and H. Pollak, *Biochem. Z.* **26**, 514 (1910).
[260] C. Neuberg and S. Sabetay, *Biochem. Z.* **162**, 479 (1925).
[261] A. Oparin and A. Kurssanoff, *Biochem. Z.* **239**, 1 (1931).
[262] C. Neuberg and H. Pollak, *Ber.* **43**, 2060 (1910).
[263] C. Neuberg and H. Pollak, *Biochem. Z.* **26**, 526 (1910).
[264] C. Neuberg and L. Liebermann, *Biochem. Z.* **121**, 326 (1921).
[265] T. Soda, *Biochem. Z.* **135**, 621 (1923).
[266] K. Djenab and C. Neuberg, *Biochem. Z.* **82**, 391 (1917).
[267] E. Fischer, *Ber.* **28**, 1160 (1895).
[268] E. Fischer, *Ber.* **47**, 1984 (1914).
[268a] H. Pottevin, *Compt. rend.* **136**, 169 (1903).
[269] R. Kuhn and T. Wagner-Jauregg, *Z. physiol. Chem.* **162**, 103 (1926).
[270] S. Hestrin and S. Avineri-Shapiro, *Biochem. J.* **38**, 3 (1944).
[271] W. T. J. Morgan, *Biochem. J.* **21**, 675 (1927).
[272] W. T. J. Morgan, *Chem. Ind.* **48**, 144 (1929).
[273] R. Robison and W. T. J. Morgan, *Biochem. J.* **22**, 1270 (1928).
[274] W. N. Haworth, C. R. Porter, and A. C. Waine, *J. Chem. Soc.* **1932**, 2254.
[275] H. H. Schlubach and G. Rauchalles, *Ber.* **58**, 1842 (1925).
[276] C. B. Purves and C. S. Hudson, *J. Am. Chem. Soc.* **56**, 702 (1934).
[277] C. B. Purves and C. S. Hudson, *J. Am. Chem. Soc.* **56**, 1969 (1934).
[278] C. B. Purves and C. S. Hudson, *J. Am. Chem. Soc.* **56**, 1973 (1934).
[279] C. B. Purves and C. S. Hudson, *J. Am. Chem. Soc.* **59**, 49 (1937).

The term β-*h*-fructosidase or β-fructofuranosidase for yeast invertase is derived from the specificity of this enzyme for fructose with β configuration and the 2,4-oxygen ring. Berner[280, 280a] claims that inulin is composed of fructofuranose residues. Actually it is not definitely established whether one of the two typical invertases is responsible for the hydrolysis of inulin and irisin. About this question and the existence of a special inulase see Sumner and Somers.[281] Inulin is hydrolyzed about 5000 times more slowly and irisin 400,000 times more slowly than sucrose. Pigman[282] gives the ratio of inulase to invertase activity as 1 to 2800–28,300 in yeasts, and 1 to 5 in *Aspergillus niger*. Asparagose and pseudoasparagose, inulinlike polysaccharides, are hydrolyzed more readily than inulin itself, according to Tanret.[283] The tetrasaccharide stachyose and the trisaccharide gentianose are equally subject to fructosidase hydrolysis. For details on the hydrolysis of stachyose by different yeasts and its effectiveness compared to that of other polysaccharide cleavages the paper of Adams *et al.*[284] should be consulted. Recently glucofructosan from asphodel was reported cleavable.[285]

VIII. Mechanism of Invertase Action

According to Kuhn[233–236] there is no reason why the same fructosidase should not cleave sucrose and raffinose. The affinity of invertase for the substrates will differ, however, and so will the dissociation constants of the complexes formed. The saccharase–sucrose affinity is given as 16 times that of the enzyme to raffinose[233] and pH activity curves below pH 4 differ markedly.[236] Even in simple glucosides the nature of the noncarbohydrate component influences the rate of hydrolysis. The size of the molecule as well as steric factors may play a part. The same applies to glucosidases. Yeast glucosidase hydrolyzes maltose about twice as rapidly as sucrose at pH 6.9. The α-glucosidase from barley malt acts on melezitose as effectively as on saccharose at its optimum pH of 4.5 to 5. Raffinose is but slightly hydrolyzed by this enzyme on account of a small β-*h*-fructosidase content. The mechanism of melezitose and turanose hydrolysis has not been completely elucidated yet (see pages 542–544). Glucosidase splits melezitose, a turanose α-glucopyranoside, into glucose and turanose.[229,230,232] Yeast invertase does not affect it.[231] Turanose, 3-α-glucopyranosylfructose,[232] is said to be hydrolyzed by a special enzyme present in bottom yeast.[287,288] Melezitase activity has been reported for preparations from *Penicillium*

[280] E. Berner, *Ber.* **66**, 1076 (1933).
[280a] E. Berner, *Ann.* **505**, 58 (1933).
[281] J. B. Sumner and G. F. Somers, Chemistry and Methods of Enzymes, 2nd ed., Academic Press, New York 1947, p. 110.
[282] W. W. Pigman, *J. Research Natl. Bur. Standards* **30**, 159 (1943).
[283] G. Tanret, *Bull. soc. chim.* (4) **5**, 899 (1909).
[284] M. Adams, N. K. Richtmyer, and C. S. Hudson, *J. Am. Chem. Soc.* **65**, 1369 (1943).
[285] H. Belval and A. de Grandchamp-Chaudun, *Compt. rend.* **226**, 1753 (1948).
[286] R. Willstätter and R. Kuhn, *Z. physiol. Chem.* **115**, 180 (1921).
[287] M. Bridel, C. Aagaard, and T. Aagaard, *Compt. rend.* **184**, 1667 (1927).
[288] M. Bridel, C. Aagaard, and T. Aagaard, *Compt. rend.* **185**, 147 (1927).

glaucum and *Aspergillus niger*[231] as well as bottom yeast, but not top yeast. Turanose, glucose, and fructose (but not sucrose) have been isolated as products of hydrolysis. A second enzyme seems to be responsible for cleavage of turanose into glucose and fructose; initial attack may take place at the turanose or the sucrose end of the molecule. Bridel and Aagard[287,288] maintain that the glucosyl fructoside bond of melezitose cannot be identical with that of sucrose, but Pringsheim,[289] supporting Kuhn,[231] points out that even when the bond is identical the enzyme substrate affinities may differ; also differences in the action of various preparations on sucrose and melezitose under distinct conditions need not be significant.

It is generally accepted today that only sucrose phosphorylase can synthesize sucrose and other disaccharides[290–293] and claims to the contrary are viewed with some skepticism. Oparin ascribes a synthesizing action to invertase absorbed on cells [294] which has allegedly been confirmed[295] and studied[296] by several Russian authors recently. Polymerative levan sucrase,[240, 297] amylosucrase, and dextran sucrase,[298] although, like invertase they set free monosaccharides, are not "sucrases" in the accepted sense and cannot be considered here.

A temporary combination of enzyme and substrate in all enzyme reactions was clearly demonstrated in 1881 by Wurtz.[299] The catalyst emerged unchanged while the substrate was hydrolyzed.[299–302] The part played in the development of this theory by Duclaux, Bodenstein, Arrhenius, V. Henri, van Slyke, Cullen, *et al.* is described in detail in Oppenheimer's book.[303] From 1899–1911 these men pointed out that enzyme and substrate combine in ratios subject to the law of mass action. In 1913 Michaelis and Menten[213, 220] postulated that the rate of inversion is a measure of the concentration of the labile enzyme-substrate complex. The cleavage products of sucrose inhibit the enzyme,[210–212] but its action is re-established when the retarding agents, first indicated by Tammann[304] in 1892, are eliminated or their concentration lowered by dilution.

[289] H. Pringsheim, Die Polysaccharide. 3rd ed., Springer, Berlin, 1931, p. 49. [290] M. Doudoroff, N. Kaplan, and W. Z. Hassid, *J. Biol. Chem.* **148**, 67 (1943). [291] M. Doudoroff, *J. Biol. Chem.* **151**, 351 (1943). [292] W. Z. Hassid, M. Doudoroff, and H. A. Barker, *J. Am. Chem. Soc.* **66**, 1416 (1944). [293] W. Z. Hassid, M. Doudoroff, H. A. Barker, and W. H. Dore, *J. Am. Chem. Soc.* **67**, 1394 (1945); **68**, 1465 (1946). [294] A. I. Oparin, *Enzymologia* **4**, 13 (1937). [295] A. L. Kursanov, E. Isaeva, and V. Popatenko, *Biokhimiya* **11**, 401 (1946); *Chem. Abstracts* **41**, 2776 (1947). [296] N. N. Sisakyan, et al., *Biokhymia* **11**, 413 (1946); **12**, 7 (1947); **9**, 104 (1944); **9**, 126 (1944); *Chem. Abstracts* **41**, 2776 (1947); **41**, 4830 (1947); **39**, 327 (1945). [297] M. Beijerinck, *Folia Microbiologica* **1**, 377 (1912); *Chem. Abstracts* **8**, 944 (1914). M. Ashner, S. Avineri-Shapiro, and S. Hestrin, *Nature* **149**, 527 (1942); **152**, 49 (1943); *Biochem. J.* **37**, 450 (1943); **39**, 167 (1945). [298] E. J. Hehre *et al.*, *Science* **93**, 237 (1941); *J. Biol. Chem.* **163**, 221 (1946); *Trans. N. Y. Acad. Sci. Ser. II*, 10 188 (1948). See also M. Stacey, *Nature* **149**, 639 (1942); W. G. C. Forsyth and D. M. Webley, *Nature* **162**, 150 (1948). [299] A. Wurtz, *Compt. rend.* **93**, 1104 (1881). [300] M. von Nencki, *Arch. exptl. Path. Pharmakol.* **21**, 299 (1886). [301] R. Wegscheider, *Monatshefte* **21**, 693 (1900). [302] K. P. Brown, *J. Chem. Soc.* **81**, 373 and 388 (1902). [303] C. Oppenheimer, Die Fermente und ihre Wirkunger, Hauptwerk 1, Thieme, Leipzig, 1924–1925, 208–214. [304] G. Tammann, *Ber.* **25**, (Ref. 686) (1892); *Z. physiol. Chem.* **16**, 271 (1892).

IX. Kinetics

Inversion of sucrose by invertase is a first order reaction for at least two thirds of the reaction and the quantity of water present may be considered constant. Deviations from the theoretical curve at the beginning (12%) and end (after 58–83% hydrolysis according to the substrate concentration) have been explained[305] as a lag period required for adsorption of the reagents and inhibition by the products adsorbed on the catalytic surface respectively. In unit time a constant amount is, however, hydrolyzed under given conditions of enzyme and substrate concentration, temperature, and pH. The optimum pH coincides with the isoelectric point or zone since Hudson showed as early as 1910 that only the undissociated part of the enzyme can effect hydrolysis. Invertase is an amphoteric electrolyte[306,307] with an acid dissociation constant of 2×10^{-7} and a basic dissociation constant of 10^{-12}. Degree of purity, state of dispersion, and nature of the invertase preparations do not measurably influence the activity, which is therefore equal for solutions or the enzyme in the living cells, for precipitates, and adsorbates. This may be due to the liberation of the adsorbed enzyme from the carrier by the substrate, as demonstrated by different authors.[106–110,308–313] This eluting effect is distinct from any specific affinity of the invertase for saccharose and takes place also with other sugars and glucosides. The affinity of the glucose and fructose produced by cleavage for the enzyme has no significant influence. The effect of temperature on saccharase kinetics has been investigated by Sizer,[314,315] who found that inversion strictly follows the Arrhenius equation between 0 and 35°. The activation energy of yeast invertase is 11,000 cal./mole between 0 and 17°, is independent of the method of extraction from the cell, and is identical in purified form or in dead or living cells. Above 17° an abrupt decrease to 8300 cal./mole was found in the case of intracellular invertase. Although the same energy of activation would be expected for invertase of different origin, malt invertase shows a higher value of 13,000 cal./mole. The difference is explained by a possible participation of other enzymes, *e.g.*, maltase, in the reaction. Different substrates, *i.e.*, saccharose and raffinose, require the same $E_{act.}$. Changes in enzyme, substrate, or electrolyte concentration or in pH between 3.2 and 7.9 have no effect.[162] The heat of formation of the enzyme–substrate complex is reported as −2000 cal./mole

[305] T. A. White, *J. Am. Chem. Soc.* **55**, 556 (1933).
[306] S. P. L. Sørensen, *Biochem. Z.* **21**, 256 (1909); *Ergeb. Physiol.* **12**, 457 (1912)
[307] L. Michaelis and M. Rothstein, *Biochem. Z.* **110**, 217 (1920).
[308] A. Eriksson, *Z. physiol. Chem.* **72**, 313 (1911).
[309] O. Meyerhof, *Arch. ges. Physiol.* (*Pflüger's*) **157**, 251 (1914).
[310] E. G. Griffin and J. M. Nelson, *J. Am. Chem. Soc.* **38**, 722 (1916).
[311] R. Willstätter and R. Kuhn, *Z. physiol. Chem.* **116**, 53 (1921).
[312] R. Willstätter, Untersuchungen über Enzyme, Springer, Berlin, 1928, p. 576.
[313] R. Willstätter, Untersuchungen über Enzyme, Springer, Berlin, 1928, p. 627.
[314] I. W. Sizer, *J. Cellular Comp. Physiol.* **10**, 61 (1937).
[315] I. W. Sizer, *J. Gen. Physiol.* **21**, 695 (1938).

or 0 cal./mole. A 350% increase in the rate of reaction for a 10° rise in temperature[167] or a temperature coefficient[316] of 1.61 have been reported. The optimum substrate concentration for maximum rate of inversion is independent of temperature and given as 5%.

Changes in the rate of hydrolysis with pressure have been less thoroughly investigated, although studies of the effect of pressure on acid sucrose inversion were initiated by Roentgen as far back as 1892. Deuticke[317] found no effect on purified invertase by 800 atmospheres, and Sander[318] reports an increase in the rate of mutarotation but no change in invertase action by pressures up to 1000 lbs. at 20°, pH 4.5. More recently Eyring *et al.*[319] reported the interrelation of pressure, temperature, and pH. While at the optimum pH for normal pressure only slight rate increases of 5% were observed at 476 atmospheres, the same pressure causes 58% increase at pH 7.5 and 38% at pH 1.5. At 40° more than 250% increase in the rate of hydrolysis was observed at 680 atmospheres.

For further details on the many kinetic studies made the reader is referred to Weidenhagen[237–239] and Nelson and Schubert.[247]

A corresponding increase in heat of activation is held responsible by Steacy[320] for the 25% decrease in the rate of hydrolysis in heavy water (D_2O).

Abnormal forms of invertase following a course of reaction that cannot be satisfactorily explained have been discussed by Nelson and coworkers.[321,322] Strangely enough, they are normalized by addition of boiled normal invertase solution but not by boiled abnormal enzyme or other proteins. Colloidal ferric hydroxide may change a normal into an abnormal form. A possible explanation has been sought on the basis of an elimination of accompanying protective substances of the pheron.[221]

X. Activity

1. Determination

Detailed descriptions of the methods of analysis have been given by Schneider[323] and in Bamann-Myrbäck[10] (pp. 1726–1730).

Polarimetric measurements have been used most frequently; Hudson[95,324] as well as Isbell and Pigman[325] emphasize the necessity of adding ammonium

[316] J. M. Nelson and G. Bloomfield, *J. Am. Chem. Soc.* **46**, 1025 (1924).
[317] H. J. Deuticke and F. Harren, *Z. physiol. Chem.* **256**, 169 (1938).
[318] F. V. Sander, *J. Biol. Chem.* **148**, 311 (1943).
[319] H. Eyring, F. H. Johnson, and R. L. Gensler, *J. Phys. Chem.* **50**, 453 (1946).
[320] E. W. R. Steacie, *Z. physik. Chem.* **B27**, 6 (1934).
[321] J. M. Nelson and F. Hollander, *J. Biol. Chem.* **58**, 291 (1923–1924).
[322] J. M. Nelson and R. W. Kerr, *J. Biol. Chem.* **59**, 495 (1924).
[323] K. Schneider *in* Oppenheimer, Die Fermente. G. Thieme, Leipzig. Vol. 3, 1929, pp. 768–790.
[324] C. S. Hudson, *J. Am. Chem. Soc.* **30**, 1160, 1564 (1908).
[325] H. S. Isbell and W. W. Pigman, *J. Research Natl. Bur. Standards* **20**, 773 (1938).

hydroxide, sodium carbonate, sodium hydroxide, or mercuric chloride to eliminate mutarotation and attain an equilibrium in the samples under investigation. A micromethod applicable to monolayers has been worked out by Sobotka and Bloch.[326] Copper reduction and colorimetric tests have also been applied,[327] as well as methods based on changes in refraction[328] or volume.[329] Although less exact, these procedures have the advantage of being more rapid and applicable to turbid liquids. To facilitate manometric determinations the use of notatin (glucose oxidase) has recently been described.[330] A chromatographic brush method may be used to demonstrate the presence of invertase.[331]

For precise polarimetric measurements the ordinary first order equation is suitably replaced by the formula developed by Hudson[332]:

$$L_{max.} = D_{max.}(0.417 - 0.005t)$$

where $L_{max.}$ and $D_{max.}$ are the maximum levo- and dextrorotations observed at temperature t.

For rapid hydrolysis preliminary to analysis of the invert sugar formed the simple and reliable method of Sumner and Howell[327] is recommended.

2. Expression

Several formulas used to express enzyme activity have been tabulated by Pigman.[232,333] The "time value" is the time in minutes required to invert 4 g. sucrose in 25 ml. solution at 15.5° and pH 4.5 by means of 0.05 g. of the invertase preparation so that the observed rotation is 0°. The reciprocal was defined as the "invertase value" (I.V.) by Willstätter and Kuhn. One invertase unit in 0.05 g. corresponds to an I.V. of 1. The "inverting power" (I.P.) is the ratio of the dry weights of sucrose to invertase times the first order constant (K) of the reaction.

XI. Technical Preparations and Applications

Uses of the enzyme for preparative and analytical purposes, in brewing, malting, and confectionery, and some patents referring to the production of stable invertase preparations can only be mentioned here. They have been discussed more fully in recent reports.[129,334]

[326] H. Sobotka and E. Bloch, *J. Phys. Chem.* **45**, 9 (1941).
[327] J. B. Sumner and S. F. Howell, *J. Biol. Chem.* **108**, 51 (1935).
[328] G. Gorbach, *Biochem. Z.* **217**, 440 (1930).
[329] D. I. Hitchcock and R. B. Dougan, *J. Phys. Chem.* **33**, 1177 (1935).
[330] D. Keilin and E. F. Hartree, *Biochem. J.* **42**, 232 (1948).
[331] L. Zechmeister and M. Rohdewald, *Enzymologia* **13**, 331 (1949).
[332] C. S. Hudson, *Ind. Eng. Chem.* **2**, 143 (1910).
[333] W. W. Pigman, *J. Research Natl. Bur. Standards* **30**, 257 (1943).
[334] C. Neuberg and I. S. Roberts, *Antonie van Leeuwenhoek. J. Microbiol. Serol.* **12**, 78 (1947).

Chapter 15

α-D-Glucosidases

By ALFRED GOTTSCHALK

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I. Mode of Contact between Enzyme and α-D-Glycoside

α-D-Glucosidases may be defined as enzymes which cleave the α-glucosidic linkage in holosides (oligosaccharides) and in heterosides (α-D-glucosides). Their specificity is directed mainly toward the glucosidic residue which is represented by the radical α-D-glucopyranosyl. It is a common feature of all α-D-glycosidases (α-D-glucosidases, α-D-galactosidases, α-D-mannosidases) that their activity is incompatible with any change in the configuration at carbon atoms 1, 2, 3, and 4 of the glycon moiety of their respective substrates or with the change from the pyranose to the furanose structure. Substitution at carbon atom 5 or 6 of the glycon, however, is tolerated by some α-glycosidases. The specificity of the α-glycosidases toward the glycon partner (aglycon) is much less marked. When this partner is the residue of a sugar (monose), substitution or other constitutional

changes may take place in the aglycon, the only effect being a reduction in the rate of the catalyzed reaction. If the substituent is the residue of another sugar, a trisaccharide is formed which, as in the case of melezitose, may be acted upon by the same α-glucosidase operative toward the nonsubstituted disaccharide sucrose. With some α-glycosidases the tolerance toward the aglycon goes so far as to allow them to hydrolyze not only the appropriate disaccharides but also the corresponding alkyl and aryl α-D-glycosides (heterosides).

The initial step of the reaction catalyzed by an α-glycosidase may be visualized as chemisorption at the enzyme protein surface of a holoside or heteroside with the glycosidic oxygen contacting the attacking group of the enzyme and with hydroxyl groups, *cis*-disposed[1] to the glycosidic oxygen, in juxtaposition to hydrogen bond-forming groups of the enzyme. Orientation of the substrate with the mean plane of the pyranose ring of the glycosyl residue parallel to the enzyme protein surface and with the glycosidic oxygen facing the enzyme will afford closest approximation (about 1 Å) of the respective groups of enzyme and substrate. By inference from the effect on enzyme activity of structural changes in the holosides or heterosides the available groups which are essential for binding the sugar to the catalyst can be identified in many cases.[2]

It is the close approach of the lower side of the pyranose ring (glycon) of the α-glycosides and the respective disaccharides to the active center of the enzyme protein which makes the α-D-glycosidases so sensitive to any configurational change at carbon atoms 1, 2, 3, and 4 of the glycon moiety of their respective substrates; for any change in the configuration of the ring carbon atoms will either remove from its proper spatial position a contacting hydroxyl group or produce a steric hindrance by displacing a hydrogen atom with the larger hydroxyl group (*cf.* Fig. 6). Both operations will impede the mutual attraction between enzyme and substrate.[2a] The relationship between the enzyme and the aglycon may vary between a nonspecific contact, effected by dispersive forces, *e.g.*, those between yeast maltase and the methyl group of methyl α-D-glucoside, and a more precise orientation, involving hydroxyl groups of the aglycon.

As may be deduced from the above discussion, a nonreducing disaccharide like sucrose (α-D-glucopyranosyl-β-D-fructofuranoside), consisting of two different hexose residues, can be hydrolyzed by an α-D-glucosidase as well as by a β-D-fructofuranosidase depending on whether the pyranosyl or furanosyl residue acts as the "glycon" moiety of the substrate, *i.e.*, provides most of the contacting groups.

[1] *Cis* and *trans* refer to the mean plane of the glycosylpyranose ring.
[2] A. Gottschalk, *Advances in Carbohydrate Chem.* **5**, 49 (1950).
[2a] A. Gottschalk, *Nature* **160**, 113 (1947).

II. Maltases

1. Historical

In 1847 Dubrunfaut[3] discovered that the interaction between kiln-dried malt and starch resulted in the production of a sugar which had a rotatory power three times as great as that of dextrose but was less soluble in alcohol than the latter. Dubrunfaut termed the sugar "*le glucose de malt.*" It was not until 1872 that this sugar was reinvestigated by O'Sullivan,[4] who proved it to be a disaccharide, yielding only dextrose on acid hydrolysis; it was then called maltose. Musculus and Gruber[5] found that potato starch when acted upon by diastase (from malt) yielded glucose (3 g. from 300 g. starch) in addition to dextrin and maltose; a similar result was obtained when starch and glycogen were digested with saliva and pancreas extract (dog), respectively.[6] The enzymatic hydrolysis of maltose into glucose was first described by Brown and Heron,[7] who used pancreas extract and dried, shredded intestinal wall (pig), respectively, as source of enzyme; they concluded that the enzyme concerned is different from malt diastase as well as from invertase. Quite independently von Mering[8] observed the hydrolytic cleavage of maltose by saliva and by malt diastase after a prolonged period of time. The presence in *Aspergillus niger* of an enzyme converting maltose into glucose was demonstrated by Bourquelot[9]; the same enzyme was found in koji (*Aspergillus oryzae*) by Kellner *et al.*[10] A first attempt to purify the maltose-splitting enzyme by repeated alcohol precipitation was made by Géduld[11]; he found the enzyme in the grains of many cereals and called it "glucase" (*cf.* also Cuisinier[12]).

Of great importance for the further progress in this special field and for developing the concept of enzyme specificity in general was Emil Fischer's[13] discovery that an extract from dried brewer's yeast was active not only toward maltose but also toward methyl α-D-glucoside; the glucose formed was identified by its osazone. Fischer established that this yeast enzyme is not identical with yeast invertase and termed it "yeast maltase,"[14] as

[3] A. P. Dubrunfaut, *Ann. chim. et phys.* [3] **21**, 178 (1847).
[4] C. O'Sullivan, *J. Chem. Soc.* **25**, 579 (1872).
[5] F. Musculus and D. Gruber, *Z. physiol. Chem.* **2**, 177 (1878–1879).
[6] F. Musculus and J. von Mering, *Z. physiol. Chem.* **2**, 403 (1878–1879).
[7] H. T. Brown and J. Heron, *Ann.* **204**, 228 (1880).
[8] J. von Mering, *Z. physiol. Chem.* **5**, 185 (1881).
[9] E. Bourquelot, *Compt. rend.* **97**, 1322 (1883).
[10] O. Kellner, Y. Mori, and M. Nagaoka, *Z. physiol. Chem.* **14**, 297 (1890).
[11] R. Géduld, *Koch's Jahresberichte Gärungs-Organismen* **1891**, p. 220, quoted by E. Fischer, *Ber.* **27**, 3479 (1894).
[12] L. Cuisinier, *Chem. Centr.* **1886**, 614.
[13] E. Fischer, *Ber.* **27**, 2985, 3479 (1894).
[14] E. Fischer, *Ber.* **28**, 1429 (1895).

previously suggested by Bourquelot.[15] Evidence for the presence in blood serum of glucase (maltase) as distinct from the enzyme transforming starch into dextrin and maltose was advanced from Röhmann's laboratory[16-19]; again the reaction product was isolated as glucosazone.

2. Occurrence

Of the α-D-glycosidases maltase is by far the most important representative, in accordance with the preponderance of D-glucose over D-galactose and D-mannose as components of naturally occurring oligo- and polysaccharides containing α-glycosidic linkages. Maltase is widely distributed throughout the vegetable and animal kingdoms; it usually occurs in association with amylase. The enzyme has been found in a great number of organisms, for example:

I. Cryptogamia

(*1*) Schizomycetes: *Escherichia coli*,[20] *Lactobacillus delbrückii*,[21] *Corynebacterium diphtheriae*, *Neisseria meningitidis*, *Streptococcus pneumoniae*,[22-26] and *Clostridium acetobutylicum*.[27]

(*2*) Fungi: *Saprolegnia* (water molds)[28]; *Mucor* spp.[29]; *Saccharomyces*[30] and *Schizosaccharomyces*[31]; *Aspergillus oryzae*[32] and *A. niger*[33]; Polyporaceae (wood-rotting polypores)[34]; *Lepiota procera* and *Coprinus radiatus*[35]; and *Alternaria solani*.[36]

II. Phanerogamia

(*1*) Monocotyledoneae: in germinated seeds of maize (*Zea*),[37] wheat (*Triti-*

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[15] E. Bourquelot, *Bull. soc. mycologie France* **9**, 189 (1893).
[16] M. Bial, *Arch. ges. Physiol.* (*Pflügers*) **52**, 137 (1892).
[17] M. Bial, *Arch. ges. Physiol.* (*Pflügers*) **54**, 72 (1893).
[18] F. Röhmann, *Ber.* **27**, 3251 (1894).
[19] C. Hamburger, *Arch. ges. Physiol.* (*Pflügers*) **60**, 543 (1895).
[20] H. Karström, *Biochem. Z.* **231**, 399 (1931).
[21] E. Hofmann, *Biochem. Z.* **275**, 320 (1935).
[22] J. M. Neill and E. L. Gaspari, *J. Exptl. Med.* **45**, 151 (1927).
[23] W. L. Fleming and J. M. Neill, *J. Exptl. Med.* **45**, 169 (1927).
[24] J. M. Neill and W. L. Fleming, *J. Exptl. Med.* **45**, 937 (1927).
[25] W. L. Fleming and J. M. Neill, *J. Exptl. Med.* **45**, 947 (1927).
[26] J. Y. Sugg, W. L. Fleming, and J. M. Neill, *J. Exptl. Med.* **46**, 909 (1927).
[27] D. J. D. Hockenhull and D. Herbert, *Biochem. J.* **39**, 102 (1945).
[28] R. K. Sakena and S. K. Bose, *J. Indian Botan. Soc.* **23**, 108 (1944); *Chem. Abstracts* **42**, 4206 (1948).
[29] S. Satina and A. F. Blakeslee, *Proc. Natl. Acad. Sci. U. S.* **14**, 229 (1928); *Chem. Centr.* **1928**, II, 455.
[30] P. Lindner, *Wochschr. Brau.* **17**, 713, 762 (1900); *Chem. Centr.* **1901**, I, 56, 404.
[31] E. Fischer and P. Lindner, *Ber.* **28**, 934, 3034 (1895).
[32] A. Compton, *Proc. Roy. Soc. London.* **B93**, 253 (1914).
[33] E. Bourquelot, *Compt. rend.* **116**, 823 (1893).
[34] S. R. Bose and S. N. Sarkar, *Proc. Roy. Soc. London.* **B128**, 193 (1937).
[35] N. N. Iwanoff, E. W. Dodonowa, and W. J. Tschastuchin, *Fermentforschung* **11**, 433 (1930).
[36] G. von Szelenyi and G. von Beeze, *Centr. Bakt. Parasitenk.* Abt. II **76**, 121 (1928); *Chem. Abstracts* **23**, 1156 (1929).
[37] W. M. Beyerinck, *Centr. Bakt. Parasitenk.* I, **1**, 221 (1896); *Chem. Centr.* **1897**, I, 112.

cum),[38] rye (*Secale*),[38] barley (*Hordeum*),[38] oat (*Avena*),[38] and in the leaves of onion (*Allium cepa*),[39] leek (*Allium porrum*),[39] and sugar cane (*Saccharum*).[40]

(*β*) Dicotyledoneæ: in germinated seeds of soy bean (*Glycine*),[41] peas (*Pisum*),[41] Cucurbitaceae,[41] *Strophanthus*,[42] and in the leaves of dock (*Rumex obtusifolius*),[39] black mustard (*Brassica nigra*),[43] potato (*Solanum*),[44] *Dahlia*,[44] beet (*Beta*),[44] rock rose (*Helianthemum*),[44] tobacco (*Nicotiana*),[45] hops (*Humulus*),[46] and silver birch (*Betula alba*).[47]

In animals the presence of maltase has been demonstrated mainly in the digestive tract (pancreas, intestine) and blood serum. Thus it has been found in invertebrates such as molluscs,[48] crustaceans,[49] and insects (Orthoptera,[50] Hymenoptera,[51] Lepidoptera[52,53]) and in vertebrates (Pisces,[54] Amphibia,[54] Reptilia,[54] Aves,[54] and Mammalia[54]). Whereas in mammals maltase is secreted by the Lieberkühn glands into the intestinal juice, in fishes the pancreas, in the absence of Lieberkühn glands, produces the enzyme.[55,56] Blood serum of the pig, dog, ox, sheep, horse, rat, and mouse contains maltase, that of the man, monkey, rabbit, guinea pig, cat, chicken, and pigeon is devoid of the enzyme.[57-60] Further, maltase is known to occur in extracts from liver (rat, cat, rabbit, pig)[61] and skeletal muscle (rabbit)[62]

[38] Z. Wierzchowski, *Biochem. Z.* **57**, 125 (1913).
[39] R. E. Chapman, *Biochem. J.* **18**, 1388 (1924).
[40] C. E. Hartt, *Proc. Hawaiian Sugar Planters' Assoc., Rept. Comm. in Charge Expt. Sta.* **57**, 110 (1937); *Chem. Abstracts* **32**, 6690 (1938).
[41] Y. Jono, *Acta Schol. Med. Univ. Imp. Kioto* **13**, 211 (1931); *Chem. Centr.* **1932**, I, 1103.
[42] A. Stoll and J. Renz, *Enzymologia* **7**, 362 (1939); *Chem. Abstracts.* **34**, 6298 (1940).
[43] A. Astruc and M. Mousseron, *Compt. rend.* **184**, 126 (1927); *Chem. Abstracts* **21**, 1666 (1927).
[44] A. J. Daish, *Biochem. J.* **10**, 49 (1916).
[45] G. P. Volgunov, A. S. Komel, and I. N. Pushkareva, *Biokhimiya* **6**, 67 (1941); *Chem. Abstracts* **35**, 6061 (1941).
[46] J. Janicki, W. V. Kotasthane, A. Parker, and T. K. Walker, *J. Inst. Brewing* **47**, 24 (1941).
[47] A. Sosa, *Ann. chim.* **14**, 5 (1940); *Chem. Abstracts* **35**, 7947 (1941).
[48] K. Oshima, *Bull. Agr. Chem. Soc. Japan.* **7**, 17 (1931); *Chem. Centr.* **1932**, II, 553.
[49] P. Krüger and E. Graetz, *Zool. Jahrb.* **45**, 463 (1928); *Chem. Abstracts* **24**, 3566 (1930).
[50] V. Wigglesworth, *Biochem. J.* **21**, 797 (1927).
[51] E. F. Philipps, *J. Agr. Research.* **35**, 385 (1927); *Chem. Centr.* **1928**, I, 937.
[52] O. Shinoda, *J. Biochem. (Japan)* **11**, 345 (1930); *Chem. Abstracts* **24**, 3566 (1930).
[53] F. H. Babers and P. A. Woke, *J. Agr. Research.* **54**, 547 (1937); *Chem. Abstracts* **31**, 5882 (1937).
[54] E. Fischer and W. Niebel, *Sitzber. kgl. preuss. Akad. Wiss.* 1896, p. 73; *Chem. Centr.* **1896**, I, 499.
[55] H. J. Vonk, Jr., *Z. vergleich. Physiol.* **5**, 445 (1927); *Biol. Abstracts* **AIII**, **3**, 12355 (1929).
[56] H. J. Vonk, Jr., *Arch. néerland. physiol.* **12**, 289 (1927); *Physiol. Abstracts* **13**, 237 (1928–1929).
[57] A. Compton, *Biochem. J.* **15**, 681 (1921).
[58] T. Kokuryo, *Japan. J. Med. Sci. II. Biochem.* **2**, 115 (1933); *Chem. Abstracts* **28**, 1063 (1934).
[59] R. H. Barnes and E. M. Mackay, *Am. J. Physiol.* **114**, 534 (1936).
[60] N. E. Goldsworthy, J. L. Still, and J. A. Dumaresq, *J. Path. Bact.* **46**, 253 (1938).
[61] G. E. Glock, *Biochem. J.* **30**, 2313 (1936).
[62] A. N. Petrova, *Biokhimiya* **12**, 209 (1947).

as well as in kidney (pig).[63] A careful study of the maltase of leucocytes was made by Willstätter and Rohdewald.[64]

In view of the wide distribution of maltase in plants and animals it is of interest that lactose yeasts, *Saccharomyces marxianus, exiguus, ludwigii* and *S. apiculatus* Reess are free from maltase.[30,31,65,66]

3. Specificity

The biological substrate for the enzyme maltase is maltose, 4-α-D-glucopyranosyl-D-glucose (Fig. 1), an end product of amylase action on amylose, amylopectin, and glycogen. Besides maltose a number of maltose derivatives and some alkyl and aryl α-D-glucopyranosides are acted upon by the enzyme. The common feature of all these compounds is a nonsubstituted α-D-glucopyranosyl residue. Yeast maltase hydrolyzes the α-glucosidic linkage in the substances recorded in Table I.

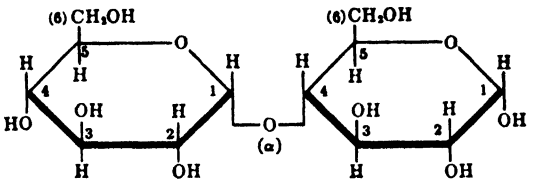


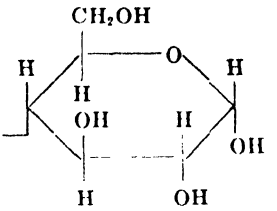
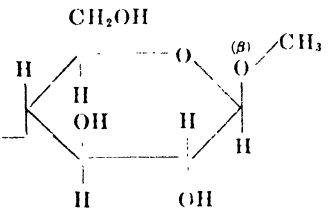
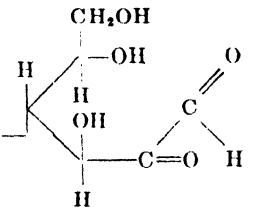
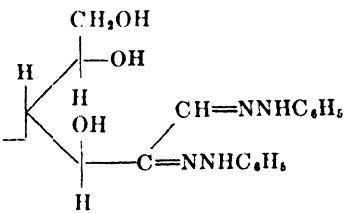
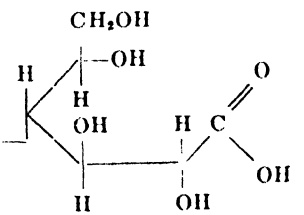
Fig. 1. Maltose (4-α-D-glucopyranosyl-D-glucose)

However, methyl α-D-isorhamnoside[67] (Fig. 2), methyl α-D-glucoside 6-methyl ether[67] (Fig. 2), methyl α-D-glucoside 6-chlorhydrin[67] (Fig. 2) and methyl α-gentiobioside[68,69] (Fig. 3), all of which may be regarded as α-D-glucosides with a substituted group at carbon atom 6 of the glucon, are not substrates for maltase. The same holds for phenyl α-lactoside and phenyl α-cellobioside,[70] *i.e.*, phenyl α-glucosides with a glycosidic residue attached to carbon atom 4. Inversion of the configuration at carbon atom 4 in methyl α-glucoside, thus changing the glucoside into the corresponding α-galactoside (Fig. 4), renders maltase inoperative.[13] Methyl α-D-2-desoxyglucoside (Fig. 5) is not acted upon by the enzyme.[71]

[63] E. Laborde, I. H. Fiszerman, and D. Fiszerman-Garber, *Bull. sci. pharmacol.* **40**, 65 (1933); *Chem. Abstracts* **27**, 3490 (1933).
[64] R. Willstätter, and M. Rohdewald, *Z. physiol. Chem.* **209**, 33 (1932).
[65] H. von Euler and K. Josephson, *Z. physiol. Chem.* **120**, 42 (1922).
[66] A. Gottschalk, *Z. physiol. Chem.* **152**, 132 (1926).
[67] B. Helferich, W. Klein, and W. Schaefer, *Ber.* **59**, 79 (1926).
[68] B. Helferich and J. Becker, *Ann.* **440**, 1 (1924).
[69] B. Helferich and J. Becker, *Ann.* **447**, 19 (1926).
[70] S. R. Petersen, *Ber. Verhandl. sächs. Akad. Wiss. Leipzig Math. phys. Klasse* **85**, 154 (1933); *Chem. Centr.* **1934**, I, 1825.
[71] M. Bergmann, H. Schotte, and W. Lechinsky, *Ber.* **55**, 158 (1922).

TABLE I

SUBSTRATE RANGE OF YEAST MALTASE

Substrate	Aglucon group	Author
Maltose		Fischer[12]
Methyl β-maltoside		Fischer, Armstrong[72]
Maltosone		Fischer, Armstrong[73]
Maltosazone		Neuberg, Saneyoshi[74]
Maltobionic acid (α-D-Glucopyranosyl-D-gluconic acid)		Neuberg, Hofmann[75]

[72] E. Fischer and E. F. Armstrong, *Ber.* **34**, 2885 (1901).
[73] E. Fischer and E. F. Armstrong, *Ber.* **35**, 3141 (1902).
[74] C. Neuberg and S. Saneyoshi, *Biochem. Z.* **36**, 44 (1911).
[75] C. Neuberg and E. Hofmann, *Biochem. Z.* **252**, 434 (1932).

as well as in kidney (pig).[63] A careful study of the maltase of leucocytes was made by Willstätter and Rohdewald.[64]

In view of the wide distribution of maltase in plants and animals it is of interest that lactose yeasts, *Saccharomyces marxianus, exiguus, ludwigii* and *S. apiculatus* Reess are free from maltase.[30,31,65,66]

3. Specificity

The biological substrate for the enzyme maltase is maltose, 4-α-D-glucopyranosyl-D-glucose (Fig. 1), an end product of amylase action on amylose, amylopectin, and glycogen. Besides maltose a number of maltose derivatives and some alkyl and aryl α-D-glucopyranosides are acted upon by the enzyme. The common feature of all these compounds is a nonsubstituted α-D-glucopyranosyl residue. Yeast maltase hydrolyzes the α-glucosidic linkage in the substances recorded in Table I.

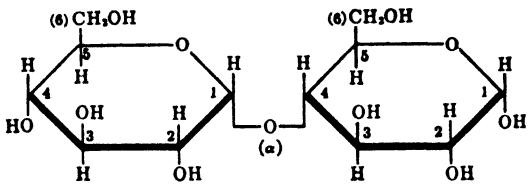


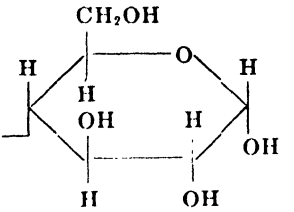
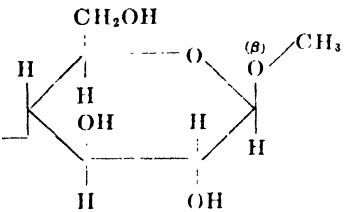
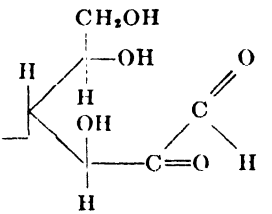
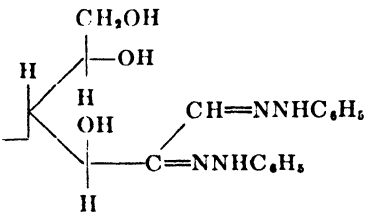
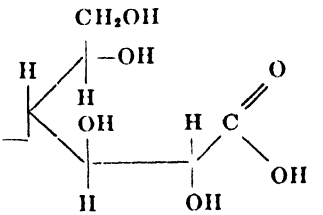
Fig. 1. Maltose (4-α-D-glucopyranosyl-D-glucose)

However, methyl α-D-isorhamnoside[67] (Fig. 2), methyl α-D-glucoside 6-methyl ether[67] (Fig. 2), methyl α-D-glucoside 6-chlorhydrin[67] (Fig. 2) and methyl α-gentiobioside[68,69] (Fig. 3), all of which may be regarded as α-D-glucosides with a substituted group at carbon atom 6 of the glucon, are not substrates for maltase. The same holds for phenyl α-lactoside and phenyl α-cellobioside,[70] *i.e.*, phenyl α-glucosides with a glycosidic residue attached to carbon atom 4. Inversion of the configuration at carbon atom 4 in methyl α-glucoside, thus changing the glucoside into the corresponding α-galactoside (Fig. 4), renders maltase inoperative.[13] Methyl α-D-2-desoxyglucoside (Fig. 5) is not acted upon by the enzyme.[71]

[63] E. Laborde, I. H. Fiszerman, and D. Fiszerman-Garber, *Bull. sci. pharmacol.* **40**, 65 (1933); *Chem. Abstracts* **27**, 3490 (1933).
[64] R. Willstätter, and M. Rohdewald, *Z. physiol. Chem.* **209**, 33 (1932).
[65] H. von Euler and K. Josephson, *Z. physiol. Chem.* **120**, 42 (1922).
[66] A. Gottschalk, *Z. physiol. Chem.* **152**, 132 (1926).
[67] B. Helferich, W. Klein, and W. Schaefer, *Ber.* **59**, 79 (1926).
[68] B. Helferich and J. Becker, *Ann.* **440**, 1 (1924).
[69] B. Helferich and J. Becker, *Ann.* **447**, 19 (1926).
[70] S. R. Petersen, *Ber. Verhandl. sächs. Akad. Wiss. Leipzig Math. phys. Klasse* **85**, 154 (1933); *Chem. Centr.* **1934**, I, 1825.
[71] M. Bergmann, H. Schotte, and W. Lechinsky, *Ber.* **55**, 158 (1922).

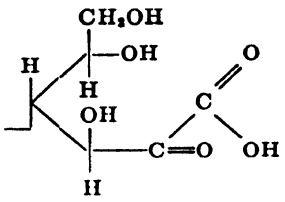
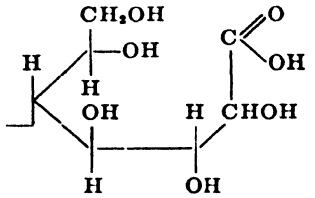
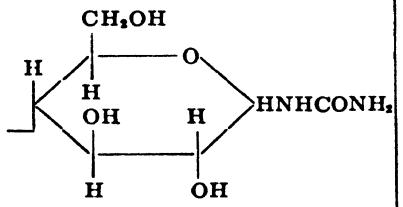
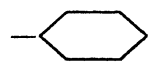
TABLE I

SUBSTRATE RANGE OF YEAST MALTASE

Substrate	Aglucon group	Author
Maltose		Fischer[19]
Methyl β-maltoside		Fischer, Armstrong[72]
Maltosone		Fischer, Armstrong[73]
Maltosazone		Neuberg, Saneyoshi[74]
Maltobionic acid (α-D-Glucopyranosyl-D-gluconic acid)		Neuberg, Hofmann[75]

[73] E. Fischer and E. F. Armstrong, *Ber.* **34**, 2885 (1901).
[73] E. Fischer and E. F. Armstrong, *Ber.* **35**, 3141 (1902).
[74] C. Neuberg and S. Saneyoshi, *Biochem. Z.* **36**, 44 (1911).
[75] C. Neuberg and E. Hofmann, *Biochem. Z.* **252**, 434 (1932).

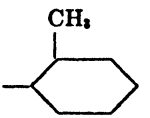
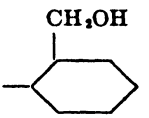
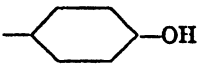
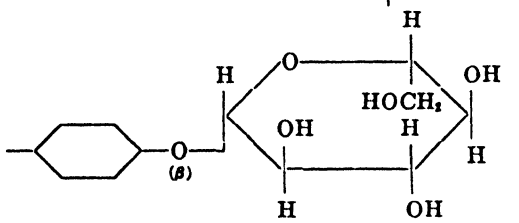
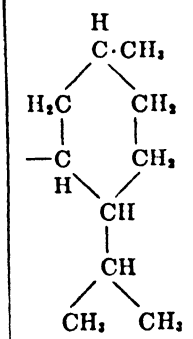
TABLE I—(*Continued*)

Substrate	Aglucon group	Author
α-Ketomaltobionic acid (α-D-Glucopyranosyl-D-fructuronic acid)		Kitasato[76]
Maltosecarboxylic acid (α-D-Glucopyranosyl-D-glucoheptonic acid)		Pratesi[77]
Ureidomaltose		Hofmann[78]
Maltosephosphoric acid	$-C_6H_{10}O_4\cdot O\cdot PO_3H_2$	Ohmiya[79]
Methyl α-D-glucopyranoside	$-CH_3$	Fischer[13]
Ethyl α-D-glucopyranoside	$-CH_2CH_3$	Fischer[13]
Propyl α-D-glucopyranoside	$-CH_2CH_2CH_3$	Bourquelot, Hérissey, Bridel[80]
Allyl α-D-glucopyranoside	$-CH_2CH{=}CH_2$	Bourquelot, Hérissey, Bridel[80]
Ethylene glycol mono-α-D-glucopyranoside	$-CH_2CH_2OH$	Bourquelot, Bridel[81]
Trimethylene glycol-α-D-glucoside[a]	$-CH_2CH_2CH_2OH$	Vintilescu, Jonescu, Kizyk[82]
D-Mannitol mono-α-D-glucoside[a]	$-C_6H_{13}O_6$	Vintilescu, Jonescu, Kizyk[82]
Phenyl α-D-glucopyranoside		Fischer, von Mechel[83]

[a] As this compound has been synthesized from glucose and the aglucon by yeast maltase, its inclusion in this table seems justified.

[76] T. Kitasato, *Biochem. Z.* **207**, 217 (1929).
[77] P. Pratesi, *Biochem. Z.* **267**, 238 (1933).
[78] E. Hofmann, *Biochem. Z.* **253**, 462 (1932).
[79] S. Ohmiya, *J. Biochem.* (*Japan*) **18**, 125 (1933); *Chem. Abstracts* **27**, 5352 (1933).
[80] E. Bourquelot, M. Hérissey, and M. Bridel, *J. pharm. chim.* [7] **7**, 525 (1913).
[81] M. Bourquelot and M. Bridel, *J. pharm. chim.* [7] **9**, 514 (1914).
[82] I. Vintilescu, C. N. Jonescu, and A. Kizyk, *Bul. Soc. Chim. Romănia* **17**, 131 (1935); *Chem. Abstracts*. **30**, 1173 (1936).
[83] E. Fischer and L. von Mechel, *Ber.* **49**, 2813 (1916).

TABLE I—(*Continued*)

Substrate	Aglucon group	Author
o-Cresyl α-D-glucopyranoside		Helferich, Lampert, Sparmberg[84]
Saligenin α-D-glucopyranoside		Helferich, Lampert, Sparmberg[84]
Hydroquinone mono-α-D-glucopyranoside		Helferich, Reichel[85]
Hydroquinone 1/α-D-glucoside 4\β-D-glucoside		Helferich, Reichel[86]
L-Menthol α-D-glucopyranoside		Neuberg, Jacobsohn, Wagner[86]

Leibowitz[87,88] has differentiated two types of maltases; one type, designated as "glucosidomaltase," is represented by yeast maltase and characterized by a high degree of tolerance toward structural changes in the

[84] B. Helferich, U. Lampert, and G. Sparmberg, *Ber.* **67**, 1808 (1934).
[85] B. Helferich and W. Reischel, *Ann.* **533**, 278 (1938).
[86] C. Neuberg, K. P. Jacobsohn, and J. Wagner, *Fermentforschung* **10**, 491 (1929).
[87] J. Leibowitz, *Z. physiol. Chem.* **149**, 184 (1925).
[88] J. Leibowitz and P. Mechlinski, *Z. physiol. Chem.* **154**, 64 (1926).

aglucon part of its substrate; it acts upon alkyl and aryl α-D-glucosides and many maltose derivatives. The second type is represented by maltase from *Aspergillus oryzae* (taka-maltase) and from barley malt; maltase from these sources, according to Leibowitz, does not tolerate any structural change in the aglucon moiety of maltose and is inoperative toward α-D-

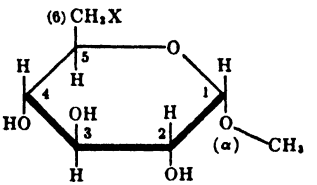


FIG. 2. Methyl α-D-isorhamnoside (X = H), methyl α-D-glucoside 6-methyl ether (X = OCH_3), methyl α-D-glucoside 6-chlorhydrin (X = Cl).

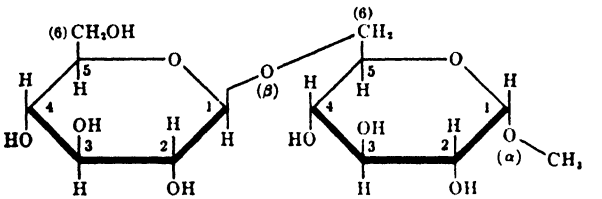


FIG. 3. Methyl α-gentiobioside

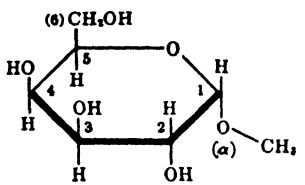


FIG. 4

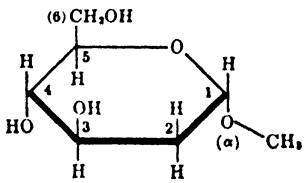


FIG. 5

FIG. 4. Methyl α-D-galactoside
FIG. 5. Methyl α-D-2-desoxyglucoside

glucosides (heterosides), indicating that its specificity is directed toward the reducing rather than the nonreducing moiety of maltose. A more thorough investigation,[75,89-91] however, has proved that the difference between yeast maltase and taka-maltase with regard to their respective specificities toward the aglucon is very small indeed. Maltase from both

[89] R. Weidenhagen, *Z. physiol. Chem.* **216**, 255 (1933).
[90] H. Pringsheim and F. Loew, *Z. physiol. Chem.* **207**, 241 (1932).
[91] S. Hestrin, *Enzymologia* **8**, 193 (1940).

sources acts on maltose derivatives as well as on heterosides (*cf.* Table II). It is evident from Table III that taka-maltase readily hydrolyzes phenyl α-D-glucoside and, though at a much lower rate, methyl α-D-glucoside.[89]

TABLE II

SUBSTRATES FOR MALTASE FROM YEAST AND FROM *Aspergillus Oryzae*

Substrate	Maltase from	
	Brewer's yeast	*Aspergillus oryzae*
Maltose	+	+
Maltosone	+	+
Maltobionic acid	+	+
Maltosazone	+	+
Phenyl α-D-glucoside	+	+
Methyl α-D-glucoside	+	±

TABLE III

HYDROLYSIS RATES[a] OF MALTOSE, PHENYL α-D-GLUCOSIDE, AND METHYL α-D-GLUCOSIDE BY TAKA-MALTASE[89]

Time, hr.	Per cent hydrolysis		
	Maltose	Phenyl α-D-glucoside	Methyl α-D-glucoside
1	30.7	—	—
2	54.4	—	—
4	67.0	8.0	—
23	—	29.6	11.2
28	92.1	—	—
95	—	75.2	19.2
196	—	97.2	28.4

[a] 0.139 *M* substrate concentration; 0.1 *N* acetate buffer (pH 4.7); 5 ml. enzyme solution (5 mg. dry substance); total volume 25 ml.; temp. 30°.

Different preparations of taka-maltase seem to vary in their activity toward methyl α-D-glucoside.[92] For barley malt maltase Weidenhagen[89] has conclusively shown that phenyl α-D-glucoside is a suitable substrate. Therefore, Leibowitz' theory of the existence in mold and barley of a "glucomaltase" which requires for action the unchanged reducing moiety of maltose, the glucosidic residue playing the role of the aglucon, is not borne

[92] K. Myrbäck, *Z. physiol. Chem.* **205**, 248 (1932).

out by the experimental findings. A subclassification of the maltases could be justified only if a detailed study of the specificity requirements of a large number of maltases from different sources would reveal a few distinct types of reaction when tested with numerous maltose derivatives and α-D-glucosides (heterosides). The available data do not suggest such a behavior. Maltase from brewer's bottom yeast, for instance, splits maltobionic acid; that from top yeast (*Presshefe*) does not.[75] Three different species of *Schizosaccharomyces* were found to have a maltase acting upon maltose and methyl α-D-glucoside.[93] Sulfatase bacteria, cultivated on maltose–agar, contain a maltase hydrolyzing maltose, and phenyl and methyl α-D-glucoside; when the same organism is grown on a meat extract–peptone medium, the bacterial maltase is inactive toward the alkyl glucoside.[94] A preparation from *Escherichia coli* cleaves maltose, but is inert toward heterosides. *Lactobacillus delbrückii* acts on maltose, and phenyl and methyl α-D-glucoside.[94] The maltase of *Solanum indicum*, a solanaceous fruit, hydrolyzes maltose readily, phenyl α-D-glucoside at a minute rate (4% in 74 hours), and methyl α-D-glucoside not appreciably.[95] Animal maltase is inactive toward methyl α-D-glycoside[54]; its behavior toward phenyl α-D-glucoside has not been investigated. It would appear from these examples that the degree of maltase specificity toward the aglucon may vary in an unpredictable manner not only from organism to organism, but also in the same organism depending on the conditions of growth. Differences in the specificity of this kind, referring to the aglucon only, may be regarded as of secondary nature and minor importance; they may be due to slight variations in the chemical structure of that part of the enzyme protein to which the aglucon is adsorbed. Certainly, yeast maltase and mold maltase are not chemically identical, as is evident from their different pH optima and their different stabilities toward acid and heat.[91] It seems advisable, therefore, to designate the individual maltases according to their origin, as proposed by Fischer.[14]

We may conclude from this discussion that all maltases, irrespective of their source, establish close contact with the glucon moiety of their specific substrates. The detrimental effect on maltase activity which is observed when the hydroxyl groups at carbon atoms 2 and 6, respectively, of the glucon are replaced by hydrogen, taken together with the lack of enzyme action on methyl α-D-galactoside, would suggest that besides the glucosidic oxygen the hydroxyl groups at carbon atoms 2, 4, and 6 are involved in the formation of the enzyme–substrate complex, as depicted in Fig. 6. The contact between enzyme protein and aglucon will vary with the chemical structure of the latter.

[93] E. Hofmann, *Biochem. Z.* **287**, 271 (1936).
[94] E. Hofmann, *Biochem. Z.* **272**, 133 (1934).
[95] H. Tauber and I. S. Kleiner, *J. Biol. Chem.* **105**, 679 (1934).

4. Preparation and Purification

Brewer's yeast is the richest source of maltase. The progress made by Willstätter in the extraction of maltase from fresh yeast lies in the recognition that under the influence of liquefying agents like toluene and chloroform, used by Fischer,[13,14] acid-producing metabolic reactions set in which destroy part or all of the maltase released from the cell. Willstätter,[96]

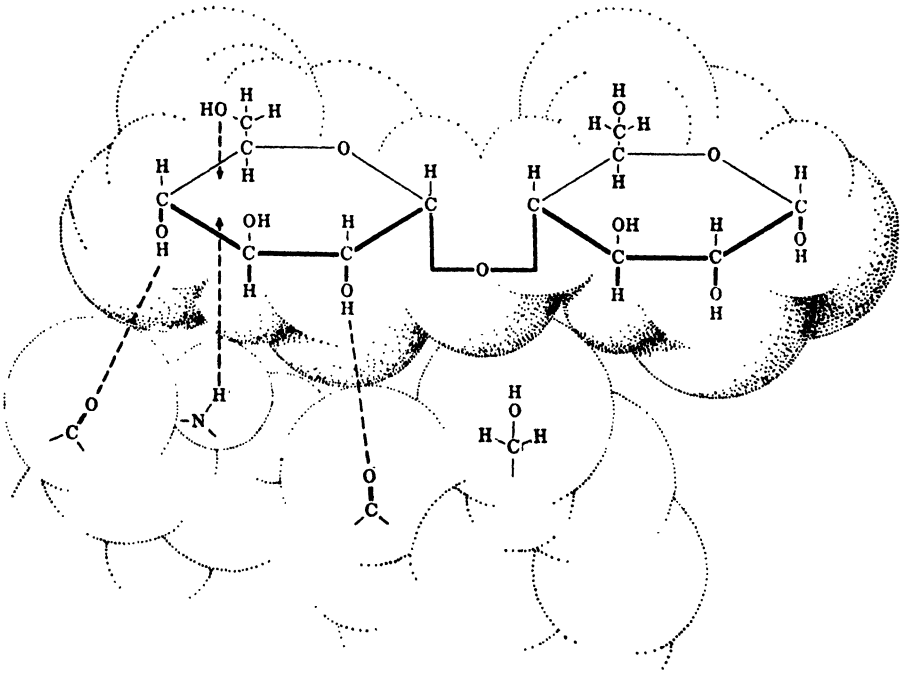


FIG. 6. Initial stage of interaction between a maltose molecule and the active center of a maltase molecule. A hydroxyl group of the enzyme protein is making an attack on the glucosidic oxygen, whereas three other groups of the enzyme are in the process of forming hydrogen bonds with hydroxyl groups of the substrate *cis*-disposed to the glucosidic oxygen.

Sugar molecules are shown as planar projections of three-dimensional objects. Though these stereoformulas represent the spatial arrangement of the addenda relative to each other only approximately, they depict accurately their position relative to the mean plane of the ring carbon atoms. There is free rotation about the C_5—C_6 single bond.

therefore, quickly liquefied the yeast cells and neutralized the acid formed during this process. He found ethyl acetate more efficient and satisfactory as liquefying agent than either toluene or chloroform. The release of maltase from fresh yeast is effected in the following manner[97]:

[96] R. Willstätter and W. Steibelt, *Z. physiol. Chem.* **111**, 157 (1920).
[97] R. Willstätter and E. Bamann, *Z. physiol. Chem.* **151**, 242 (1926).

The yeast is washed, pressed, and then stirred while ethyl acetate is added (1 ml. per 10 g. pressed yeast). Stirring is continued for the next 10 minutes during which liquefaction takes place. The mixture is allowed to stand for about 30 minutes, diluted with water, and neutralized with 0.1 *N* ammonia (litmus paper as indicator). After less than 24 hours at room temperature approximately 95% of the yeast maltase is in solution. The optimum period of autolysis is controlled by analyzing samples for their maltase activity. If autolysis is carried out at 0°, longer periods are required. Satisfactory results were also obtained with diammonium phosphate which acts simultaneously as liquefying and neutralizing agent (for details see Weidenhagen[98]).

The autolyzate thus obtained contains saccharase (β-fructofuranosidase) in addition to maltase. Willstätter and Bamann[99] have described a method for the almost quantitative separation of the two enzymes. They found that meta-aluminium hydroxide (AlOOH) readily adsorbs maltase, whereas its adsorptive affinity for β-fructofuranosidase is low. The selective adsorption of maltase on AlOOH is followed by selective elution. After pre-elution of the residual β-fructofuranosidase with potassium dihydrogen phosphate the maltase is eluted with diammonium phosphate. Weidenhagen[100] recommended freshly prepared aluminium hydroxide B in small amounts as adsorbent.

Barley malt, kiln-dried at low temperature, is another source of maltase. The dried malt is digested for several days at 0° with toluene–water, the extract concentrated under diminished pressure, and eventually dialyzed against water.[89]

Maltase preparations from *Aspergillus oryzae* may be obtained by dialyzing commercial taka-diastase or an extract from pure cultures of the organism against water and by concentrating the residue *in vacuo*.[89] In order to destroy the saccharase present in such preparations, Feigenbaum[101] treats the residue for 24 hours at room temperature with $Na_2S_2O_4$ and then removes the reducing agent by dialysis.

5. Measurement of Activity

In order to determine the maltase activity the yeast is treated in a manner similar to that described in Section 4:

One ml. of acetic acid ethyl ester is added to 10 g. of fresh yeast (corresponding to about 2.5 g. dried substance) and the mixture stirred for 10–15 minutes until liquefaction is complete. The yeast is then well mixed with 20 ml. water, followed by neutralization with 0.1 *N* ammonia. The suspension is allowed to stand for 10 minutes, after which neutralization, if necessary, is completed and the total volume made up to 50 ml. with water.[97] Ten ml. of the suspension is pipetted into a glass vessel containing 2.5 g. maltose hydrate and 0.5 g. of a mixture of primary and secondary phosphate

[98] R. Weidenhagen *in* Bamann-Myrbäck, Die Methoden der Fermentforschung. Thieme, Leipzig, 1941; Academic Press, New York, 1945, p. 1746.

[99] R. Willstätter and E. Bamann, *Z. physiol. Chem.* **151**, 273 (1926).

[100] R. Weidenhagen, *Z. Ver. deut. Zucker-Ind.* **80**, 155 (1930); *Chem. Abstracts* **25**, 5437 (1931).

[101] J. Feigenbaum, *Science* **96**, 521 (1942).

(in such a proportion as to give a pH of 6.8) and the volume adjusted with water to 50 ml. A few drops of toluene are added; the temperature is maintained at 30° (standard conditions[102]). At appropriate intervals samples of 5 ml. are withdrawn and added to 10 ml. of 2 *N* sodium carbonate solution. In the filtrate the change in optical rotation or in reducing power due to the conversion of 1 molecule maltose hydrate into 2 molecules D-glucose is determined; from the figures obtained the percentage of hydrolysis is calculated.

Units.[97] "Time value" is defined as the time in minutes required for the hydrolysis of one-half the maltose present by 1 g. of the material (dry weight) containing the enzyme. "Maltase value" denotes the reciprocal of the "time value" multiplied by 1000. "Apparent maltase unit' is the amount of enzyme in 1 g. dried material hydrolyzing half the maltose present in 1 minute.

6. KINETICS

a. Effect of Enzyme Concentration

As may be seen from Table IV, at low and medium concentrations of the enzyme the hydrolysis rate is proportional to enzyme concentration; at higher concentrations the velocity is proportional to the square root of the amount of enzyme present.[97,103-105]

b. Effect of Substrate Concentration

The effect of substrate concentration on the hydrolysis rate of maltose at different levels of enzyme concentration has been carefully investigated by Jsaiev.[103] The experiments were arranged in series in such a manner that in all assays of one series the concentration of maltose was the only variant; the series differed from each other in the concentration of the enzyme, which increased regularly. The time curve of the enzymatic hydrolysis of maltose was found to be markedly influenced by the substrate concentration, the reaction proceeding relatively more slowly in more concentrated solutions of maltose. No simple relationship between rate and substrate concentration could be observed; moreover, this relationship was varying continually during the reaction. Figs. 7 and 8 illustrate how different the course of the reaction is according to the relative amounts of enzyme, substrate, and reaction product present.[97,104] Jsaiev deduces from the experimental data that maltase combines with both the substrate and the reaction product, the active mass of the enzyme thus being progressively decreased. That glucose, indeed, strongly inhibits the enzymatic hydrolysis

[102] R. Weidenhagen, *Ergeb. Enzymforsch.* **1**, 168 (1932).
[103] V. J. Jsaiev, *J. Inst. Brewing* **32**, 552 (1926).
[104] R. Willstätter, T. Oppenheimer, and W. Steibelt, *Z. physiol. Chem.* **110**, 232 (1920).
[105] R. Willstätter, R. Kuhn, and H. Sobotka, *Z. physiol. Chem.* **134**, 224 (1924).

TABLE IV

VARIATION OF HYDROLYSIS RATE WITH ENZYME CONCENTRATION (AT 30°)[103]

A

Time, min.	Percentage of 0.08 *M* maltose hydrolyzed					
	E[a]: 1	2	4	6	8	12
10	1.3	4.0	5.9	7.8	9.0	10.1
20	2.4	6.1	9.0	11.6	13.3	14.9
30	4.0	8.0	11.6	14.5	16.7	18.4
45	5.0	10.5	14.3	17.7	20.2	21.5
60	6.1	12.1	16.3	19.9	22.8	25.0
90	8.4	15.3	19.9	24.1	27.0	29.4
180	12.7	21.0	27.5	32.8	36.7	40.1

EVALUATION OF TABLE A

Hydrolysis, per cent	$T \times E$					$T \times \sqrt{E}$	
	E: 2	4	6	8	12	*E*:8	12
10	84	92	96	96	120	33.9	34.6
15	174	200	222	192	240	67.9	69.3
20	342	360	348	352	432	124.5	124.7
25	—	584	582	600	720	212.1	207.9
30	—	—	876	920	1110	325.3	322.5

B

Time, min.	Percentage of 0.16 *M* maltose hydrolyzed					
	E: 1	2	4	6	8	12
10	1.0	3.2	5.1	5.5	7.4	8.0
20	2.0	4.4	7.5	9.6	11.0	14.1
30	2.8	6.5	9.4	11.7	13.4	14.9
45	4.1	8.1	11.5	14.3	16.5	18.2
60	5.1	9.6	13.2	16.5	19.0	21.3
90	5.5	12.1	16.5	20.5	23.1	25.5
180	10.7	17.7	22.4	27.1	30.4	33.4

EVALUATION OF TABLE B

Hydrolysis, per cent	$T \times E$						$T \times \sqrt{E}$	
	E: 1	2	4	6	8	12	*E*: 8	12
5	60	42	40	—	—	—	—	—
10	165	130	132	132	136	168	50	48
15	—	264	300	288	296	360	104	105
20	—	—	548	516	528	636	184	187
25	—	—	—	852	784	1032	298	277
30	—	—	—	—	1400	1632	471	495

[a] *E* is relative concentration of enzyme. *T* is time necessary to reach a definite degree of hydrolysis.

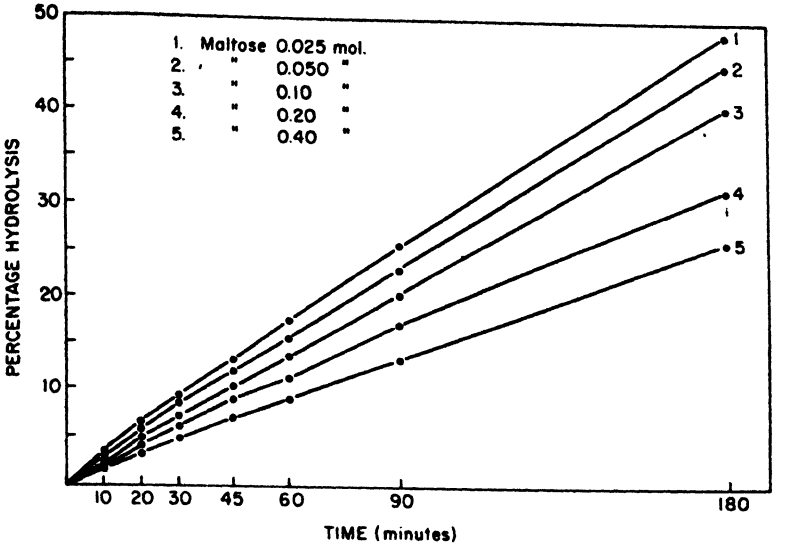


FIG. 7. Time curve of enzymatic hydrolysis of maltose.[103] Lower enzyme concentration.

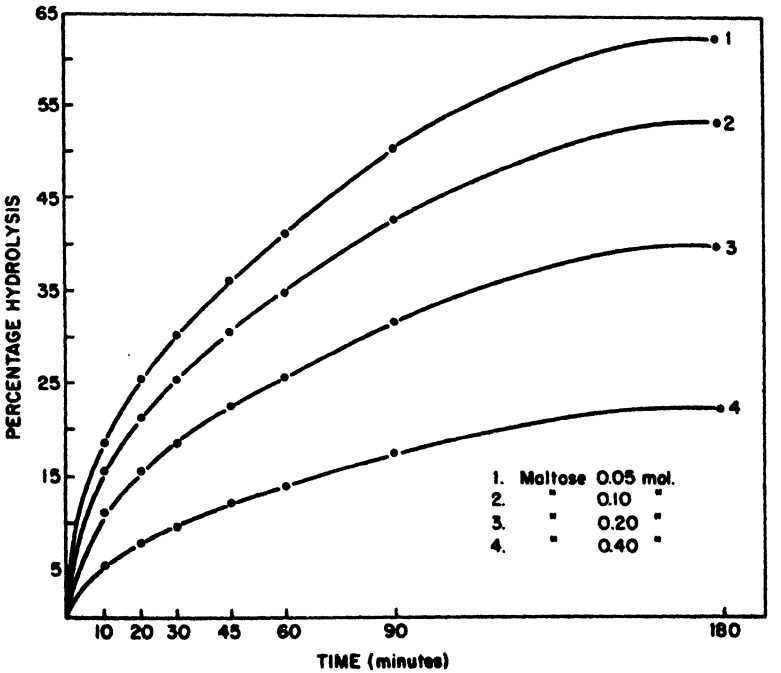


FIG. 8. Time curve of enzymatic hydrolysis of maltose.[103] Higher enzyme concentration.

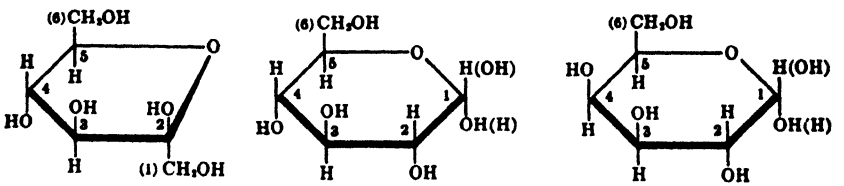
of maltose was first observed by Armstrong[106]; D-fructose, too, inhibits maltase activity[103,107], whereas D-galactose is without any effect on this reaction.[103] Table V summarizes the evidence.

TABLE V

EFFECT OF GLUCOSE, FRUCTOSE, AND GALACTOSE ON THE ENZYMATIC HYDROLYSIS OF MALTOSE[103]

Substrate mixture	Per cent maltose hydrolyzed		
	1 hr.	4.5 hr.	21 hr.
5% maltose only	19.7	40.2	62.2
5% maltose + 6% glucose	—	13.9	32.3
5% maltose + 7% glucose	2.4	8.8	29.2
5% maltose + 8% glucose	—	—	23.3
5% maltose + 5% fructose	11.7	28.6	52.5
5% maltose + 5% galactose	19.3	39.7	63.3

The inhibitory power of D-fructose on the enzymatic hydrolysis of maltose may be explained on the assumption that it is D-fructofuranose which competes with the substrate for a position at the receiving area of the enzyme surface. Gottschalk[108] has shown that at 20° a D-fructose solution at equilibrium contains about 20% of the ketose in the furanose form. From Fig. 9 it is evident that the steric configuration of β-D-fructofuranose is such as to provide hydroxyl groups for contact with the enzyme protein in a similar manner to maltose (*cf.* Fig. 6) and D-glucopyranose. D-galactose, on the other hand, does not afford a contacting group at carbon atom 4 (Fig. 9).



β-D-Fructofuranose α(β)-D-Glucopyranose α(β)-D-Galactopyranose

FIG. 9

According to Jsaiev[103] the dissociation constant of the maltase–maltose compound, determined by the method of Michaelis and Menten,[109] varies between 0.1349 and 0.1995 depending on the concentration of maltase, though it should be independent of the latter and characteristic for the

[106] E. F. Armstrong, *Proc. Roy. Soc. London.* **B73**, 516 (1904).
[107] R. O. Herzog, *in* C. Oppenheimer, Die Fermente. 4th ed., Thieme, Leipzig, 1913, p. 987.
[108] A. Gottschalk, *Australian J. Exptl. Biol. Med. Sci.* **21**, 139 (1943).
[109] L. Michaelis and M. L. Menten, *Biochem. Z.* **49**, 333 (1913).

enzyme. The dissociation constant of the maltase–glucose compound was found to be 0.0160 to 0.0200 (average 0.0178) for solutions containing 0.142 *M* maltose and 0.05 to 0.222 *M* glucose. This means that the affinity of maltase for D-glucose is about ten times greater than its affinity for maltose.

Furthermore, Jsaiev[103] has investigated whether in the case of maltase the effect of substrate concentration on the hydrolysis rate can be adequately expressed by the Michaelis-Menten equation, taking into account the part played by the reaction product, D-glucopyranose. The equation assumes the form:

$$kt = \left[\frac{1}{a} + \frac{2}{K_g}\right] a \ln \frac{a}{a - x} + \left[\frac{1}{K_m} - \frac{2}{K_g}\right] x,$$

where K_g and K_m are the respective dissociation constants of the maltase–glucose and maltase–maltose compounds, and where a and $a - x$ are the respective maltose concentrations (molar) at time 0 and time t. The values for k at time t with increasing substrate concentration and with different concentrations of the enzyme are recorded in Table VI. During the first half of the hydrolysis and at low concentration of maltase the values for k are fairly steady (Table VIA); at higher concentration k rises after a period of constancy (Table VIB); with very high enzyme concentration the values for k have a marked tendency to fall as the reaction proceeds (Table VIC). It would appear from these results that the dissociations of the maltase–maltose and maltase–glucose complexes correspond only in a rough approximation to the dissociation of weak acids.

Willstätter *et al.*[105] determined the dissociation constants of the maltase–substrate intermediate compounds using maltose, phenyl α-D-glucoside, and methyl α-D-glucoside as substrates. As may be seen from the figures in Table VII the affinity of maltase for its substrates decreases in the order phenyl α-D-glucoside, methyl α-D-glucoside, maltose; like β-glucosidase (from almonds) maltase has a higher affinity for phenyl than for methyl glucoside. The dissociation constant for the same substrate differs from yeast to yeast. It is most probably due to this fact that the ratio of the "maltase values" methyl α-D-glucoside/maltose, obtained with 0.14 *M* substrate concentration, varies considerably from yeast to yeast; the variation is somewhat smaller for the ratio phenyl α-D-glucoside/maltose (*cf.* Table VIII). When, however, the substrate concentration is infinite ($[S] = \infty$), the ratios of the "enzyme values" methyl α-D-glucoside to maltose and phenyl α-D-glucoside to maltose, respectively, become fairly constant, *i.e.*, independent of the kind of yeast, indicating that the concentration of the maltase–substrate intermediate compound is a major factor determining the ratio of the hydrolysis rates.

Summarizing the available evidence it would appear that the Michaelis-

Menten equation does not exactly describe the enzymatic hydrolysis of maltose. Factors seem to take part in the control of the reaction for which provision is not made in the Michaelis-Menten equation. One may consider, as pointed out by Michaelis[110], that the enzyme is present in the form of multimolecular micelles rather than in a molecular dispersion. Under

TABLE VI

TEST OF THE VALIDITY OF THE MICHAELIS-MENTEN EQUATION FOR THE ENZYMATIC HYDROLYSIS OF MALTOSE[103]

Time, min.	Values for $k \times 10^3$ at maltose concentrations of				
	0.025 *M*	0.05 *M*	0.10 *M*	0.20 *M*	0.40 *M*
A. Enzyme concentration 1; $K_m = 0.1513$; $K_g = 0.01784$					
10	1.68	1.46	1.85	1.94	1.79
20	1.72	1.80	1.91	1.98	1.97
30	2.08	1.90	1.98	2.07	2.02
45	1.82	1.93	2.01	2.35	2.10
90	1.76	1.94	2.09	2.24	1.92
180	1.34	1.51	1.75	1.97	1.83
B. Enzyme concentration 8; $K_m = 0.1995$; $K_g = 0.01784$					
10	4.21	4.35	5.47	5.37	5.67
20	3.97	4.11	5.34	5.45	5.41
30	4.00	4.40	5.05	5.61	6.75
45	4.19	4.69	5.66	6.39	7.36
60	4.43	4.91	6.06	6.39	7.84
90	4.83	7.26	7.13	8.56	9.12
180	6.69	8.46	11.63	11.58	14.29
C. Enzyme concentration very high; $K_m = 0.1513$; $K_g = 0.01784$					
10	—	41.1	42.5	43.1	34.3
20	—	32.2	37.4	34.0	22.2
30	—	27.8	30.4	29.8	19.6
45	—	25.2	27.4	27.1	18.2
60	—	23.8	25.6	25.6	16.7
90	—	23.6	25.3	24.8	13.7
180	—	19.2	19.9	18.9	10.2

these conditions the fixation of a maltose or glucose molecule to one active center of the micelle may influence the fixation of another molecule, whether maltose or glucose, to another site of the micelle. In such a system K_m does not stand for one fundamental dissociation constant but for the average

[110] L. Michaelis, *Advances in Enzymol.* **9**, 1 (1949).

of various dissociation constants under a given set of experimental conditions; the same would hold for K_σ (cf. Haldane[111]).

c. *Reversion of Maltose Hydrolysis*

The enzymatic hydrolysis of maltose is a reversible reaction. This was shown by Hill,[112] to whom goes the credit of having demonstrated for the first time an enzymatic synthesis *in vitro*. In order to reduce the active mass of water Hill used concentrated (about 40%) solutions of glucose which he submitted to the action of yeast extract. Hill claimed the disaccharide

TABLE VII

DISSOCIATION CONSTANTS OF MALTASE-SUBSTRATE COMPOUNDS[105]

Maltase extract	Dissociation constants		
	Maltose	Methyl α-D-glucoside	Phenyl α-D-glucoside
Löwenbräu yeast	0.12	0.075	0.050
Hofbräu yeast	0.145	0.028	0.021
Copenhagen yeast	0.30	0.037	—

TABLE VIII

RATIO OF "MALTASE VALUES" FOR EQUAL CONCENTRATION OF SUBSTRATES[a] AND FOR EQUAL CONCENTRATION OF MALTASE-SUBSTRATE INTERMEDIATE COMPOUNDS[b105]

Maltase extract	Methyl α-glucoside/maltose		Phenyl α-glucoside/maltose	
	$Q_{0.14}$	Q_∞	$Q_{0.14}$	Q_∞
Löwenbräu yeast	0.25	0.21	7.0	5.1
Hofbräu yeast	0.31	0.18	10.7	6.1
Copenhagen yeast	0.56	0.23	—	—

[a] Observed.
[b] Calculated.

formed to be maltose. Though Hill's statement that maltose is formed in the above reaction was contested by Emmerling,[113] Armstrong,[114] and others, it was shown beyond doubt by Pringsheim and Leibowitz[115] that purified yeast maltase acting at pH 6.4 and 37° upon a concentrated solution of D-glucose for several weeks catalyzes the formation of maltose, which was

[111] J. B. S. Haldane, Enzymes. Longmans, Green and Co., London, 1930, p. 43.
[112] C. Hill, *J. Chem. Soc.* **73**, 634 (1898).
[113] O. Emmerling, *Ber.* **34**, 600 (1901).
[114] E. F. Armstrong, *Proc. Roy. Soc. London.* **B76**, 592 (1905).
[115] H. Pringsheim and J. Leibowitz, *Ber.* **57**, 1576 (1924).

isolated in the crystalline state. Hill[116] admitted to his critics, however, that maltose is not the only product of reversion under these conditions. Gentiobiose, another disaccharide formed by yeast extract in the presence of glucose and identified by its octaacetate derivative,[103] owes its synthesis to the activity of a β-glucosidase; with regard to revertose, the third product formed by the action of yeast extract on glucose, its structure is as yet undecided.[103,117-119]

The reversible reaction:

$$\underset{\text{maltose}}{C_{12}H_{22}O_{11}} + H_2O \overset{\text{maltase}}{\rightleftharpoons} 2\ \underset{\text{glucose}}{C_6H_{12}O_6}$$

can be studied also with low concentrations of maltose and glucose, respectively, provided that glycerol is added to the reaction mixture, thus diminishing the active mass of water. With this arrangement Jsaiev[103] found that at equilibrium about 15% maltose is present, the same position being reached from both sides. The equilibrium does not vary with enzyme concentration or temperature.

In accord with the hydrolyzing effect of yeast maltase on alkyl α-D-glucosides the enzyme catalyzes also the synthesis of these heterosides. In a large number of fascinating investigations Bourquelot and coworkers have synthesized from α-D-glucopyranose and various alcohols a series of alkyl α-D-glucosides using yeast extract (maltase) as catalyst. Thus methyl,[120] ethyl,[120] propyl,[80] and allyl[80] α-D-glucosides were prepared and isolated as crystals. Ethylene glycol[81] yielded a mono-α-D-glucoside, whereas both propylene glycol[121] and glycerol[122] formed a mixture of two different mono-α-D-glucosides, probably due to the substitution in the one case of a primary, in the other of a secondary alcohol group. Of the α-D-glucosides with polyhydric alcohols as aglucons only ethylene glycol mono-α-D-glucoside was obtained in crystalline form by Bourquelot. The enzymatic synthesis of these alkyl α-D-glucosides was shown to be a true reversible process, the same equilibrium being attained from both sides of the reaction. The optimal conditions for the enzymatic synthesis of alkyl α-D-glucosides have been described in detail.[123,123a,b] Recently two more crystalline α-D-glucosides were prepared with yeast maltase (yeast extract) as catalyst; these are trimethylene glycol mono-α-D-glucoside and D-mannitol mono-α-D-glucoside.[82]

[116] C. Hill, *J. Chem. Soc.* **83**, 578 (1903).
[117] H. Pringsheim, J. Bondi, and J. Leibowitz, *Ber.* **59**, 1983 (1926).
[118] A. Georg and A. Pictet, *Helv. Chim. Acta* **9**, 612 (1926).
[119] H. Berlin, *J. Am. Chem. Soc.* **48**, 1107 (1926).
[120] E. Bourquelot, H. Hérissey, and M. Bridel, *J. pharm. chim.* [7] **7**, 145 (1913).
[121] E. Bourquelot and A. Aubry, *J. pharm. chim.* [7] **12**, 283 (1915).
[122] E. Bourquelot, M. Bridel, and A. Aubry, *Compt. rend.* **161**, 41 (1915).
[123] M. Bourquelot and A. Aubry, *J. pharm. chim.* [7] **12**, 15, 182 (1915).
[123a] A. Aubry, *J. pharm chim.* [7] **9**, 19 (1914).
[123b] A. Aubry, *J. pharm. chim.* [7] **10**, 202 (1914).

7. Effect of pH and Temperature

Aqueous solutions of maltase, prepared from yeast, are rather unstable; glycerol has a protective effect.[103] Contact of the enzyme with weak acids or alkalis for a longer period of time destroys irreversibly its activity[96,103]; after a short contact (30–60 minutes) with these agents activity may be restored by neutralization.[103]

The pH optima of the maltases from different sources are recorded in Table IX. The optimum pH for yeast maltase is identical at 25 and 35° (Table X).

TABLE IX

pH Optima of Maltases from Various Sources

Source	pH Optimum	Author
Escherichia coli	7.0	Karström[20]
Lactobacillus delbrücki	6.5	Hofmann[21]
Sulfatase bacteria	6.2–6.5	Hofmann[94]
Brewer's bottom yeast	6.1–6.8	Michaelis, Rona[124]
" " "	6.75–7.25	Willstätter, Bamann[97]
" " "	6.1–6.7	Jsaiev[103]
Schizosaccharomyces octosporus	4.5	Hofmann[125]
S. pombe	4.5	Hofmann[125]
S. mellacei	4.5	Hofmann[125]
Aspergillus oryzae (taka-diastase)	4.5	Leibowitz, Mechlinski[68]
A. niger	4.2–4.6	Hofmann[21]
Barley malt	4.5	Pringsheim, Leibowitz[126]
Sorghum vulgare	4.2	Acharya[127]
Solanum indicum	5.5	Tauber, Kleiner[95]
Bombyx mori (intestine)	6.8	Shinoda[52]
Intestine (pig)	5.2–7.2	van Nieuwenhoven *et al.*[128]
Intestinal mucosa (pig)	6.5	Steensholt, Veibel[129]
Mammary gland (cow)	6.8–7.0	Kleiner, Tauber[130]
Skeletal muscle (rabbit)	5.6–6.8	Petrova[62]
Blood serum (dog, pig)	6.6	Kokuryo[58]

Maltase is only slightly resistant to heat; the enzyme is destroyed at 55°.[131] The optimum temperature for yeast maltase[103] at pH 6.5 is about 35°; the temperature optimum does not vary with the concentration of enzyme

[124] L. Michaelis and P. Rona, *Biochem. Z.* **57**, 70 (1913).
[125] E. Hofmann, *Biochem. Z.* **272**, 417 (1934).
[126] H. Pringsheim and J. Leibowitz, *Biochem. Z.* **161**, 456 (1925).
[127] C. N. Acharya, *Indian J. Agr. Sci.* **4**, 476 (1934); *Chem. Abstracts* **28**, 6737 (1934).
[128] L. M. van Nieuwenhoven, D. P. Noordmans, and H. J. Vonk, *Proc. Acad. Sci. Amsterdam* **45**, 302 (1942); *Chem. Abstracts* **37**, 6025 (1943).
[129] G. Steenholt and S. Veibel, *Acta Physiol. Scand.* **6**, 62 (1943); *Chem. Abstracts* **39**, 4636 (1945).
[130] I. S. Kleiner and H. Tauber, *J. Biol. Chem.* **99**, 241 (1932).
[131] C. J. Lintner and E. Kroeber, *Ber.* **28**, 1050 (1895).

or substrate.[132] The effect of temperature on the hydrolysis rate of maltose by yeast maltase at different pH values is shown in Table X. Calculation from the tabulated figures gives a value of 1.3 for the temperature coefficient (25–35°) of the enzymatic maltose hydrolysis at optimum pH.

Alcohol destroys yeast maltase[103,133] nearly completely, acetone reduces its activity markedly.[103] Whereas toluene has a damaging effect on yeast maltase, sodium fluoride concentrations up to 1% do not appreciably change the rate of the enzymatic cleavage of maltose.[103]

TABLE X

EFFECT OF TEMPERATURE ON ENZYMATIC HYDROLYSIS OF MALTOSE AT DIFFERENT pH VALUES[103]

pH	Percentage of maltose hydrolyzed					
	25°			35°		
	0.5 hr.	1 hr.	2 hr.	0.5 hr.	1 hr.	2 hr.
5.57	10.83	15.97	22.92	18.34	26.67	37.09
5.97	19.86	28.20	37.92	26.11	35.84	47.09
6.47	21.40	30.56	40.14	27.36	37.92	48.90
6.98	16.81	25.14	35.84	23.48	32.78	45.56

8. THE PROBLEM OF MALTOLYSIS WITHOUT MALTASE ACTION

In 1895 Fischer[14] advanced the theory that the alcoholic fermentation of maltose by yeast is initiated by the enzymatic hydrolysis of the disaccharide to glucose; the basis of this theory was his discovery that an extract from dried brewer's yeast readily splits maltose to glucose. Willstätter *et al.*[97,134,135] opposed this concept on the strength of the following evidence:

(*1*) Autolyzates of brewer's and distiller's yeasts, prepared in a similar manner to that described in Section II-5 (0.5–1.0 g. dry weight of yeast in 100 ml. of the final buffer solution), exhibited scarcely any maltase activity when the pH of the final mixture was adjusted to 4.5. The intact cells, however, suspended in a buffer solution of pH 4.5, fermented maltose, brewer's yeast at a higher rate than distiller's yeast.

(*2*) With some kinds of distiller's yeast, even at the optimal pH 6.8 for maltase, no appreciable maltose hydrolysis was effected by the autolyzate, whereas the intact cells fermented the disaccharide, though at a low rate.

From these results Willstätter deduced that yeast cells are able to fer-

[132] A. Compton, *Ann. Inst. Pasteur* **30**, 497 (1916).
[133] E. Fischer, *Z. physiol. Chem.* **26**, 60 (1898).
[134] R. Willstätter and W. Steibelt, *Z. physiol. Chem.* **116**, 211 (1921).
[135] R. Willstätter and E. Bamann, *Z. physiol. Chem.* **152**, 202 (1926).

ment maltose "directly," *i.e.*, without previous hydrolysis by maltase. In a recent discussion[136] of the problem three objections against Willstätter's arguments were raised:

(*a*) As was shown by Gottschalk,[137] the pH of the interior of the yeast cell, determined by the glass electrode, is 6.0; the buffer system of the yeast cell is so efficient that addition of 5.0 ml. of 0.045 *N* sulfuric acid to 50 g. baker's yeast, liquefied by freezing and thawing, lowered the pH value only from 6.0 to 5.6. Therefore, living yeast suspended in a buffer solution of pH 4.5 will maintain its physiological pH of 6.0. When, however, autolyzed yeast is added to 25-fold volume of buffer (pH 4.5), this buffer will control the pH of the resulting suspension.

(*b*) In the assay for determining the maltase content of yeast the enzymes made soluble during the process of autolysis are diluted by addition of the buffer more than 30 times compared with their intracellular concentration. In such high dilutions enzymes are usually unstable. Willstätter *et al.* have not tested whether under the conditions prevailing in very dilute autolyzates the hydrolysis rate of maltose is proportional to the enzyme concentration.

(*c*) In the autolyzate glucose produced by the action of maltase on maltose accumulates and inhibits the hydrolysis rate, whereas in the fermentation of maltose by living yeast the glucose formed is fermented and thus withdrawn from the maltase-maltose system.

Obviously, the conditions under which Willstätter tested for maltose fermentation (by living yeast cells) and for maltase activity (in dilute autolyzate) were so different that an identical response could not be expected.

More recently Leibowitz and Hestrin[138,139] claimed to have obtained strong evidence in favor of a "direct" mechanism of maltose fermentation by yeast. These authors approached the problem by comparing the fermentation rates of maltose and methyl α-D-glucoside by living yeast under various conditions. The fermentation rate of methyl α-D-glucoside was taken as a measure of the maltase activity of the living and actually fermenting cell on the assumption that methyl α-D-glucoside can be fermented only after a preliminary hydrolysis. Leibowitz and Hestrin made the following observations:

(*1*) The fermentation rates of glucose, maltose, and methyl α-D-glucoside, observed with pH as the variant, are recorded in Table XI.

(*2*) With brewer's bottom yeast the ratio of the fermentation rate of maltose to that of methyl α-glucoside was found to be 4.2 at 35°, 31 at

[136] A. Gottschalk, *Wallerstein Labs. Communs.* **12**, 55 (1949).
[137] A. Gottschalk, *Australian J. Exptl. Biol. Med. Sci.* **21**, 133 (1943).
[138] J. Leibowitz and S. Hestrin, *Enzymologia* **6**, 15 (1939).
[139] J. Leibowitz and S. Hestrin, *Biochem. J.* **36**, 772 (1942).

25° and greater than 144 at 4° with 10% substrate concentration, whereas the ratio of the fermentation rate of maltose to the fermentation rate of glucose was unity at all temperatures with the substrate concentrations tested (Table XII).

TABLE XI

EFFECT OF pH ON FERMENTATION RATE OF GLUCOSE, MALTOSE, AND METHYL α-GLUCOSIDE BY LIVING YEAST AT 32°[139]

Type of yeast	Substrate (6% concn.)	Ml. CO_2/10 min./g. fresh yeast at pH of						
		3.0	4.0	5.0	6.0	6.5	7.0	7.6
Brewer's	Maltose	3.3	5.6	6.0	5.5	—	3.3	—
	Methyl α-glucoside	0.1	0.2	1.0	2.1	—	2.6	2.3
Baker's	Maltose	3.8	5.9	6.0	5.0	3.2	0	—
	Methyl α-glucoside	0	0	0	0	0	0	0
	D-Glucose	8.7	8.7	8.7	8.7	—	8.7	—

TABLE XII

EFFECT OF TEMPERATURE AND SUBSTRATE CONCENTRATION ON FERMENTATION RATE OF GLUCOSE, MALTOSE, AND METHYL α-GLUCOSIDE BY LIVING YEAST AT pH 6.8[138]

Brewers' bottom yeast	Temp., °C.	Substrate concentration,[a] %	Reciprocal of maximum fermentation rate[b]		
			Glucose	Maltose	Methyl α-glucoside
Sample A	35	2.5	1.2	1.2	200
		10.0	1.2	1.2	5
Sample B	25	2.0	1.3	1.3	460
		5.0	1.3	1.3	140
		10.0	1.3	1.3	40
Sample C	35	2.5[c]	1.7	1.6	5
	4	2.5[c]	22	20	>2880

[a] Referred to glucose units in the substrate.

[b] Expressed as time, in minutes, required for the fermentation of 1 mg. glucose in the substrate, determined after the end of the induction period.

[c] Concentration of methyl α-glucoside 10%.

(3) The fermentation rate by living brewer's yeast of methyl α-D-glucoside varied over a much wider range with substrate concentration than that of glucose or maltose (Table XII).

The differential effect of pH, temperature, and substrate concentration on the fermentation rate of maltose and methyl α-glucoside is regarded by

Leibowitz and Hestrin as proof for the existence of a direct mechanism of maltose fermentation. Additional evidence for this conclusion is seen in the fact that the pH–activity curve of maltose fermentation by yeast differs markedly from that of cell-free maltase.

The evidence presented by the authors, however, cannot be regarded as conclusive. A coincidence of the pH optima of cell-free maltase and of maltose fermentation by intact cells cannot be expected, as pointed out above (*a*). The interpretation by Leibowitz and Hestrin that cell-bound maltase is subject to the improbably high temperature coefficient $V_{35}/V_4 > 576$ is incompatible with the observation that yeast maltase has a rather low temperature coefficient—1.90 for temperature interval 10–20°, 1.44 for 20–30°, and 1.28 for 30–40°.[103,131]

Differences in the permeability of the yeast cell membrane for maltose and methyl α-glucoside seem to be a more likely factor in determining the different fermentation rates by the living cell of the respective substrates than differences in the enzyme systems concerned. Leibowitz and Hestrin have themselves published data supporting this view. Thus, it was shown that living brewer's yeast at 4° did not ferment methyl α-glucoside at all and at 25° fermented this compound (2% concentration) only at a minute rate. In contrast to this, cell-free maceration juice prepared from the same yeast fermented methyl α-D-glucoside at 4° as well as at 28°, even at 2% concentration, at the same rate as maltose. Furthermore, baker's yeast, which failed to ferment maltose at pH 7.0 and methyl α-glucoside at any pH, did so after drying. These results strongly suggest that the rates at which maltose and methyl α-glucoside penetrate the cell membrane are different and that the ratio of these rates varies with the type of yeast. Such a concept would account for the differential effect of pH and temperature on the fermentation rate of the two substrates (*cf.* Orskov,[140] Dawson and Danielli,[141] and Gottschalk[142]). We may conclude, therefore, that there is no valid evidence for the theory that the industrial yeasts are able to ferment maltose by another mechanism than that initiated by maltase action.

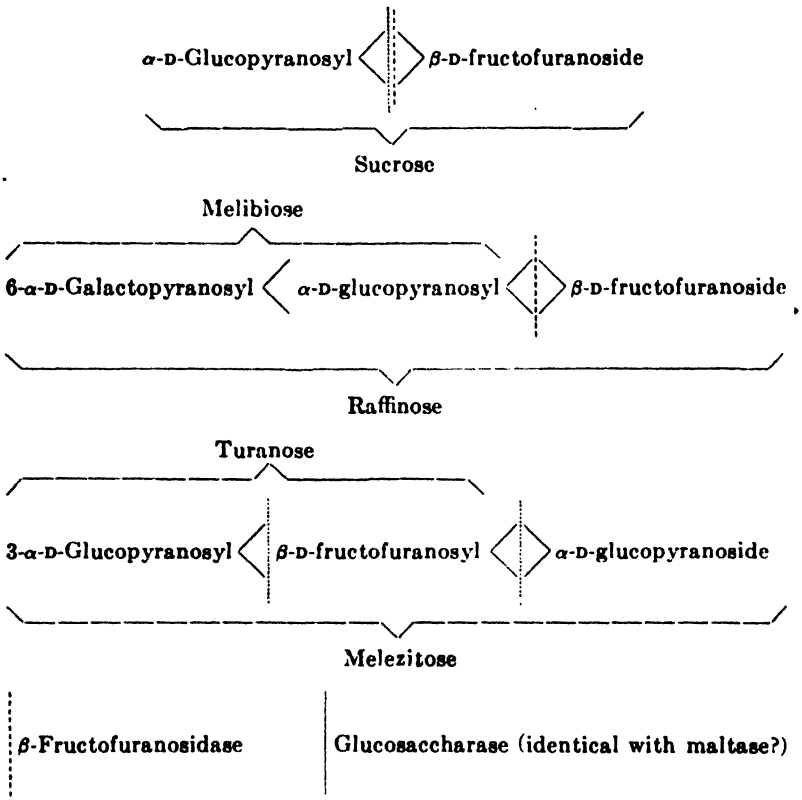
III. Glucosaccharase

There exist in nature enzymes which hydrolyze sucrose by a mechanism different from that of the classical yeast saccharase. Yeast saccharase has been shown to be a β-fructofuranosidase, acting on a number of β-D-fructofuranosides (holosides and heterosides) and requiring an unsubstituted β-D-fructofuranosyl residue. Since it establishes contact mainly with the

[140] S. L. Orskov, *Acta Path. Microbiol. Scand.* **23**, 523 (1946).
[141] H. Davson and J. F. Danielli, The Permeability of Natural Membranes. The University Press, Cambridge, 1943.
[142] A. Gottschalk, *Biochem. J.* **41**, 276 (1947).

unsubstituted fructose moiety of the disaccharide, it is inert toward melezitose[143-145] (*cf.* Table XIII) and has no affinity for α-D-glucopyranose.[146] The enzyme acts as a fructosaccharase. Kuhn[147,148] has described the existence in *Aspergillus oryzae* of a glucosaccharase which combines predominantly with the unsubstituted glucose part of sucrose. Glucosaccharase hydrolyzes

TABLE XIII



sucrose and melezitose (into glucose and fructose), but is inoperative toward raffinose.

There is, however, some doubt whether glucosaccharase is an enzymatic entity. Weidenhagen[149] has advanced the attractive theory that all α-D-

[143] R. Kuhn and C. E. von Grundherr, *Ber.* **59**, 1655 (1926).
[144] M. Bridel and T. Aagaard, *Compt. rend.* **184**, 1667 (1927).
[145] R. Weidenhagen, *Z. Ver. deut. Zucker-Ind.* **78**, 781 (1928); *Chem. Abstracts* **23**, 4485 (1929).
[146] R. Kuhn and H. Münch, *Z. physiol. Chem.* **163**, 1 (1927).
[147] R. Kuhn, *Z. physiol. Chem.* **129**, 57 (1923).
[148] R. Kuhn, *Naturwissenschaften* **11**, 732 (1923).
[149] R. Weidenhagen, *Fermentforschung* **11**, 155 (1930).

glucosidic linkages in naturally occurring oligosaccharides (maltose, sucrose, turanose, melezitose, and trehalose) and in synthetic α-D-glucosides (heterosides) are split by the same type of enzyme, α-D-glucosidase. The theory is based on his observation[150,151] that yeast maltase, purified and freed from β-fructofuranosidase as described in Section II-4, readily hydrolyzes maltose, sucrose, and melezitose at pH 6.9; under these conditions sucrose is split about twice as fast as maltose and the enzyme's affinity for sucrose is twice that for maltose. At pH 4.7 (optimum pH for fructofuranosidase) sucrose is not acted upon by yeast maltase. If the eluate, prepared according to Willstätter (see Sect. II-4), contains, as Weidenhagen assumes, one enzyme only, namely, α-glucosidase, this enzyme is endowed with a singular versatility as far as its "unspecificity" toward the aglucon group is concerned. It would then be necessary to add sucrose,[150] melezitose,[150] and turanose[144] to the long list of substrates for yeast maltase (*cf.* Table I). The biological distribution of the two enzymes hydrolyzing sucrose by different mechanisms seems to favor Weidenhagen's assumption that glucosaccharase is identical with maltase (α-glucosidase). Contrary to Kuhn's first statement that brewer's yeast contains only fructosaccharase (fructofuranosidase) and *Aspergillus oryzae* only glucosaccharase, further investigations, partly by Kuhn himself, have shown that both organisms contain or can contain both types of enzymes.[91,145,146,152,153]

If yeast maltase splits sucrose at pH 6.9, it cannot be claimed that this function is a general feature of plant and animal maltases. Maltase preparations obtained from *Schizosaccharomyces octosporus*,[125] *Neisseria meningitidis*,[22] and *Escherichia coli*[20,154-157] are without any effect on sucrose, and it is well known that the maltase present in blood serum (horse, ox, sheep, rat) and in extracts from gastric mucosa (horse, ox) and mammary glands (cow) does not act on sucrose[54, 130].

Summarizing the available evidence it may be regarded as established that the disaccharide sucrose, in the glucosidic linkage of which the reducing groups of both constituents are involved, can be hydrolyzed by β-fructofuranosidase and an α-glucosidase. By parallel tests with raffinose and melezitose, respectively, it is possible to determine the type of enzyme responsible for the cleavage (*cf.* Table XIII). Whether α-glucosidase is identical with maltase (Weidenhagen) or a separate enzyme (glucosac-

[150] R. Weidenhagen, *Naturwissenschaften* **16**, 654 (1928).
[151] R. Weidenhagen, *Z. Ver. deut. Zucker-Ind.* **78**, 539 (1928); *Chem. Abstracts* **23**, 3237 (1929).
[152] R. Kuhn and H. Münch, *Z. physiol. Chem.* **150**, 220 (1925).
[153] R. Weidenhagen, *Z. Ver. deut. Zucker-Ind.* **78**, 406 (1928); *Chem. Abstracts* **23**, 3237 (1929).
[154] A. I. Virtanen, *Biochem. Z.* **235**, 490 (1931).
[155] K. Myrbäck, *Z. physiol. Chem.* **198**, 196 (1931).
[156] H. Tauber and I. S. Kleiner, *J. Gen. Physiol.* **16**, 767 (1933).
[157] M. Hotchkiss, *J. Bact.* **29**, 391 (1935).

charase of Kuhn) will be decided by further experimental work. Not until Weidenhagen's α-glucosidase (maltase) preparation is shown by physicochemical methods to be homogeneous will the identity of maltase and glucosaccharase be regarded as proved; this holds for yeast maltase, mold maltase, and the maltase of the intestinal mucosa of the pig. On the other hand, the mere fact that maltases from some sources are not operative toward sucrose does not disprove the identity of maltase and glucosaccharase; as pointed out in Section II-3, maltases of different origin may vary considerably with regard to the range of their appropriate substrates.

IV. Trehalase

1. Historical

The sugar known as trehalose was discovered, in 1832, by Wiggers[158] in *Secale cornutum* (*Claviceps purpurea*) and called "saccharum spermodiae." Berthelot[159] obtained the same substance from trehala manna (cocoons of the parasitic insect *Larinus nidificans* found on *Echinops persicus*) and termed it "trehalose." Since then trehalose has been found in a great number of fungi where it may play a role similar to that of sucrose in higher plants (*cf.* Iwanoff[160]). An enzyme hydrolyzing trehalose was first observed in *Aspergillus niger* by Bourquelot[15,33] and described as trehalase. Bourquelot, who had previously[9] found maltase in the same organism, differentiated the two enzymes by their different heat stabilities; whereas trehalase was completely destroyed at 63°, maltase was still active at this temperature losing its activity between 74 and 75°. This was the first proof that trehalase is an enzyme distinct from maltase.

2. Occurrence

The occurrence of trehalase in nature is summarized in Table XIV.

3. Specificity

Trehalase splits the glucosidic linkage of the nonreducing saccharide trehalose, *i.e.*, α-D-glucopyranosyl α-D-glucopyranoside (Fig. 10). Since yeast maltase does not act on trehalose,[161] there can be no doubt that trehalase is an individual enzyme; therefore Weidenhagen's theory in its broadest form (*cf.* page 578) does not hold. The findings by Helferich[162] and Baba,[163] respectively, that 6-methanesulfonyltrehalose and trehalose monophosphoric ester, which most likely is a 6-ester, are substrates for trehalase

[158] H. A. L. Wiggers, *Ann.* **1**, 129 (1832).
[159] M. Berthelot, *Ann. chim. phys.* [3] **55**, 269 (1859).
[160] N. N. Iwanoff, *Biochem. Z.* **162**, 455 (1925).
[161] R. Weidenhagen, *Z. Ver. deut. Zucker-Ind.* **78**, 788 (1928); *Chem. Centr.* **1929**, I, 2312.
[162] B. Helferich and F. von Stryk, *Ber.* **74**, 1794 (1941).
[163] T. Baba, *Biochem. Z.* **273**, 207 (1934).

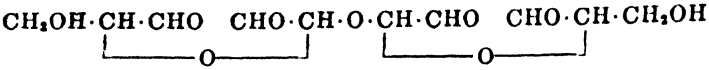
TABLE XIV

OCCURRENCE OF TREHALASE

Organism	Author
Plants	
Myxomycetes	
Lycogala	Iwanoff[160]
Fungi	
Mucor spp	Satina, Blakeslee[89]
Saccharomyces	Fischer,[14] Kalanthar,[164] Lindner[165]
Aspergillus niger	Bourquelot[15,33]
Penicillium glaucum	Bourquelot[15,33]
Monilia candida	Lindner[165]
Boletus edulis, *B. aurantiacus*, *B. luteus*, *B. badius*; *Amanita muscaria*, *A. rubescens*; *Lactarius turpis*, *L. torminosus*; *Paxillus involutus*; *Russula delica*; *Cortinarius elatior*	Bourquelot, Hérissey[166,167]
Volvaria speciosa	Bourquelot[15,33]
Phanerogamia	
Hordeum sativum (green malt)	Fischer[14]
Amygdalus communis (sweet almond)	Weidenhagen[102]
Animals	
Insects	
Gryllotalpa gryllotalpa	Frèrejacque[168]
Doryphora sp	Frèrejacque[168]
Crustaceans	
Homarus vulgaris (blood serum)	Willstädt, Borggård[169]
Fishes	
Perca fluviatilis (blood serum); *Esox lutius* (blood serum); *Cyprinus carpio* (blood serum)	Fischer, Niebel[84]
Raniceps raninus (blood serum and intestinal mucosa); *Labrus berggylta* (blood serum and intestinal mucosa); *Pleuronectes platessa* (intestinal mucosa); *Cottus scorpius* (intestinal mucosa)	Willstädt, Borggård[169]
Mammals	
Horse (mucosa of small intestine); ox (mucosa of small intestine)	Fischer, Niebel[84]

[164] A. Kalanthar, *Z. physiol. Chem.* **26**, 88 (1898).
[165] P. Lindner, *Wochschr. Brau.* **28**, 61 (1911); *Chem. Centr.* **1911**, I, 831.
[166] E. Bourquelot and H. Hérissey, *Compt. rend.* **139**, 874 (1904).
[167] E. Bourquelot and H. Hérissey, *Bull. soc. mycologie France* **21**, 50 (1905).
[168] M. Frèrejacque, *Compt. rend.* **213**, 88 (1941).
[169] H. Willstaedt and M. Borggård, *Arkiv Kemi Mineral. Geol.* **23**, No. 3, B1, (1946).

indicate that the enzyme tolerates substitution of one primary alcohol group of the substrate. It seems reasonable to assume that the 6-substituted glucose residue is the "aglucon" moiety of the substrate. If, however, the structure of both glucose residues of the disaccharide is changed, as is the case on periodic oxidation of trehalose, the resulting tetra-aldehyde:



is not acted upon by trehalase.[170]

A production of trehalose by yeast juice (prepared by maceration of dried brewer's yeast) fermenting glucose was demonstrated recently[170a]. It is as yet unknown whether yeast trehalase takes part in the synthesis of

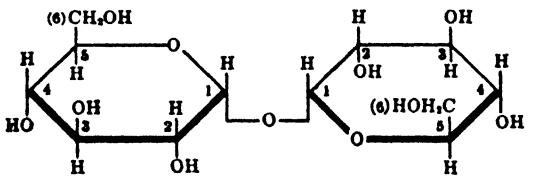


FIG. 10. Trehalose (α-D-glucopyranosyl α-D-glucopyranoside)

trehalose monophosphoric ester formed in the fermentation of glucose and fructose by dried brewer's bottom yeast.[171]

4. PREPARATION

Trehalase from Aspergillus niger.[16,172] A pure culture of *Aspergillus niger*, after it has been washed and minced, is added to four times its weight of 95% ethanol. After 3 hours the ethanol is filtered off and the residue dried *in vacuo*. The dried product is thoroughly mixed with water in order to extract the enzyme; after some time the mixture is filtered and the filtrate precipitated with ethanol. The precipitate is washed with ethanol and finally dried *in vacuo*. Optimum pH for the *Aspergillus* trehalase[169] is about 4.0.

Trehalase from Yeast.[173] To 500 g. pressed baker's yeast (*Presshefe*) are added 300 ml. water and 50 ml. ethyl acetate; the mixture is allowed to stand at room temperature for 5 days and then filtered. 1 g. of trehalose dissolved in 10 ml. of the filtrate is hydrolyzed in 47 hours at 20° to the extent of 57%. Optimum pH is about 5.4.

It may be pointed out that besides trehalase both preparations contain other enzymes, e.g. maltase.

5. MEASUREMENT OF ACTIVITY

The activity of the enzyme is measured by determining in the assay the reducing power or the change in optical rotation.

[170] J. Courtois and A. Valentino, *Bull. soc. chim. biol.* **26**, 93 (1944).
[170a] M. Elander and K. Myrbäck, *Arch. Biochem.* **21**, 249 (1949)
[171] R. Robinson and W. T. J. Morgan, *Biochem. J.* **22**, 1277 (1928).
[172] P. Harang, *J. pharm. chim.* [6] **23**, 16 (1906).
[173] K. Myrbäck and B. Örtenblad, *Biochem. Z.* **291**, 61 (1937).

Chapter 16

β-Glucosidase

By STIG VEIBEL

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I. Occurrence

1. Plants

The term emulsin was originally used to designate the enzyme which is able to split the amygdalin present in bitter almonds into glucose, benzaldehyde, and hydrocyanic acid. It was soon found to be present not only in almonds, but in the kernels of other plants belonging to the *Rosaceae* (apricots, cherries, hips, etc.). Later experiments have proved the presence of β-glucosidase in many other higher plants, *e.g.*, different members of the *Allium* genus,[1] in sweet cherry, and in the mandarin. In most instances the β-glucosidase is accompanied by a β-galactosidase which in special cases may be the sole β-glycosidase present (*e.g.*, soybeans, alfalfa seeds, see page 624). Yeast may contain considerable amounts of β-glucosidase,[2] and also molds, *e.g.*, *Aspergillus oryzae* (taka-diastase)[3] and *A. niger*[4] are able to catalyze the hydrolysis of β-glucosides. In sulfatase bacteria Hofmann[5] was able to show the presence of a β-glucosidase, but other bacteria examined did not contain β-glucosidase even if β-galactosidase was present. No systematic investigation has, however, been carried through.

2. Animals

Warm-Blooded Animals. Neuberg and Hofmann[6] prepared β-glucosidase-active powders from kidney and liver of horse ans cattle. Steensholt and Veibel[8] examined mucosa of the small intestine and the duodenum of pig and found a β-glucosidase present.

Cold-Blooded Animals. Only the digestive juice of *Helix pomatia* has, to the knowledge of the reviewer, been examined. It contains β-glucosidase and at the same time cellase (cellulase).[9] The question whether these two enzymes are really different or if they are to be considered one enzyme will be discussed in Section IV-2-*b*.

II. Preparation and Purification

1. Extraction and Precipitation

a. Almond Emulsin

As sweet almonds seem to be the best source of β-glucosidase a description of the extraction and isolation of dry preparations of β-glucosidase from sweet almonds will be given here.[10]

[1] R. E. Chapman, *Biochem. J.* **18**, 1388 (1924).
[2] C. Neuberg and E. Hofmann, *Biochem. Z.* **256**, 450 (1932).
[3] J. Hatano, *Biochem. Z.* **151**, 501 (1924).
[4] E. Hofmann, *Biochem. Z.* **275**, 320 (1934–1935).
[5] E. Hofmann, *Biochem. Z.* **272**, 133 (1934).
[6] C. Neuberg and E. Hofmann, *Biochem. Z.* **231**, 431 (1935). cf. ref.[51]
[7] R. Willstätter and E. Waldschmidt-Leitz, *Z. physiol. Chem.* **125**, 132 (1923).
[8] G. Steensholt and S. Veibel, *Acta physiol. Scand.* **6**, 62 (1943).
[9] P. Karrer, *Kolloid-Z.* **52**, 304 (1930).
[10] B. Helferich, S. Winkler, R. Goots, O. Peters, and E. Günther, *Z. physiol. Chem.* **208**, 91 (1932).

One kg. finely powdered press cake of sweet almonds is dispersed in a solution of 50 g. zinc sulfate (commercial hydrate, 7 H_2O) in 4.5 l. water and left standing at 0° for 4–5 hours. The cold solution is then filtered through a cloth and well pressed on the filter. To the filtrate is added cautiously a solution of 1.4 g. tannin in 500 ml. water. A precipitate consisting mostly of impurities is removed by centrifugation and discarded. The bulk of the enzyme is then precipitated by slowly adding a solution of 15 g. tannin in 500 ml. water. The precipitate is isolated by centrifugation, freed from tannin by repeatedly dispersing it in acetone in the beakers, centrifuging it, and finally drying it in a desiccator over sulfuric acid. The crude enzyme *Rohferment* of Helferich, obtained in this way, has an activity of 1.25, expressed as β-glucosidase value (see Sect. III-2).

Other methods of isolation of raw preparations are indicated by Rabaté[11] and by Helferich and Bredereck.[12] β-Glucosidase is isolated in a similar manner from other higher plants.

b. Yeast Emulsin

Yeast is plasmolyzed by addition of ethyl acetate. Neuberg and Hofmann[3] indicate that different strains of yeast are plasmolyzed with varying ease. Usually the addition of 10% by weight of ethyl acetate is sufficient to break the cell walls completely in 5 minutes. The dark colored liquid is then diluted with 4 volumes of water, 1% toluene is added, and the solution is kept in the icebox for 2 days. After centrifugation the clear solution may be used for experiments or the enzyme may be isolated as dry preparation[13] by adding the solution to 25 volumes of an ethanol–diethyl ether mixture (4:1). In 3–5 minutes the enzyme is precipitated, isolated by decantation, centrifugation, or filtration, repeatedly washed with ethanol and diethyl ether, and finally dried in a vacuum desiccator. Five g. air-dried yeast give some 0.4 g. enzyme powder, slowly but completely soluble in water.

c. β-Glucosidase of Animal Origin

β-Glucosidase of animal origin is prepared[4] by mincing the glands shortly after the animal has been killed (1–2 hours), mixing the paste with the equal weight of water, adding 1% toluene, and keeping the mixture in closed bottles for 2 days at 37°. The enzyme is liberated by the autolysis of the cells. The autolyzed liquid is centrifuged and may be used directly as enzyme solution or may be transformed into a dry preparation by adding 1 volume of the solution to 4 volumes of an ethanol–ether mixture (4:1). The precipitate is isolated by suction, washed repeatedly with the ethanol–ether mixture, and finally with ether, and dried in a vacuum desiccator over calcium chloride, phosphorus pentoxide, and paraffin.

A more direct method for producing dry preparations of different enzymes is indicated by Willstätter and Waldschmidt-Leitz.[7] The minced organs are freed from water by slowly mixing them with 10 volumes of acetone. The precipitate is treated once more with 10 volumes of acetone, then with 10 volumes of an ether–ethanol mixture (1:1) and finally two times with 10 volumes of ether. The material is then placed on filter paper in a thin layer and air-dried. It still contains some coarse remnants of cell membranes which are done away with by milling the powder in an efficient mill and sieving through a fine-mesh sieve.

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[11] J. Rabaté, *in* Bamann-Myrbäck, Die Methoden der Fermentforschung. Thieme, Leipzig, 1941, Academic Press, New York, 1945, p. 1819.

[12] B. Helferich and H. Bredereck, *Z. physiol. Chem.* **189**, 273 (1930).

[13] E. Hofmann, *Biochem. Z.* **256**, 462 (1932).

From the mucosa of intestines Steensholt and Veibel[9] prepared enzyme solutions by scraping off the mucosa, mixing it with sea sand, and carefully grinding it in a mortar. The mixture was then extracted with water (some 50 ml per g. mucosa) for several hours and centrifuged. The clear solution was used directly as enzyme solution. Glycerol, which for other enzymes may be more efficient than water as solvent, proved to be unsuitable for the extraction of β-glucosidase.

2. Purification

a. Precipitation Methods

The above-mentioned preparation of crude enzyme includes a purification of the enzyme by precipitating it with tannin and removing the tannin by washing with acetone. A further purification may be obtained by precipitating the enzyme with silver acetate, followed by a rapid treatment of the precipitate with hydrogen sulfide in order to avoid the destruction of the enzyme by the silver ions.[14]

Two g. crude enzyme are dissolved in 200 ml. water and the solution cooled in ice water. An addition of 8.0 ml. 0.02*N* silver acetate immediately followed by the dropwise addition of 15.5 ml. 0.01*N* sodium hydroxide produces a precipitate practically free of β-glucosidase. It is removed by centrifugation and discarded. Without raising the temperature 14 ml. 0.02*N* silver acetate and then 27.5 ml. 0.01*N* sodium hydroxide are added. The precipitate is rapidly isolated by centrifugation, washed twice with ice-cold water without stirring and dissolved in about 50 ml. ice-cold 1/800 *N* ammonia, which is added in portions of about 2 ml. with efficient stirring. If some of the precipitate remains undissolved after 2–3 minutes it is removed by centrifugation. The somewhat turbid ice-cold liquid is precipitated by dropwise addition of 3.3 ml. 0.2*N* silver acetate. The precipitate contains the bulk of the enzyme and is isolated by centrifugation. The supernatant liquid is removed by decantation; the precipitate is suspended in 30 ml. ice-cold water and hydrogen sulfide is introduced, just sufficient to cause a smell of hydrogen sulfide. The silver sulfide is removed by centrifugation and the enzyme is precipitated with acetone and diethyl ether. The yield is 60–80 mg. β-glucosidase with a β-glucosidase value of 10–12, *i.e.*, 40–50% of the enzyme content of the crude enzyme (*Rohferment*), but only if the temperature is kept low and all operations are carried through rapidly.

b. Adsorption Methods

Willstätter and Csanyi[15] and Josephson,[16–18] have shown that purification of β-glucosidase solutions may be obtained by adsorption of the enzyme on aluminium hydroxide or on kaolin. Kritschewskaja[19] obtained no separation of β-glucosidase, saccharase, maltase, and lactase by adsorption on alumina Cγ[20] and elution with phosphates, ammonia, or sodium carbonate.

[14] B. Helferich and S. Winkler, *Z. physiol. Chem.* **209**, 269 (1932).
[15] R. Willstätter and W. Csányi, *Z. physiol. Chem.* **117**, 172 (1921).
[16] K. Josephson, *Z. physiol. Chem.* **147**, 1 (1925).
[17] K. Josephson, *Ber.* **58**, 2726 (1925).
[18] K. Josephson, *Ber.* **59**, 821 (1926).
[19] E. I. Kritschewskaja, *Biochem. Z.* **272**, 348 (1934).
[20] R. Willstätter, H. Kraut, and O. Erbacher, *Ber.* **58**, 2448 (1925).

By elution with dilute solutions of different glycosides it was possible, however, to obtain a specific elution. β-Glucosidase may thus be eluted with a 1% amygdalin solution.

Helferich and Pigman[21] use Carboraffin C (Leunawerke) in order to get rid of some impurities by adsorption, after which they adsorb the β-glucosidase on silica gel, followed by an elution with ammonia. 60% of the β-glucosidase is recovered, the β-glucosidase value rising from 6.5 to 12 (*cf.* Helferich[21a]). The most specific separation is, however, obtained by chromatography on bauxite or alumina.[22-24] From the glycosidases present in almond emulsin, β-glucosidase is first adsorbed, whereas α-galactosidase and chitinase pass through the column. Using two columns of bauxite Zechmeister *et al.*[22-24] succeeded in obtaining from the first column an adsorbate yielding by elution 78% of the β-glucosidase content of the original preparation, but only 20% α-galactosidase and no chitinase. From the second column was recovered by elution 78% of the α-galactosidase and in the filtrate was found 80% of the chitinase with no β-glucosidase and only 2.5% α-galactosidase.

c. Dialysis

An early report by Ohta[25] claims that protein-free solutions of β-glucosidase may be obtained by digestion of crude solutions of β-glucosidase with pancreatin and dialysis of the digested solution for 5–10 days. The inner solution is then protein-free but still contains the β-glucosidase. These results have never been confirmed. Tauber,[26] on the contrary, indicates that pancreatin and other proteases (pepsin, trypsin) are without effect on β-glucosidase. In repeating Ohta's experiment he obtained after dialysis a solution which gave no color reaction for proteins, but when the solution was concentrated in vaccum the protein reactions reappeared. Tauber is, therefore, of the opinion that the negative protein reaction of Ohta is due only to the great dilution arrived at during the dialysis. That dialysis is no efficient method of purification is proved by Edman and Jorpes,[27] who after a very efficient dialysis obtained preparations with no greater activity than ordinarily obtained. In the opinion of the reviewer it seems obvious that β-glucosidase, being as other enzymes of protein nature, cannot be efficiently separated and purified by dialysis, as the main problem is not to get rid of dialyzable impurities but to separate proteins with β-glucosidase properties from those without these properties.

d. Cataphoresis (Electrophoresis)

Edman and Jorpes[27] experimented to find out whether a purification could be arrived at by cataphoresis, but the result was not encouraging.

[21] B. Helferich and W. W. Pigman, *Z. physiol. Chem.* **259**, 253 (1939).
[21a] B. Helferich, *Ergeb. Enzymforsch.* **9**, 70 (1943).
[22] L. Zechmeister, G. Tóth and M. Balint, *Enzymologia* **5**, 302 (1938–1939).
[23] L. Zechmeister and G. Tóth, *Enzymologia* **7**, 165 (1939).
[24] L. Zechmeister, G. Tóth and P. Fürth, *Enzymologia* **9**, 155 (1940–1941).
[25] K. Ohta, *Biochem. Z.* **58**, 329 (1914).
[26] H. Tauber, *J. Biol. Chem.* **99**, 257 (1932).
[27] P. Edman and E. Jorpes, *Acta physiol. Scand.* **2**, 41 (1941).

They determined the isoelectric point to be at pH 5.7 to 5.8 and examined the result of cataphoresis at pH 3.78 and pH 8.72. In both instances the β-glucosidase properties followed the protein and no purification was obtained. Further investigations of the effect of electrophoresis should, however, be of value, as not all the possibilities of this efficient method of separation of (even related) proteins are exhausted by the experiments mentioned.

3. Chemical Nature

The protein nature of β-glucosidase is, as mentioned above, not seriously questioned. The general conception of an enzyme as composed of an colloidal carrier and a prosthetic group as the seat of the specific properties of the enzyme is by some authors considered valid also for the glycosidases.

Kostytschew[28] raised some doubt as to the individuality of the different glycosidases and considered them mixtures of substances with special physical and colloid-chemical behavior. An enzyme is, according to him, rather a condition than an individual. Neuberg[29] opposed this assumption vigorously. The view of Willstätter and Rohdewald[30] may be considered as a reconciliation of the two different interpretations. The enzyme, the symplex, is composed of a carrier and a prosthetic group, but one prosthetic group may be able to combine with different carriers, thus acquiring somewhat different specificity. Besides, accompanying substances may be able to influence the colloidal carrier, thus producing differences in the specificity of the symplex. Examples of this kind are known, *e.g.*, among the oxidation enzymes, but until now no definite proof of the presumed dissociability of β-glucosidases into carrier and prosthetic group has been reported. Helferich, Hiltmann, and Pigman[31] have tried to obtain a dissociation by dialysis of β-glucosidase solutions at different pH values from 2.75 to 10.35. In the region pH 3.6 to 8.25 the activity remained constant during 4 days of dialysis; beyond these limits the activity was diminished, but that this was not due to a dissociation was proved by the fact that neither the addition of the outer solution nor the addition of boiled enzyme solution was able to restore the activity of the enzyme.

In direct opposition to this result Malaguzzi-Valeri[32] claims to obtain a far-reaching separation of β-glucosidase into apoenzyme and coenzyme by dialysis for 24 hours at pH 6.1. The dissociation of the enzyme is reversible, since the β-glucosidase activity is restored by mixing the inner and the outer solution after dialysis. The nature of the coenzyme, the prosthetic group, has not been elucidated by the Italian author, he notes, however, that it is neither an amino acid nor a polypeptide and that it does not contain sulfhydryl, sulfide, or amino groups, The combination of apoenzyme and coenzyme is saltlike. As ampho ion or as cation the holoenzyme dissociates spontaneously into apoenzyme and coenzyme; as anion it is stable. To the knowledge of the reviewer, no confirmation of these very astonishing results has been reported and they

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[28] S. Kostytschew, *Z. physiol. Chem.* **154**, 263 (1926).
[29] C. Neuberg, *Z. physiol. Chem.* **157**, 299 (1926).
[30] R. Willstätter and M. Rohdewald, *Z. physiol. Chem.* **229**, 241 (1934).
[31] B. Helferich, H. Hiltmann and W. W. Pigman, *Z. physiol. Chem.* **259**, 150 (1939).
[32] C. Malaguzzi-Valeri, *Arch. sci. biol. Italy* **25**, 261 (1939); quoted from *Chem. centralbl.* **1940** I, 878; *Chem. Abstracts* **34**, 122 (1940).

are to be regarded with scepticism, being in opposition to all previously obtained experience with regard to the behavior of β-glucosidase.

All β-glucosidase preparations so far examined contain carbohydrate. Helferich and Pigman[21,21a] have examined the effect of purification of β-glucosidase upon its content of carbohydrate and have found that even the most active preparations (β-glucosidase values 14–16) contain 3–4% carbohydrate but that there is no direct correlation between the activity and the carbohydrate content. The carbohydrate is very firmly bound to the protein. Inactivation of the enzyme by treatment with ozone, ultraviolet radiation, or heat does not make the carbohydrate dialyzable. Helferich, Richter, and Grünler[33] found that the carbohydrate is a mixture of mannose and arabinose or possibly of glucose, mannose, and arabinose. They suggested that the role of the carbohydrate groups in the enzyme molecule is to function as the prosthetic group, or holding group, responsible for the adsorption of the glucoside molecule to the enzyme, but their suggestion is so far not supported by direct experimental evidence.

The inhibition of the β-glucosidase by carbonyl group reagents may be due to an inactivation of the prosthetic carbohydrate group but this is by no means the only possible explanation. The undialyzability of the carbohydrate seems to indicate that it is part of the structure of the protein. According to this the active area responsible for the affinity of the sugar part of a glycoside to β-glucosidase[34,35] would consist of a carbohydrate molecule, which according to Helferich and associates[33] may be either a hexose or a pentose molecule, since not only β-glucosides but also β-xylosides and α-arabinosides are hydrolyzed by β-glucosidase. A further discussion of these consequences of the assumption of carbohydrates as an integral part of the enzyme molecule will be given later (Sect. IV).

The theory of a carbohydrate molecule as a prosthetic group has been supported by Lettré,[36,36a] who compares the formation of an enzyme–substrate complex with the formation of a partial racemate. Such compounds are formed only when the two molecules are of approximatively identical structure, and as the glycosides, by definition, contain a carbohydrate group it follows that the prosthetic group of a glycosidase must be a carbohydrate or a closely related group. The aldehyde group of the carbohydrate must be free, since carbonyl group reagents inhibit the action of glycosidases. Veibel and Lillelund[37] object to this assumption, first because the pyranose ring postulated presumes the formation of an ether, not of a semiacetal, and secondly because the presence of such a ring, being of a structure too different from the pyranose ring of the glycoside, would prohibit the formation of the partial racemate necessary for the hydrolysis of the glycoside.

[33] B. Helferich, W. Richter, and S. Grünler, *Ber. Verhandl. sächs. Akad. Wiss. Leipzig, Math.-phys. Klasse* **89**, 385 (1938).
[34] W. W. Pigman, *J. Research Natl. Bur. Standards* **27**, 1 (1941).
[35] W. W. Pigman, *Advances in Enzymol.* **4**, 41 (1944).
[36] H. Lettré, *Angew. Chem.* **50**, 581 (1937).
[37] S. Veibel and H. Lillelund, *Z. physiol. Chem.* **253**, 55 (1938).

III. Standardization

The action of β-glucosidase is usually followed polarimetrically or reductometrically.[38] Different methods of assay are in use.

1. Enzyme Efficiency

For the comparison of the efficiency of different enzyme preparations of maltase Willstätter and associates[39] suggested estimation of the "time value" (*Zeitwert*), *i.e.*, the time necessary to obtain 50% hydrolysis of a solution containing 2.500 g. maltose and 1 g. enzyme in 50 ml. at 30° and optimal pH. For β-glucosidase Weidenhagen[40,41] proposed fixing standard conditions corresponding to these values, taking salicin as the standard substrate. The dependence of the velocity of hydrolysis on not only the concentration of the enzyme, but also on the concentration of the substrate, the pH, and the temperature of the solution makes it necessary to standardize all these factors. Practically it is impossible to obtain a solution containing 1 g. enzyme in 50 ml. but in most instances there is a strict proportionality between velocity of hydrolysis and concentration of enzyme so that the enzyme concentration usually is selected so that the velocity of hydrolysis is conveniently measurable, and calculation of the time value for the enzyme concentration 1 g. in 50 ml. will cause no essential error.

The dependence on substrate concentration is much more complicated and therefore the standard concentration must be observed. The standard solution contains 1.986 g. salicin in 50 ml., *i.e.*, it is 0.139*M*. The standard temperature is 30°; the standard pH is 4.4 or 5.0. The β-glucosidase value is the reciprocal value of the time value and indicates thus the number of enzyme units in 1 g. dry substance of the enzyme.

2. Salicin Factor

Independent of the proposal of Willstätter and of Weidenhagen Josephson[16] suggested indicating the efficiency of β-glucosidase preparations by the velocity constant for hydrolysis of a 2% salicin solution at 30° and pH 4.4 by an enzyme concentration of 1 g. in 50 ml. The value k/e (e = g. enzyme in 50 ml. solution) he termed salicinfactor (sal.f.). It is seen that his standard solution is more dilute than that proposed by Willstätter, and the velocity constants found by him are therefore considerably greater than those found with the above-mentioned standard solution. Josephson does not indicate to what extent the hydrolysis has to proceed in determining the velocity constant, but as the velocity constant for the hydrolysis of salicin is by no means a constant, showing a marked decrease with increasing hydrolysis, it seems necessary to standardize the degree of hydrolysis. Most conveniently 50% hydrolysis is fixed as

[38] S. Veibel *in* Bamann-Myrbäck, Die Methoden der Fermentforschung. Thieme, Leipzig, 1941, Academic Press, New York, 1945, p. 1775.

[39] R. Willstätter, T. Oppenheimer, and W. Steibelt, *Z. physiol. Chem.* **110**, 232 (1920).

[40] R. Weidenhagen, *Z. Ver. deut. Zucker-ind.* **79**, 591 (1929).

[41] R. Weidenhagen, *Ergeb. Enzymforsch.* **1**, 168 (1932).

standard degree of hydrolysis, since then the following relation between time value, β-glucosidase value, and sal.f. allows the comparison of preparations standardized by different methods:

$$\beta\text{-glucosidase value} = 1/\text{time value} = \text{sal.f.}/\log 2 = k_{50\%}/e \times \text{sal.f.}$$

This relation is only valid when sal.f. and time value are determined with salicin solutions of identical concentration. The velocity constants are the unimolecular constants determined with the minute as unit of time and calculated with common logarithms.

3. Salicin Value

Since the standard salicin solution mentioned (0.139*M*) is not far from saturation at 30°, it may be inconvenient to prepare the standard reaction solution by mixing an enzyme solution with a more concentrated salicin solution because this will often be supersaturated at 30°. Another inconvenience is the decline of the velocity constants with increasing hydrolysis, due at all events partially to the inhibiting action of the products of hydrolysis. Veibel and Lillelund[42,43] proposed to avoid these inconveniences by using not the directly determined velocity constants, $k_{obs.}$ but a velocity constant termed by them k_3, which is the calculated velocity constant for the dissociation

TABLE I

k_3 Values for Various β-Glucosides and Factors for Conversion to Salicin Value and Sal.f.

Glucoside	$k_3 \times 10^2$	Proportionality factor for transformation into Salicin value	Sal.f. $\times 10^2$
Salicin	18.9	1.0	5.3
Methyl gluco ide	1.89	10.0	53
Ethyl glucoside	1.58	12	63
Propyl glucoside	5.08	3.7	20
Isopropyl glucoside	7.51	2.5	13
n-Butyl glucoside	2.25	8.4	45

of the enzyme–substrate complex, formed instantaneously, into enzyme. glucose, and saligenin. The determination of k_3 implies knowledge of the dissociation constants K_m, K_{m_1}, and K_{m_2} of the addition compounds enzyme–glucoside, enzyme–glucose, and enzyme–aglucon. It was possible to show that the expression:

$$k_{obs.}[K_m + c + (K_m/K_{m_1} + K_m/K_{m_2} - 1)x]/e = \text{constant}$$

is approximatively valid for all values of c. $k_{obs.}$ is determined from point to point, x being the mean value of the concentration of the products of hydrolysis at the points used for the calculation of $k_{obs.}$. For details concerning the determination of K_m, K_{m_1}, and K_{m_2} and the calculation of k_3, see Veibel.[38,43] Veibel and Lillelund termed this constant the salicin value. It is related to the salicin factor through the relation:

$$\text{salicin value} = k_3(\text{sal.f.})$$

[42] S. Veibel and H. Lillelund, *Enzymologia* **5**, 129 (1938).

[43] S. Veibel and H. Lillelund, *K. Danske Videnskab. Selskab. Mat.-fys. Medd.* **17**, no. 6 (1940).

[44] B. Helferich and H. Appel, *Z. physiol. Chem.* **205**, 231 (1932).

The salicin value has the advantage over the other values in use that every glucoside for which k_3 has been determined may be used for the standardization of emulsin preparations. Veibel and Lillelund[43] indicate for some of the alkyl glucosides proportionality factors which may be applied for the transformation of k_3 into either salicin value or salicin factor (Table I).

4. Influence of pH and Neutral Salts

The activity of a certain preparation of β-glucosidase is dependent on the nature of the buffer solution,[44,45] the enzyme being more active in phosphate–citrate buffer than in acetate buffer. Also the pH optimum seems to some extent to be dependent on the nature of the buffer. Addition of neutral salts may be of considerable activating effect.[46,47] The influence of the anion is greater than the influence of the cation. In most cases the activation increases with increasing salt concentration to a certain limit and then remains constant even if the salt concentration is further increased, but in some instances a maximum activation is reached and further increase in salt concentration causes a decrease in the activation cr even a decrease of the original enzyme activity.

The reason for this action of neutral salts is not quite clear. According to the experiments of Veibel and Lillelund[45] no influence of the ionic strength is to be found, and the experiments of Helferich and Schmitz-Hillebrecht[46] cannot be explained by consideration of the ionic strength of the salt solutions. The action of the different anions varies in accordance with the lyotropic series, and Helferich and Schmitz-Hillebrecht are inclined to look for a colloid-chemical explanation, *e.g.*, a variation in the degree of peptization of the enzyme. As a matter of fact some neutral salts are able to cause a coagulation of the enzyme, as of other proteins, when present in sufficient concentration. It is, therefore, natural to presume that even more dilute salt solutions may influence the degree of peptization, but the question seems very complicated. Veibel and Lillelund[45] were able to show that the affinity between enzyme and substrate is dependent on the nature of the buffer solution, but the influence of neutral salts on the affinity was not investigated. Further investigation seems necessary before the question of the influence of neutral salts on the activity of the enzyme can be regarded as solved.

Not only neutral salts but also other neutral substances may influence the activity of a β-glucosidase preparation. Veibel[47,48] could show that a saturation of the solution with toluene (in order to prevent the growth of microorganisms) caused an increase of the activity of β-glucosidase from almond emulsin. The action is dependent on the substrate but seems to be independent of the purity of the enzyme preparation. Also, the redox

[45] S. Veibel and H. Lillelund, *Enzymologia* **9**, 161 (1940–1941).

[46] B. Helferich and E. Schmitz-Hillebrecht, *Z. physiol. Chem.* **234**, 54, (1935).

[47] Yu Hsieh and Tsung-Hyui Koo, *J. Chinese Chem. Soc.* **8**, 49 (1941). quoted from *Chem. Abstracts* **37**, 222 (1943).

[48] S. Veibel, *Enzymologia* **2**, 367 (1937–1938).

potential of the solution seems to influence the activity of β-glucosidase. Koch and Gagnon[49] have examined the influence of small amounts of reducing or oxydizing substances on the hydrolysis of salicin by β-glucosidase from sweet almonds and have found a maximum of activity at Eh 93 mv. Below 60 mv. and above 230 mv. the enzyme is irreversibly damaged. The explanation of the action of variation in Eh may be either physical or chemical. A physical explanation might be the same as that mentioned by Helferich and Schmitz-Hillebrecht,[46] *viz.*, an influence on the dispersity of the enzyme. In order to explain the influence chemically Koch and Gagnon suggest that certain groups of importance for the enzyme activity are themselves part of redox systems and therefore liable to be influenced by the Eh of the solution. Such systems may be a mercapto or a phenol group in equilibrium with the corresponding disulfide or quinone system. This explanation may be correct but it does not seem to be supported experimentally by the authors, and the evidence found for the necessity of the presence of mercapto or phenol groups in the enzyme molecule is by no means convincing. Also here further investigation is necessary before the significance of the Eh of the solution is clear.

The pH optimum is dependent on the source of the β-glucosidase and to a minor degree on the substrate and the buffer solution. For β-glucosidase of sweet almonds it is 4.4 to 5.0,[16,44] for β-glucosidase from *Populus* and *Salix* species[50] 5.6 to 7.0, from lactose yeast[2,13] 6.3, and from liver and kidney.[6,51] Veibel and Lillelund[45] found that the pH optimum for β-glucosidase from sweet almonds was at 4.4 for *n*-butyl glucoside both in acetate buffer and in phosphate–citrate buffer, whereas with *o*-cresyl glucoside as substrate the optimum was at pH 5.0 in acetate buffer, and at pH 4.4 to 4.5 in phosphate–citrate buffer.

IV. Specificity

1. Absolute Specificity

a. Sugar Specificity

Originally the concept was that each naturally occurring glycoside required a special enzyme, *e.g.*, amygdalase, salicinase, arbutainase. However, as the artificially prepared glycosides proved to be hydrolyzable under the influence of such enzymes as emulsin or invertase, it became clear that the specificity of the enzymes was not as great as at first presumed, but that on the other hand these preparations were not single enzymes but mixtures of different glycosidases.[52] Further experiments led to the assumption that all β-glucosides are hydrolyzed by one enzyme, a β-glucosidase.

[49] P. Koch and A. Gagnon, *Rev. Can. biol.* **6**, 52 (1947).
[50] A. W. Blagoweschenski and N. J. Sossiedow, *Biochem. J.* **21**, 1206 (1927).
[51] E. Hofmann, *Biochem. Z.* **281**, 438 (1935).
[52] R. Willstätter and G. Oppenheimer, *Z. physiol. Chem.* **121**, 183 (1922).

Willstätter and Kuhn[53] and later Weidenhagen[54] postulated that only five types of glycosidases exist, *viz.*, α- and β-glucosidases, α- and β-galactosidases, and β-*h*-fructosidase. Glycosides of all other sugars are assumed to be hydrolyzed by one of these types. Emulsin was found to contain at all events four of these five types, and the generally used terminology of emulsin as identical with β-glucosidase had to be abandoned. On the other hand, it had become usual to designate as emulsins glycosidase preparations from other sources than almonds, *e.g.*, snail emulsin, milk sugar yeast emulsin, but to understand thereby the β-glucosidase of these preparations. In order to avoid this ambiguity Helferich[55] proposed to term as emulsin all preparations able to catalyze the hydrolysis of glycosides, independent of the nature of the sugar, and to use the unambiguous names α-glucosidase, β-glucosidase, etc. when an individual enzyme is disucussed. This proposition has been generally acknowledged and the designation emulsin as equivalent to β-glucosidase has disappeared.

The sugar specificity of β-glucosidase has been studied most thoroughly by Helferich[56] and by Pigman.[57] The main result of their investigations will be discussed briefly but first the methods applied for comparing the hydrolyzability of different glycosides must be described. To this aim Helferich[55] has proposed to determine the "*Wertigkeit*", the enzyme efficiency, of the glycosides, *i.e.*, the value k/e (log 2), k and e having the significance mentioned above and the substrate concentration being $0.052M$. Evidently the determination of the velocity constants allows the ranging of β-glucosides in a series with decreasing velocity of hydrolysis if the accuracy of the determination is sufficient, but this does not seem to be the case with the methods applied by Helferich. In many cases the calculation of k is based upon a single determination of the degree of hydrolysis after, *e.g.*, 30 minutes. If the degree of hydrolysis then incidentally is in the neighborhood of 50%, the corresponding k value may be applied, being encumbered only with the usual experimental uncertainty, but if it differs essentially from 50% the error may be considerable, the velocity constants usually decreasing rapidly with increasing hydrolysis, at all events partially due to the inhibiting action of the products of hydrolysis. In order to avoid this source of uncertainty Pigman[58] calculates k from a number of samples taken from one solution at different times, covering the range 30–50% of hydrolysis. But even so, the determination of the velocity constants at an arbitrarily chosen substrate concentration pays no attention to the differences in affinity between enzyme and substrate, which may vary considerably even

[53] R. Willstätter, R. Kuhn, and H. Sobotka, *Z. physiol. Chem.* **139**, 33 (1924).
[54] R. Weidenhagen, *Fermentforschung* **11**, 154 (1930).
[55] B. Helferich, *Ergeb. Enzymforsch.* **7**, 83 (1938).
[56] B. Helferich, *Ergeb. Enzymforsch.* **2**, 74 (1933).
[57] W. W. Pigman, *J. Research Natl. Bur. Standards* **30**, 257 (1943).
[58] W. W. Pigman, *J. Research Natl. Bur. Standards* **30**, 159 (1943).

for glucosides of a homologous series of aglycons. Veibel and Lillelund[38,42,43] therefore suggested the comparison not of the $k_{obs.}$ values but the k_2 values (see Sect. III-3) in order to eliminate the influence of differences in affinity. Pigman recently,[35] discussing the two principles, expresses the opinion that both are of value and should be applied simultaneously when possible, but that in comparing the k_3 values the most important factor of enzyme-catalyzed reactions, *viz.*, the ease of formation and the stability of the enzyme–substrate complex, probably is eliminated from consideration.

This seems to the present reviewer rather obscure. The determination of k_2 implies experimental evidence sufficient for the calculation of $k_{obs.}$ and implies also the determination of the dissociation constant for the enzyme–substrate complex, this constant being the relation between the velocities of dissociation and formation of this complex and thus a measure just of the stability of the complex, no hydrolytic reaction being taken into account. The ease of formation is, according to Medwedew,[59] great. According to Veibel and Eriksen[60,61] the combination of enzyme and substrate proceeds without heat of activation and the velocity must therefore be considered unmeasurably great. The determination of k_3 (including the determination of K_m) thus provides us with material for the comparison of the stability of the enzyme–substrate complex with regard to both dissociation (K_m) and hydrolytic cleavage (k_3).

The limit between glycosides hydrolyzable and unhydrolyzable by almond emulsin is indefinite. Arbitrarily glycosides with enzyme efficiency 10^{-5} or less are considered unhydrolyzable, since even with β-glucosidase preparations of the highest obtainable β-glucosidase value (about 16) the enzyme efficiency 10^{-5} is the limit of the experimentally traceable hydrolysis.

The specificity with regard to carbon atom number 1 seems to be absolute. β-Glucosidase is without action on α-glucosides. The few examples of hydrolysis of α-glucosides by β-glucosidase preparations reported may be explained as resulting from a content of α-glucosidase in the β-glucosidase preparations (taka-diastase and other mold emulsins, but not almond emulsin). Also with regard to carbon atom number 2 the specificity is absolute. Glycosides can be hydrolyzed by β-glucosidase only when the hydroxyl groups at carbon atoms 1 and 2 are in *trans* position, and, since the specificity with regard to carbon atom 1 is absolute, it follows that the carbon atom 2 specificity also must be absolute.

Since in α-D-mannosides the hydroxyl groups at carbon atoms 1 and 2 are in *trans* position and carbon atoms 3–5 have the same configuration as

[59] G. Medwedew, *Enzymologia* **2**, 53 (1937–1938).
[60] S. Veibel and F. Eriksen, *Kgl. Danske Videnskab. Selskab, Mat-fys. Medd.* **13**, No. 17 (1936).
[61] S. Veibel and F. Eriksen, *Kgl. Danske Videnskab. Selskab. Mat.-fys. Medd.* **14**, No. 15 (1937).

in D-glucose, the α-D-mannosides might be hydrolyzed by β-glucosidase; this, however, does not seem to happen. The hydrolysis of α-D-mannosides by, *e.g.*, almond emulsin seems to be due to an α-mannosidase, not to the β-glucosidase.

If the hydroxyl group at carbon atom 2 is replaced by a hydrogen atom, 2-desoxyglucosides are formed. These might be hydrolyzed by both glucosidases and mannosidases. Helferich and Iloff[62] have shown that phenyl 2-desoxy-α-D-glucoside is slowly hydrolyzed by almond emulsin (enzyme efficiency 2.9×10^{-4}) but that the α-mannosidase is responsible for the hydrolysis. The corresponding phenyl 2-desoxy-β-D-glucoside has not been investigated, but it would be presumed that it might be hydrolyzed by β-glucosidase. This is the only change at carbon atom 2 which does not inhibit the hydrolysis completely, possibly due to the fact that, the volume of the hydrogen atom being small compared with the volume of the hydroxyl group, the substitution has only a negligible influence on the conformation[63] of the pyranose ring and therefore does not alter completely the conditions for the attack on the glycoside molecule.

Esterification of the 2-hydroxy group also makes the β-glucoside unhydrolyzable. Helferich and Grünler[64] prepared the 2-tosyl derivative of vanillin β-D-glucoside and found no hydrolyzing action of β-glucosidase in 100 hours. The tosyl group being a large one it would be of interest to examine the influence of a smaller group, *e.g.*, a methyl group, but 2-methyl β-D-glucosides have not yet been prepared. Replacement of the 2-hydroxy group with an amino group (β-D-glucosaminide) does not completely inhibit the hydrolyzing action of almond emulsin if the amino group is made less likely to capture a proton by acetylating it; Helferich and Iloff,[62] however, could show that not β-glucosidase but another enzyme, β-*N*-acetylglucosaminidase, is responsible for the hydrolysis. The hydrolyzability of *N*-acetylglucosaminides is of interest since the *N*-acetylamino group has a rather great volume.

At carbon atom 3 also the specificity seems absolute. 3-Methylphenyl β-D-glucoside was found unhydrolyzable by Helferich and Lang.[65] Here the methyl group is on the same side of the pyranose ring as the glucoside linkage. It would be of interest to examine the corresponding 2- and 4-methyl derivatives having the methyl group on the opposite side of the ring, but these compounds have not been investigated so far. Epimerization at carbon atom 3 would transform the β-D-glucosides into β-D-allosides. These compounds have not been examined.

The specificity at carbon atom 4 has been examined very thoroughly.

[62] B. Helferich and A. Iloff, *Z. physiol. Chem.* **221**, 252 (1933).
[63] W. W. Pigman, *J. Research Natl. Bur. Standards* **26**, 197 (1941).
[64] B. Helferich and S. Grünler, *J. prakt. Chem.* [2] **148**, 107 (1937).
[65] B. Helferich and O. Lang, *Z. physiol. Chem.* **216**, 123 (1933).

Epimerization here means transformation of β-D-glucosides into β-D-galactosides, which are hydrolyzable by almond emulsin and by β-glucosidase preparations of almost every source, even if the velocities are only about 10% of the velocity of hydrolysis of the corresponding β-glucosides. It has, therefore, been generally assumed that β-glucosidase may also hydrolyze β-galactosides. Since, however, β-glucosidase preparations have been found which do not hydrolyze β-galactosides,[66,67] the question is still open. A more detailed discussion will be given in Chapter 17. The replacement of OH at carbon atom 4 with OR seems to be reconcilable with a retention of the hydrolyzability, since β-methyl maltoside (4-α-D-glucopyranosidomethyl--β-D-glucoside) may be hydrolyzed by β-glucosidase.[56] This is interesting because the substituent has a volume as great as the glucoside, but the glucoside linkage and the substituent are on opposite sides of the pyranose ring.

At carbon atom 5 the specificity is not quite absolute. The —CH_2OH— group may be substituted by a hydrogen atom without total loss of hydrolyzability. That means that a β-D-xyloside may be hydrolyzed by β-glucosidase, even if the enzyme efficiency drops from 0.33 (phenyl β-D-glucoside) to 0.0018 (β-D-xyloside).[55] If in β-D-galactosides the —CH_2OH— group is replaced by a hydrogen atom an α-L-arabinoside is formed, which is hydrolyzed by β-glucosidase (or β-galactosidase) of almond emulsin; in this case most of the enzyme efficiency is retained, decreasing only to some 70% of the efficiency in the hydrolysis of the β-galactoside.

Finally, at carbon atom 6 no absolute specificity seems to exist. Substitutions may cause increase or decrease in the velocity of hydrolysis and in the enzyme efficiency of a certain β-glucosidase preparation as compared with the enzyme efficiency toward the corresponding β-glucoside.

Pigman[63] summarizes the evidence concerning the sugar specificity of β-glucosidase (and other glycosidases) in saying that glycosides with identical ring conformation are hydrolyzable by one glycosidase, but difference in conformation (furanose ring instead of pyranose ring, different configuration of one or more of the ring carbon atoms) implies a special glycosidase for each conformation. As to the possibility of finding an enzyme able to hydrolyze synthetically prepared glycosides the following condition must be fulfilled: Glycosides with unsubstituted rings having the same conformation as those in a naturally occurring sugar may be expected to be hydrolyzed by enzymes, and, conversely, naturally occurring enzymes will hydrolyze only naturally occurring hexoside types (*cf.* Gottschalck,[67a] who thinks that for glucosides it is essential that the hydroxyl group at carbon atom 3 is free). In this connection is must also be mentioned that the sub-

[66] C. Antoniani, *Rend. inst. lombardo sci.* [2] **68**, 355 (1935).
[67] S. Veibel, C. Møller, and J. Wangel, *Kgl. Danske Videnskab. Selskab Mat.-fys. Medd.* **22**, No. 2 (1945).
[67a] A. Gottschalck, *Nature* **160**, 113 (1947).

stitution of the ring oxygen atom with a sulfur atom or an NH- group makes the glycosides resistant to hydrolysis by β-glucosidase. Fischer and Delbrück[66] had found that thioglucosides are unhydrolyzable with almond emulsin. Pigman[63] has reinvestigated the enzymatic hydrolysis of the thioglucosides and has found that even with the more active enzyme preparations now available these substances are quite unhydrolyzable. The same holds for *N*-glucosides.[57] This may be due to (*1*) diminished affinity between enzyme and substrate when oxygen is substituted by sulfur, or (*2*) diminished velocity of hydrolysis of the enzyme–substrate complex. (*1*) Presumes that the oxygen and the sulfur atoms in the pyranose ring are of significance for the union of enzyme and substrate. This may be possible, but the alternative, too, must be taken into account as the thioglucosides are resistant not only to enzymatic hydrolysis but also to hydrolysis catalyzed by acids. No determination of the affinity of the *S*- or the *N*-glycosides to β-glucosidase seems to have been reported in the literature.

b. Aglycon Specificity

No case of real absolute specificity caused by the aglucon seems to have been reported, even if the velocity of hydrolysis of different β-glucosides may differ by a factor 10^4 or 10^5. Among the β-glucosides most slowly hydrolyzed are glucosides of aliphatic tertiary alcohols.[37] The trimethylcarbinol β-D-glucoside has an enzyme efficiency of about 2.10^{-4}, *i.e.*, it is in the neighborhood of the nonhydrolyzable glycosides (enzyme efficiency $< 10^{-5}$). The aglucon specificity will therefore be discussed under the heading "relative specificity."

2. Relative Specificity

a. Sugar Specificity

It was mentioned above that the only modification of the glucose molecule which does not completely annihilate the hydrolyzability of β-glycosides by β-glucosidase is at carbon atom 4 (if β-glucosidase and β-galactosidase may be regarded as one enzyme; *cf.* Chapter 17), carbon atom 5 (substitution of $-CH_2OH$ by $-H$), and at carbon atom 6. Here we shall discuss exclusively the specificity caused by substitution at carbon atom 6, since specificity at carbon atoms 4 and 5 has been sufficiently treated above.

Substitution of the hydroxyl group by a hydrogen atom, *i.e.*, formation of a 6-desoxy-β-D-glucoside or a β-D-isorhamnoside causes for the phenol glycosides an increase of enzyme efficiency from 0.3 to 0.56. The volume of the substituents at carbon atom 6 seems to be the determining factor as shown in Table II.[69]

[66] E. Fischer and K. Delbrück, *Ber.* **42**, 1476 (1909).
[69] B. Helferich, S. Grünler, and A. Gnüchtel, *Z. physiol. Chem.* **248**, 85 (1937).

Esterification of the hydroxyl group at carbon atom 6 with long acyl radicals such as mesyl or tosyl radicals causes a decrease of the enzyme efficiency to the neighborhood of the glycosides termed unhydrolyzable. The rule of the radical volume as determining factor is, however, not without exceptions. Substitution of the hydroxyl group at carbon atom 6 by an amino group causes a very great decrease in enzyme efficiency,[55,70] but by acetylating the amino group, whereby the radical volume is increased, a considerable increase in enzyme efficiency may be produced. Pigman[71] is of the opinion that here, as in the case of the 2-amino β-D-glucoside and its acetyl derivative, it is the positive charge on the free amino group which inhibits the hydrolysis of the amino glucoside. This means that the electrochemical conditions are of greater significance than the radical volume.

TABLE II

ENZYME EFFICIENCIES OF 6-SUBSTITUTED β-D-GLUCOSIDES

Glucoside	Enzyme efficiency
Phenyl β-D-isorhamnoside	0.56
Phenyl β-D-glucoside	0.3
Phenyl β-D-glucoside 6-fluorohydrin	0.03
Phenyl β-D-glucoside 6-bromohydrin	0.003
Phenyl β-D-glucoside 6-methyl ether	0.0023

b. Aglycon Specificity

In investigating the aglycon specificity of β-glucosidase a great amount of work has been done by Helferich and associates,[55,72–74] by Veibel and associates,[37,75] and by Pigman and Richtmyer.[76] Helferich and associates have studied the hydrolysis of aryl β-D-glucosides thoroughly and have found that these generally are hydrolyzed much faster than the classical glucoside of Emil Fischer, methyl β-D-glucoside. Table III gives some examples of the enzyme efficiency of aryl β-D-glucosides. For the sake of comparison the value for methyl β-D-glucoside is indicated too. The β-glucosidase value of the sweet almond emulsin used in this investigation is 1.2. It is seen that vanillin β-D-glucoside is hydrolyzed more than 100 times faster

[70] B. Helferich, A. Iloff, and H. Streeck, *Z. physiol. Chem.* **226**, 258 (1934).
[71] W. W. Pigman, *Advances in Enzymol.* **4**, 50 (1944).
[72] B. Helferich and H. Lutzmann, *Ann.* **541**, 1 (1939).
[73] B. Helferich, O. Lang, and E. Schmitz-Hillebrecht, *J. Prakt. Chem.* [2] **138**, 275 (1933).
[74] B. Helferich and J. Goerdeler, *Ber. Verhandl. sächs. Akad. Wiss. Leipzig, Math.-phys. Klasse* **92**, 75 (1940).
[75] S. Veibel and E. Frederiksen, *Kgl. Danske Videnskab. Selskab, Mat.-fys. Medd.* **19**, No. 1 (1941).
[76] W. W Pigman and N. K. Richtmyer, *J. Am. Chem. Soc.* **64**, 369 (1942).

than *p*-cresyl β-D-glucoside and more than 400 times faster than methyl β-D-glucoside. An interesting feature in this table is the increase in enzyme efficiency caused by a methyl group in ortho position to the hydroxyl group responsible for the glucoside formation. *o*-Cresyl β-D-glucoside is hydrolyzed some 13 times faster than phenyl β-D-glucoside. But if the other ortho hydrogen atom is also replaced by a methyl group a decrease results. Phenyl β-D-glucoside is hydrolyzed some 3.5 times as fast as *o*, *o'*-xylenyl β-D-glucoside.[77] This effect of an *o*-methyl-group is not found to the same extent in β-glucosidases from other sources than sweet almonds. The table shows clearly that there is no correlation between enzyme efficiency and molecular volume of the aglycon.

In further investigations Helferich[78-80] has determined the enzyme efficiency of glucosides of divalent alcohols and phenols. If the two hydroxyl groups are at neighboring carbon atoms the *bis*-glucoside is hydrolyzed

TABLE III

ENZYME EFFICIENCY OF SOME ARYL β-D-GLUCOSIDES

Aglycon	Enzyme efficiency	Aglycon	Enzyme efficiency
Methanol	0.034	*o*-Hydroxybenzaldehyde	8.6
Phenol	0.33	Caffeic acid	8.4
Salicylic alcohol	1.7	Protocatechualdehyde	10
o-Cresol	4.3	Isovanillin	11
m-Cresol	0.55	Isobourbonal	2.5
p-Cresol	0.12	Vanillin	13

much more slowly than the monoglucoside, even if the statistical possibility of hydrolysis is doubled. Without determining the affinity constants of the glucosides to β-glucosidase Helferich expresses the opinion that the affinity of the *bis*-glucoside is greatly decreased as compared with the affinity of the monoglucoside. His explanation of this is that the two glucose molecules are so near to each other that the affinity forces of glucose, which in monoglucosides are partially responsible for the formation of the enzyme–substrate complex, in the *bis*-glucosides are saturated between the two glucose units so that no or only very slight binding of the glucose to the enzyme surface takes place. He thinks this hypothesis is corroborated by the fact that one of the glucose molecules may be substituted by a galactose molecule without increase in the enzyme efficiency. If, on the other hand, one of the glucose molecules is esterified in the hydroxyl group at

[77] B. Helferich and H. Scheiber, *Z. physiol. Chem.* **226**, 272 (1934).
[78] B. Helferich, H. Scheiber, R. Streeck, and F. Vorsatz, *Ann.* **518**, 211 (1935).
[79] B. Helferich and C. P. Burt, *Ann.* **520**, 156 (1935).
[80] B. Helferich and R. Hiltmann, *Ann.* **531**, 160 (1937).

carbon atom 6 by methanesulfonic acid (which makes this glucoside linkage more resistant to the action of β-glucosidase) the other glucoside linkage is hydrolyzed nearly as fast as the monoglucoside. He thinks, therefore, that the mesylation of the glucose molecule makes the establishment of affinity bonds between the two glucose molecules impossible so that the affinity forces of the not mesylated glucose molecule may be used undisturbed for binding the glucoside to the enzyme. This explanation may be correct, but as it is so far unsustained by determination of affinity constants, great precaution has to be observed with regard to deductions drawn from the hypothesis. The possibility of some form of attractive forces between two glucose molecules very close to one another (hydrogen bonds?) is by no means far-fetched, but, *e.g.*, molecular weight determination of carbohydrates in aqueous solution does not show any sign of association of the molecules. The affinity of glucose to β-glucosidase is not very great,[61] K_m being 0.18, and therefore it is difficult to understand that the decrease in enzyme efficiency caused by the second glucose molecule should be dependent only on the diminished affinity between glucose and β-glucosidase. Further investigation seems necessary here.

Veibel and associates have dealt mainly with alkyl β-D-glucosides. They are determining not only the unimolecular velocity constants of hydrolysis, $k_{obs.}$, but also the affinity constants, K_m, and the k_3 values. The $k_{obs.}$ values increase with increasing number of carbon atoms in the aglycon (alkyl groups from C_1 to C_6 were examined). The K_m values decrease, which means that the affinity to the β-glucosidase is increasing. Pigman and Richtmyer[76] have found that the $k_{obs.}$ values reach a maximum at C_7. They have made no K_m determinations so that it cannot be seen from their data if the decrease in $k_{obs.}$ is due to a diminished affinity with increasing chain length, to an increased stability of the enzyme-substrate complex (decrease of k_3), or possibly to an inhibition by the products of hydrolysis. Pigman and Richtmyer[76] have examined the hydrolysis of benzyl and cyclohexyl β-D-glucosides and homologs and have found a similar increase of the $k_{obs.}$ values with increasing number of methylene groups between the phenyl (cyclohexyl) ring and the hydroxyl group. Maximum values have in these series not been reached but the insolubility of glucosides of higher members of these series have restricted the investigation to alcohols with not more than three methylene groups.

The comparison of the results of Veibel on the one hand and of Helferich, and of Pigman on the other hand is complicated by the lack of identity not only in standard concentration but in standardization of the enzyme preparation. The standard substrate is in all cases salicin and the standard concentration for determining the enzymatic force $0.139N$ so that only a proportionality factor 1/log 2 should be the difference between sal.f. (Veibel) and β-glucosidase value (Helferich, Pigman). The β-glucosidase

value being by definition $k_{50\%}/e(\log.2)$ the hydrolysis has to be followed to this point, as the constants for the hydrolysis of salicin decrease rapidly with increasing hydrolysis.[41] From some experiments described by Helferich and associates it may be concluded that either in determining the enzyme efficiency or in determining the β-glucosidase value insufficient attention has been payed to this. Helferich[55] indicates, *e.g.*, that with an emulsin preparation of β-glucosidase value 1.2 the enzyme efficiency of salicin is found to be 1.7. Now Veibel and Lillelund[42] have found K_m for salicin to be 0.014. From this it may be calculated that the value of the velocity constant for $0.052M$ solution is 2.3 times the velocity constant for $0.139M$ solutions but the value indicated by Helferich is only 1.7/1.2 = 1.4 times the value for the $0.139M$ solution (*cf.* Pigman,[34] who indicates that the enzyme efficiency of salicin, measured with almond emulsin of β-glucosidase value 1.0, is 2.07.

Pigman and Richtmyer have tried to recalculate the values of Veibel and associates to standard concentration $0.052M$ by graphic interpolation and have reduced the interpolated value for the enzyme efficiency of *n*-butyl glucoside to the value found by them for this glucoside. The proportionality factor thus found they then have used for the other alkyl glucosides too. In Table IV are given the values of Veibel and associates published up to now and some unpublished results, recalculated in another way, by means of the equation $k(K_m + c)$ = constant, to the concentration $0.052M$. The values for methyl and *n*-butyl glucosides are then compared with the values indicated by Pigman and Richtmyer. The proportionality factors are 0.40 and 0.44, mean value 0.42. All the values of Veibel are then multiplied by this proportionality factor; in Fig. 1 these values are plotted against the number of carbon atoms in the aglycon together with some results of Pigman and Richtmyer and of Helferich.

Table IV also contains the values of K_m and k_3 for the glucosides investigated by Veibel, and for some of them is indicated too the values of K_{m_2}, the dissociation constant of the compound β-glucosidase–aglycon, a means of estimating the inhibiting action of the products of hydrolysis which is dependent on the relation K_m/K_{m_2}. In Figs. 3 and 4 the values of K_m and k_3 are plotted against the number of carbon atoms in the aglycon. From the Table IV and Fig. 1 is first seen the increase of velocity of hydrolysis with increasing number of carbon atoms in the aglycon discussed by Pigman. It is seen too that a single branching of the carbon chain slightly decreases the rate of hydrolysis and that it is of no particular significance whether the branching is at carbon atom 2 or 3, the influence of the configuration of an asymmetric carbon atom in position 2 being greater than the influence of shifting the branching from 2 to 3. A double branching at carbon atom 2 as in neopentyl β-D-glucoside decreases the velocity of hydrolysis very considerably, however.

β-Glucosides of secondary alcohols are hydrolyzed very much faster than those of primary alcohols, isopropyl glucoside being an exception, possibly on account of the two methyl groups at the atom with the glucosidic linkage, since methyl groups linked to the carbon atom responsible for the glucosidic linkage always seems to have a retarding influence on

TABLE IV

VALUES OF VELOCITY CONSTANTS, ENZYME EFFICIENCIES, K_m, k_2, AND K_{m_2} FOR SOME ALKYL β-D-GLUCOSIDES

Aglycon	$k_{obs.} \times 10^2$	Enzyme efficiency	K_m	$k_2 \times 10^2$	K_{m_2}
Methanol	2.7	0.037	0.62	1.78	∞
Ethanol	5.3	0.071	0.25	1.54	∞
n-Propanol	22.6	0.29	0.16	4.52	0.18
n-Butanol	24.5	0.29	0.03	1.71	0.03
n-Pentanol[a]	27.3	0.32	0.024	1.75	—
Isobutyl alcohol	23.6	0.27	0.017	1.35	0.032
2-Methylbutanol,[a] D-	28.3	0.32	0.013	1.53	—
2-Methylbutanol,[a] L-	23.9	0.27	0.013	1.28	—
Isoamyl[a]	23.3	0.27	0.015	1.28	—
3-Methylpentanol,[b] D-	19.2	0.27	—	—	—
3-Methylpentanol,[b] L-	17.9	0.25	—	—	—
Isopropyl alcohol	16.9	0.23	0.40	7.45	0.40
Butanol-2, D-	48.3	0.59	0.041	3.91	0.10
Butanol-2, L-	140	1.72	0.048	12.4	0.014
Pentanol-3	220	2.56	0.020	13.2	0.022
Hexanol-2,[b] D-	228	2.68	—	—	—
Hexanol-2,[b] L-	57	0.72	—	—	—
Trimethylcarbinol	0.02	0.00066	1.46	0.04	0.37
Dimethylethylcarbinol	0.56	0.0073	0.14	0.11	—
Methyldiethylcarbinol	6.2	0.079	0.079	0.74	—
Triethylcarbinol	2.5	0.031	0.057	0.24	—
Neopentyl alcohol	4.5	0.054	0.046	0.39	0.10

[a] Unpublished results.

[b] S. Veibel, *Biochem. J.* **28**, 1733 (1934).

the velocity of hydrolysis. As soon as one of the methyl groups is substituted by an ethyl group the velocity of hydrolysis increases considerably, and again the influence of the configuration of the asymmetric carbon atom is very pronounced. By substitution of the other methyl group with an ethyl group a new increase in velocity of hydrolysis is produced, and, as first pointed out by Pigman and Richtmyer, some of these alkyl glucosides

are hydrolyzed considerably faster than phenyl β-D-glucoside, so that no general rule with regard to the velocity of hydrolysis of alkyl and aryl glucosides seems to exist.

β-Glucosides of tertiary alcohols are hydrolyzed very slowly; trimethylcarbinol β-D-glucoside, with three methyl groups at the glucoside-linked

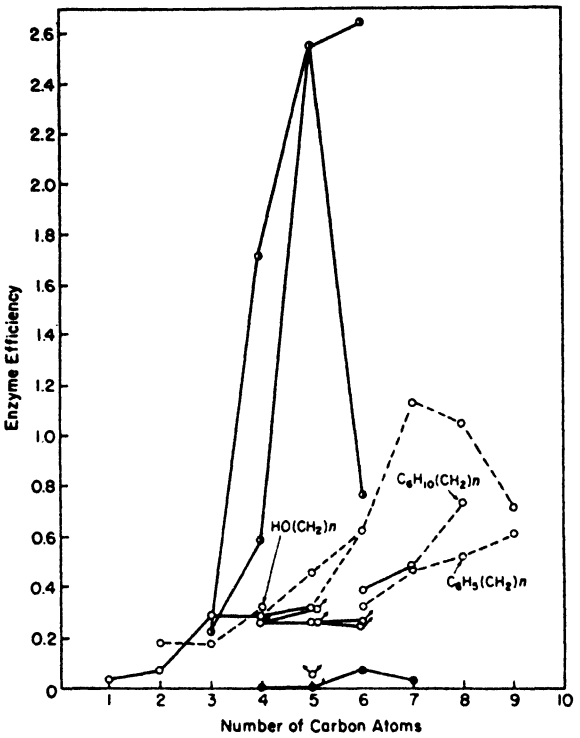

FIG. 1. Enzyme efficiencies for different β-glucosides

○——○ Primary alcohols
◑——◑ Secondary alcohols
●——● Tertiary alcohols
○ Unbranched carbon chains
♂ Single-branched carbon chains
↖♂ Double-branched carbon chains
Full lines: Experiments of Veibel and associates
Dotted lines: Experiments of others.

carbon atom, is so slowly hydrolyzed that the glucoside is in the neighborhood of the glycosides termed unhydrolyzable. Substitution of one or two methyl groups by ethyl groups increases the velocity, but if the third methyl group also is substituted by an ethyl group the velocity is again somewhat decreased. Further investigation of glucosides of secondary and tertiary alcohols seems necesssary in order to examine whether an optimal number

of carbon atoms in the aglucon exists in these series as in the series of glucosides of primary alcohols.

In order to explain the mechanism of the enzymatic hydrolysis of glucosides Pigman[34,35] refers to the conception of von Euler[81] of the union of the substrate with the enzyme through two active areas on the surface of the enzyme molecule, one capable of binding the sugar part of the glucoside molecule through adsorption bonds, the other binding the agycon through adsorption. Fig. 2 is Pigman's presentation of this conception. In all β-glucosides the affinities of the β-glucose part of the molecule to the β-glucosidase molecule must be assumed identical, so that the relative specificity depends upon the affinity of the aglycon to the area II. Pigman

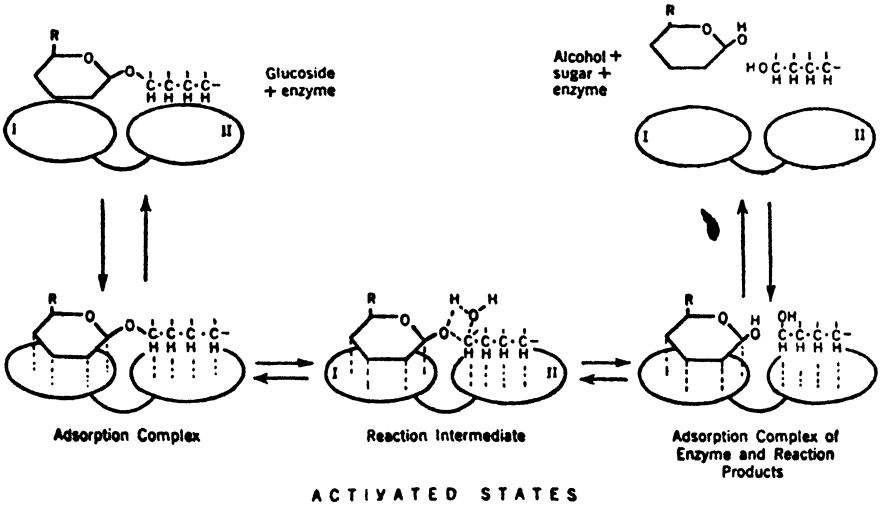

FIG. 2. Postulated mechanism for the enzymic hydrolysis of an alkyl glucoside (from W. W. Pigman, Chemistry of the Carbohydrates, Academic Press, New York, 1948).

thinks it reasonable to assume that the possibility of adsorption bonds between the aglycon and area II increases with increasing number of carbon atoms, and that consequently the velocity of hydrolysis will also increase. But whereas the increase in adsorption will increase continually, the velocity of hydrolysis has been found to reach a maximum and then decrease with further prolongation of the carbon chain, not on account of diminished velocity of hydrolysis of the adsorption complex but because the aglycon liberated during the hydrolysis will block the area II so that neither the formation of the adsorption complex nor its hydrolysis are the rate-determining processes but the desorption of the aglycon from the enzyme surface. The fact that a branching of the carbon chain in the agly-

[81] H. von Euler, *Z. physiol. Chem.* **143**, 79 (1925).

con molecule will diminish the velocity of hydrolysis as compared with the hydolysis of the isomeric unbranched glycoside may be explained as resulting from a greater difficulty in accommodation of the branched aglycon to the enzyme surface than the unbranched. A double branching at one carbon atom will produce still greater difficulties.

But the great velocity of hydrolysis of glucosides of secondary alcohols is not explained by this hypothesis. With regard to the =CHOH group the carbon chain is branched, but notwithstanding this the velocity generally is considerably greater than for the isomeric glucosides of unbranched primary alcohols. The difference in velocity of hydrolysis of D- and of L-alkyl β-D-glucosides may be explained by the hypothesis that in the two cases it is the two different alkyl groups which may be adsorbed to the enzyme surface. But still it is difficult to understand why diethylcarbinol β-D-glucoside is hydrolyzed some 35 times faster than ethyl β-D-glucoside.

The slow hydrolysis of the glucosides of the tertiary alcohols is interpreted as the result of a diminished adsorption of the aglycon. Pigman and Richtmyer[76] advance the hypothesis that the diminished adsorption is due to the lack of a hydrogen atom at the carbon atom responsible for the glucoside linkage. This carbon atom is therefore not adsorbed to the enzyme surface and the contact between the glycosidic linkage and the enzyme is a more indirect one than in the other glucosides. This explanation is, in the opinion of the reviewer, very improbable since the aryl glucosides, formally being tertiary glucosides and thus also lacking the connecting hydrogen atom, are hydrolyzed very much faster than the tertiary alkyl glucosides.

An estimate of the degree of adsorption may be gained by determining the K_m values for the glucosides. Veibel and associates have made such determinations. The results are indicated in Table IV and plotted against the number of carbon atoms of the aglycon in Fig. 3. It will be seen that the adsorption increases with increasing number of carbon atoms and that, for isomeric glucosides, it decreases from primary through secondary to tertiary alkyl glucosides. This fits well into the hypothesis mentioned above but gives no explanation of the great velocity of hydrolysis of glucosides of secondary alcohols, and the difference of the K_m values of D- and L-methylethylcarbinol β-D-glucoside is not great enough to explain the very great difference in enzyme efficiency toward the two glucosides. Also, the influence of branching of the carbon chain on the K_m values does not correspond to the influence on the enzyme efficiencies toward the corresponding glucosides, isobutyl and isoamyl glucoside being adsorbed more strongly than the corresponding *n*-alkyl glucosides, whereas the enzyme efficiencies toward the *n*-alkyl glucosides are greater than toward the isoalkyl glucosides. The effect of double branching corresponds to the hypothesis; both the adsorption and the enzyme efficiency are

strongly diminished as compared with the isomeric *n*-alkyl glucoside. Further investigation is badly wanted, however, before the correlation between adsorption and enzyme efficiency can be regarded as sufficiently elucidated.

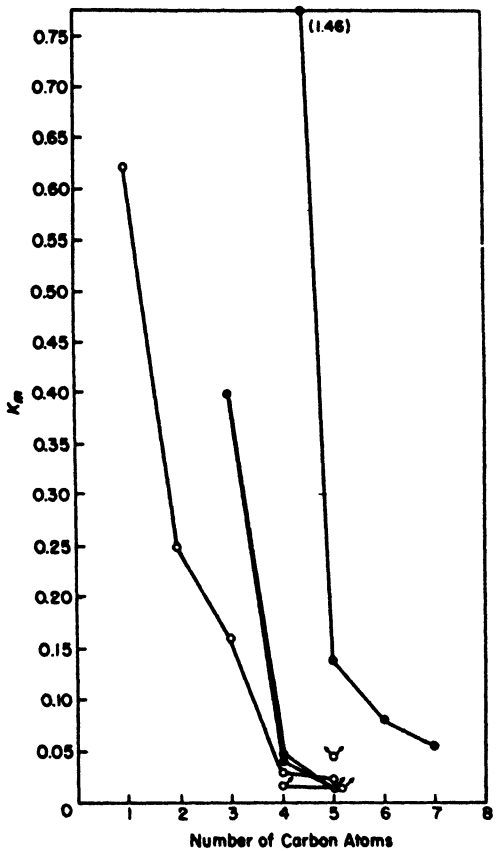

FIG. 3. K_m values for different alkyl β-D-glucosides

○——○ Primary alcohols
◑——◑ Secondary alcohols
●——● Tertiary alcohols
○ Unbranched carbon chains
♂ Single-branched carbon chains
♂♂ Double-branched carbon chains

The hypothesis here discussed presumes that the hydrolysis of the glucoside takes place by the reaction of the adsorption complex with water. As the velocity of formation of this adsorption complex must be unmeasurably great (the K_m values being independant of temperature,[43,60,61] *i.e.*, the heat of activation for the formation of the adsorption complex

~0, it is the hydrolysis of the reaction intermediate which is measured (or for the higher alkyl glucosides the desorption of the aglycon from the enzyme). Measurements of the heat of activation would here be of interest for the elucidation of this question.

The velocity constant of the hydrolysis of the reaction intermediate is the constant termed k_3 by Veibel and Lillelund[42], and in Table IV values

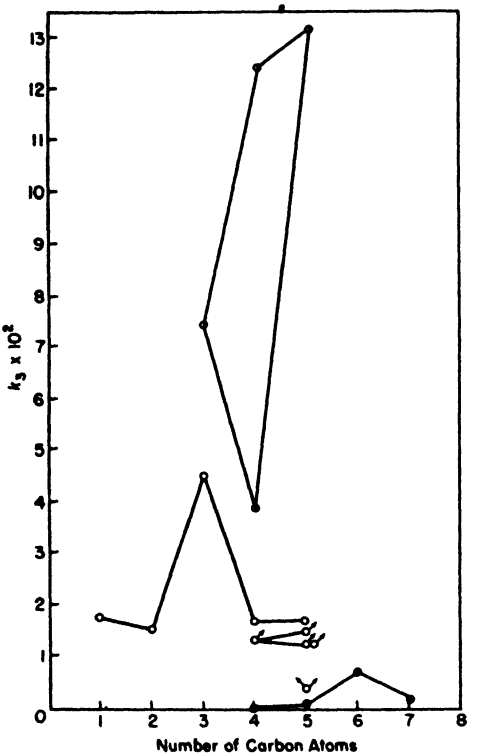

FIG. 4. k_3 values for different alkyl β-D-glucosides

○——○ Primary alcohols
◑——◑ Secondary alcohols
●——● Tertiary alcohols
○ Unbranched carbon chains
♂ Single-branched carbon chains
Double-branched carbon chains

of k_3 are indicated together with the constants mentioned above. In Fig. 4 these values are plotted against the number of carbon atoms in the aglycon, and it may be seen that for glucosides of primary alkyl groups there is no great differences in the k_3 values (with the exception of propyl glucoside, for which the value of k_3 is abnormally great). Simple branching of the carbon chain does not decrease the k_3 values significantly, but double

branching at one carbon atom causes a great decrease in k_3. For the secondary alkyl glucosides the k_3 values are very much greater than for the primary, and the individual differences are considerable, not only depending on the number of carbon atoms but on the configuration of the asymmetric carbon atom as well. Finally, the tertiary alkyl glucosides have very small k_3 values, but it is interesting to note that the value for methyldiethylcarbinol β-D-glucoside is double that for neopentyl β-D-glucoside, so that double branching of the carbon chain may be of greater effect on the hydrolyzability of the glucoside than the shift from a primary to a tertiary alkyl group.

In some interesting papers Bonhoeffer and associates[82,83] have investigated the influence of substitution of water as solvent with deuterium oxide on the rate of enzymatic hydrolysis of β-glucosides. In some instances the velocity increases, in other it decreases. Bonhoeffer explains this as due to a double effect of the deuterium oxide. The affinity of the substrate for the enzyme is greater in D_2O than in H_2O, the rate of hydrolysis of the enzyme-substrate complex on the other hand smaller. This means that if the K_m value in water is great (slight affinity) it seems possible that the actual concentration of the enzyme-substrate complex may be so much greater in D_2O than in H_2O that the $k_{obs.}$ value may increase even if the k_3 value is smaller in D_2O than in H_2O, the relation between k_3, $k_{obs.}$ and K_m being $k_3 = k_{obs.}(K_m + c)$. If, on the other hand, the affinity in water is great (K_m small) the increase in affinity cannot be sufficient to compensate for the decrease in k_3. Experimentally Bonhoeffer and associates found that the velocity of hydrolysis of methyl β-D-glucoside is greater in D_2O than in H_2O, that of salicin smaller. The corresponding K_m values in water are 0.62 and 0.014. In deuterium oxide they have not been determined. Steacie,[83a] on the contrary, found the velocity of hydrolysis of salicin greater in D_2O than in H_2O.

Veibel and Frederiksen[75] have determined the heat of activation and the value of B in the Arrhenius equation $\ln k = -Q/RT + B$ for the enzymatic hydrolysis of alkyl glucosides. They have found that for glucosides of primary and secondary alcohols the heat of activation is, within the limits of the experiment, identical, *ca.* 12,300 cal. The B values, too, are identical for these two types of alkyl glucosides. The glucosides of tertiary alcohols, on the other hand, have heats of activation about 19,900 cal. and B values some 50% higher than the two other types. The neopentyl β-D-glucoside with double-branched carbon chain resembles both in the value of heat of activation and in B value the glucosides of tertiary alcohols.

In the light of the hypothesis proposed by Pigman[34,35] the heat of activation will be dependent on the nature of the adsorption bonds between the aglycon and the enzyme, and the tertiary alkyl glucosides, having no direct adsorption bonds from the glucoside-linked carbon atom may, according to him, be expected to have greater heat of activation than the

[82] F. Salzer and K. F. Bonhoeffer, *Z. physik. Chem.* **176A**, 304 (1936).
[83] K. F. Bonhoeffer, *Ergeb. Enzymforsch.* **6**, 47 (1937).
[83a] E. W. R. Steacie, *Z. Physik. Chem.* **28B**, 236 (1935).

other types. In order to examine the validity of this hypothesis it should be of interest to compare the heats of activation of tertiary alkyl glucosides and aryl glucosides, but, to the knowledge of the reviewer, no investigation of the influence of temperature on the hydrolysis of aryl glucosides has been published. The hypothesis mentioned here presumes a great resemblance between the mechanism of enzyme-catalyzed and hydrogen ion-catalyzed hydrolysis of glucosides. Kuhn and Sobotka[84] and Moelwyn-Hughes[85,86] are of the opinion that in both cases it is the velocity of the reaction between the adsorption complex (of β-glucoside and enzyme or hydrogen ion) and water which is measured. When the heat of activation for the enzyme-catalyzed hydrolysis is only about a third of the heat of activation for the acid-catalyzed hydrolysis, the cause is, according to Moelwyn-Hughes,[85] an increase in the number of degrees of freedom caused by the enzyme. Another explanation, the physical meaning of which possibly is more obvious, is that the heat of activation, which in the

TABLE V

COMPARISON OF ACID AND ENZYMATIC HYDROLYSIS OF SOME ALKYL β-D-GLUCOSIDES[a]

Aglycon	Acid hydrolysis, 0.1*N* HCl			Enzymatic hydrolysis, 30°			
	$k \times 10^5$, 60°	*Q*	*B*	$k'_{obs.} \times 10^3$	$k_2 \times 10^3$	*Q*	*B*
CH_3	0.35	32.610	16.0	1.0	0.73	12.200	5.7
$CH_3CH_2CH_2$	0.46	32.430	16.0	8.7	1.95	13.500	7.0
$(CH_3)_2CH$	0.79	32.090	16.0	6.5	2.88	13.100	6.9
$(C_2H_5)_2CH$	1.18	31.520	15.8	84.0	5.10	10.600	5.3
$(CH_3)_3C$	9.97 (40°)	30.810	17.5	0.011	0.02	19.960	9.7
$(CH_3)_2C(C_2H_5)$	42.9 (40°)	29.870	17.5	0.22	0.042	19.960	10.0
$(CH_3)_3CCH_2$	0.67	31.540	15.5	1.7	0.15	18.060	9.2

[a] Velocity constants are calculated with natural logarithms and the second as unit of time.

acid-catalyzed hydrolysis has to be removed by collision with other molecules, in the enzyme-catalyzed hydrolysis has the opportunity of being dispersed over the great enzyme molecule. But irrespective of which explanation is correct a close correlation between the rate of the acid- and of the enzyme-catalyzed reactions might be expected. This correlation is not found, however, as shown by several investigations, most recently by Veibel and Frederiksen,[75] from whose paper the values given in Table V are taken. The most striking difference is that in acid the velocity of hydrolysis of tertiary alkyl glucosides is 200–2000 times that of primary or secondary alkyl glucosides at the same temperature, whereas in the enzymic reactions, as indicated above, the tertiary alkyl glucosides are hydrolyzed much more slowly than the primary or secondary alkyl glucosides.

[84] R. Kuhn and H. Sobotka, *Z. physik. Chem.* **109**, 65 (1924).
[85] E. A. Moelwyn-Hughes, *Trans. Faraday Soc.* **25**, 81 (1929).
[86] E. A. Moelwyn-Hughes, *Ergeb. Enzymforsch.* **2**, 1 (1933).

The heat of activation is 2.5 to 3 times as great for the acid hydrolysis as for the enzymatic hydrolysis when primary or secondary alkyl glucosides are examined but only 1.5 times as great for the tertiary alkyl glucosides. For acidic hydrolysis the heat of activation of the tertiary alkyl glucosides is slightly lower than that of the primary and secondary alkyl glucosides, for enzymatic hydrolysis it is 1.5 times as great.

The *B* values cannot be compared since the molecular weight and the degree of purity of the enzyme are not known, but it may be seen that for acidic hydrolysis the *B* values for tertiary alkyl glucosides are some 10% greater than for primary and secondary alkyl glucosides, for enzymic hydrolysis some 50% greater. An interpretation of these results for the time being is not possible. The behavior of neopentyl glucoside is interest

TABLE VI

COMPARISON OF RATES OF ACIDIC AND ENZYMATIC HYDROLYSIS OF SOME AROMATIC β-GLUCOSIDES

Aglycon	Enzyme efficiency	Acid hydrolysis $k \times 10^4$
C_6H_5	0.34	23
o-$CNCH_2C_6H_4$	2.0	4.9
p-$CNCH_2C_6H_4$	1.2	16
o-$CH_3COC_6H_4$	3.3	110
p-$CH_3COC_6H_4$	1.1	8
o-$CHOC_6H_4$	8.6	9
p-$CHOC_6H_4$	4.2	8
o-OH, p-$CHOC_6H_3$	11	13
6-OCH_3, 3-$CHOC_6H_3$	10	43
o-OCH_3, p-$CHOC_6H_3$	13	35
p-$CH_2C_6H_4$	0.12	21
o-$CH_3C_6H_4$	4.3	18
o-$CH_2OHC_6H_4$	1.9	11

ing. For the acid hydrolysis of this substance, the values of *k*, *Q*, and *B* fall in the range of the primary alkyl glucosides; for the enzymatic hydrolysis, on the contrary, the values are in the range of the tertiary alkyl glucosides. For aryl β-D-glucosides no determinations of the heat of activation or the *B* values seem to have been published, but in Table VI are listed the velocity constants for the acid and the enzymatic hydrolysis of some aryl β-D-glucosides.[77,78] Also from this table it is seen that there is no parallelism between the two types of hydrolysis.

Stearn[87] proposes calculating ΔH and ΔS, the heat and entropy change during the reaction, hoping to utilize these values for the interpretation of the mechanism of the hydrolysis. So far the material which may be used for

[87] A. E. Stearn, *Ergeb. Enzymforsch.* 7, 1 (1938).

such calculations is, however, not sufficient to permit the drawing of reliable conclusions.

The hydrolyzability of disaccharides by β-glucosidase has been investigated by Helferich and associates [88,89] and by Pigman.[34] All disaccharides with β-glucosidic linkages are hydrolyzed by almond emulsin but the velocities vary considerably, as may be seen in Table VII. Even slight changes in the aglycon may markedly influence the hydrolyzability. In cellobiose the aglycon is glucose, but if it is substituted by mannose the enzyme efficiency decreases to one seventh. A comparison of the enzyme efficiency of cellobiose and gentiobiose shows that the shift of the glucosidic linkage from carbon atom 4 to 6 produces a decrease of the enzyme efficiency to half. With the two-affiniity theory of Pigman as wroking hypothesis the great influence of the structure of the aglycon seems quite

TABLE VII

EASE OF ENZYMATIC HYDROLYSIS OF DISACCHARIDES WITH β-GLUCOSIDIC LINKAGES

Substrate	Enzyme efficiency × 10^3
Cellobiose, 4-glucose-β-glucoside	159, 180
Gentiobiose, 6-glucose-β-glucoside	75
Celtrobiose, 4-altrose-β-glucoside	(23)[a]
4-Mannose-β-glucoside	2.3
Phenyl α-cellobiose, 4-(phenyl α-glucoside)-β-glucoside	160
Phenyl β-glucoside	330

[a] Not measured originally under standard conditions. The values given were calculated from the corresponding values for cellobiose, measured under the same conditions, and are approximately those to be expected under the standard conditions. The data for celtrobiose were derived from the data of N. K. Richtmyer and C. S. Hudson, *J. Am. Chem. Soc.* **61**, 1834 (1939).

logical. It is to be assumed that the hydroxyl groups of the aglycon are more closely adsorbed to the enzyme surface than the hydrogen atoms and therefore the distribution of the hydroxyl groups on the two sides of the pyranose ring in the aglycon is essential. But also the aldehyde group of the aglycon-linked hexose molecule is of significance for the hydrolyzability of the disaccharide. If it is reduced to a hexitol or oxidized to a hexonic acid the enzyme efficiency is decreased to a small fraction of its original value (for examples see Chapter 17). A substitution of glucose by, *e.g.*, phenyl α-glucoside as in phenyl α-cellobioside, on the other hand, does not decrease the enzyme efficiency toward β-glucosidase.

Helferich and Werner[90] have shown that the monoglucoside of ethylene

[88] B. Helferich and R. Griebel, *Ann.* **544**, 191 (1940).

[89] S. A. Petersen, *Ber. Verhandl. sächs. Akad. Wiss. Leipzig, Math.-phys. Klasse* **85**, 154 (1933).

[90] B. Helferich and J. Werner, *Ber.* **75**, 949 (1942).

glycol, which is easily hydrolyzed by emulsin, becomes resistant toward the enzyme if it is transformed into an anhydride by formation of an ether with the hydroxyl group at carbon atom 2 in the glucose molecule. This is quite natural since it is a modification of the pyranose ring, which, as mentioned in Section IV-1 destroys the hydrolyzability of the glucoside. If, however, the anhydride is formed within the aglycon the resistance to enzymatic hydrolysis is not absolute. Helferich and Thiemann[91] have prepared glycol β-D-celloside anhydride and have found that it is hydrolyzed by the β-glucosidase of sweet almond emulsin, even if the enzyme efficiency is only a tenth of the efficiency of cellobiose. This means that the existence of a special glycobiosidase, a cellase, responsible for the hydrolysis of biosides, is not necessary, since the hydrolysis may start with the hydrolysis of the bioside into glucose and glucoside; the latter in turn is hydrolyzed by the β-glucosidase. Since many naturally occurring glycosides are biosides or polyosides it is a question of some significance whether the results mentioned here have general validity, but even if Helferich and Werner[92] found glycol lactoside anhydride hydrolyzable by the β-galactosidase of sweet almond emulsin, Helferich and Thiemann stress that the evidence so far produced is too scanty to allow a generalization.

The influence of acidic or basic groups in the aglycon has been investigated by Helferich and associates. Fischer[93] showed that the presence of a carboxyl group in the aglucon reduces the enzyme efficiency, whereas an amide group is without this effect. Helferich, Pigman, and Isbell[94] fully corroborated this result and Helferich and Lutzmann,[72] extending the investigation to the influence of the sulfonic acid group, found a still greater influence of this group than of the carboxyl group. Esterification of the sulfonic acid group restored the hydrolyzability of the glucoside. Helferich and Schnorr,[95] investigating the problem more closely, found that the effect of the sulfonic acid group decreases rapidly with increasing number of carbon atoms between the acid group and the glucosidic linkage. Their results are given in Table VIII. Helferich, Pigman, and Isbell[94] assumed that the negative charge on the carboxylic ion was responsible for the decrease in enzyme efficiency, since salts of carbonic acids were as slowly hydrolyzed as the free acids. Helferich and Schnorr,[95] discussing this explanation, find it corroborated by the greater influence of the sulfonic acid group than of the carbonic acid group. The experiments are conducted at pH 5.0 where the carbonic acids are only to a minor degree dissociated, whereas the sulfonic acids, being considerably stronger, are correspondingly

[91] B. Helferich and K. Thiemann, *Z. physiol. Chem.* **281**, 126 (1944).
[92] B. Helferich and J. Werner, *Ber.* **76**, 595 (1943).
[93] E. Fischer, *Z. physiol. Chem.* **107**, 176 (1919).
[94] B. Helferich, W. W. Pigman, and H. S. Isbell, *Z. physiol. Chem.* **261**, 189 (1939).
[95] B. Helferich and H. Schnorr, *Ann.* **547**, 201 (1941).

more dissociated. The values of the enzyme efficiency of the halogen-substituted glucosides show that the presence of an electronegative substituent (without an electrical charge) does not decrease the hydrolyzability. Helferich and Schnorr, therefore, are of the opinion that the electrical charge is a hindrance for the adsorption of the substrate on the enzyme surface. When the sulfonic acid is esterified the charge disappears and the adsorption takes place undisturbed, *i.e.*, the hydrolyzability is restored. A positive charge on the aglycon acts in the same manner.

Helferich and associates[96,97] have examined the hydrolysis of aminomethyl-substituted phenyl glucosides and have found the enzyme efficiency reduced for both the ortho, meta, and para compounds. Acetylation of

TABLE VIII

ENZYME EFFICIENCY OF β-D-GLUCOSIDES OF α-HYDROXYALKYL HALIDES, SULFONIC ACIDS, OR ESTERS

Alkyl group	Chloride	Iodide	Sulfonic acid	Sulfonic ester	$E.e._{acid}/E.e._{ester}$
Ethane	0.44	0.94	0.0009	0.32	1/340
Propane	0.53	0.44	0.08	0.8	1/10
n-Butane	0.63	1.1	0.36	1.2	1/3

TABLE IX

ENZYME EFFICIENCY OF SOME AMINOMETHYL- AND *N*-ACETAMINOMETHYL-SUBSTITUTED PHENYL GLUCOSIDES

Substituent	Ortho	Meta	Para
CH_3	4.29	0.55	0.12
NH_2CH_2	0.036	0.05	0.027
$CH_3CONHCH_2$	0.88	1.48	0.13

the amino group partially or completely restores the enzyme efficiency. Some results are given in table IX. It is seen that the effect of the amino group in the ortho position is very much greater than in the meta and especially greater than in the para position. The explanation is, according to Helferich, the same as for the negatively charged substituents: The amino group will, at pH 5, be partially transformed into ammonium ions and the positive charges are just as inhibitory for the adsorption of the substrate on the enzyme surface as the previously discussed negative charges. The validity of the explanation has not been sustained by determination of the dissociation constants of the enzyme-substrate complexes.

[96] B. Helferich, E. Günther, and S. Winkler, *Ann.* **508**, 192 (1933).
[97] B. Helferich and F. Philipp, *Ann.* **514**, 228 (1934).

3. Specificity of β-Glucosidase from Various Sources

The enzyme used for the specificity studies mentioned above is sweet almond emulsin. It has been shown, however, that for β-glucosidases from other sources the specificity may be different from that of almond emulsin. In a very interesting study Miwa, Cheng, Fujisaki, and Toishi[98] have compared the action of β-glucosidase from different sources on four different β-glucosides. Their results are summarized in Table X. An interesting

TABLE X

Comparison of Specificities of β-Glucosidases from Different Sources[a]

Source of emulsin	β-Glucoside							
	Phenyl		Salicyl		*o*-Cresyl		*p*-Cresyl	
	f	Ratio	*f*	Ratio	*f*	Ratio	*f*	Ratio
Prunus armeniaca (apricot)	2.47	1.0	30.7	12.5	59.8	24.2	1.27	0.51
Amygdalis communis (sweet almond)	2.10	1.0	29.1	13.9	52.5	25.0	1.20	0.57
A. persica (peach)	0.315	1.0	3.16	10.0	5.84	18.5	0.169	0.53
Cycas revoluta (sago palm)	0.0226	1.0	0.176	7.65	0.394	17.4	0.0114	0.50
Papaver somniferum (opium poppy)	0.00459	1.0	0.0163	3.54	0.0268	5.82	0.00411	0.89
Glycine hispida (soy bean)	0.00073	1.0	0.00181	2.48	0.00071	0.97	0.00146	2.0
Cucurbita moschata (squash)	0.00913	1.0	0.00503	0.55	0.00421	0.46	0.00897	1.0
Aspergillus oryzae (taka-diastase)	0.110	1.0	0.0962	0.88	0.0139	0.13	0.0722	0.59
Aspergillus oryzae	1.058	1.0	0.843	0.80	0.013	0.12	0.749	0.71
Aspergillus niger	2.99	1.0	0.965	0.32	0.0772	0.025	4.34	1.46
Ergot	0.148	1.0	0.0523	0.35	0.0349	0.24	0.148	1.0

[a] *f* is a measure of the ease of hydrolysis similar to the enzyme value and enzyme efficiency, but 0.012*M* substrate solutions have been employed and the enzyme concentration is expressed as g. in 8 ml. For each enzyme preparation the ease of hydrolysis of the phenyl glucoside is taken as unity.

feature of this table is that the marked effect of an ortho methyl group on the hydrolyzability of phenyl glucoside first discovered by Helferich and associates for almond emulsin is found only for some of the emulsins from higher plants, above all the *Prunus* species and sago palm, whereas for β-glucosidase from fungi the opposite is found: phenyl glucoside and also

[98] T. Miwa, C. Cheng, M. Fujisaki, and A. Toishi, *Acta Phytochim. Japan* 10, 155 (1937).

p-cresyl glucoside are hydrolyzed with greater velocity than *o*-cresyl glucoside. β-Glucosidases from other plants are intermediate in type and are much less affected by substitution in the aglycon group than almond emulsin β-glucosidase. Also in β-glucosidase from the digestive juice of *Helix pomatia* the great *o*-methyl effect is lacking, according to Helferich and Goerdeler,[74] and the snail β-glucosidase is rather insensitive to substitution in the aglycon group as shown in Table XI.

For β-glucosides of animal origin Hofmann[99] has found some differences in specificity. Whereas almond β-glucosidase hydrolyzes salicin rapidly but phlorizin very slowly, β-glucosidase from horse kidneys hydrolyzes phlorizin more easily than salicin or phenyl glucoside. Other animal β-glucosidases (from pig and cattle kidneys, from horse liver, and horse, cattle, and pig intestinal mucosa), however, resemble almond emulsin in their action on phlorhizin and salicin.

TABLE XI

COMPARISON OF SPECIFICITIES OF β-GLUCOSIDASES OF ALMOND AND SNAIL EMULSIN[a]

Aglycon	Sweet almond emulsin		Snail emulsin	
	e.e.	ratio	e.e.	ratio
Phenol	0.33	1.0	0.028	1.0
Salicyl alcohol	1.7	5.2	0.034	1.2
Vanillin	13	39	0.151	5.4
p-Cresol	0.12	0.36	0.026	0.9
o-Cresol	4.3	13	0.035	1.3
Ethanol	0.045	0.14	0.0056	0.2

[a] For each enzyme preparation the e.e for phenyl glucoside is taken as unity.

From the fact that the specificity of β-glucosidase (and other glycosidases) from different sources shows these great differences, Pigman[57] found it necessary to substitute the specificity theory of Weidenhagen[47] of one enzyme responsible for the hydrolysis of all β-glucosides by a theory according to which β-glucosidase is not a single enzyme, strictly speaking, but rather a class of closely related enzymes, which all show a specific ability to hydrolyze β-glucosidase linkages. Pigman does not give any hint as to the nature of the differences between the members of this class of β-glucosidases but as he, as mentioned above (page 605), supports the idea of two active areas on the surface of the enzyme molecule from which area II is responsible for the aglycon specificity, it seems to the reviewer reasonable to assume that the class differences are dependent on differences in shape or size of area I or possibly on the distance between the two areas.

[99] E. Hofmann, *Biochem. Z.* **285**, 429 (1936).

V. Inactivation

1. By Salt Formation

The activity of β-glucosidase is abolished by the addition of salts of heavy metals, especially of mercury, silver, and copper. The inactivation is probably due to salt formation with the enzyme, since the activity may be restored if the heavy metal is precipitated by hydrogen sulfide. If, however, the action of the metal ions continues for too long a time an irreversible inactivation (denaturation of the protein) takes place.

2. By Oxidation

Helferich and associates[100,101] have examined the action of ozone on β-glucosidase and have found an almost instantaneously irreversible inactivation. The degree of inactivation is proportional to the amount of ozone, but the purity of the enzyme is of significance, the purer the enzyme, the smaller the quantity of ozone necessary for the destruction of an enzyme unit. For a preparation with β-glucosidase value 9.6, 0.0031 g. ozone will destroy one enzyme unit at pH 5.0. The degree of inactivation of the enzyme is parallel to a decrease of its tryptophan content, and addition of tryptophan to the solution before the treatment with ozone will partially protect the enzyme.

The inactivation caused by osmium tetroxide is of another type. Helferich and Vorsatz[102] found that the action is not instantaneous. If the solution is treated with hydrogen sulfide or cysteine within some few minutes after the addition of osmium tetroxide no, or only little, inactivation is observed. Parallel to the inactivation goes a decrease in the amount of active oxygen. Helferich and Vorsatz are therefore of the opinion that the first action of the osmium tetroxide is a competitive inhibition, an adsorption of osmium tetroxide to the enzyme surface, rapidly followed by an oxidation of the enzyme molecule. During the adsorption period reducing agents may restore the activity of the enzyme but if it has been oxidized it cannot be reduced to an active compound. Tryptophan has no protective action against osmium tetroxide.

3. By Irradiation

Helferich and Brieger[103] have shown that irradiation with mercury vapor light inactivates β-glucosidase. Jena glass and Uviol glass protects against the action of the irradiation; quartz does not. It must be assumed, therefore, that it is the line 245 mμ which causes the inactivation since it is the only one of the mercury lines which is absorbed by Uviol glass and not by quartz. The purer the enzyme the stronger the inactivation, which probably is caused by absorption of light by the enzyme molecule.

4. By Formaldehyde

Formaldehyde inactivates β-glucosidase rapidly,[104,105] but if sodium hydrogen sulfite is added without too great delay the enzyme activity may be restored. This might be explained as an action of formaldehyde on amino groups in the enzyme molecule. It is known that formaldehyde denatures proteins, so that the result of prolonged action of formaldehyde will be an irreversible inactivation. Mascré and Paris[106] have studied

[100] B. Helferich, S. Winkler, E. Schmitz-Hillebrecht, and H. Bach, *Z. physiol. Chem.* **229**, 112 (1934).
[101] B. Helferich and S. R. Petersen, *Z. physiol. Chem.* **233**, 75 (1935).
[102] B. Helferich and F. Vorsatz, *Z. physiol. Chem.* **239**, 241 (1936).
[103] B. Helferich and G. Brieger, *Z. physiol. Chem.* **221**, 94 (1933).

the action of formaldehyde more closely. Their opinion is that even if the addition of formaldehyde involves the liberation of carboxyl groups (as in formol titration) no real formation of methylene compounds take place, as the enzyme may be recovered with only slight loss of activity by treatment with ethanol. They are inclined to find an explanation in the colloid-chemical nature of the enzyme. The addition of formaldehyde is believed to produce a change in degree of peptization. No definite explanation is given by them, however.

5. By Carbonyl Group Reagents

According to Lettré[96] and other investigators β-glucosidase is assumed to contain one or more carbohydrate molecules, as prosthetic group or as part of the protein molecule. Carbonyl group reagents, therefore, might be able to inhibit β-glucosidase action, and some observations to this effect may be found in the literature (inhibition by sodium hydrogen sulfite and by phenylhydrazine but not by hydrogen cyanide). The question has not been systematically investigated, however, and nothing can be said as to the nature of the inhibition.

VI. Enzymatic Synthesis

If β-glucosidase is a real catalyst it will catalyze not only the hydrolysis of β-glucosides but also their synthesis. Hérissey, investigating this problem about 1900, was able to present evidence for the synthesizing action of emulsin (β-glucosidase) and Bourquelot and associates, in a long series of papers[107] carried out a very thorough qualitative examination of the synthetic properties of the enzyme. This synthetic action does not need to be taken into account in investigations of the enzymatic hydrolysis of β-glucosides, since the equilibrium between glucoside, glucose, aglycon, and water is such that in dilute aqueous solutions the hyrolysis will proceed until less that 1% of the glucoside is left unhydrolyzed.

In order to obtain a greater amount of glucoside in the equilibrium mixture, *i.e.*, to perform an enzymatic synthesis of the glucoside, it is necessary to use glucose dissolved or suspended in a very concentrated solution of the aglycon or in the pure aglycon. The enzyme is only slightly soluble in such mixtures but it may act as contact catalyst nevertheless. The reaction under these conditions is a very slow one, but if the solutions are rotated so that the enzyme cannot establish an equilibrium at its surface, making the diffusion of the glucoside from the saturated layer the rate-determining process the equilibrium generally will be established within 2–3 weeks.

No serious doubt seems to exist as to the identity of the enzyme causing synthesis and hydrolysis. Bersin[108] mentions that a difference in degree of dispersity may decide if the action of the enzyme is a synthesis or a hydrolysis, speaking of macroheterogeneous synthesizing and microheterogeneous hydrolyzing enzymes, but this conception must be disapproved

The question has been discussed whether the enzymic synthesis follows the law of mass action. Von Euler and Josephson,[109] calculating some experiments of Bourquelot and associates, have found that in the synthesis of methyl β-D-glucoside the law of mass action is followed to a methyl alcohol concentration of 50%, but in more

[104] T. Bokorny, *Biochem. Z.* **94**, 69 (1919).
[105] B. Helferich and S. Winkler, *Z. physiol. Chem.* **221**, 98 (1933).
[106] M. Mascré and A. Paris, *Bull. soc. chim. biol.* **15**, 918 (1933).
[107] E. Bourquelot, *Ann. chim.* [8] **29**, 145 (1913).
[108] T. Bersin, Kurzes Lehrbuch der Enzymologie. Akadem. Verlagsgesellschaft, Leipzig, 1938, p. 30.
[109] H. von Euler and K. Josephson, *Z. physiol. Chem.* **136**, 30 (1924).

concentrated solutions of methyl alcohol the synthesis does not reach the calculated degree. They are of the opinion that the enzyme is inactivated by prolonged contact with concentrated alcohol, and Shipitsina[110] also takes this view. Pronin and Krylova[111] have made the observation that at constant alcohol concentration the glucose concentration may be varied without deviation from the law of mass action, but if the concentration of alcohol (ethyl) is changed from 35 to 81% another equilibrium constant is found. Vintilesco, Ionesco, and Kisyk,[112] on the other hand, calculating the same experiments of Bourquelot as von Euler, thinks that the deviation from the law of mass action is only apparent inasmuch as not the total glucose-concentration but only the concentration of β-glucose has to be taken into account, and with increasing alcohol concentration the amount of β-glucose in the equilibrium decreases, which may be seen from the increase of the specific rotation of glucose with increasing alcohol concentration. Veibel,[113] however, was able to show that not only is the specific rotation of glucose modified by increasing alcohol concentrations, but also that of β-glucosides, and here no possibility of variation of the ratio β-glucoside: α-glucoside does exist. The examination of the validity of the law of mass action is, there-

TABLE XII

EQUILIBRIUM CONSTANTS AND HEATS OF REACTION FOR SOME GLUCOSIDES

Aglycon	$K_{30°}$	$K_{4°}$	Q, cal.
Methanol	0.311	0.296	314
Ethanol	0.519	0.466	700
Propanol	0.657	0.587	722
Butanol	0.624	0.568	602
Isobutyl alcohol	0.785	0.738	397
Isopropyl alcohol	1.495	1.253	1131
sec.-Butanol	1.391	1.221	835
tert.-Butanol	3.761	2.967	1524

fore, very complicated, but not reason of doubting its validity for enzymatic glucoside synthesis seems to exist.

Veibel[113] has examined the position of the equilibrium in dependence on the type of the aglycon (primary, secondary, tertiary alkyl). In order to be able to use identical concentrations of glucose, alcohol, and water in all experiments acetone was used as a neutral component of the solutions, which were 0.15*M* with regard to glucose, 3.00*M* with regard to alcohol, and 15.00*M* with regard to water. The equilibrium was determined at 30° and at 4°. Table XII gives the results. It is seen that the amount of glucoside formed depends both on the number of carbon atoms in the aglycon and on the type of the alcohol. For primary alcohols the degree of glucoside formation decreases with increasing number of carbon atoms (K being the equilibrium constant for the hydrolysis, $K = 1$ corresponds to about 16% synthesis, $K = 2$ to 9%, and K

[110] G. K. Shipitsina, *Biokhimiya* **7**, 1 (12) (1942). quoted from *Chem. Abstracts* **37**, 4411 (1943).

[111] S. A. Pronin and S. A. Krylova, *Arch. sci. biol. U. S. S. R.* **44**, 167 (175) (1936). quoted from *Chem. Abstracts* **32**, 6535 (1938).

[112] I. Vintilescu, C. N. Ionescu, and A. Kisyk, *Bul. Chim. Soc. Chim. România* **17**, 137 (1935).

[113] S. Veibel, *Enzymologia* **1**, 124 (1936–1937).

= 3 to 6%). The degree of glucoside formation decreases in the series primary through secondary to tertiary alcohols. The equilibrium constant for *sec.* butyl glucoside is probably more uncertain than the others since the alcohol contains an asymmetric carbon atom and the velocity of synthesis presumably is different for the D- and the L component of the alcohol. Even if, according to Kuhn,[114] it is to be expected that the glucoside of the racemic alcohol will result if the time of reaction is sufficiently prolonged, this stage is certainly not reached in the experiments described, inasmuch as the last phase of the reaction is very slow as compared with the initial rates of synthesis. Helferich and Lampert[115] are of the opinion that the initial velocity of synthesis increases with increasing number of carbon atoms in the aglycon (for unbranched aliphatic alcohols). Veibel[113] could not corroborate this result, but further investigation of the kinetics of the enzymatic glucoside synthesis is necessary before the dependence of the velocity on size and structure of the alcohol may be regarded as elucidated.

Vintilesco, Ionesco, and Solomon[116] investigated disaccharide synthesis catalyzed by β-glucosidase, but only preliminary results have been published.

The degree of glucoside formation is greater at 4° than at 30° (see Table XII). Veibel[113] calculated the heat of reaction for the glucoside formation from the expression:

$$Q = \frac{RT_1T_2}{T_1 - T_2} \ln \frac{K_{T_1}}{K_{T_2}}$$

It is seen that the heat of reaction is greater for secondary alkyl glucosides than for primary and still greater for tertiary alkyl glucosides. This is in accordance with the order in which the degree of glucosidification decreases.

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[114] W. Kuhn, *Ergeb. Enzymforsch.* **5**, 1 (1936).

[115] B. Helferich and U. Lampert, *Ber.* **68**, 2050 (1935).

[116] I. Vintilescu, C. N. Ionescu, and M. Solomon, *Bul. Chim. Soc. Chim. România* **17**, 267, 283 (1935).

CHAPTER 17

Hydrolysis of Galactosides, Mannosides, and Thioglycosides

BY STIG VEIBEL

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I. α-Galactosidase

1. Occurrence, Preparation and Purification

The best source of α-galactosidase is brewer's yeast[1-4]; the enzyme is absent from baker's yeast. Bierry[5] mentions that the digestive juice of *Helix pomatia* hydrolyzes not only β-galactosides but α-galactosides as well. The digestive juice therefore contains an α-galactosidase, which was, however, not further investigated by Bierry. Helferich and Appel[6] found that sweet almond emulsin also contains an α-galactosidase which is different from β-glucosidase (and β-galactosidase), since the α-galactosidase action disappears with progressive purification of the almond emulsin preparation. This was more closely investigated and corroborated by Helferich and associates,[7] who found, in accordance with the specificity rules of Weiden-

[1] E. Fischer and P. Lindner, *Wochschr. Brau.* **12**, 959 (1895).
[2] A. Bau, *Wochschr. Brau.* **12**, 1062 (1895).
[3] A. Bau, *Wochschr. Brau.* **20**, 560 (1903).
[4] A. Bau, *Wochschr. Brau.* **20**, 595 (1903).
[5] H. Bierry, *Compt. rend.* **156**, 265 (1913).
[6] B. Helferich and H. Appel, *Z. physiol. Chem.* **205**, 231 (1932).
[7] B. Helferich, S. Winkler, R. Gootz, O. Peters, and E. Günther, *Z. physiol. Chem.* **206**, 91 (1932).

hagen,[8,9] that the effect of purification was a decrease of the enzyme efficiency for α-D-galactosides and β-L-arabinosides. Methods for the isolation of the α-galactosidase were not indicated.

Hill[10] found that β-galactosidase from alfalfa seed also contains α-galactosidase. The two galactosidases are not efficiently separated by purification with tannin, but by adsorption on alumina the β-galactosidase is more strongly adsorbed than the α-galactosidase, so that a solution relatively richer in α-galactosidase may be obtained. The β-galactosidase is more resistant to heat than the α-galactosidase. In a 0.25% aqueous solution of alfalfa seed emulsin about 56% of the β-galactosidase activity but only 20% of the α-galactosidase activity remain after heating to 45° for 3 hours. Against ultraviolet radiation the stability of the two galactosidases is identical. The pH optimum of the α-galactosidase is not sharp, an optimal zone at pH 3.3–5.0 is found, whereas the β-galactosidase has a fairly sharp pH optimum at pH 3.4–3.5 (*cf.* Veibel and Østrup[11]).

Coffee emulsin, which in properties resembles alfalfa seed emulsin very much, also contains an α-galactosidase.[12] Helferich,[13] investigating kefir emulsin, found it able to hydrolyze-α-galactosides, so that this emulsin, too, contains an α-galactosidase.

The difference between β-glucosidase and α-galactosidase of sweet almond emulsin is seen when the crude enzyme preparation is purified with carboraffin C according to Helferich and Pigman.[14,15] The enzyme efficiency of the β-glucosidase may increase 200%, that of α-galactosidase only 50%. By purification with silver oxide the α-galactosidase is completely destroyed. The best separation of the α-galactosidase in sweet almond emulsin is obtained by chromatography on bauxite[16] (see Chapter 16). Also the cellobiase (chitinase) which accompanies the β-glucosidase and the galactosidases is in this way separated from the α-galactosidase. In the standardization of α-galactosidase, melibiose is used as substrate. The standard concentration of Willstätter and associates[17] is used, *i.e.*, 1.3125 g. melibiose in 25 ml. solution. Standard pH is 4.62 (acetate buffer).

Even if it is questionable whether β-glucosidase and β-galactosidase are two different enzymes (see Sect. II-2) no doubt exists as to the difference between α-glucosidase and α-galactosidase. α-Glucosidase is isolated from

[8] R. Weidenhagen, *Naturwissenschaften* **16**, 654 (1928).
[9] R. Weidenhagen, *Z. Ver. deut. Zucker-Ind.* **78**, 417 (1928).
[10] K. Hill, *Ber. Verhandl. sächs. Akad. Wiss. Leipzig, Math.-phys. Klasse* **86**, 115 (1934).
[11] S. Veibel and G. Østrup, *Biochim. et Biophys. Acta* **1**, 1 (1947).
[12] B. Helferich and F. Vorsatz, *Z. physiol. Chem.* **237**, 254 (1935).
[13] B. Helferich, *Ber. Verhandl. sächs. Akad. Wiss. Leipzig, Math.-phys. Klasse* **95**, 135 (1943).
[14] B. Helferich and W. W. Pigman, *Z. physiol. Chem.* **259**, 253 (1939).
[15] W. W. Pigman, *Z. physiol. Chem.* **261**, 82 (1939).
[16] L. Zechmeister, G. Tóth, and M. Bálint, *Enzymologia* **5**, 302 (1938).
[17] R. Willstätter, Tr. Oppenheimer, and W. Steibelt, *Z. physiol. Chem.* **110**, 232 (1920).

brewer's yeast by plasmolyzing the yeast with ethyl acetate at 0° and pH 7.0. Only traces of α-galactosidase are liberated from the cells by this procedure, and the α-glucosidase may be freed from these traces by adsorbing them on aluminum hydroxide B.[18] An α-glucosidase-free solution of α-galactosidase is, on the other hand, obtained[18] by plasmolyzing brewer's yeast with toluene at 30° and pH 7.0.

2. Specificity

The typical substrate for α-galactosidase is melibiose, α-galactosido-6-glucose. α-Galactosidase is one of the five glycosidase types presumed by Weidenhagen[8,9] to be responsible for the hydrolysis of all glycosides. α-Galactosidase will, according to this theory, hydrolyze not only α-galactosides but also glycosides derived from α-galactosides by substitution at carbon atoms 5 and 6. Pigman[19] has slightly modified the system of Weidenhagen. According to Pigman α-galactosidase is responsible for the hydrolysis of α-D-galactosides, β-L-arabinosides, α-D-fucosides (?), melibiose, α-D- and L-*glycero*-D-*galacto*-aldoheptosides (D- and L-*glycero* refer to the configuration at carbon atom 6 in the heptosides, D-*galacto* to the configuration at carbon atoms 2–5). But even if no absolute specificity towards these glycosides seems to exist the relative specificity may be great. Also a relative aglycon specificity is found, but it is not always as pronounced as for β-glucosidase and β-galactosidase. Both alkyl[20,21] and aryl α-D-galactosides[18] and the trisaccharide raffinose with an α-galactosidic linkage are hydrolyzed by α-galactosidase, and raffinose is presumably the only naturally occurring substrate for this enzyme. But whereas both β-glucosidase and β-galactosidase will hydrolyze the *o*-cresyl β-D-glycosides considerably faster than the corresponding phenyl β-D-glycosides, *o*-cresyl and phenyl α-D-galactoside are hydrolyzed with nearly the same velocity.

3. Enzymatic Synthesis

Bourquelot and Aubry[20] and Bourquelot[22] have investigated the enzymatic synthesis of α-galactosides, using the technique indicated by Bourquelot for the synthesis of β-glucosides (see page 618). A series of alkyl α-D-galactosides have been prepared in this way.

II. β-Galactosidase

1. Occurrence, Preparation, and Purification

Practically all β-glucosidase preparations are able to hydrolyze β-galactosides. Until now it has been impossible to separate the two glycosidases

[18] R. Weidenhagen and A. Renner, *Z. Wirtschaftsgruppe Zuckerind.* **86**, 22 (1936).
[19] W. W. Pigman, *J. Research Natl. Bur. Standards* **30**, 257 (1943).
[20] E. Bourquelot and A. Aubry, *J. pharm. chim.* [7] **9**, 225 (1914).
[21] R. Weidenhagen, *Z. Ver. deut. Zucker-Ind.* **77**, 696, 707 (1927).
[22] E. Bourquelot, *J. pharm. Chim.* [7] **10**, 393 (1914).

and a special description of the preparation of β-galactosidase is therefore not necessary, as it is identical with the preparation of β-glucosidase (page 584). But in addition to sources from which both glycosides may be isolated β-galactosidase may be isolated from materials which do not contain β-glucosidase. Hofmann[23] found that emulsin from the kernels of *Rosa canina* is able to hydrolyze β-galactosides but β-glucosides only very slowly and also that emulsin from different strains of bacteria (*Lactobacillus delbrückii*, *Escherichia coli*)[24] and from soybeans[25] will hydrolyze β-galactosides but not β-glucosides. The best source for β-glucosidase-free β-galactosidase seems to be seeds of alfalfa[10] or coffee.[12]

10 kg. alfalfa seeds are soaked with water for 24 hours, allowed to germinate for 48 hours at 25°, dried in a thin layer (0.5 cm.) for 3–4 days, and milled to a fine powder (raw enzyme 0). Ten kg. of this powder are dredged with 50 l. water; 3 kg. toluene are added and the mixture is left over night. Next day the liquid is pressed from the powder (40 l.) and precipitated with a solution of 200 g. tannin in 2 l. normal sodium acetate, 0.5 l. 2*N* acetic acid, and 7.5 l. water. After 20 hours the precipitate is isolated by decantation and centrifugation. Tannin is removed by four times repeated dredging in 10 l. acetone followed by centrifugation (yield 320 g., enzyme I). For further purification 50 g. enzyme I are dredged in 2.5 l. 0.1 *N* acetate buffer, pH 5.0. The insoluble part is isolated by decantation after some hours, washed with acetone till free from tannin, and dried (yield 3.7 g., enzyme II). The β-galactosidase value of raw enzyme 0 is 0.0038, of enzyme I 0.029, and of enzyme II 0.105. Purification with silver acetate[26] cannot be employed, since alfalfa β-galactosidase is practically destroyed by this procedure.

2. Specificity

In Chapter 16 the general problem of the specificity of glycosidases is treated. It suffices therefore to mention here that it is to be expected that β-galactosidase will hydrolyze β-D-galactosides, α-L-arabinosides, β-D-fucosides (?), lactose, and heptosides with β-D-galactose configuration (*i.e.* β-D- and L-*glycero*-D-*galacto*-aldoheptosides).[19] Originally it was thought that lactase and β-galactosidase were two different enzymes, but convincing evidence has been produced to prove that lactose and lactose derivatives (lactose ureide, lactobionic acid) are hydrolyzed by β-galactosidase, so that this question will not be discussed here.

But another question of specificity exists. It was mentioned that sweet almond emulsin and most other β-glucosidase preparations contain β-galactosidase, and in Chapter 16 it is mentioned that it was not proved that the specificity for carbon atom 4 of β-glucosidase is absolute, but that it may be only relative. In the last case β-glucosidase and β-galactosidase are one enzyme. In order to elucidate this question Helferich and associates have tried to determine whether different purification or inactivation manipula-

[23] E. Hofmann, *Biochem. Z.* **267**, 309 (1933).
[24] E. Hofmann, *Biochem. Z.* **272**, 133 (1934).
[25] E. Hofmann, *Biochem. Z.* **272**, 426 (1934).
[26] B. Helferich and S. Winkler, *Z. physiol. Chem.* **209**, 269 (1932).

tions were able to alter the ratio between the enzyme efficiency of phenyl β-D-glucoside and β-D-galactoside or the *o*-cresyl glycosides. From their results it may be mentioned that purification to an enzyme efficiency 740 times greater than the crude enzyme,[7,15] partial inactivation by heat,[27] treatment with ozone,[28,29] osmium tetroxide,[30] ultraviolet light,[31] and formalin[32,33] have produced no variation in the ratio between the two enzyme efficiencies. Helferich, therefore, is of the opinion that β-glucosidase and β-galactosidase of sweet almond emulsin are one enzyme.

Hofmann[34] opposes this, noting that the difference between the ratio β-glucosidase:β-galactosidase in enzyme preparations from different sources are so great that one enzyme cannot be responsible for the hydrolysis of both types of glycosides. But, as shown by Miwa and associates,[35] real β-glucosidases from different sources may also show considerable differences in the ratio between the efficiencies of, *e.g.*, salicin and phenyl β-D-glucoside (see Chapter 16, Table X), this ratio being 25.0 for sweet almond emulsin and 0.025 for emulsin from *Aspergillus niger*. Notwithstanding this no question has been raised as to the responsibility of a β-glucosidase for the hydrolysis in both instances. This led Helferich to maintain his assumption of the identity of β-galactosidase and β-glucosidase at least for almond emulsin. Since, however, Helferich and associates[10,12,36] found in alfalfa seed emulsin and in coffee emulsin enzymes capable of hydrolyzing β-galactosides but practically without effect towards β-glucosides, it was no longer possible to maintain the theory of the identity of the two β-glycosidases unrestricted, and Helferich modified it in postulating that in almond emulsin one enzyme is responsible for the hydrolysis of both β-glycosides; in alfalfa and coffee emulsin, on the contrary, the β-galactosidase is different from the β-galactosidase in almond emulsin. His argument for this postulate is first, that, whereas the β-galactosidase of almond emulsin hydrolyzes *o*-cresyl β-D-galactoside some 13 times faster than phenyl β-D-galactoside, the β-galactosidase of alfalfa emulsin will hydrolyze the two β-galactosides with apparently the same velocity, and second, that the treatment of the somewhat purified enzyme with silver oxide has proved to be a very efficient way of purification of almond emulsin, whereas alfalfa seed emulsin is not at all

[27] B. Helferich, H. Heyne, and R. Gootz, *Z. physiol. Chem.* **214**, 139 (1932).
[28] B. Helferich, S. Winkler, E. Schmitz-Hillebrecht, and H. Bach, *Z. physiol. Chem.* **229**, 112 (1934).
[29] B. Helferich and S. R. Petersen, *Z. physiol. Chem.* **233**, 75 (1935).
[30] B. Helferich and F. Vorsatz, *Z. physiol. Chem.* **239**, 241 (1936).
[31] B. Helferich and G. Brieger, *Z. physiol. Chem.* **221**, 94 (1933).
[32] B. Helferich and S. Winkler, *Z. physiol. Chem.* **221**, 98 (1933).
[33] B. Helferich, *Ergeb. Enzymforsch.* **7**, 83, (1938).
[34] E. Hofmann, *Naturwissenschaften* **22**, 406 (1934).
[35] T. Miwa, C. Cheng, M. Fujisaki, and A. Toishi, *Acta Phytochim. Japan* **10**, 155 (1937).
[36] B. Helferich and R. Griebel, *Ann.* **544**, 191 (1940).

precipitated by silver oxide, which on the contrary tends to destroy this β-galactosidase.

Curiously enough Helferich and Goerdeler[37,38] by investigating the β-glucosidase of snail emulsin (*Helix pomatia*) have established that a quite similar difference exists between this β-glucosidase and the β-glucosidase of almond emulsin (nearly the same velocity of hydrolysis of *o*-cresyl and of phenyl β-D-glucoside, no purification of the enzyme by treating it with silver oxide), but here they do not assume that the two β-glucosidases are different.

Antoniani[39] isolated from the seeds of *Sorghum saccharatum* an enzyme which was able to hydrolyze β-glucosides but not β-galactosides, and Veibel and associates[40] found, contrary to the statement of Hofmann,[41] that milk sugar yeast emulsin (from *Saccharomyces fragilis Jørgensen*) too was able to hydrolyze β-glucosides but not β-galactosides. The β-glucosidase present in this enzyme preparation hydrolyzes phenyl β-D-glucoside twice as fast as *o*-cresyl β-D-glucoside. If these results may be verified it seems necessary to presume the existence of two β-glucosidases as well as two β-galactosidases. Hofmann (see Addendum, p. 634) maintains that the milk sugar yeast emulsin hydrolyzes β-galactosides and thinks Veibel and associates have overlooked the β-galactosidase. Even if this were true it does not authorize considering the β-glucosidase of milk sugar yeast identical with β-galactosidase, and the assumption of at least two different enzymes, a β-glucosidase and a β-galactosidase, seems a necessity. If the reasons of Helferich for postulating two different β-galactosidases must be considered compelling it seems necessary to assume two β-glucosidases as well, but the idea of Pigman[19]—of a β-glycosidase not as a single enzyme but as a class of very related enzymes, having the same absolute specificity towards different glycosides but allowing for relative specificities towards different members of a definite class of glycosides—may possibly be a more correct representation of our knowledge about the nature and the identity of glycosidases than the more rigid conception of Helferich. The idea of Pigman seems to be reconcilable with the assumption of Gottschalck[42] of the adaptation of the pyranose ring of a glycoside (in chair position) to the surface of the enzyme through the bridge oxygen atom and one of the ring hydroxyl groups. The proteins of the different sources of glycosidases may differ in the shape and the size of the active part of the surface, but as long as the

[37] B. Helferich and J. Goerdeler, *Ber. Verhandl. sächs. Akad. Wiss. Leipzig, Math.-phys. Klasse* **92**, 75 (1940).
[38] B. Helferich, *Ergeb. Enzymforsch.* **9**, 70 (1943).
[39] C. Antoniani, *Rend. ist. Lombardo sci.* [2] **68**, 355 (1935).
[40] S. Veibel, C. Møller, and J. Wangel, *Kgl. Danske Videnskab. Selskab. Mat.-fys. Medd.* **22**, No. 2 (1945).
[41] E. Hofmann, *Biochem. Z.* **256**, 462 (1932).
[42] A. Gottschalck, *Nature* **160**, 113 (1947).

bridge oxygen atom and, *e.g.*, the hydroxyl group at carbon atom 4 may be associated with the active area, only relative specificities will be found.

In order to substantiate the principal postulate of Helferich that in sweet almond emulsin a single enzyme is responsible for the hydrolysis of both β-glucosides and β-galactosides, Helferich and Göller[43] investigated the simultaneous hydrolysis of two glycosides, first *n*-butyl β-D-glucoside and phenyl β-D-glucoside and then *n*-butyl β-D-glucoside and *m*-cresyl β-D-galactoside. The three glycosides have approximately the same enzyme efficiency towards sweet almond emulsin. From the Table I it may be seen that the hydrolysis of *n*-butyl β-D-glucoside is not inhibited by phenyl β-D-glucoside nor by *m*-cresyl β-D-galactoside, whereas both phenyl β-D-glucoside and *m*-cresyl β-D-galactoside are considerably inhibited by *n*-butyl β-D-glucoside. Helferich and Göller draw from these experiments the conclusion

TABLE I

MUTUAL INFLUENCE OF GLUCOSIDES AND GALACTOSIDES ON ENZYME EFFICIENCY

Substrate[a]	Enzyme efficiency of		
	n-Butyl glucoside	Phenyl glucoside	*m*-cresyl galactoside
Butyl glucoside	0.26, 0.24	—	—
Phenyl glucoside	—	0.33	—
m-Cresyl galactoside	—	—	0.13
Butyl-glucoside + phenyl glucoside	0.26	0.20	—
Butyl-glucoside + *m*-cresyl galactoside	0.24	—	0.048

β-Glucosidase value of sweet almond emulsin in all cases 1.2.
[a] Concentration of each substance 0.052 *N*.

that there is no reason to assume the existence of two different glycosidases. This is obviously correct. But it is very questionable if the problem can be solved in the way described. Without knowledge of the dissociation constants of the enzyme–substrate complexes it seems impossible from experiments carried out with only one substrate concentration to derive any conclusions.

Veibel and associates determined for β-glucosidase[44] and for β-galactosidase[45] of almond emulsin the values of $k_{obs.}$, K_m, K_{m_1}, K_{m_2}, and k_3 (for the significance of these constants see Chapter 16, p. 591) at various pH values and buffer compositions, using *o*-cresyl β-D-glucoside and β-D-galactoside as substrates. They found rather great differences in the variations of these

[43] B. Helferich and W. Göller, *Z. physiol. Chem.* **247**, 220 (1937).
[44] S. Veibel and H. Lillelund, *Enzymologia* **9**, 161 (1941).
[45] S. Veibel, J. Wangel, and G. Østrup, *Biochim. et Biophys. Acta* **1**, 126 (1947).

constants for the two substrates, but the ratio of $k_{obs.}$ of glucoside to $k_{obs.}$ of galactoside showed no greater variation than might be explained by experimental error, even if the figures in Table II seem to indicate a regular decrease in the ratio with increasing pH. The variation of the ratio of k_3 of glucoside to k_3 of galactoside with pH in phosphate–citrate buffer, on the other hand, is so great that it cannot be explained as experimental error. Veibel, Wangel, and Østrup[45] mention the possibility that the colloidal carriers in the two enzymes are different, but since for none of the enzymes has

TABLE II

pH Effect on $k_{obs.}$ and k_3 Ratios of β-Glucosidase to β-Galactosidase

Glucoside: galactoside ratio	Buffer	pH					
		3.6	4.0	4.4	4.8	5.2	5.6
$k_{obs.}$	Acetate	8.4	8.3	7.8	7.2	6.8	6.8
	Phosphate–citrate	7.9	7.4	7.1	6.5	6.4	6.3
k_3	Acetate	4.9	—	—	—	4.2	4.5
	Phosphate–citrate	10.5	—	—	3.6	—	4.8

TABLE III

Affinity Constants of Glucose and Galactose to β-Glucosidase and β-Galactosidase from Sweet Almond Emulsin

Substrate	K_{m_1} (glucose)	K_{m_1} (galactose)
o-Cresol-β-D-glucoside	0.21	00
o-Cresol-β-D-galactoside	0.41	0.41

a dissociation into colloidal carrier and prosthetic group been realized it seems more adequate to ascribe the differences between the two enzymes to a different location of the active areas on the enzyme surface (*cf.* Gottschalck[42]).

Veibel and associates[45,46] determined the inhibitory action of glucose and galactose on the hydrolysis of *o*-cresyl β-D-glucoside and *o*-cresyl β-D-galactoside. If the β-glucosidase and the β-galactosidase are identical, the K_{m_1} values for glucose and galactose should be independent of the substrate used. Table III shows that this is not the case, and therefore it is concluded that the two glycosidases are different.

In other experiments[46] the β-galactosidases of almond emulsin and of alfalfa seed emulsin were compared and it was found that the pH optimum and the values of the different dissociation constants of the complexes of

[46] S. Veibel and G. Østrup, *Biochim. et Biophys. Acta* **1**, 1 (1947).

enzyme–substrate, enzyme–galactose, and enzyme–aglycon differ considerably. Glucose, which has some affinity for almond emulsin β-galactosidase, has no affinity at all for alfalfa seed emulsin β-galactosidase and does not inhibit at all the hydrolysis of *o*-cresyl β-D-galactoside by this enzyme, whereas galactose, which as seen from Table III has the same affinity for almond emulsin β-galactosidase as glucose, has so great an affinity for alfalfa seed emulsin β-galactosidase that an exact value cannot be determined. These results, too, may be explained by the hypothesis of Pigman[19] and Gottschalck,[42] but it may be regarded as a question of definition whether the two β-galactosidases shall be considered one or two enzymes, the difference

TABLE IV

ENZYME EFFICIENCY OF SOME DISACCHARIDES AND RELATED COMPOUNDS WITH β-GALACTOSIDIC LINKAGES

Substrate	Structure	Enzyme efficiency $\times 10^3$
Lactose	4-Glucose β-galactoside	11.2
Lactulose	4-Fructose β-galactoside	(14)[a]
Neolactose	4-Altrose β-galactoside	(2.8)[a]
Lactositol	4-Sorbitol β-galactoside	0.84
Lactobionic acid	4-(Gluconic acid) β-galactoside	0.41
Phenyl β-lactoside	4-(Phenyl β-glucoside) β-galactoside	23
Protocatechuic aldehyde β-lactoside	4-(Protocatechuic aldehyde-β-glucoside) β-galactoside	80
Phenyl β-galactoside		32–49

[a] Not measured originally under standard conditions. The values given were calculated from the corresponding value for lactose, measured under the same conditions, and are approximately those to be expected under the standard conditions.

between them being in many respects as great as, *e.g.*, the difference between β-glucosidase and β-galactosidase of almond emulsin.

From experiments with hydrolysis of disaccharides with β-glucosidic linkages it may be seen how great an influence relatively small changes in the aglycon have on the enzyme efficiency of the substrate. Table IV contains some results obtained by Helferich, Pigman, and associates.[47] It is seen that reduction of the aldehyde group in lactose reduces the enzyme efficiency to a thirteenth of the original value.

3. ENZYMATIC SYNTHESIS

Bourquelot and associates[48] have investigated the synthetic action of almond emulsin on solutions of galactose in aqueous alcohols. The results are

[47] W. W. Pigman, *Advances in Enzymol.* **4**, 41 (1944).
[48] E. Bourquelot, *J. pharm. chim.* [7] **10**, 361, 393 (1914).

comparable with those found for the enzymatic syntheses of β-glucosides (see page 618), except that β-galactosidase seems to be more easily inactivated by the alcohols than β-glucosidase so that the true equilibrium is obtained with great difficulty.[49]

Vintilescu and associates[50] examined the syntheses in mixtures of water, ethanol, acetone, and galactose and found that, if the alcohol concentration is too low, synthesis of galactobiose will complicate the evaluation of the extent of the alkyl β-D-galactoside synthesis. Not only has almond emulsin been investigated, but Bourquelot and Hérissey[51] have also examined the action of kefir on solutions of galactose in aqueous ethyl alcohol and have found a synthesis of ethyl β-D-galactoside.

The isolation of the galactosides is somewhat complicated by the presence of great quantities of galactose. In the enzymatic glucoside syntheses glucose may be disposed of by addition of baker's yeast, which ferments the glucose, but galactose is unfermentable by baker's yeast. Brewer's yeast will ferment the galactose, but it will at the same time contaminate the solution with so much protein that it is very difficult to isolate the galactosides as pure compounds.

III. α-Mannosidase

1. Occurrence, Preparation and Purification

α-Mannosidase is found in most of the vegetable materials used for the preparation of β-glucosidase. It will therefore be found in the common β-glucosidase preparations. Fischer had found it present in almond emulsin. Hérissey[52] found that various molds also contain α-mannosidase but that the best source for this enzyme seems to be alfalfa seed. The investigation of Hill[10] on alfalfa seed emulsin corroborated this result. The enzyme efficiency of an alfalfa seed emulsin preparation towards different glycosides are given in Table V.

That α-mannosidase is different from β-glucosidase and β-galactosidase is proved by the influence of different purification and inactivation manipulations on the ratio of β-galactosidase to α-mannosidase.[10] The purification of alfalfa seed emulsin by fractional precipitation with tannin does not lead to a separation of β-galactosidase and α-mannosidase (Table VI). But by adsorption on alumina a partial separation is obtained (Table VII). Still greater is the difference in resistance towards heat (Table VIII) and towards ultraviolet radiation (Table IX).

[49] M. Bridel, *J. pharm. chim.* [7] **24**, 209 (1921).
[50] I. Vintilescu, C. N. Ionescu, and M. Solomon, *Bul. Chim. Soc. Române Chim.* **20**, 115 (1938).
[51] E. Bourquelot and H. Hérissey, *J. pharm. chim.* [7] **7**, 110 (1913).
[52] H. Hérissey, *Compt. rend.* **172**, 766 (1921).

TABLE V

ENZYME EFFICIENCY OF ALFALFA SEED EMULSIN TOWARDS DIFFERENT GLYCOSIDES

Substrate	Enzyme efficiency
Phenyl β-D-galactoside	0.177
Phenyl *N*-acetyl β-D-glucosaminide	0.125
Phenyl α-D-mannoside	0.10
Phenyl α-D-galactoside	0.086
Phenyl β-D-isorhamnoside	0.006
Phenyl β-D-glucoside	0.003
Phenyl α-D-glucoside	0.0004
Melibiose	0.004
Lactose	0.017

TABLE VI

INFLUENCE OF PRECIPITATION WITH TANNIN ON RATIO OF β-GALACTOSIDASE TO α-MANNOSIDASE

Step	β-Galactosidase		α-Mannosidase	
	E.e.[a]	Weight × E.e.	E.e.	Weight × E.e.
Crude enzyme	0.076	0.133	0.084	0.151
1st precipitation	0.023	0.010	0.042	0.019
2nd precipitation	0.228	0.126	0.254	0.139
3rd precipitation	0.037	0.004	0.0	0.0

[a] Enzyme efficiency.

TABLE VII

ADSORPTION OF β-GALACTOSIDASE AND α-MANNOSIDASE ON ALUMINA

Preparation	β-Galactosidase: α-mannosidase
Crude enzyme	2.00: 1.26
Solution after adsorption	0.93: 0.60
Eluate	6.8 :12.3

TABLE VIII

ACTION OF HEAT ON β-GALACTOSIDASE AND α-MANNOSIDASE

Preparation	Enzyme efficiency of	
	Phenyl β-galactoside	Phenyl α-mannoside
Crude enzyme solution	0.165	0.105
Heated 3 hr. at 45°	0.079	0.101
Heated 7 hr. at 45°	0.057	0.095
Heated 3 hr. at 60°	0.0	0.031

Coffee emulsin[12] also resembles alfalfa emulsin with regard to the content of α-mannosidase. In almond emulsin α-mannosidase may be separated from β-glucosidase by heating the enzyme solution for 40 hours to 45° at pH 5.0.[27] Hereby the β-glucosidase action practically disappears whereas the α-mannosidase action is undisturbed. α-Mannosidase is, on the other hand, sensitive to the action of silver oxide or silver acetate, which, as shown by Helferich and Winkler,[26] causes an efficient purification of β-glucosidase from almond emulsin. α-Mannosidase is practically destroyed by this treatment.

The purification of almond emulsin β-glucosidase with carboraffin C[14,15] (see Chapter 16) is not so effective towards α-mannosidase as towards β-glucosidase. The enzyme efficiency of the mannosidase increases from 0.10 to 0.22, that of β-glucosidase from 0.24 to 0.63.

TABLE IX

ACTION OF ULTRAVIOLET RADIATION ON β-GALACTOSIDASE AND α-MANNOSIDASE

Preparation	Enzyme efficiency of	
	Phenyl β-galactoside	Phenyl α-mannoside
Crude enzyme solution	0.147	0.13
Irradiated 20 min	0.132	0.09
Irradiated 40 min	0.102	0.10
Irradiated 80 min	0.102	0.10
Irradiated 160 min	0.062	0.10

2. SPECIFICITY

The specificity of α-mannosidase has been investigated only in a preliminary way. The sugar specificity has been outlined by Pigman[19,47]; the enzyme will hydrolyze α-D-mannosides, α-D-lyxosides,[53] and, presumably, heptosides with α-D-mannose configuration at carbon atoms 2–5. As to the aglycon specificity, it is known that the enzyme efficiency toward phenyl α-D-mannoside is considerably greater than toward methyl α-D-mannoside. Here, too, there seems to be great resemblance to the more thoroughly investigated glycosidases mentioned above.

3. ENZYMATIC SYNTHESES

The synthetic properties of α-mannosidase have been investigated by Hérissey.[54–58] α-Mannosidase is easily inactivated at high concentrations

[53] W. W. Pigman, *J. Am. Chem. Soc.* **62**, 1371 (1940).
[54] H. Hérissey, *Compt. rend.* **173**, 1406 (1921).
[55] H. Hérissey, *Compt. rend.* **176**, 779 (1923).
[56] H. Hérissey, *Compt. rend.* **178**, 123, 1372 (1924).
[57] H. Hérissey, *Bull. soc. chim. biol.* **4**, 480 (1922).
[58] H. Hérissey, *Bull. soc. chim. biol.* **5**, 501 (1923).

of the alcohol examined, so that solutions stronger than 10% cannot be used. The amount of α-mannoside in the equilibrium mixture at this alcohol concentration is only slight and in order to isolate the mannoside it is necessary to separate it from relatively great amounts of mannose. This is done by fermentation of the mannose by baker's yeast.

IV. β-Mannosidase

In an investigation of the enzymes present in highly purified invertase preparations Adams, Richtmyer, and Hudson[59] describe a hitherto unknown enzyme, β-mannosidase, which is able to hydrolyse β-mannosides but not α-mannosides. The new enzyme was found both in baker's and in brewer's yeast. The hydrolysis of phenyl β-D-mannoside was investigated; the enzyme efficiency towards β-mannoside of the brewer's yeast enzyme was 2.1×10^{-5}, and of the baker's yeast preparation 1.1×10^{-5}, *i.e.*, the hydrolyzing properties are just discernable. Attempts at purification of the enzyme or further investigation of its specificity have so far not been published.

V. Thioglucosidase

An enzyme capable of catalyzing the hydrolysis of naturally occurring thioglucosides has been known for a long time under the designation myrosin, myrosinase, or sinigrase. These names were given because the best known substrate is the glycoside sinigrin (the potassium salt of myronic acid), present in the seeds of *Sinapis alba* and of many other Cruciferae.

The enzyme myrosinase may be prepared[60] by extraction for 1 hour of 100 g. finely milled seeds of *S. alba* with 300 ml. water at room temperature. After centrifuging, the liquid is precipitated with an equal volume of 90% ethanol and centrifuged once more. The precipitate is washed in the beaker with 70% ethanol and again centrifuged. It is then suspended in 100 ml. water and after some 12 hours at room temperature filtered. The solution still contains small amounts of organically bound sulfate (sinapin), which is hydrolyzed by keeping it in the ice box for 3–4 days after addition of 1% toluene. The solution is then ready for use. The precipitation with alcohol may be repeated, but generally no purification is obtained, on the contrary, the solution loses somewhat in activity. Some plant protein may separate out during the first 1 or 2 weeks, but the solution is stable at pH 5–6, which is the pH of the solution obtained in the method described.

A dry preparation may be obtained by precipitation with ethanol. The vacuum-dried precipitate is said to be very stable. The isolation of thioglucosidase from sources other than *Sinapis* is described by Costa.[61] The myrosinase solution contains two different enzymes, a sulfatase, which hydrolyzes the ester linkage through which the sulfuric acid in sinigrin is bound, and a thioglucosidase, responsible for the splitting of sinigrin into allyl mustard

[59] M. Adams, N. K. Richtmyer, and C. S. Hudson, *J. Am. Chem. Soc.* **65**, 1369 (1943).

[60] C. Neuberg and J. Wagner, *Biochem. Z.* **174**, 457 (1926).

[61] O. A. Costa, *Rev. quim. e farm Rio de Janeiro* **2**, 71 (1937); *Chem. Abstracts* **33**, 4614 (1939).

oil and glucose. The two enzymes are active over a very broad pH range (4.4 to 7.0), but the enzyme solution has its maximum stability at pH 5–6. The sulfatase is more stable than the thioglucosidase at about pH 3, but less stable than the thioglucosidase at an alkaline reaction (above pH 7).

A separation of the two enzymes may be obtained by fractional precipitation with mercuric acetate.[62,63] When 0.05% mercuric acetate is added to the myrosinase solution a precipitate is formed which is isolated by centrifugation and which by elution with phosphate gives an eluate with the ratio thioglucosidase:sulfatase of 20:100. Renewed addition of mercuric acetate precipitates only sulfatase and the centrifugate contains the thioglucosidase with only a trace of sulfatase. Adsorption on kaolin or ferric hydroxide, but not on aluminium hydroxide, also results in a change of the ratio thioglucosidase:sulfatase.

For the standardization of thioglucosidase, sinigrin is used as standard substrate. According to Neuberg and Schönbeck[63] a thioglucosidase unit is the quantity of enzyme (in mg.) which in 20 ml. of sinigrin solution containing 62.3 mg. sinigrin at pH 7.0 and at 40° in 1 hour hydrolyzes 35% of the sinigrin.

The specificity of the thioglucosidase is very pronounced. The enzyme is able to hydrolyze only the naturally occurring mustard oil glucosides but not synthetically prepared thioglucosides. On the other hand, neither the mustard oil glycosides nor the thioglucosides are hydrolyzable by other known glycosidases.[64] According to Pigman[19,47] thioglucosidase is a β-glucosidase which presumably will be able to hydrolyze thioxylosides also. But it seems very unlikely that thioxylosides prepared by synthesis should be hydrolyzable. Only if naturally occurring mustard oil thioxylosides should be found it may be reasonable to assume that they will prove to be hydrolyzable by the thioglucosidase. No further investigation of the specificity of thioglucosidase has been reported, to the knowledge of the reviewer. Lack of substrates opposes the investigation both of sugar specificity and of aglycon specificity. No investigation of the enzymatic synthesis of thioglucosides has been reported.

Hofmann (*Biochem. Z.* **319**, 522, 1949) was able to show that the discrepancy between the results of Hofmann[41] and Veibel *et al.*[40] was due to the fact that β-glucosidase and β-galactosidase are two different enzymes and that the latter is more firmly attached to the cell-walls than the former. Veibel plasmolyzed the centrifuged yeast-cells without previously transforming them into a dry preparation, and only the not too firmly bound enzymes are liberated. Hofmann used so sharply centrifuged cells that they, by plasmolysis, behave as dry preparations, and in this case the firmly bound enzymes are liberated also.

By repeating his experiments, isolating the enzymes once *ad modum* Hofmann and then *a.m.* Veibel, Hofmann was able to corroborate both his own and Veibel's results.

[62] C. Neuberg and O. von Schönbeck, *Naturwissenschaften* **21**, 404 (1933).
[63] C. Neuberg and O. von Schönbeck, *Biochem. Z.* **265**, 223 (1933).
[64] W. W. Pigman, *J. Research Natl. Bur. Standards* **26**, 197 (1941).

CHAPTER 18

β-Glucuronidase

BY WILLIAM H. FISHMAN

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I. Introduction

The enzymatic splitting of conjugated glucuronides was observed as early as 1905 by Neuberg and Niemann[1] who reported the hydrolysis of euxanthic acid and phenol glucuronic acid by emulsin. Other preparations of emulsin have been found to split *l*-camphoglucuronic acid, by Hämäläinen,[2] syringaglucuronic and vanillinglucuronic acids by Hildebrandt,[3] campho-and men-

[1] C. Neuberg and C. Neimann, *Z. Phys. Chem.* **44**, 114 (1905).
[2] J. Hämäläinen, *Skand. Arch. Physiol.* **23**, 297 (1910).
[3] A. Hildebrandt, *Hofmeister Beitr.* **7**, 438 (1905).

thol glucuronic acids by Ishidate,[4] baicalin by Miwa.[5] In most cases the hydrolysis was not marked.

Helferich and Sparmberg[6] concluded that emulsin (almonds) contained a glucuronide-splitting enzyme distinct from β-glucosidase. The same con-

TABLE I

DISTRIBUTION OF β-GLUCURONIDASE IN ANIMAL TISSUES

Organ	Mouse	Rat	Rabbit	Dog	Ox	Man
Liver	25	26	27	13,28	13	22,20
Kidney	25	26	27	13,28	13	20
Spleen	25	26	27	13,28	13,28	20
Lung		26		13	13	24
Heart				13	13	
Intestine				13	13	22
Pancreas				26	13	22
Seminal vesicle		26				
Stomach						22
Prostate		26				
Testis	25	26		13,28	13	
Ovary	25	26		13,28	13	20
Breast						22
Uterus	25	26		28	13	22,20
Endometrium						22,20
Thymus					13	
Parotid gland					13	
Adrenal		26		13	13	
Thyroid		26	13	13		
Lymph nodes						22
Brain					13	
Blood plasma	16					19
Blood serum						20
White blood cells						29
Muscle		26				
Placenta						20
Amniotic fluid						30
Urine						30
Tears						29
Body fluids						31
Saliva						29

clusion was reached by a number of authors whose work has been reviewed by Veibel.[7] Shibata[8] and later Miwa[5,9,10] investigated an enzyme, baicalin-

[4] M. Ishidate, *J. Pharm. Soc. Japan* **49**, 336 (1929).
[5] T. Miwa, *Acta Phytochim. Japan*, **9**, 89 (1936).
[6] B. Helferich and G. Sparmberg, *Z. Physiol. Chem.* **221**, 92 (1933).
[7] S. Veibel *in* Bamann-Myrbäck, Die Methoden der Fermentforschung. Vol. 2, Thieme, Leipzig, 1941, Academic Press, New York, 1945, p. 1

ase, which had the pronounced ability to hydrolyze baicalin, the glucuronide of 5,6,7-trihydroxyflavone. In its behavior, it differed in many important ways from emulsin β-glucosidase and was characterized by a marked specificity for the oxyflavon glucuronides. Miwa[5] identified bacalein and glucuronic acid as the products of baicalin hydrolysis by baicalinase.

The study of animal glucuronidases began with the work of Sera,[11] Masamune[12] and Oshima.[13,14] In these studies animal β-glucuronidase was established as an enzyme (distinct from β-glucosidase) which accomplished the hydrolysis of a variety of glucuronides. Studies were made of the distribution of the enzyme, its partial purification and its reaction-kinetics.

Later Fishman[15,16] extended these studies and demonstrated a relationship of β-glucuronidase to estrogen action. Correlations of tissue β-glucuronidase activity with processes of cell proliferation have been found in Levvy's laboratory[17,18] and interest in serum glucuronidase changes in human pregnancy has arisen from the observations of Fishman[19] and McDonald and Odell.[20, 21] A recent development relates to the presence of enhanced amounts of β-glucuronidase in human cancer tissues.[19,22,23,24]

The references (Table I) indicate publications in which positive indications of glucuronidase activity are given. With the use of the more sensitive methods, erythrocytes and platelets can be excluded as sources of glucuronidase. Oshima's negative results in some tissues should not be accepted until a search has been made with the methods now available.

II. Properties of Glucuronidase

1. Occurrence

In plants, β-glucuronidase activity has been found in roots of *Scutellaria baicalensis*[5,8–10] and in various emulsin preparations.[1–4] A more widespread distribution has been reported in animal tissues (Table I). Certain mucases

[8] K. Shibata, *Acta Phytochim. Japan* **1**, 105 (1923); **5**, 117 (1930).
[9] T. Miwa, *Acta Phytochim. Japan* **8**, 231 (1935).
[10] T. Miwa, *Acta Phytochim. Japan* **9**, 89 (1936).
[11] Y. Sera, *Z. physiol. Chem.* **92**, 261 (1914).
[12] H. Masamune, *J. Biochem. Japan* **19**, 353 (1934).
[13] G. Oshima, *J. Biochem. Japan* **20**, 361 (1934).
[14] G. Oshima, *J. Biochem. Japan* **23**, 305 (1936).
[15] W. H. Fishman and L. W. Fishman, *J. Biol. Chem.* **152**, 487 (1944).
[16] W. H. Fishman, *J. Biol. Chem.* **7**, 159 (1947).
[17] L. M. H. Kerr and G. A. Levvy, *Nature* **160**, 463 (1947).
[18] G. A. Levvy, L. M. H. Kerr, and J. G. Campbell, *Biochem. J.* **42**, 462 (1948).
[19] W. H. Fishman, *Science* **105**, 646 (1947).
[20] D. F. McDonald and L. D. Odell, *J. Clin. Endocrinol.* **7**, 535 (1947).
[21] L. D. Odell and D. F. McDonald, *Am. J. Obstet. Gynecol.* **56**, 1 (1948).
[22] W. H. Fishman and A. J. Anlyan, *Science* **106**, 66 (1947).
[23] W. H. Fishman and A. J. Anlyan, *J. Biol. Chem.* **169**, 449 (1947).
[24] W. H. Fishman, A. J. Anlyan, and E. Gordon, *Cancer Research* **7**, 808 (1947).

(see chapter on mucopolysaccharides) of animal and bacterial origin may contain glucuronidases.

The following generalizations seem justified from the data which have appeared on the mouse, rat, rabbit, dog, ox, and man. Spleen appears to be consistently the richest source of the enzyme, with liver and many of the endocrine tissues exhibiting marked activities. With the more sensitive methods now available, it is usually possible to demonstrate β-glucuronidase activity in all fresh tissues including blood, urine, white blood cells, tears, and skeletal muscle. This extremely wide distribution and enhanced concentration in endocrine tissues are two facts which deserve attention with regard to considerations of its physiological role.

2. Substrates

Two substrates, baicalin (5,6,7-trihydroxyflavone glucuronide) and scutellarin (4′,5,6,7-tetrahydroxyflavone glucuronide) were hydrolyzed by baicalinase, which surprisingly did not hydrolyze menthylglucuronic acid. On the other hand, emulsin, which has been found by several investigators[1–5] to hydrolyze a variety of conjugated glucuronides, did not split[5] baicalin or scutellarin. These findings point to the aglucuron radical as an important factor in determining enzyme–substrate specificities.

β-Glucuronidases prepared from animal tissues have been found to hydrolyze the glucuronides of orcinol,[12] phloroglucinol,[12] menthol,[12,13,28] phenol,[12,32] borneol,[12,28] β-naphthol,[12] phenolphthalein,[26,33] pregnanediol,[34] 1-*o*-hydroxyphenylazo-2-naphthol,[35] 8-hydroxyquinoline,[35] and estriol.[25] The following substances were not attacked by β-glucuronidase: α-menthyl-glucuronic acid,[12] β-menthyl glucoside,[12] β-phenyl glucoside,[12,36] α- and β-methyl glucosides,[12] α-menthyl glucoside,[12] salicin,[36] and cellobiose.[36] The conclusion seems justified that the presence of the terminal carboxyl group on position 6 appears to be the important factor in determining a specificity which is markedly different from that of β-glucosidase.

Three substrates, L-menthyl glucuronide, phenyl glucuronide, and phenolphthalein glucuronide have come into wide use for measuring animal β-glucuronidase activity. In these, it should be noted that the nature of the aglucuron and its linkage differ. Thus, in menthyl glucuronide, glucuronic

[25] W. H. Fishman, *J. Biol. Chem.* **136**, 229 (1940).
[26] P. Talalay, W. H. Fishman, and C. Huggins, *J. Biol. Chem.* **166**, 757 (1946).
[27] G. T. Mills, *Biochem. J.* **40**, 283 (1946).
[28] W. H. Fishman, *J. Biol. Chem.* **127**, 367 (1939).
[29] W. H. Fishman, B. Springer, and R. Brunetti, *J. Biol. Chem.* **173**, 449 (1948).
[30] L. D. Odell, W. H. Fishman, and W. R. Hepner, *Science* **106**, 355 (1948).
[31] A. J. Anlyan and W. H. Fishman, *Bull. Am. Coll. Surgeons* **32**, 262 (1947).
[32] L. M. H. Kerr, A. F. Graham, and G. A. Levvy, *Biochem. J.* **42**, 191 (1948).
[33] A. A. Di Somma, *J. Biol. Chem.* **133**, 277 (1940).
[34] N. B. Talbot, J. A. Ryan, and J. E. Wolfe, *J. Biol. Chem.* **151**, 607 (1943).
[35] J. S. Friedenwald and B. Becker, *J. Cellular Comp. Physiol.* **31**, 303 (1948).
[36] A. F. Graham, *Biochem. J.* **40**, 603 (1946).

acid is connected by a β-glucosidic linkage to a saturated cyclic terpene alcohol, in phenyl glucuronide to an unsaturated benzenoid phenol, and in phenolphthalein to a diphenol of larger molecular size than the other two. How important these differences may be in influencing the enzyme specificity and reaction rates remains to be determined.

3. Preparation of β-Glucuronidase

Baicalinase[5] was extracted from finely cut roots of *Scutellaria baicalensis* with five times its weight of 70% glycerol for 3 days. The filtrate was made weakly acid and the enzyme was adsorbed by kaolin and eluted from it with ammonia. Data on the degree of purification achieved are lacking.

The preparation of β-glucuronidase from animal tissues was first described by Masamune[12] and was improved by Oshima.[14] Briefly, autolysis of ox spleen or kidney liberated the enzyme which was precipitated with ethanol. Extraction of the enzyme from this precipitate was made with water. After a second alcohol treatment, the enzyme was concentrated *in vacuo* and filtered. It was adsorbed by kaolin at pH 3.6 and eluted with phosphate and dialyzed. While active extracts of the enzyme were obtained which permitted the study of some of its properties, the method of preparation was accompanied by large losses in activity and had other disadvantages. Difficulty in repeating these preparations was encountered.[28]

Fishman[28] introduced two steps, an isoelectric precipitation at pH 5.0 and an ammonium sulfate fractionation procedure, which have been applied with success to the purification of the enzyme. By eliminating the autolysis procedure and other steps which were not essential, the process of preparing β-glucuronidase has been made much more convenient. The process gave a 140-fold purification of the enzyme. The use of acetone as an enzyme precipitant and of long periods of evaporation were steps which have been deleted in a simplified process described later.[37] Both Graham[36] and Mills[38] found that the use of acetone was helpful in the initial treatment of the tissue pulp but was definitely deleterious in later stages. Graham[36] defined the conditions of pH and of ammonium sulfate concentration required for fractionation and concentration of β-glucuronidase, while Mills[38] drew attention to the temperature as an important limiting factor governing the precipitation of the enzyme. Graham's procedure resulted in a product (purified 315-fold) which contained at least two components with closely similar electrophoretic mobilities.

By selecting conditions of pH, temperature, and ammonium sulfate concentration, Mills was able to separate two protein fractions termed β-glucuronidase I and β-glucuronidase II which had different pH optima. Thus, β-glucuronidase I exhibited a pH optimum of 4.5 with the substrates L-menthyl glucuronide, phenyl glucuronide, and phenolphthalein glucu-

[37] W. H. Fishman and P. Talalay, *Science* **105**, 131 (1947).
[38] G. T. Mills, *Biochem. J.* **43**, 125 (1948).

ronide; β-glucuronidase II had a pH optimum of 5.0 with L-menthly glucuronide, of 5.2 with the glucuronides of phenol and phenolphthalein. The conditions employed by Fishman and Graham eliminated one of these protein components so that their purified preparations probably contained only one β-glucuronidase.

4. Methods of Assay

a. Reductimetric

The aldehyde group of glucuronic acid is liberated during the hydrolysis of the glucosidic linkage of the substrate, increasing the reducing power of the digest. On the basis of this reaction Masamune[12] had measured the hydrolysis[39] of the glucuronides of borneol, menthol, phenol, β-naphthol, and phloroglucinol. In the case of the last three compounds, it was necessary to remove the liberated aglucuron from the digest by extraction with organic solvents because of its reducing properties. The validity of the reductimetric methods was established by Miwa,[5] who isolated from a baicalinase hydrolyzate chemically pure baicalein and glucuronic acid in amounts which left no doubt that these were the products of hydrolysis. Masamune[12] estimated simultaneously the menthol and glucuronic acid liberated by the enzymatic hydrolysis of menthylglucuronic acid and found that they were equivalent. This excluded the possibility of a nonhydrolytic decomposition of the substrate. Levvy[40,41] prepared the dibenziminazole derivative of saccharic acid after oxidizing glucuronic acid which had been separated from an enzymatic hydrolyzate of menthylglucuronic acid. The ceric sulfate titration method for glucose of Miller and Van Slyke[42] as adapted by Fishman[28,49] to the measurement of glucuronic acid has been generally employed for the purpose of measuring the increase in reducing power of β-glucuronidase digests. Units were usually expressed on the basis of milligrams glucuronic acid liberated per gram tissue or volume of fluid per hour.

Levvy[43] proposed the use of the Conway microburet in the cerimetric determination of 10–300 γ glucuronic acid in protein-free filtrates prepared with copper tungstate and barium carbonate reagents. Criticism was directed at the use of trichloroacetic acid as a protein precipitant and of phenolphthalein as an indicator in neutralizing the protein-free filtrate.

Mills[38] found that Levvy's alkaline copper tungstate deproteinization technique was unsatisfactory at a pH less than 4.6. He recommended the retention of Fishman's trichloroacetic acid deproteinization with the addi-

[39] H. Masamune, *J. Biochem. Japan* **18**, 259, 277 (1933).
[40] G. A. Levvy, *Nature* **160**, 54 (1947).
[41] G. A. Levvy, *Biochem. J.* **42**, 2 (1948).
[42] B. F. Miller and D. D. Van Slyke, *J. Biol. Chem.* **114**, 583 (1936).
[43] G. A. Levvy, *Biochem. J.* **40**, 396 (1946).

tion of excess sodium carbonate, which eliminated the use of an acid-base indicator. The details of Mills' method[38] are given below:

Acetate buffer (0.2 *M*, 1.0 ml.), 0.5 ml. 0.02 *M* L-menthyl glucuronide solution (pH 5.0), and 0.5 ml. of a suitable dilution of the enzyme solution are incubated in 15 ml. centrifuge tubes for 2 hours at 38°. The reaction is then stopped and protein removed by the addition of 1 ml. 20% trichloroacetic acid followed by centrifugation; 2 ml. of the supernatant fluid is transferred to 4 × 5/8 in. Pyrex tubes and 1 ml. 10% Na_2CO_3 solution followed by 1 ml. alkaline ferricyanide solution added. The tubes are closed with glass bulbs, heated in a boiling water bath for 15 minutes, and then cooled in running water. Sulfuric acid (18 *N*, 0.25 ml.) is added, followed by a drop of 0.05% Lissamine green solution, and the mixture titrated with 0.01 *N* ceric sulfate solution using a 2 ml. microburet. Stirring is performed by a slow stream of nitrogen and a daylight lamp is used during the titration.

The limitations of the reductimetric method are these: (*a*) the increment in reducing power must be large compared to the control because of the presence of significant amounts of reducing substances in tissue extracts. This necessitates the undesirable use of very long incubation times in the case of tissues with low enzymatic activities. (*b*) The possibility exists that the tissue extracts contain other enzymes which split their specific naturally occurring substrates, liberating reducing groups. False high values may be obtained therefore if heat-inactivated controls are used exclusively. (*c*) If the controls are composed of enzyme and buffer without added substrate, preformed glucuronides in the tissue extract may provide substrate which can be split. However, substances giving the naphthoresorcinol reaction[41] were absent in tissue extracts incubated in the absence of substrate.

b. Naphthoresorcinol Reaction

The Tollens naphthoresorcinol reaction has been applied by Florkin[44,45] to the study of the enzymatic synthesis of borneol glucuronide. In his technique, free glucuronic acid was removed quantitatively from the digest by a "desugaration" with cupric sulfate and calcium hydroxide, and the conjugated glucuronic acid in the filtrate determined by means of the Tollens reaction.

Levvy[41] has utilized the naphthoresorcinol method for measuring glucuronic acid remaining in digests from which the conjugated glucuronic acid has been removed by continuous liquid-liquid extraction with diethyl ether.

In general, the naphthoresorcinol methods are not as convenient nor as free of technical limitations as are the reductimetric or aglucuronometric procedures. Nevertheless, they avoid some of the difficulties attached to

[44] M. Florkin and R. Crismer, *Bull. acad. roy. méd. Belg.* **5**, 50 (1940).
[45] M. Florkin, R. Crismer, G. Duchateau, and R. Houet, *Enzymologia* **10**, 220 (1942).

the preparation of proper controls since the color reaction is rather specific for glucuronic acid. Their use should be continued.

c. Aglucuronometric Methods

The measurement of the nonglucuronic acid moiety (aglucuron) liberated in the course of enzymatic hydrolysis of conjugated glucuronides offered another approach to the assay of β-glucuronidase activity. Basing their method on the observations of Di Somma,[25] conditions were described by Talalay, Fishman, and Huggins[26] for the assay of β-glucuronidase using phenolphthalein β-glucuronide as the substrate. It was found that the glucuronide has only 0.18% of the light absorption of free phenolphthalein at 552 mμ which made it possible to determine the phenolphthalein liberated by enzymatic hydrolysis with negligible interference from the presence of substrate. This method is very sensitive since quantitative measurements can easily be made of a few micrograms of phenolphthalein. This made it possible to reduce the time of incubation very considerably, a step which is desirable for reasons of theoretical nature and of convenience. Since phenolphthalein is not found in animal or plant tissues, one avoids having to make two measurements, a procedure in which errors may be magnified. In applying the method to preparations which were either turbid or which absorbed light near 552 mμ, a deproteinization step was found necessary. The following procedure is now widely used:

Into two Wassermann tubes were pipetted 0.1 cc. β-glucuronidase-containing solution, 0.8 ml. 0.1 *M* acetate buffer (pH 4.5), and 0.1 ml. 0.01 *M* phenolphthalein glucuronide. A third Wassermann tube containing the enzyme and buffer but not the substrate served as the control. The digests were then incubated at 38° and the time recorded. The length of the incubation period varied between 1 to 5 hours for tissues depending on the dilution of the enzyme; for blood plasma, serum, and cells between 15 and 24 hours. When the enzyme aliquot was neither turbid or colored, the reaction was stopped and phenolphthalein color developed by adding the following reagents in the order given: glycine buffer (pH 10.4) 2 ml., sodium hydroxide solution (0.5 *N*) 0.5 ml., trichloroacetic acid (5%) 1 ml., distilled water 1.5 ml. No protein precipitate appears. When the enzyme solution was turbid or pigmented, the reagents are added in the following order: trichloroacetic acid (1.0 ml.) (the protein precipitate is centrifuged off and washed at least three times in small amounts of distilled water to remove adsorbed phenolphthalein; the supernatant and washings are combined), glycine buffer (pH 10.4) 2 ml., and sodium hydroxide solution (0.5 *N*, 0.5 ml.). The phenolphthalein was measured photocolorimetrically and the results expressed as micrograms phenolphthalein liberated per gram tissue per 100 ml. fluid per hour.

Kerr, Graham, and Levvy[46] successfully applied the method of King and Armstrong[47] for phosphatase determination to the assay of β-glucuronidase, using biosynthetic phenyl glucuronide as substrate. The value of the con-

[46] L. M. H. Kerr, A. F. Graham, and G. A. Levvy, *Biochem. J.* **42**, 191 (1948).
[47] E. J. King and A. R. Armstrong, *J. Can. Med. Assoc.* **31**, 376 (1934).

trol incubated without substrate was kept to a low figure by first partially purifying the enzyme.

Preparations from mouse tissues were partially purified according to the method of Fishman and Talalay[47] and 0.4 ml. of the extracts were added to 0.2 ml. 0.1 *M* citrate buffer (pH 5.2) and 0.2 ml. 0.06 *M* phenyl glucuronide. After incubation for 1 hour at 37°, 2 ml. of the diluted Folin-Ciocalteu reagent were measured into the tube. Protein was removed by centrifuging for 3 minutes, and 2 ml. supernatant transferred to a 10 ml. stoppered centrifuge tube containing 4 ml. sodium carbonate solution. The contents of the tube were mixed and the color developed by incubating for 20 minutes at 37°. Every assay was controlled by incubating mixtures of enzyme and buffer containing 0.2 ml. water in place of substrate. A small rise in the blank occurs during incubation.

The assay of tissue β-glucuronidase by the use of any of the above methods requires the consideration of a number of important factors. One must choose the extent of treatment of the tissue homogenate—whether to assay the crude homogenate, its supernatant, or a purified solution obtained after ammonium sulfate fractionation. The dilution of the homogenate is also important. The optimum pH of hydrolysis should be determined once for each tissue prior to its study in order to deal with the presence perhaps of two or possibly more glucuronidases, as pointed out by Mills[38] and Kerr.[48] While the full significance of these considerations is not yet completely known, it would seem most important that in all biological experiments, such as those discussed in this chapter, the conditions be made identical as far as possible and be limited to the study of the effect of one variable at a time.

In the assay of blood β-glucuronidase, reductimetric methods are not suitable, as pointed out by Talalay *et al.*[26] The naphthoresorcinol reaction has apparently not been applied to the determination of blood β-glucuronidase. Aglucuronometric methods seem best suited and reports have appeared in which blood β-glucuronidase activity was measured with phenolphthalein glucuronide as the substrate. Precautions are necessary to prevent errors due to excessive buffering capacity of plasma, turbidity in the final digests, and adsorption of phenolphthalein in the protein precipitate.

5. Factors Influencing Rate of Enzymatic Hydrolysis of Glucuronides

a. pH

In all instances examined so far, the enzymes hydrolyzing β-glucuronides have their optimum pH in the acid range. Emulsin has its optimum for menthyl glucuronide at pH 4.0 and that of spleen β-glucuronidase is 5.0, a point of difference in the two enzymes. Attention is again drawn to the

[48] L. M. H. Kerr and G. A. Levvy, *Nature* **162**, 219 (1948).

observations of Mills (Table II) who found two proteins in spleen with β-glucuronidase activity with consistently different pH optima for three substrates. As a rule, the pH activity curves fall off very sharply on either side of the optimum.

TABLE II

β-GLUCURONIDASES AND THEIR pH OPTIMA

Enzyme	Source	Substrate	pH Optimum	Reference
Baicalinase	*Scutellaria baicalensis*	Baicalin	6.0	Miwa[9]
		Scutellarin	5.7	Miwa[9]
Emulsin	Almonds	Menthyl glucuronide	4.0	Masamune[12]
β-Glucuronidase	Ox kidney	Menthyl glucuronide	5.3	Masamune[12]
	Ox kidney	Phenyl glucuronide	5.3–5.6	Masamune[12]
	Ox spleen	Menthyl glucuronide	5.0–5.2	Oshima[14]
	Ox spleen	Menthyl glucuronide	5.0	Fishman[49]
	Ox spleen	Bornyl glucuronide	4.4	Fishman[49]
	Ox spleen	Estriol glucuronide	4.3	Fishman[49]
	Ox spleen	Phenyl glucuronide	4.5–5.2	Kerr *et al.*[44]
	Ox liver	Phenyl glucuronide	4.5–5.2	Kerr *et al.*[44]
	Pooled tissues	Phenolphthalein glucuronide	4.5	Talalay *et al.*[26]
β-Glucuronidase I	Ox spleen	Menthyl glucuronide	4.5	Mills[23]
	Ox spleen	Phenyl glucuronide	4.5	Mills[23]
	Ox spleen	Phenolphthalein glucuronide	4.5	Mills[23]
β-Glucuronidase II	Ox spleen	Menthyl glucuronide	5.0	Mills[23]
	Ox spleen	Phenyl glucuronide	5.2	Mills[23]
	Ox spleen	Phenolphthalein glucuronide	5.2	Mills[23]

b. Enzyme Concentration

The enzymatic hydrolysis of β-glucuronides has been shown to be a linear function of enzyme concentration for the hydrolysis of bornyl glucuronide,[45] baicalin,[9] phenolphthalein glucuronide,[26] and phenyl glucuronide.[46]

c. Substrate Concentration

The relation of the initial velocity of hydrolysis to substrate concentration was shown by Fishman[49] to proceed linearly to a maximum velocity in the case of menthyl, bornyl, and estriol glucuronides. Similar findings

[49] W. H. Fishman, *J. Biol. Chem.* **131**, 225 (1939).

were made for phenolphthalein glucuronide by Talalay *et al.*[26] and for phenyl glucuronide by Kerr *et al.*[46] Michaelis constants have been reported and are listed in Table III.

Both estriol and phenolphthalein glucuronides have a relatively marked affinity ($1/K_m$) for the enzyme as compared to the values for the other substrates listed. It may be relevant that these two conjugated glucuronides have functional groupings in another portion of the aglucuron moiety. Perhaps these serve as points of attachment to the active surface of the enzyme protein.

Inhibition by excess substrate was noticeable in the case of estriol glucuronide[49] and marked for phenyl glucuronide,[46] the curves for the latter being somewhat anomalous.

TABLE III

MICHAELIS CONSTANTS OBSERVED IN THE ENZYMATIC HYDROLYSIS OF β-GLUCURONIDES

Glucuronide of	K_m	$1/K_m$	Author
Estriol	0.0005	2000	Fishman[49]
Borneol	0.01	100	Fishman[49]
Menthol	0.004	250	Fishman[49]
Phenol	0.0035	286	Kerr[46]
Phenolphthalein	0.00005	20,000	Talalay[26]

d. Effect of Temperature

For baicalinase, Miwa[10] found various temperature coefficients (Q_{10}) depending on the temperature range. Thus, Q_{10} was 1.42 for the range 30–40°, 1.17 for 40–50°, and 1.01 for 50–60°. Masamune[12] reported that β-glucuronidase was inactivated at 70°. Oshima[14] reported a Q_{10} value of 2.66 for the temperature range 20 to 50° (spleen β-glucuronidase acting on menthyl glucuronide).

e. Observations on Crude Tissue Extracts

Masamune[12] and Oshima[13] reported that the yield of enzyme was increased considerably when minced spleen was autolyzed at 38° for a period of days. In both papers, no record of controls can be found. Upon repeating these experiments using the difference in reducing power between heat inactivated controls and the experimental digests, Fishman[50] found no change in the amount of enzyme present after 1 hour or 5 days of autolysis. Obviously, in autolyzates large amounts of reducing material are present which are not derived from the hydrolysis of the substrate. These inter-

[50] W. H. Fishman, Thesis, Univ. Toronto (1939).

fering substances are eliminated by purification with ammonium sulfate fractionation.

According to Graham,[36] an enhancement of crude spleen β-glucuronidase activity did take place with autolysis, but dialysis was required to demonstrate this effect. No evidence in the dialyzate of an inhibitor was observed. He also found an increase in dialyzable reducing material in the digests without substrate.

An increase in reducing power takes place when blood plasma has been incubated in the absence of added substrate.[26] The nature of the reactions which manifest themselves in this increase in reducing power in the absence of substrate is unknown. The possibility of mucopolysaccharide hydrolysis suggests itself.

On the basis of the action of β-glucuronidase upon estriol glucuronide, Fishman[49] proposed conditions for the application of β-glucuronidase in lieu of strong mineral acid to the hydrolysis of urinary steroid glucuronides. Success in the use of rat liver preparations for this purpose was reported in the case of pregnanediol glucuronide[34] and of a mixture of the glucuronides of pregnanediol, pregnanetriol and various ketosteroids.[54] Bacterial glucuronidase[64,65] preparations were successfully applied to the hydrolysis of the glucuronides of estrogens, ketosteroids, and corticosteroids in previously untreated urine.

III. Physiological Role of β-Glucuronidase

1. Effect of Extrinsic Factors

a. Administration of Drugs and Toxic Chemicals

Fishman[25] observed an increase in the β-glucuronidase activity of liver, kidney, and spleen but not of ovary, uterus, and pancreas in dogs fed borneol. A similar finding was made in mice receiving menthol. On the basis of the assumption that the synthesis of glucuronides was being catalyzed by β-glucuronidase, it was suggested that the increase in enzyme activity might have been due to an adaptation of β-glucuronidase to its substrate in a manner analogous to the increase in enzyme found in the substrate adaptation of bacterial enzymes. Oshima[13] earlier considered that the enzyme was accelerating the synthesis of conjugated glucuronic acids in the animal body and suggested that the reason the tissues of the dog contained more β-glucuronidase than those of the ox was due to the greater ability of herbivorous animals to detoxicate alcohols as compared to carnivorous ones.

Florkin[45] and associates were able to demonstrate the *in vitro* synthesis of bornylglucuronic acid in a system containing purified β-glucuronidase,[28] buffer, borneol, and glucuronic acid in four instances tested. The evidence indicated this to be an enzyme catalyzed condensation. Although the

amount of glucuronide synthesized was small and the time of incubation long, this evidence of synthesis cannot be dismissed in considerations of the physiological role of β-glucuronidase.

Levvy[17,18] and his group have confirmed Fishman's findings for mice fed menthol in the liver and kidney but not in the spleen. Administration of chloroform, carbon tetrachloride, mercuric nitrate, yellow phosphorus, phenylarsenoxide, uranyl acetate, menthyl glucuronide, and sulfathiazole caused changes in β-glucuronidase in the liver or kidney in an analogous fashion to menthol. Levvy observed the occurrence in these tissues of cellular proliferation and repair processes which took place in the organs damaged by menthol and the other toxic agents studied. He has suggested, therefore, that the increased β-glucuronidase activity observed was not related to its glucuronidogenic property but was related secondarily to the processes of cell division. De Meio and Arnolt[51] observed that, if the phenol and borneol are fed to rats, phenol conjugation increases as measured *in vitro* in surviving slices of liver and kidney. Monoiodoacetic acid inhibited phenol conjugation, which was re-established by the addition of glucuronic acid but not of lactate. After cyanide inhibition or under anaerobic conditions, glucuronic acid does not re-establish conjugation. They have suggested the possibility that glucuronic acid combines directly with phenol to form the conjugate, which, however, is dependent upon a coupled reaction requiring the integrity of the cell and the presence of oxygen. The picture has become somewhat confused by De Meio's later observations[52] of genetic strain differences in the degree and type of phenol conjugation. It would be very desirable that this be reinvestigated using a substance (other than phenol) whose conjugation was limited only to glucuronic acid.

Mills[27] found that in the rabbit the normal output of glucuronic acid and the per cent conjugation of L-menthol with glucuronic acid were both increased for the first two weeks after splenectomy. Normal values were found by the fourth week. On the basis of the work of Talbot *et al.*,[34] who were successful in preparing a β-glucuronidase from rat liver but not from rat spleen which would hydrolyze pregnanediol glucuronide, Mills was led to believe that the function of the liver and spleen enzymes were different. Accordingly, he explained the increased urinary menthyl glucuronide following splenectomy as due to the absence of an organ (spleen) with marked glucuronide hydrolyzing power. The failure of Talbot *et al.* to obtain active spleen β-glucuronidase cannot be accepted as evidence of a functional difference specific to the spleen enzyme in view of the many observations of others (Table I) showing similar hydrolytic activities for spleen and liver. In Mill's experiments, it is possible that there may have

[51] R. H. De Meio and R. I. Arnolt, *J. Biol. Chem.* **156**, 577 (1944).
[52] R. H. De Meio, *Arch. Biochem.* **7**, 323 (1945).

taken place a physiological compensatory process following splenectomy (perhaps a change in renal reabsorptive capacities for menthyl glucuronide) which was reflected by the urinary increase in conjugated glucuronides.

2. Relationships with Estrogen Metabolism

a. In Animals

Fishman and Fishman[15] in 1944 reported that uterine β-glucuronidase was reduced in mice following castration. The injection of estrogenic hormones into ovariectomized mice resulted in an increase in the concentration of β-glucuronidase in the uterus but not in the liver. No change in enzyme concentration in both organs followed the injection of progesterone, pregnanediol, sodium pregnanediol glucuronide, borneol, and menthol. Later, Fishman[16] found that the β-glucuronidase response could be elicited by amounts of estrogens well within the physiological range. Stilbestrol was also as effective as the natural estrogens. Testosterone propionate did not counteract the stimulation of uterine glucuronidase by estrogens. From the relationship of enzyme activity to uterine nitrogen in these experiments it was deduced that the enzyme response to hormones was a physiologically significant one and not a result merely of the formation of more cell protoplasm *per se*. It was hypothesized that the enzyme synthesized the glucuronide of the hormone as the initial step in its utilization by the tissue. The possibility was pointed out, in another study,[24] that under special circumstances hydrolysis of glucuronides *in vivo* may take place in tissues with probably important biological consequences.

Kerr and Levvy[48] confirmed Fishman's findings with regard to the fall in uterine β-glucuronidase following ovariectomy and its response to the subsequent injection of estrone. In addition it was demonstrated that the uterine enzyme had an optimum pH of 4.5 (substrate, phenyl glucuronide). Levvy *et al.*[18] found greater β-glucuronidase activity in spleen, liver, and kidney in young mice (1 to 15 days) than in normal adults. Following hepatectomy, the regenerating liver was found to contain large amounts of β-glucuronidase. According to Levvy these changes and those seen in tissue following administration of extrinsic agents are associated with processes of cell poliferation.

Odell *et al.*[30] found infants' blood plasma to contain more β-glucuronidase than maternal blood. Kerr *et al.*[53] very recently discovered an elevated enzyme activity in mouse liver after administration of estrone. This was observed in castrated males and females, normal males, but not in normal females. In the livers of the rats given estrone, active mitosis was seen but no evidence for tissue damage was found. One week after injection of chloroform into ovariectomized mice, there occurred increases in uterine weight

[53] L. M. H. Kerr, J. G. Campbell, and G. A. Levvy, *Biochem. J.* **44**, 487 (1949).
[54] H. L. Mason and E. J. Kepler, *J. Biol. Chem.* **161**, 235 (1945).

and β-glucuronidase activity which were found to be secondary to liver regeneration. These results were explained on the basis of a hitherto unsuspected production of an extraovarian growth hormone for the uterus.

From the data of Fishman and of Levvy, the conclusion seems warranted that β-glucuronidase is indeed closely related to estrogen metabolism in the mouse. It is too early to draw final conclusions with respect to the exact role of the enzyme in this relationship. Perhaps, as Fishman suggests, it participates in the conjugation of the hormone as it exerts its physiological action or it may be that, according to Levvy, its alterations are a manifestation of one or more process of cell division which results from estrogen action, independent, however, of any synthetic activity of β-glucuronidase. At present, both of these working hypotheses possess great usefulness in the planning of experiments. They should be either retained, modified, or abandoned in the future as new information becomes available.

b. In Humans

With a view to exploring a possible relationship of β-glucuronidase to estrogen metabolism in the human, Fishman[22] studied the blood β-glucuronidase during pregnancy. The plasma level was correlated with the events of pregnancy exhibiting high values before parturition. McDonald and Odell,[20] using the methods of Talalay *et al.*[26] made similar observations and found that cord blood was lower in β-glucuronidase activity than was maternal blood.

Odell and McDonald[21] extended their observations to pregnant women with diseases of pregnancy and frequently encountered extremely high values in women who later developed eclampsia. Fishman[55] found that the elevated serum β-glucuronidase could be made to persist by the postpartum administration of stilbestrol.[55]

Values for human breast tissue (lactating) and human placenta were moderately high (Table I). Changes in β-glucuronidase of human endometrium were related with the events of the menstrual cycle, suggesting hormonal control.[56] The uterus and vagina of women after the menopause exhibited very low β-glucuronidase activities.[24]

3. Mucin Metabolism

Circumstantial evidence for the most part points to a role of β-glucuronidase in mucin metabolism. The occurrence of β-glucuronidase in the mucous secretions[29] and its presence (as demonstrated histochemically[35]) in the secreting glands is suggestive. In addition, Meyer *et al.*[57] observed that

[55] W. H. Fishman, L. D. Odell, J. E. Gill, and R. A. Christensen, *Am. J. Obstet. Gynecol.* **59**, 414 (1950).

[56] L. D. Odell and W. H. Fishman, *Am. J. Obstet. Gynecol.* **59**, 200 (1950).

[57] K. Meyer, E. Chaffee, G. L. Hobby, and M. H. Dawson, *J. Exptl. Med.* **73**, 309 (1941).

spleen β-glucuronidase did (in one case) hydrolyze hyaluronic acid. A relationship between estrogen action, edema, and tissue mucin has been suggested[55] and this field would seem to hold much promise for future investigation.

4. General Considerations

In the first experiments[15, 25] on the physiological role of the enzyme, the data were most readily explained on the basis that β-glucuronidase (*a*) catalyzed the conjugation reaction and (*b*) had "adaptive" characteristics similar to those observed in the case of some bacterial enzymes. Direct evidence in support of these views has been contributed by the *in vitro* observations of Florkin and De Meio. Circumstantial and indirect evidence has come from other sources. Since then other observations have been made which have led to the opinion by some that the enzyme functions solely as a hydrolytic enzyme *in vivo* without the necessity for ascribing an adaptive property to β-glucuronidase.

Thus, Levvy concluded that the sole activity of β-glucuronidase in the organism can only be that of hydrolysis mainly because of (*a*) the occurrence of an increase in β-glucuronidase activity following the administration of various toxic substances which, in his opinion, cannot form glucuronides,[18] (*b*) the ability of extrinsic menthyl glucuronide to produce a change in tissue β-glucuronidase,[18] (*c*) the inability of saccharic acid (an inhibitor of the hydrolysis of phenyl glucuronide) to prevent glucuronide synthesis by mouse liver slices,[58] (*d*) the demonstration by Lipschitz and Bueding[59] that glucuronic acid synthesis *in vitro* was promoted to a much greater extent by lactic acid and other three-carbon sugar derivatives than by glucuronic acid, and (*e*) the observation that *in vitro* glucuronide synthesis was not related necessarily to the extent of the tissue's ability to hydrolyze glucuronides.[58]

It is probably too early to reach a decision with regard to the ability or inability of β-glucuronidase to synthesize conjugated glucuronides in tissue on the basis of *in vitro* experiments alone. Indeed, as pointed out previously, there is positive evidence (both direct and indirect) supporting both contentions. It may develop that both may be correct for certain phenomena and that wide generalizations as to the function of β-glucuronidase may not be warranted for some time yet.

While some of Levvy's criticisms may be met by proposing alternative explanations (*e.g.*, the extrinsic toxic agents may be first converted into another product which may be conjugated), other of his objections along with information supplied by Lipschitz and De Meio serve to make it unlikely that the synthesis of glucuronides is a simple reaction, catalyzed

[58] M. C. Karunairatnam and G. A. Levvy, *Biochem. J.* **44**, 599 (1949).
[59] W. L. Lipschitz and E. Bueding, *J. Biol. Chem.* **129**, 333 (1939).

in reverse by β-glucuronidase. Rather it appears that glucuronide synthesis is a more complicated process which in Lipschitz's studies is difficult to distinguish from processes of glucuronic acid formation. The importance of the aglucuron in determining synthesis was observed. However, the trioside hypothesis of Lipschitz and Bueding seems inadequate in view of the fact that the triosides have never been detected in biological systems.

Analogous situations have arisen elsewhere in which argument is encountered as to whether or not the same enzyme which hydrolyzes the substrate will also effect its synthesis *in vivo*. This question is of fundamental importance. The necessity of an energy source is stressed greatly by others and is widely considered to require the presence of substances with high-energy phosphate linkages. However, in the case of phosphotransferase[60] and in the enzymatic synthesis of dextrans[61–63] the catalysis of synthesis is performed by the enzyme in the absence of energy-rich phosphorus compounds.

In view of these observations, consideration should be given to other possible sources of energy for synthesis. The data of Bergmann and Fruton indicate the possibility of the formation of an insoluble product which will favor a reaction in the direction of synthesis. Of course, rapid diffusion of the product from the site of its synthesis will also favor the reaction. These last two possibilities can be visualized as occurring in the normal living organism and may be extremely important.

Although the *in vitro* evidence for the synthetic role of the enzyme is not conclusive in one direction or the other, the observations *in vivo* are more readily explained on the basis of glucuronide synthesis than on the basis of glucuronide hydrolysis, although it is conceivable that both reactions take place in the body. The great substrate specificity shown in the hydrolytic reactions of β-glucuronidase would make it seem important to include the enzyme in considerations of *in vivo* reactions. A similar view has been expressed by Fruton[66] with regard to peptidase action.

IV. Implications in Human Cancer

Recently, Fishman *et al.*[19,22–24] have reported the presence of greater amounts of β-glucuronidase in human cancer tissue than in the tissues of origin. Studies have been carried out on carcinomas of the breast, stomach, colon, pancreas, uterus, ovary, penis, bladder, lung, and on lymphadenop-

[60] B. Axelrod, *J. Biol. Chem.* **172**, 1 (1948).
[61] E. J. Hehre, *J. Biol. Chem.* **177**, 267 (1949).
[62] S. Hestrin, S. Avineri-Shapiro, and M. Aschner, *Biochem. J.* **37**, 450 (1943).
[63] S. Hestrin and S. Avineri-Shapiro, *Biochem. J.* **38**, 2 (1944).
[64] H. J. Buehler, P. A. Katzman, and E. A. Doisy, *Federation Proc.* **8**, 189 (1949).
[65] H. J. Buehler, P. A. Katzman, P. P. Doisy and E. A. Doisy, *Proc. Soc. Biol. Med.* **72**, 297 (1949).
[66] J. S. Fruton *in* Green, Currents in Biochemical Research, 1946, Interscience, New York, 1946 p. 123.

athies. Of importance is the observation that lymph nodes to which cancer has metastasized contained in many cases high β-glucuronidase activities. Ascitic and pleural fluids from patients with carcinomatosis involving the pleura and peritoneum often contained increased amounts of β-glucuronidase as compared to body fluids from patients without cancer.

While, as a working hypothesis, it has been suggested that the increased β-glucuronidase in tumors might reflect an enhanced activity of estrogen or other steroid substances, considerably more data are required in order to evaluate the exact significance of these results.

V. Summary

β-Glucuronidase is a constituent of many animal tissues and of some plants. Many of its enzymatic properties have been studied, especially the effects of pH, substrate, enzyme, and temperature. It has been purified considerably and the existence has been detected of more than one protein with β-glucuronidase activity.

β-Glucuronidase seems to play an important role in the metabolism and action of the estrogenic hormones and the evidence indicates its close association with processes of cell division and multiplication. The exact mechanism of its *in vivo* behavior is still unsettled.

CHAPTER 19

Amylases and the Hydrolysis of Starch and Glycogen

BY KARL MYRBÄCK AND GUNNAR NEUMÜLLER

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I. The Substrates

1. Starch

Starch[1,2] is the chief reserve carbohydrate of the higher plants just as glycogen is in the animal kingdom and in certain microorganisms. During germination of the starch-rich plant seeds the starch is broken down, presumably by the accompanying amylases, to sugars, which constitute the chief source of energy in the early development of the plant. The belief seems well founded that plant amylases play a fundamentally important role in plant metabolism.

Starch is quantitatively the most important constituent of human food; it is saccharified by amylases in saliva and pancreatic juice. The sugars pass into the blood stream whence they are taken up by the tissues, partly used in respiration, and partly stored in the liver as glycogen.

Starch is of immense industrial importance. In most cases, as in the fermentation industries, degradation by amylases is an integral part of the procedure.

From a structural point of view starch and glycogen belong to the group of homopolysaccharides, *i.e.*, they are built up exclusively of residues of one simple sugar. This fact was first stated by de Saussure, who by acid hydrolysis converted starch almost completely to glucose.[3] Enzymatic treatment converts about 80% of starch to fermentable sugars, chiefly maltose, the balance being made up of oligosaccharides of a molecular weight higher than that of maltose, the so-called dextrins.[4]

Starch occurs in the plant as microscopic, anisotropic granules of varying size and shape. For information concerning the properties and the identification of starch granules and the methods used, the reader is referred to the literature cited.[5,6] Intact granules are insoluble in cold water. Treated with hot water the granules swell and at a sufficiently high temperature, the gelatinization temperature, they burst and form viscous

[1] The literature to 1927 is collected *in* R. P. Walton, A Comprehensive Survey of Starch Chemistry. Chemical Catalog Co., New York, 1928.

[2] A. Purr *in* Bamann-Myrbäck, Die Methoden der Fermentforschung, Thieme, Leipzig, 1941; Academic Press, New York, 1945, p. 173. M. Samec, *Ergeb. Enzymforsch.* **9**, 89 (1943). R. W. Kerr, Chemistry and Industry of Starch, 2nd ed., Academic Press, New York, 1950. T. J. Schoch, *Advances in Carbohydrate Chem.* **1**, 247 (1945). K. Myrbäck, *Advances in Carbohydrate Chem.* **3**, 252 (1948). See also *Ann. Rev. Biochem.*

[3] T. de Saussure, *Bull. pharm.* **6**, 499 (1814).

[4] T. de Saussure, *Trans. Roy. Soc. London* **109**, 29 (1819).

[5] N. P. Badenhuizen, *Protoplasma* **28**, 293 (1937); *ibid.* **29**, 246 (1938).

[6] A. Frey-Wyssling, Submicroscopic Morphology of Protoplasm and its Derivatives. Elsevier, Amsterdam, New York, 1948.

solutions or gels. It has, however, been claimed that even above the gelatinization temperature the granules still persist although enormously swollen, and that paste viscosity is due to frictional effects between the swollen granules.[7] As evidence it has been pointed out that the viscosity of a potato starch paste is strongly diminished by mechanical treatment of the paste, as for instance in a colloid mill.

Starch granules and the commercial starches always contain small amounts of noncarbohydrate substances.[8] Such substances, other than water, are higher fatty acids, other lipides, and inorganic compounds, of which phosphoric acid has attracted most interest.[9]

Fatty acids occur in cereal starches in a range of about 0.6 to 0.8%.[10] It is possible by use of certain fat solvents with hydrophilic character to remove the fatty acids, which seem to be only adsorbed on the starch molecules.[11-13] For this extraction lower aliphatic alcohols may be used, preferrably 85% methanol, or aqueous dioxane or the Cellosolves.

The phosphoric acid content of starch does not exceed about 0.2% as phosphorus. The phosphorus constituents can be removed from most cereal starches by extraction. In tuber starches, however, especially in potato starch, the phosphorus occurs in esterified form, as judged by its isolation after acid hydrolysis of the starch as glucose-6-phosphate.[14]

As already mentioned, starch is built up of glucose units. These are joined in the majority of cases through glucosidic 1,4 linkages, *i.e.*, the same linkage which occurs in maltose, the "maltose linkage." These facts emerge from results obtained by use of the exhaustive methylation technique.[15-17] From the fact that starch is strongly dextrorotatory and from the kinetics of hydrolysis and acetolysis it is concluded that the linkages have the alpha configuration as in maltose.[18,19]

It has long been recognized that starch is heterogeneous. The term starch covers a group of closely related but chemically and physically different substances. It is possible to fractionate most starches into two

[7] C. L. Alsberg, *Plant Physiol.* **13**, 295 (1938).

[8] A table is given in R. W. Kerr, Chemistry and Industry of Starch, 2nd ed., Academic Press, New York, 1950, p. 229.

[9] M. Samec and M. Blinc, *Kolloid Beihefte* **47**, 371 (1938); *ibid.* **40**, 449 (1934).

[10] T. C. Taylor and J. M. Nelson, *J. Am. Chem. Soc.* **42**, 1726 (1920). T. C. Taylor and L. Lehrman, *ibid.* **48**, 1739 (1926). T. C. Taylor and J. H. Werntz, *ibid.* **49**, 1584 (1927). T. C. Taylor and R. P. Walton, *ibid.* **51**, 3431 (1929). T. C. Taylor and R. T. Sherman, *ibid.* **55**, 258 (1933).

[11] L. Lehrman and E. A. Kabat, *J. Am. Chem. Soc.* **55**, 580 (1933).

[12] T. J. Schoch, *J. Am. Chem. Soc.* **60**, 2824 (1938); *ibid.* **64**, 2954 (1942).

[13] L. Lehrman, *J. Am. Chem. Soc.* **64**, 2144 (1942).

[14] T. Posternak, *Helv. Chim. Acta* **18**, 1351 (1935).

[15] J. C. Irvine and J. Macdonald, *J. Chem. Soc.* **1926**, 1502.

[16] W. N. Haworth, E. L. Hirst, and J. I. Webb, *J. Chem. Soc.* **1928**, 2681.

[17] E. L. Hirst and G. T. Young, *J. Chem. Soc.* **1939**, 951.

[18] K. Freudenberg, G. Blomqvist, L. Ewald, and K. Soff, *Ber.* **69**, 1258 (1936).

[19] K. H. Meyer, H. Hopff, and H. Mark, *Ber.* **62**, 1103 (1929).

main fractions, one of which is composed of straight-chain molecules, analogous to cellulose in configuration. The other fraction is composed of molecules with a complicated structure, a highly branched or ramified configuration. The branching involves a linkage of another kind than the 1,4 bond mentioned. Evidence has been brought forward that this linkage is an α-glucosidic 1,6 linkage.[20-22] The occurrence of linkages of a further type has been proposed, but at present there is not much evidence for this.[23,24]

The heterogeneity of starch is still questioned occasionally by some investigators who state that the fractions obtained are degradation products of the native starch, formed during dissolution or in its manufacture.[25,26] Many experimental facts, however, do not support this statement, and it has been contradicted.[27] In this connection, however, it is necessary to point out that the commercial manufacture of starch includes some chemical treatment, which in some cases may cause chemical degradation, a fact to keep in mind in investigations designed to provide fundamental information concerning the starch molecule.[28]

In the earlier literature several names for the fractions of starch have been used, sometimes in a rather confusing way. This is due to the fact that earlier methods of fractionation were ineffective and that no satisfactory methods were available for characterization of the fractions and estimation of their purity. The names most commonly accepted at present are those proposed by Maquenne[29] and later reproposed and defined by Meyer.[30] According to Meyer amylose is defined as a polysaccharide made up of straight-chain molecules, a polymerized maltose, while amylopectin is considered to consist of branched-chain molecules. It does not seem unlikely, however, that molecules exist which are intermediate in structure between these two extremes.[31,32]

An essential condition for a quantitative separation of the starch components is a physically homogeneous solution. It seems possible to break

[20] K. Freudenberg and H. Boppel, *Ber.* **73**, 609 (1940).
[21] K. Myrbäck, B. Örtenblad, and K. Ahlborg, *Biochem. Z.* **307**, 53 (1940). K. Myrbäck and K. Ahlborg, *ibid.* **307**, 69 (1940). K. Ahlborg and K. Myrbäck, *ibid.* **308**, 187 (1941).
[22] C. C. Barker, E. L. Hirst, and G. T. Young, *Nature* **147**, 296 (1941).
[23] Y. Nakamura, *J. Faculty Agr. Hokkaido Imp. Univ.* **49**, 95 (1942); *Chem. Abstracts* **42**, 6145 (1948).
[24] T. G. Halsall, E. L. Hirst, J. K. N. Jones, and A. Roudier, *Nature* **160**, 899 (1947).
[25] E. Pacsu and L. A. Hiller, *Textile Research J.* **16**, 243 (1946). E. Pacsu, *J. Polymer Sci.* **2**, 565 (1947).
[26] R. Sutra, *Bull. soc. chim. biol.* **29**, 221 (1947).
[27] T. G. Halsall, E. L. Hirst, and J. K. N. Jones, *Nature* **159**, 97 (1947).
[28] R. W. Kerr, Chemistry and Industry of Starch, 2nd ed., Academic Press, New York, 1950, Sect. II.
[29] L. Maquenne and E. Roux, *Compt. rend.* **140**, 1303 (1905).
[30] K. H. Meyer, *Naturwissenschaften* **28**, 397, 564, 722 (1940).
[31] R. W. Kerr, *Paper Trade J.* **115**, 30 (1942).
[32] R. W. Kerr, *Cereal Chem.* **7**, 377 (1945).

up the granule structure completely without chemical degradation, provided some precautions are taken. First, it is necessary to remove the fatty acids completely, since they interfere with the fractionation procedure. Second, it is necessary to maintain the pH within narrow limits during autoclaving. According to Schoch a defatted corn starch in water gives a pH of about 6, and this value is constant during autoclaving. Consequently no degradation occurs.[33]

For the separation of the components in solution several methods have been used. Most of them, however, are ineffective. The best method available and one which represents an important advance in the preparation of pure starch fractions is the one described by Schoch.[33,34] As first described, an autoclaved suspension of defatted starch while still hot is treated with butanol to the saturation point. On cooling slowly an amylose–butanol complex crystallizes and is collected by centrifuging. The product is purified by repeated recrystallizations from butanol–water mixtures. The amylopectin fraction is isolated by treating the mother liquid with water-soluble alcohols.

The method of selective precipitation of the starch components has achieved widespread use and has been modified in some details.[35,36] It is based on the fact that amylose adsorbs polar substances forming complexes which separate or crystallize, while amylopectin either forms no complex or the complex is soluble. Several organic substances with a hydrophilic group attached to a hydrophobic residue have the ability of forming complexes with amylose. The list of substances used for selective precipitation include aliphatic alcohols,[37] fatty acids,[38] nitroparaffins,[39] and thymol.[40]

Another method of fractionation is the leaching-out method.[41,42] The starch is suspended in water at a temperature near the gelatinization point. The extraction is continued at constant temperature for 1 hour with slow stirring. After centrifuging, the amylose is in a clear solution. The extractions are repeated until no more soluble substance is removed. A water solution of amylose is unstable in a colloidal sense and on standing most of the amylose crystallizes or retrogrades. Such a retrograded amylose is insoluble in water and not attacked by amylases (see page 673). The mechanism of retrogradation in its essential parts is still un-

[33] T. J. Schoch, *J. Am. Chem. Soc.* **64**, 2957 (1942).
[34] T. J. Schoch, *Cereal Chem.* **18**, 121 (1941).
[35] E. J. Wilson, Jr., T. J. Schoch, and C. S. Hudson, *J. Am. Chem. Soc.* **65**, 1380 (1943).
[36] R. H. Hopkins and B. Jelinek, *Biochem. J.* **43**, 28 (1948).
[37] T. J. Schoch, *Advances in Carbohydrate Chem.* **1**, 259 (1945).
[38] T. J. Schoch and C. B. Williams, *J. Am. Chem. Soc.* **66**, 1232 (1944).
[39] R. L. Whistler and G. E. Hilbert, *J. Am. Chem. Soc.* **67**, 1161 (1945).
[40] W. N. Haworth, S. Peat, and P. E. Sagrott, *Nature* **157**, 19 (1946).
[41] M. E. Baldwin, *J. Am. Chem. Soc.* **52**, 2907 (1930).
[42] K. H. Meyer, W. Brentano, and P. Bernfeld, *Helv. Chim. Acta* **23**, 845 (1940).

known. Probably the retrogradation is due to hydrogen bond formation. The simple molecules form aggregates and become insoluble.[43]

The leaching-out method does not seem to allow a complete separation of the starch components. The yield of amylose is low and it is always contaminated with amylopectin. The method is tedious since it involves the use of large volumes and furthermore the isolation of amylose without retrogradation is difficult. A combination of the leaching-out method with the precipitation method has led to the isolation of a crystallized amylose, which has served as a reference substance.[44]

Further purification of the fractions may be achieved by adsorption on cotton or filter paper. The amylose fraction is preferentially adsorbed and is subsequently removed by elution with hot water. The method is of less value for preparation of amylose but may be useful to free an amylopectin solution from small amounts of contaminating amylose.[45, 46] However, recent investigations tend to give a different explanation of the action of cotton.[46a]

In order to decide whether degradation has occurred during fractionation the alkali lability method is applied. Since the starch and glycogen molecules are built up of linear or branched chains of glucose units, there must be one end chain of each molecule having a free pseudoaldehydic group. The reducing power of this group, owing to the high molecular weight, is very slight and thus not detectable by use of the common sugar reagents; starch is said to be "nonreducing." This is in fact not true. By use of certain sugar reagents it is possible to detect a very slight reducing power.[47,48] On treatment of starch with hot alkali, acids are formed from the reducing end of the molecules, such as formic acid, acetic acid, etc. The first stage of this reaction is probably an enolization of the terminal aldehydic group.[49] Since under the conditions used the interior 1,4-glucosidic linkage is relatively stable against alkali, the formation of acidic material proceeds progressively from the reducing end.[50] A degraded starch will, thus, under the same conditions liberate more acidic material than an undegraded starch due to the higher amount of free aldehyde groups exposed to the attack of alkali. An estimation of the rate of acid forma-

[43] R. W. Kerr, Chemistry and Industry of Starch, 2nd ed., Academic Press, New York, 1950, p. 468.

[44] R. W. Kerr and G. M. Severson, *J. Am. Chem. Soc.* **65**, 193 (1943).

[45] C. Tanret, *Compt. rend.* **158**, 1353 (1914).

[46] E. Pacsu and J. W. Mullen, *J. Am. Chem. Soc.* **63**, 1168 (1941).

[46a] K. H. Meyer and G. C. Gibbons, *Helv. Chim. Acta* **33**, 210 (1950.)

[47] F. F. Farley and R. M. Hixon, *Ind. Eng. Chem., Anal. Ed.* **13**, 616 (1941).

[48] W. A. Richardson, R. S. Higginbotham, and F. D. Farrow, *J. Textile Inst.* **27**, 131 (1936).

[49] W. L. Evans and M. P. Benoy, *J. Am. Chem. Soc.* **52**, 294 (1930). W. L. Evans, *Chem. Revs.* **31**, 537 (1942).

[50] T. J. Schoch, E. J. Wilson, Jr. and C. S. Hudson, *J. Am. Chem. Soc.* **64**, 2871 (1942).

tion by titration of the acids under specified conditions will give the alkali number. This method gives characteristic figures for the components of starch and has proved valuable for detecting even small hydrolytic changes in the starch molecule.[51]

The separation of the starch components by the selective precipitation method seems to work almost quantitatively and the method has thus been used for the analysis of the amounts of the components in starches of different origin.

The values found by the last-mentioned method are supported by the results obtained by a quantitative estimation of the iodine activity in starch–iodine solutions. As has long been recognized the fractions of starch are characteristically colored by iodine. Amylose solutions are colored an intense blue, while amylopectin solutions exhibit reddish-purple colors. Amylose has a much greater affinity for iodine as measured by the

TABLE I

AMYLOSE CONTENT OF VARIOUS STARCHES

Starch	Amylose, %
Cereals[53]	25–29
Roots and tubers[53]	17–22
Waxy varieties of cereal starches[52,54]	0–2
Lily bulbs[52]	30–35
Wrinkled garden peas[52,55,56]	60–70 98

potentiometric method of Bates *et al.*[52] The iodine activity of a solution is estimated by measuring the E.M.F. of the cell $Pt|I_2,KI||$calomel. On addition of succesive portions of iodine to an amylose solution the activity of iodine rapidly rises to a characteristic value and then remains fairly constant until a certain amount of iodine has been added. The amount of iodine adsorbed is about 20% of the weight of amylose. Further addition of iodine will rapidly increase the iodine activity. Since amylopectin has no or at least only a slight influence on the iodine activity the method may be used for the estimation of the amount of amylose present in various starches. The results obtained agree well with those obtained by the precipitation method. The activity of iodine or the potential is a function of the molecular magnitude of the amylose; consequently it is possible to determine the relative molecular magnitude of different amylose preparations. For example, iodine added to corn amylose gives, *ceteris paribus*, a higher potential than iodine added to potato amylose, due to the lower

[51] T. J. Schoch and C. C. Jensen, *Ind. Eng. Chem., Anal. Ed.* **12**, 531 (1940).
[52] F. L. Bates, D. French, and R. E. Rundle, *J. Am. Chem. Soc.* **65**, 142 (1943).

molecular weight of the former. In Table I approximate figures of the amylose content of various starches are collected.

The absorption spectra of the complexes between iodine and the starch components provide another means of analyzing for the two components in whole starch.[56,57,58] The distinction between the iodine colorations of amylose and amylopectin consists essentially in the intensity of the blue. While amylopectin shows no great variation in absorption from 500–800 mμ, amylose exhibits a marked maximum absorption between 600–620 mμ.

The chemical evidence for the structure of starch is for the most part based on the results of the methylation method. Methylation of unfractionated starch or amylopectin, followed by hydrolysis and an estimation of the methylglucoses formed gives the following figures: 85–90% of 2,3,6-trimethylglucose, 4–5% of 2,3,4,6-tetramethylglucose, and varying amounts of dimethylglucose.[59–63] The trimethylglucose fraction is derived from the intermediate glucose units, except those involved in branching. Consequently at least 85–90% of the linkages in starch are of the 1,4-glucosidic type. The tetramethylglucose originates from the terminal nonreducing glucose units. The amount of tetramethylglucose obtained corresponds to one end group for 20–28 glucose units. The physical properties of starch and the behavior of starch when treated with amylases, however, indicate a very high molecular weight, as an average some 1000 glucose units at least. To connect this with the end group content, it is necessary to assume a branched structure for starch or, more correct in the light of modern concepts, for the amylopectin fraction of starch. In fact several such structures have been suggested.

Staudinger proposed a formula consisting of a relatively short principal chain with branches linked to the principal chain with glucosidic 1,6 and 1,3 linkages. Each branch is thought to consist of 24–28 glucose units[64,65]

[57] L. Simerl and B. Browning, *Ind. Eng. Chem., Anal. Ed.* **11**, 125 (1939). R. M. McCready and W. Z. Hassid, *J. Am. Chem. Soc.* **65**, 1154 (1943). R. W. Kerr and O. R. Trubell, *Paper Trade J.* **117**, No. 15, 25 (1943). R. R. Baldwin, R. S. Bear, and R. E. Rundle, *J. Am. Chem. Soc.* **66**, 111 (1944).

[58] L. H. Lampitt, C. H. F. Fuller, and N. Goldenberg, *J. Soc. Chem. Ind. London* **66**, 142 (1947); *ibid.* **67**, 97 (1948).

[59] W. N. Haworth and E. G. V. Percival, *J. Chem. Soc.* **1932**, 2277.

[60] W. N. Haworth, E. L. Hirst, and M. D. Woolgar, *J. Chem. Soc.* **1935**, 177.

[61] E. L. Hirst and G. T. Young, *J. Chem. Soc.* **1939**, 951, 1471.

[62] S. Peat and J. Whetstone, *J. Chem. Soc.* **1940**, 276.

[63] W. Z. Hassid and R. M. McCready, *J. Am. Chem. Soc.* **63**, 1632 (1941).

[64] G. E. Hilbert and M. M. McMasters, *J. Biol. Chem.* **162**, 229 (1946).

[64] E. J. Bourne and S. Peat, *J. Chem. Soc.* **1949**, 5.

[65] S. Peat, E. J. Bourne, and M. J. Nicholls, *Nature* **161**, 206 (1948).

[65] J. P. Nielsen and P. C. Gleason, *Ind. Eng. Chem., Anal. Ed.* **17**, 131 (1945).

[64] H. Staudinger and E. Husemann, *Ann.* **527**, 195 (1937).

[65] H. Staudinger and E. Husemann, *Ber.* **71**, 1057 (1938).

(see Fig. 1A.) At about the same time, from considerations on limit dextrin formation, Myrbäck postulated a similar branched structure.[66] Haworth, discussing the origin of the dimethylglucose fraction, suggested that the starch molecule is composed of parallel chains. Each chain containing 24–28 glucose units is linked to a neighboring chain by a glucosidic 1,6 linkage[67] (Fig. 1B.) Meyer, however, has proposed a more complicated structure, a quite irregular molecule with multiple branching. This structure includes no principal chain.[68,69] This formula was founded on experiments with different enzymes. (See below, page 697, and Fig. 1C.)

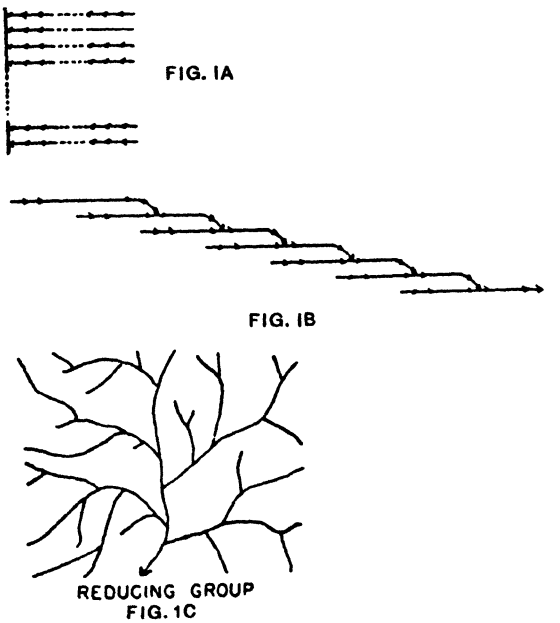

FIG. 1. (A) Staudinger formula; (B) Haworth formula; (C) Meyer formula (Myrbäck[2])

Support for an irregular structure with multiple branching is found in the results of the calculations concerning the possible mechanism of synthesis of starch carried out by Myrbäck and Sillén.[70] The end chains are supposed to contain 11 glucose units as an average, whereas the interior chains between the branching points should contain about 5 units each.

The dimethylglucose fraction from methylated starch is composed of

[66] K. Myrbäck, *Current Sci. India* **6**, 47 (1937). K. Myrbäck and K. Ahlborg, *Svensk Kem. Tid.* **49**, 216 (1937).
[67] W. N. Haworth, E. L. Hirst, and E. Oliver, *J. Chem. Soc.* **1934**, 1917. W. N. Haworth, E. L. Hirst, and F. A. Isherwood, *ibid.* **1937**, 577.
[68] K. H. Meyer, *Naturwissenschaften* **28**, 397 (1940).
[69] K. H. Meyer and P. Bernfeld, *Helv. Chim. Acta* **23**, 875 (1940).
[70] K. Myrbäck and L. G. Sillén, *Svensk Kem. Tid.* **55**, 311, 354 (1943).

2,3-dimethyl- and 2,6-dimethylglucose. The former probably originates from the points of branching and it is noteworthy that 2,3-dimethylglucose is found in an amount approximately equivalent to that of tetramethylglucose, *i.e.*, every point of branching corresponds to an end group. The 2,6-dimethylglucose seems to be a secondary product formed during hydrolysis from trimethylglucose.[70,71]

The methylation technique applied to amylose, however, gives only about one tenth of the amount of tetramethylglucose isolated from methylated amylopectin. This corresponds to one end group for about 250–300 glucose units. Since the molecular magnitude of amylose is of the same order it is concluded that the amylose molecule is an unbranched straight chain.[72–74]

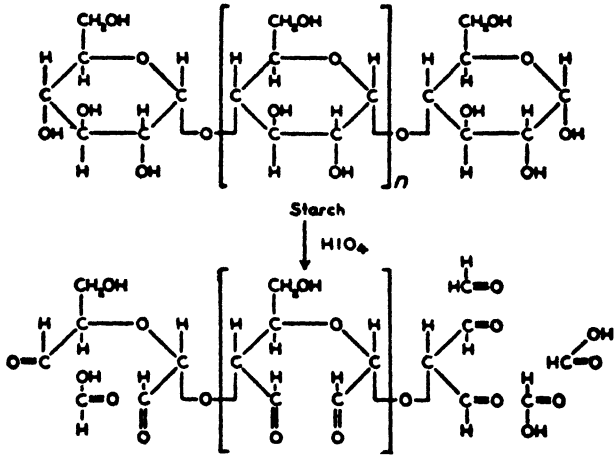

FIG. 2. Periodic acid oxidation of starch (Kerr[8])

The above mentioned end group estimations are in good agreement with those obtained by the periodate oxidation end group assay. Periodate, having a highly specific action on glycol configurations, oxidizes every intermediate glucose unit in starch or glycogen by splitting the carbon–carbon bond between carbon atoms 2 and 3 forming a dialdehyde.[75] A reducing terminal glucose unit will give rise to two moles of formic acid and one mole of formaldehyde, while the nonreducing terminal glucose unit will give one mole of formic acid (Fig. 2).

[71] K. Freudenberg, *Naturwissenschaften* **28**, 264 (1940).
[72] K. Hess and B. Krajnc, *Ber.* **73**, 976 (1940).
[73] K. H. Meyer, M. Wertheim, and P. Bernfeld, *Helv. Chim. Acta* **23**, 865 (1940); *ibid.* **24**, 378 (1941).
[74] W. Z. Hassid and R. M. McCready, *J. Am. Chem. Soc.* **65**, 1157 (1943).
[75] E. L. Jackson and C. S. Hudson, *J. Am. Chem. Soc.* **59**, 2049 (1937); *ibid.* **60**, 989 (1938).

Quantitative formaldehyde estimations after periodate oxidations have been used to find the end group content and thus the molecular magnitudes of starches and dextrins, and these values have been compared with the molecular weight values calculated from the reducing power.[76]

Recently the estimation of formic acid has been used for an end group assay and a comparison also made with the results obtained by the methylation method.[77-79] For a certain sample of starch both methods gave almost identical results. Consequently, few if any glucose units linked solely by carbon atoms 1 and 6 are present in starch.

2. Glycogen

Glycogen[80,81] includes a group of homologous polysaccharides occurring in cell plasma, particularly in liver and muscle cells and serving as a reserve carbohydrate in animal metabolism. Glycogen is found distributed throughout the protoplasm and part of it is said to be bound to proteins. By treatment with hot alkali in an oxygen-free atmosphere the glycogen is set free and extracted. It is isolated from the extract by ethanol precipitation.

Glycogen is water-soluble and gives opalescent solutions which are colored red to reddish-brown by iodine. Glycogen is completely converted to glucose by acid hydrolysis, yields heptacetylmaltosyl bromide when treated with acetyl bromide, and gives considerable amounts of maltose when digested with amylases.

End group estimations carried out with the methylation method give a large amount of tetramethylglucose. Two different types of glycogen seem to exist. Most glycogens have one end group for each 12 glucose units.[59,67,82-84] Certain specimens of rabbit liver glycogen, however, are reported to have one terminal group for 18 glucose units.[67,85] Almost identical figures of the end group content are obtained with the periodic acid method[86] (see page 662).

The above-mentioned facts point to a structure for glycogen similar to that of amylopectin or the limit dextrin remaining after β-amylase treatment of amylopectin. The differences are the higher degree of branching

[76] C. G. Caldwell and R. M. Hixon, *J. Biol. Chem.* **123**, 595 (1938).
[77] F. Brown, T. G. Halsall, E. L. Hirst, and J. K. N. Jones, *J. Chem. Soc.* **1948**, 27.
[78] A. L. Potter and W. Z. Hassid, *J. Am. Chem. Soc.* **70**, 3488 (1948).
[79] K. H. Meyer and P. Rathgeb, *Helv. Chim. Acta* **31**, 1545 (1948); *ibid.* **32**, 1102 (1949).
[80] See review by K. H. Meyer, *Advances in Enzymol.* **3**, 109 (1943).
[81] D. J. Bell, *Biol. Revs. Cambridge Phil. Soc.* **23**, 256 (1948).
[82] D. J. Bell, *Biochem. J.* **29**, 2031 (1935); *ibid.* **31**, 1683 (1937).
[83] W. Z. Hassid and I. L. Chaikoff, *J. Biol. Chem.* **123**, 755 (1938).
[84] W. N. Haworth, E. L. Hirst, and F. Smith, *J. Chem. Soc.* **1939**, 1914.
[85] D. J. Bell, *Biochem. J.* **30**, 1612, 2144 (1936).
[86] T. G. Halsall, E. L. Hirst, and J. K. N. Jones, *J. Chem. Soc.* **1939**, 1399.

and the higher molecular weight for glycogen. Glycogen contains no linear fraction (amylose).

Considerations concerning the mechanism of the breakdown of glycogen with β-amylase has led to a picture of the molecule in principle similar to that of the amylopectin (Fig. 1C.). The end chains of glycogen are supposed to contain 5 to 7 glucose units each, united by interior chains with about 3 glucose units between the branching points.[80,87]

II. Enzymatic Degradation of Starch and Glycogen

1. Introduction

The amylases are enzymes which hydrolyze starch and its components (amylose and amylopectin), glycogen, and certain degradation products originating from these polysaccharides. (The term "diastase" is sometimes used for these enzymes; it is, however, misleading since in certain languages diastase means enzyme in general.) The specificity of the various amylases will be dealt with later on, but it should be made clear from the beginning that amylase action is a purely hydrolytic one, the sole action of the amylases being a cleavage of the α-glucosidic 1,4 linkage, the "maltose linkage." (The *Bacillus macerans* amylase has an action differing somewhat from that of the true amylases; the *B. macerans* enzyme is treated in Sect. VII.)

The degradation products formed by the action of the amylases (except the enzyme from *B. macerans*) on the polysaccharides are: (*a*) reducing, fermentable sugars, chiefly maltose; and (*b*) reducing, nonfermentable (or extremely slowly fermentable) dextrins, which are to be regarded as originating from parts of the polysaccharide molecules which for certain reasons are not completely degraded.

It is necessary to distinguish carefully between the hydrolysis of polysaccharides and other types of degradation which may be confused with hydrolysis. For instance, enzymes have been described under names as "phosphate-requiring amylases"[88] which certainly are not amylases at all but which, in the light of modern knowledge, are to be classed as phosphorylases. Phosphorylase preparations containing isomerases or phosphatases may cause the formation of reducing esters of phosphoric acid or even free reducing sugars. The true amylases are not influenced characteristically by phosphates.

2. Synthetic Action of Amylases

So far no conclusive evidence of a synthetic action of the true amylases has been brought forward. (An exception, in certain respects, is the *Bacillus macerans* enzyme.) The true amylases seem to be concerned exclusively with the hydrolysis of the substrates, while the phosphorylases catalyse hydrolysis and synthesis as well.

[87] K. H. Meyer, *Naturwissenschaften* **29**, 287 (1941).
[88] R. Willstätter and M. Rohdewald, *Z. physiol. Chem.* **221**, 13 (1933).

A synthesizing action of several crude amylase preparations has been observed by Pigman.[89] Whereas none of the preparations had any action on glucose solution, some produced varying amounts of nonfermentable saccharides from maltose. There was some activity in preparations from mold (*Aspergillus*), from *Bacillus subtilis*, and from pancreas, but the cereal amylases showed no activity. Pigman points out that the preparations exhibiting the synthesizing action were surely mixtures of various enzymes and the synthesis may possibly be due to enzymes other than amylases. Since the experiments were performed in phosphate buffer, phosphorylase action is perhaps not excluded.

3. Classification of Amylases

The action of amylases on starch paste is accompanied by the following three, easily demonstrable phenomena: (*a*) decrease in viscosity, "liquefaction"; (*b*) change in the color formation with iodine and liberation of reducing groups, "dextrinization"; and (*c*) formation of fermentable sugars "saccharification." The same phenomena are observed, more or less markedly, in amylase action on glycogen. The sole action of the amylases resulting in the three phases mentioned, is the hydrolysis of α-glucosidic 1,4 linkages. As far as we know at the present time, no other reaction can be made responsible for any of the observed phenomena: liquefaction, change in color with iodine, formation of reducing groups and fermentable sugars; all are consequences of the disruption of glucosidic linkages between the unit residues of the chain molecules of the substrates. There is, for instance, no decrease in viscosity of a starch paste which is not accompanied by the appearance of reducing groups. However, it must be kept in mind that a very slight increase in reduction value may correspond to an extremely marked shift in viscosity.

a. Amylophosphatase

It should be mentioned in this connection that the occurrence of chemically bound phosphoric acid in many starches may be connected with the viscosity. This question has been extensively treated by Samec.[90] The existence of an "amylophosphatase" in barley malt, displaying a liquefying action without rupture of glucosidic linkages, was claimed by Waldschmidt-Leitz *et al.*[91,92] Other authors, however, failed to reproduce these results. In any case the liquefaction of starch paste by malt enzymes has nothing to do with the liberation of phosphoric acid. On the contrary the substitution of a glucose residue by a phosphoric acid radical makes the adjoining glucosidic linkages difficultly accessible to the amylases.[93] All

[89] W. W. Pigman, *J. Research Natl. Bur. Standards* **33**, 105 (1944).

[90] M. Samec and M. Blinc, Handbuch der Kolloidwissenschaft in Einzeldarstellungen; Band VIII, Steinkopff, Dresden, Leipzig, 1941. M. Samec, *Ergeb. Enzymforsch.* **9**, 89 (1943).

[91] E. Waldschmidt-Leitz and K. Mayer, *Z. physiol. Chem.* **236**, 168 (1935).

[92] E. Waldschmidt-Leitz, M. Samec, and K. Mayer, *Z. physiol. Chem.* **250**, 192 (1937).

[93] T. Posternak, *Helv. Chim. Acta*, **24**, 921 (1941).

phosphoric acid in potato starch is recovered in bound form in the limit dextrins[94] (see below).

b. Different Types of Amylases

If the velocities of liquefaction, dextrinization, and saccharification are determined for different amylase preparations, the fact is immediately apparent that the ratios between the velocity values are by no means constant. Therefore different types of amylases obviously exist; some are mainly dextrinizing, and in others the saccharifying activity is predominant. Crude preparations may be mixtures of different amylases; extracts of germinated cereals contain, *e.g.*, two amylases. After the enzymatic homogeneity of barley malt extracts had been questioned by several authors in the past (Bourquelot is said to be the first), Ohlson[95,96] demonstrated conclusively that "malt amylase" contains two distinct amylases, termed by him "dextrinogenic" and "saccharogenic amylase."

All amylases (with the possible exception of the *Bacillus macerans* enzyme) are saccharifying in the sense that sooner or later fermentable sugars are formed as a result of the degradation process catalyzed by them. The chief end product of amylase action is always maltose; this fact was demonstrated as early as 1876 by O'Sullivan.[97] The conversion of starch, however, is seldom or never complete; as pointed out early by O'Sullivan, other saccharides, "dextrins," are produced together with maltose. The yield of maltose and the composition of the dextrin fraction vary with the amylase preparation, and the pathway from the high-molecular substrates to fermentable sugar is clearly different.

In some cases the increase in reducing value during enzymatic action corresponds precisely to the formation of maltose, as determined by fermentation experiments. The explanation is that the enzyme attacks the substrate molecules from chain ends, liberating every time a maltose unit. Enzymes of this kind are the true saccharifying amylases in the sense that besides maltose no low-molecular degradation products with determinable reducing value are formed. These enzymes, acting from chain ends of the substrate molecules, may be designated as exoamylases.

In other cases the increase in reducing value is, especially in the first stages of the reaction, much larger than the simultaneous production of fermentable sugars. The decrease in viscosity of a starch paste accompanying these early stages of the amylase action is most conspicuous. The explanation is that the amylases of this type break up the large polysaccharide molecules into smaller parts, the dextrins, and these compounds are of such low molecular weights that their solutions have no appreciable

[94] K. Myrbäck and B. Kihlberg, *Biochem. Z.* **315**, 250 (1943).
[95] E. Ohlsson, *Compt. rend. soc. biol.* **87**, 1183 (1922).
[96] E. Ohlsson, *Compt. rend. trav. lab. Carlsberg Sér. chim.* **16**, No. 7 (1926).
[97] J. O'Sullivan, *J. Chem. Soc.* **29**, 478 (1876); **30**, 125 (1876).

viscosity, but on the other hand are large enough to be nonfermentable. The amylases displaying this type of action are the dextrinizing or liquefying amylases. For their attachment to the substrate molecules they are not dependent on end groups of substrate chains; they are endoamylases.

Brown and Heron[98] found that "malt amylase" (which they regarded as a single enzyme) liberates maltose of beta-configuration, mutarotating upward. Their results were verified by von Euler and Helleberg[99] and by Kuhn,[100] who found, furthermore, that, whereas "malt amylase" yields β-maltose, the degradation products of other amylases mutarotate downward. His conclusion that these amylases yield α-maltose may be generalized as follows: probably all reducing groups set free by these enzymes, whether in maltose or in dextrins, have the α-configuration.[101] Determination of the direction of the mutarotation of the hydrolysis products thus allows a classification of the amylases into α- and β-amylases.

c. α- and β-Amylases

Determination of the mutarotation of the degradation products requires particular techniques. The reaction mixture must be clear enough to allow polarimetric readings. Ohlson used a starch solution cleared by electrodecantation. Other authors have used more or less degraded soluble starches. The enzyme concentration must be so high that the velocity of hydrolysis is great compared to that of the mutarotation at the pH in question. Samples are taken from the reaction mixture, read in the polarimeter, made alkaline with sodium carbonate for instance, and read again Determination of the reducing value gives the degree of hydrolysis, and fermentation experiments furnish values of the actual amounts of maltose and other fermentable sugars.

It should be emphasized that the formation of α- and β-maltose, respectively, does not justify the conclusion that in the polysaccharides α- and β-glucosidic linkages alternate. It must be regarded as fully proved that all linkages in starch and glycogen which are ruptured by the amylases are α-glucosidic in configuration. Then we have to conclude that the rupture of such a linkage by β-amylase is accompanied by a complete Walden inversion, whereas the hydrolysis by α-amylases takes place without inversion. It seems plausible that this is due to the different mode of attachment of the enzymes to the substrate molecules, but the connection is not at all clear.

The upward mutarotation of the products of "malt amylase" action was shown by Ohlson[102] to be due to the saccharifying enzyme in malt. This enzyme therefore is a β-amylase, which is at the present time the most widely used designation. The dextrinogenic enzyme in malt was

[98] H. T. Brown and J. Heron, *J. Chem. Soc.* **35**, 596 (1879); *Ann.* **204**, 228 (1880).
[99] H. von Euler and K. Helleberg, *Z. physiol. Chem.* **139**, 24 (1924).
[100] R. Kuhn. *Ber.* **57**, 1965 (1924); *Ann.* **443**, 1 (1925).
[101] K. Myrbäck, *Biochem. Z.* **307**, 140 (1941).
[102] E. Ohlsson, *Z. physiol. Chem.* **189**, 17 (1930).

shown by Ohlson to be an α-amylase. α-Amylases are found in all kinds of organisms and tissues, β-amylase chiefly if not exclusively in higher plants.

From the foregoing we conclude that the amylases may be divided into two main classes: (*a*) exoamylases, saccharogenic or saccharifying amylases, β-amylases; (*b*) endoamylases, dextrinogenic or dextrinizing amylases, liquefying amylases, or α-amylases.

4. Determination of Amylase Action

a. Determination of Decrease in Substrate Concentration

Since the natural substrates of the amylases are not homogeneous and no sharp limit exists between them and their degradation products, it seems impossible to indicate a method for the quantitative determination of unaltered substrate. However, determinations of certain fractions of a high degree of polymerization are possible, of course. A method of this type has been devised by Caldwell and Hildebrand.[103] Starch (presumably together with certain high-molecular degradation products) is precipitated by addition of ethanol to 55% by volume, the minimum alcohol concentration sufficient to completely precipitate starch solutions. In the case of β-amylase, where the sole products are maltose and high-molecular β-dextrin (see below), the quantitative precipitation of the latter should be possible in this way.

b. Measurement of Changes in Color with Iodine

The classical method is that described by Wohlgemuth.[104] Innumerable modifications of this method are in use. Measurements are made under standard conditions of the time required to change the blue color of the reaction mixture with iodine to a certain shade or intensity, or of the amount of enzyme necessary to produce the change in a given time. In any case the experiments have to be performed so that the activity values are proportional to the amount of enzyme used in the experiment. As an end point of the experiments one may choose the "achroic point" (no color with iodine) or any violet or reddish color definable by aid of a standard.[105] The measurements may of course be considerably refined by using suitable colorimeters or spectrophotometers. The reader is referred for details to other publications.[90,106]

The change in color with iodine from dark blue through violet and red to colorless is characteristic for the change in the starch substrate brought about by the action of the α-amylases and may be used for the quantitative estimation of α-amylase activity. In combination with other methods the iodine methods are of considerable value for the characterization of

[103] M. C. Caldwell and F. C. Hildebrand, *J. Biol. Chem.* **111**, 411 (1935).

[104] J. Wohlgemuth, *Biochem. Z.* **9**, 1 (1908).

[105] C. S. Hanes and M. Cattle, *Proc. Roy. Soc. London* **B125**, 387 (1938). R. M. Sandstedt, E. Kneen, and M. J. Blish, *Cereal Chem.* **16**, 712 (1939). S. Redfern, *ibid.* **24**, 259 (1947). P. Bernfeld and M. Fuld, *Helv. Chim. Acta* **31**, 1420 (1948).

[106] E. Bamann and K. Myrbäck, Die Methoden der Fermentforschung. Thieme, Leipzig, 1941; Academic, New York 1945.

amylase preparations. The enzymatic homogeneity of an α-amylase preparation or the presence of β-amylase as well may, for instance, be inferred from such experiments. Otherwise the value of the iodine methods in scientific work appears limited. Little is known about the relation between color with iodine and the nature of the degradation products. When the achroic stage is reached it can be concluded, however, that no products with chains longer than something like 20 glucose units are present.

c. Measurement of Change in Viscosity

As mentioned above α-amylase action is characterized by an initial rapid drop in viscosity of starch paste, and the decrease in viscosity, properly evaluated, can be used as a measure of enzyme activity. The viscometric methods have one great advantage at least; they give evidence of amylase action even at extremely low degrees of hydrolysis. Myrbäck and Gjörling[107] found for instance in an experiment with malt α-amylase that when the viscosity of a certain starch paste had dropped to half the initial value only about 0.1% of the glucosidic linkages were ruptured.

The viscometric methods, in combination with reductometric determination of the degree of hydrolysis, are of considerable use for the determination of enzymatic homogeneity of α-amylase preparations. It cannot be denied, however, that the viscometric methods have serious drawbacks. The results are strongly dependent on the preparation of the substrate solution, and the reproducibility of the substrate preparations is generally far from satisfactory. Nevertheless many viscometric methods are in use; generally very detailed prescriptions for substrate preparation and performance of the viscosity determinations are given.[106,108]

When the object of an investigation is to explore the mechanism of the amylase action, the colorimetric and viscometric methods alone are of limited value. They must in any case be supplemented with reductometric determinations of the degree of hydrolysis and with determination of the products of the degradation.

d. Determination of Degree of Hydrolysis

The number of free, reducing pseudoaldehydic groups is determined. A great many different methods have been used for this purpose. Theoretically the hypoiodite method of Willstätter and Schudel[109] or any method of the same type would be best suited, since hypoiodite reacts stoichiometrically with the pseudoaldehydic groups with formation of "-onic" acids. On the other hand small amounts of iodine may be consumed in side reactions. The errors introduced herewith have no significance in the

[107] K. Myrbäck and L. G. Gjörling, *Arkiv Kemi Mineral. Geol.* **20A**, No. 5 (1945).
[108] Q. Landis and S. Redfern, *Cereal Chem.* **24**, 157 (1947). E. Hultin, *Acta Chem. Scand.* **1**, 269 (1947).
[109] R. Willstätter and G. Schudel, *Ber.* **51**, 780 (1918). J. Blom and C. O. Rosted, *Acta Chem. Scand.* **1**, 32 (1947).

determination of low-molecular sugars but may be of importance in the determination of saccharides with very low reducing value. However, in the case of starch and glycogen the errors should be small since the polysaccharides themselves do not consume measurable quantities of hypoiodite.

Several copper-containing reagents, reagents of the Hagedorn-Jensen type *etc.*, have been used in amylase work and reproducible values are often obtained. But since these reagents do not react stoichiometrically, the "sugar values" must be derived from standard curves or tables. The only available standard substances are glucose and maltose. Since even these two sugars in many cases do not yield the identical factor for the calculation of the number of reducing groups it seems improbable that an accurate calculation could be made in the case of saccharides with very low reducing values. Certain copper reagents, however, give the same factor for glucose and maltose, and reagents of this type in any case would be preferable.[110]

Colorimetric methods depend on the determination of the reducing groups by means of reagents which yield colored reduction products. The dinitrosalicylic acid method introduced by Sumner[111] has been widely applied. A modification by Meyer *et al.*[112] is said to give reliable values even for saccharides of a very high molecular weight (amylose).

If the reducing value calculated as mg. glucose is x, the degree of hydrolysis, *i.e.*, the relative number of ruptured linkages, is:

$$\alpha = x/(\text{mg. starch})1.111$$

The reducing values are often calculated as maltose. If the yield of anhydrous maltose in a certain experiment is y mg., the degree of hydrolysis is:

$$\alpha = y/2(\text{mg. starch})1.053$$

In these expressions "mg. starch" denotes the amount of starch in the samples used for sugar determination. The expression:

$$100\ y/(\text{mg. starch})1.053$$

is often called the "degree of saccharification in per cent." This expression however should not be used without a statement that the reducing values have been calculated as anhydrous *maltose*. In the case of starch or glycogen degradation with β-amylase y is the real amount of maltose, but in all experiments with α-amylases it represents only the apparent amount of this sugar.

e. Determination of Degradation Products

(*1*). *Fermentable Sugars.* It must be kept in mind that the "maltose values" calculated from reductometric measurements only in exceptional cases (with β-amylase) correspond to the real amount of maltose. In all

[110] M. Somogyi, *J. Biol. Chem.* **117**, 771 (1937).
[111] J. B. Sumner, *J. Biol. Chem.* **62**, 287 (1925).
[112] K. H. Meyer, G. Noelting, and P. Bernfeld, *Helv. Chim. Acta.* **31**, 103 (1947).

other cases the real yield of maltose is smaller than the apparent value. The real amounts of glucose and maltose are conveniently determined by fermentation. Yeasts should be used which distinguish between glucose, maltose, etc. Many "milk sugar yeasts," as *Saccharomyces fragilis*, *Torula lactosa*, and others, ferment glucose but not maltose and the same holds for *Saccharomyces marxianus*. Some types of baker's yeast ferment maltose so slowly compared to glucose that the determination of both sugars in one experiment is possible.[101]

An interesting method for determination of glucose production in biological systems has been devised by Keilin and Hartree;[113] glucose oxidase (notatin) is used and the determination is performed manometrically.

Maltotriose (4-α-maltosidoglucose) is formed by the amylases under certain conditions. This sugar is fermented by ordinary yeasts.[114] The rate may be almost equal to that of maltose; sometimes it is much lower. The presence of appreciable amounts of the sugar may be inferred from fermentation experiments in which not only the amount of sugar fermented is determined by measuring the amount of carbon dioxide evolved but also the decrease in reducing value caused by fermentation. Certain yeasts (*Saccharomyces uvarum*) are reported to ferment only glucose and maltose but no trisaccharides.[115]

After removal of the fermentable sugars by fermentation (with baker's yeast, for instance) the "dextrins" may be precipitated from relatively concentrated solutions with water-miscible solvents such as ethanol or acetone. A certain fractionation can be obtained and repeated fractional precipitation can result in saccharide fractions which are homogeneous, at least with respect to molecular weight.[116, 117] Whether, for instance, two tetrasaccharides containing two 1,4 linkages and one 1,6 linkage with different position in the chain can be separated by fractional precipitation seems doubtful.

For separation of saccharide mixtures and for testing the homogeneity of preparations obtained, paper chromatography and, on a larger scale, the Tiselius adsorption apparatus have been successfully applied.[117]

(*2*). *Nonfermentable or Slowly Fermentable Products.* Since starch and glycogen contain not only the "normal" maltose linkages, which under certain circumstances are split by the amylases, but also "anomalous" 1,6 linkages (isomaltose linkages) to a lesser extent, which are not, or at the utmost are extremely slowly attacked by amylases (α-amylases), there are formed from the natural substrates besides the fermentable sugars reducing degradation products also containing 1,6 linkages. These

[113] D. Keilin and E. F. Hartree, *Biochem. J.* **42**, 230 (1948).
[114] K. Myrbäck and E. Leissner, *Arkiv Kemi Minerai. Geol.* **17A**, No. 18 (1943).
[115] J. Blom and C. O. Rosted, *Acta Chem. Scand.* **1**, 233 (1947).
[116] B. Örtenblad and K. Myrbäck, *Biochem. Z.* **303**, 335 (1940).
[117] A. Tiselius and L. Hahn, *Kolloid-Z.* **105**, 177 (1943).

include the so called limit dextrins. The simplest product of this kind is isomaltose, 6-α-glucosidoglucose. It is formed from starch after prolonged action of certain amylases.[118,119] Isomaltose is fermented extremely slowly if at all by ordinary yeasts. The fermentation rate is so slow that no confusion with maltose is possible.

In addition to isomaltose the formation of nonfermentable saccharides containing one isomaltose linkage together with one or more normal maltose linkages is to be foreseen. A trisaccharide containing one maltose and one isomaltose linkage was isolated and characterized by Myrbäck and Ahlborg[120] (type A, Fig. 3).

No doubt saccharides with one, or possibly in rare cases more than one, isomaltose linkage together with a certain number of maltose linkages constitute the mixture of nonfermentable saccharides, left over by the amylases, which has been designated for many years "residual dextrins"

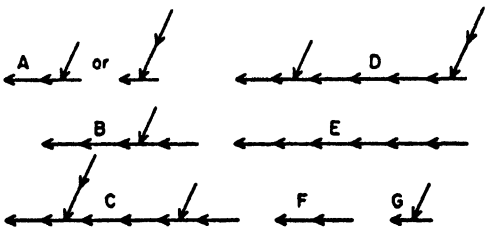

FIG. 3. Different types of reducing dextrins. In this and the following figures of the same kind the glucose residue is represented by an arrow, the arrowhead denoting carbon atom 1. The normal maltose linkage is represented by the combination arrowhead-arrowtail, the anomalous isomaltose linkage arrowhead-arrowshaft. (A) Trisaccharide with one maltose and one isomaltose linkage; (B) – (D) Anomalous α-dextrins; (E) normal α-dextrin; (F) maltose; (G) isomaltose.

or "limit dextrins."[121] It is certainly not improbable that such limit dextrins may partly contain branched chains (type B, Fig. 3), even if no such products have been identified as yet. But saccharides of this type will certainly appear among the products of incomplete degradation with α-amylases (α-dextrins). In this case, furthermore, we have to consider the occurrence of products with more than one isomaltose linkage, which may or may not signify the existence of more than one side chain (types C and D, Fig. 3). In incomplete degradation of starch or glycogen, especially in the case of starch, products will also occur having maltose linkages exclusively (type E, Fig. 3). The author has termed such dextrins "normal

[118] K. Ahlborg and K. Myrbäck, *Biochem. Z.* **308**, 187 (1941).
[119] E. M. Montgomery, F. B. Weakley, and G. E. Hilbert, *J. Am. Chem. Soc.* **69**, 2249 (1947); **71**, 1682 (1949).
[120] K. Myrbäck and K. Ahlborg, *Biochem. Z.* **307**, 69 (1940).
[121] K. Myrbäck, *Svensk Kem. Tid.* **58**, 119 (1946).

α-dextrins" in contradistinction to the "anomalous α-dextrins" (A–D, Fig. 3) containing isomaltose linkages.

If a dextrin has been isolated in homogeneous form (which admittedly is difficult to prove), its constitution may be ascertained in the conventional way by methylation, hydrolysis, and identification of the hydrolysis products. The number of isomaltose linkages and the number of end groups can in any case be obtained. It is difficult, however, to determine the position of the isomaltose linkages and the side chains, respectively. In some cases oxidation with periodic acid will give information concerning the position of the branching points.[122]

5. Substrate Preparations

Raw starch granules are generally very resistant to amylase action (page 720), and solutions or pastes have to be used. The solutions may be prepared in the ordinary way by boiling or autoclaving or by dissolving the polysaccharide in sodium hydroxide and adding acetic acid in excess, calculated to give the desired pH. In many cases different types of "soluble starches" may be used with advantage. It would seem that products prepared by action of acids on starch (Lintner starch and the like) are to be preferred to substances like Zulkowski starch or products obtained by dry heating of starch. The Zulkowski starch contains chemically bound glycerol[123] and dry-heated products may easily contain anhydrides. The Lintner starches, when sufficiently treated with acid, do not "retrograde," and the complications introduced by this phenomenon are avoided.

Otherwise the retrogradation of the substrate may play a role in experiments with amylases, and special difficulties arise in the case of amylose on account of its low solubility and tendency to "retrograde." It is well known that amylose solutions, if not extremely dilute, are unstable; they turn turbid and finally deposit amylose in a more or less crystalline state. The solid amylose is not accessible for the enzymes, and it may well be that even submicroscopic particles are more or less stable against enzyme action. This point has been stressed by Meyer *et al.* and others.[124] The fact that several authors have found incomplete saccharification of amylose by β-amylase may be caused by retrogradation of the substrate (provided it was free from amylopectin). Meyer has devised experimental procedures in which the retrogradation of the substrate is prevented as far as possible; amylose is dissolved in weak sodium hydroxide solution and this solution is added drop by drop to a strong solution of the amylase preparation in a buffer solution of suitable pH and a concentration calculated to give no serious change of the pH on introduction of the alkaline substrate solution. Methods of this kind are useful especially when the purpose is the deter-

[122] K. Ahlborg, *Svensk Kem. Tid.* **54**, 205 (1942).

[123] E. Berner and F. Melhus, *Ber.* **66**, 1333 (1933).

[124] K. H. Meyer, E. Preiswerk and R. Jeanloz, *Helv. Chim. Acta* **24**, 1395 (1941). P. Bernfeld and P. Gürtler, *ibid.* **31**, 106 (1948). G. Noelting and P. Bernfeld, *ibid.* **31**, 286 (1948). R. H. Hopkins, B. Jelinek, and L. E. Harrison, *Biochem. J.* **43**, 32 (1948).

mination of the limit of saccharification. On the other hand this method has the disadvantage that, since the substrate is brought into contact with the enzyme gradually, kinetic measurements and studies of intermediary products are rendered difficult. Generally only the last, and sometimes least interesting, phase of the reaction can be determined with accuracy.

If in a certain experiment the enzyme action ceases because of retrogradation of the substrate, it is clear that undegraded substrate must be present. In experiments with β-amylase it is admittedly difficult to decide if this is the case, but otherwise it should be easy. If in experiments with α-amylase the iodine test is negative (in a boiled and chilled sample) there can have been no retrogradation, causing stability against the amylases.

6. Buffer Solutions

Certain ions, especially the halogen ions, have a powerful activating action on animal amylases (page 691). However, most of the common buffer substances seem to exert no specific actions, at least not at the pH optimum. Since the action of the amylases is appreciable only between pH 3 and 9 most of the pH range in question is covered by the usual acetate and phosphate buffers, which seem to have only a nonspecific effect due to acidity.

Phosphate and certain other buffers are of course unsuitable in experiments on inhibition by heavy metals for instance, since the metal may be precipitated by the buffer ions. Acetate causes no disturbances of this kind. The buffer should not interfere with the sugar determinations. Borate buffers may be regarded as somewhat suspicious in this respect. Besides, borate has a retarding effect on some amylases.

The amylases in general are extremely sensitive to inactivation by heavy metals, especially copper. Even traces of copper arising from defective tinning of a still for instance, may cause very severe poisoning. Poisonous effects of this kind are strongly dependent on the purity of the enzyme preparation.

7. Units of Activity

The fundamental method for determination of amylase activity is the determination of the increase in reducing groups. In the case of β-amylase this is the only practicable method. With α-amylase all three methods mentioned above are complementary to each other. If the object of the investigation is the mechanism of α-amylase action, the determination of the reducing value in any case must be applied, but if the experiment is only a means for determination of the enzymatic activity or "amount of enzyme," any of the three methods can be of value. It is only necessary to find an expression for the velocity of the reaction which is proportional to the enzyme concentration. The increase in reducing value in experiments with starch or other substrates is proportional to the enzyme concentration

for all kinds of amylases, but it must be remembered that this holds strictly only for relatively small degrees of hydrolysis.

Since the mechanism of amylolysis is rather complicated and differs among amylases from different sources it seems best to avoid all expressions of the reaction velocity based on theories regarding the type of reaction. There is, for example, no reason why enzymatic amylolysis should be of the monomolecular type. When the sole object of the investigation is to find an accurate and well reproducible expression for the reaction rate it seems best to limit the determination to the very first stages of the reaction where, under defined conditions, the number of reducing groups set free by the enzyme is directly proportional to the reaction time. In this case the expression:

$$\text{per cent hydrolysis}/t$$

is used as a measure of the velocity. The expression:

$$\text{per cent hydrolysis}/tg$$

is a measure of the activity of the enzyme preparation, if g is the amount of enzyme used in the experiment. The reducing values may of course be calculated as mg. glucose, or mg. maltose, etc.

Even if the calculation of a reaction constant has no theoretical foundation it has been shown that the course of amylolysis in many instances is, to a certain stage of hydrolysis, approximately monomolecular. The monomolecular reaction constant, k, in these cases, may be used as a measure of the reaction rate and the value k/g as a measure of the activity of the enzyme. The *Sf* unit (saccharifying faculty) of von Euler and Svanberg and the "Amylasewert" of Willstätter are units of this kind.[106] They have a certain practical value since a relatively large portion of the hydrolysis curve can be used in the calculation, but theoretically they are by no means to be preferred to expressions of the type "mg. maltose/min." This is illustrated for instance by the fact that in the formula:

$$k = \frac{1}{t} \log \frac{a}{a - x}$$

the value of a to be used is not 100, if x is calculated as per cent hydrolysis, but a lower value thought to correspond to the "saccharification limit" of the amylase preparation. In the Willstätter determination the value 75 is used. In the case of β-amylase one would have to use, *ceteris paribus*, the value 60, or perhaps an even lower value.

A word must be said about the determination of β- and α-amylase when mixed as in malt extracts. This problem has been completely treated by Sandstedt, Kneen, and Blish.[125] The Wohlgemuth values for a certain amount of α-amylase are shown to depend on the presence of β-amylase. α-Amylase, therefore, is determined colorimetrically in one experiment in presence of a large excess of β-amylase. In this case the "decoloration time" is really a measure of the α-amylase activity. Since there is a constant ratio between the α-amylase activities as determined by the colori-

[125] R. M. Sandstedt, E. Kneen, and M. J. Blish, *Cereal Chem.* **16**, 712 (1939). L. E. Ehrnst, G. J. Yakish, and W. Olson, *ibid.* **16**, 724 (1939).

metric method on one hand and by the reduction method on the other, it is possible to calculate a "saccharification equivalent" of α-amylase. When, in a second experiment, the total saccharifying power is determined by the reduction method the true β-amylase activity of the preparation is obtained by subtraction of the "saccharification equivalent" of the α-amylase.

III. Chemical Nature of Amylases[90]

As far as we know at the present time all amylases like other hydrolases are proteins without discernible prosthetic groups. The pure enzyme preparations give the usual protein reactions. Earlier statements to the contrary are to be regarded as erroneous.[126]

The amylases are thermolabile, but the heat stability varies markedly with the type of amylase and with the degree of purity of the enzyme preparation. Indication of so-called optimum temperatures has little meaning. The velocity of the substrate degradation increases with temperature in the usual way. Instability of amylase preparations may be caused by accompanying proteolytic enzymes attacking the amylase proteins.[127]

The activity–pH optima have different positions and the activity–pH curves different shapes with different amylases. This is doubtless a consequence of the fact that the different amylase proteins have different isoelectric points, but the connection is not at all clear. Animal amylases are activated in a characteristic manner by certain negative ions (page 691).

1. Exoamylase, β-Amylase

This enzyme occurs abundantly in seeds and certain other parts of higher plants; the common cereals generally are rich in β-amylase, sometimes enzymatically almost homogeneous but often mixed with small amounts of α-amylase and other enzymes. The not infrequent presence of maltase may cause mistakes concerning the degradation products and the mechanism of the action. In certain materials phosphorylases may interfere with the amylase determination. Since the α-amylase of the cereals is formed or activated during germination, ungerminated samples should be used for the preparation of β-amylase. However, the removal of contaminating α-amylase is easy. Ohlsson[95,96,102] found that α-amylase is much more sensitive to acid than is β-amylase, and on his results Blom[128] and Kneen [129] and associates have based convenient methods for the preparation of barley or wheat β-amylase free from α-amylase. Contaminating α-amylase may be derived from the microflora on the grains.[130]

[126] E. Waldschmidt-Leitz and M. Reichel, *Z. physiol. Chem.* **204**, 197 (1932).
[127] E. H. Fischer and P. Bernfeld, *Helv. Chim. Acta* **31**, 1839 (1948).
[128] J. Blom, A. Bak, and B. Braae, *Z. physiol. Chem.* **241**, 273 (1936).
[129] E. Kneen, R. M. Sandstedt, and C. M. Hollenbeck, *Cereal Chem.* **20**, 399 (1943).
[130] R. H. Hopkins and T. F. S. Cooper, *J. Inst. Brewing* **52**, 188 (1946).

a. Barley β-Amylase

Barley is probably the most widely used raw material. Different barley varieties show distinct differences in amylase content.[131,132] Six-row barley is generally much richer in amylase than is two-row barley. The β-amylase is extracted from ground barley with water or dilute salt solutions with toluene or thymol as disinfectant. A substantial part of the enzyme of the grains is inactive and insoluble in water.[133–135] The "latent" enzyme, which evidently is bound to insoluble protein, can be activated and brought into solution with proteolytic enzymes, especially papain. Treatment of barley meal with papain solution (if necessary activated with hydrogen cyanide) results in highly active solutions, which, however, are rich in nitrogenous

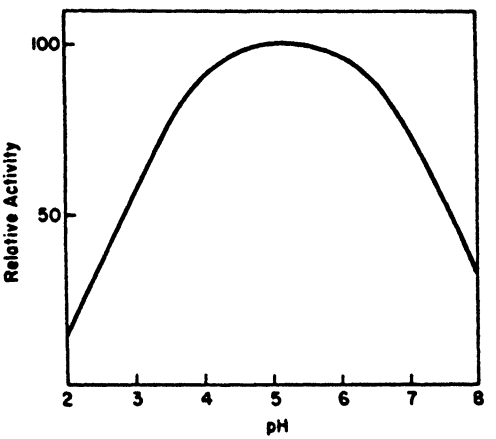

FIG. 4. Activity–pH curve of barley β-amylase

substances. Short extraction of the barley meal with cold water yields solutions of higher activity per mg. nitrogen.

In germinated barley, *i.e.*, malt, all β-amylase is active and water soluble. The total amount of β-amylase, however, does not increase materially during germination, though the activity of a water extract of the barley increases 2 to 3 times because of the proteolytic liberation of the "latent" enzyme. Because of the simultaneous formation or activation of α-amylase, extraction of barley malt yields a mixture of β- and α-amylase. This mixture was regarded earlier as a single enzyme and termed "malt amylase." The two enzymes in malt extracts can be separated by partial inactivation[95,96,102,136] or by adsorption technique.[106,137–139]

[131] K. Myrbäck, *Enzymologia* **1**, 280 (1936–1937).
[132] E. Kneen and H. L. Hads, *Cereal Chem.* **22**, 407 (1945).
[133] J. L. Baker and H. F. E. Hulton, *J. Chem. Soc.* **121**, 1929 (1922).
[134] K. Myrbäck and B. Örtenblad, *Enzymologia* **2**, 305 (1937).
[135] S. R. Snider, *Cereal Chem.* **18**, 186 (1941).

Linderstrøm-Lang and Engel[140] have studied the distribution of amylase in the outer parts of the barley grain. The aleuron cells do not contain much enzyme, but the layer between these and the endosperm cells are rich in β-amylase and contain about 15% of the total amount in the grain.

The pH optimum of barley and malt amylases and most other cereal amylases lies at about pH 5 and is fairly broad (Fig. 4). The influence of variation of the buffer anion on the enzyme of wheat has been studied by Ballou and Luck.[141] No effect is found at the pH optimum but on the acid side a specific influence is observed (Fig. 5).

The inactivation of β-amylase by chemical reagents has been investigated by Weil and Caldwell[142] and others.[143] They found an inactivation by nitrous acid, which, however, was abolished by hydrogen sulfide. These and other experiments suggest that sulfhydryl groups are essential to the enzyme action. Iron salts have little effect on the enzyme.[144]

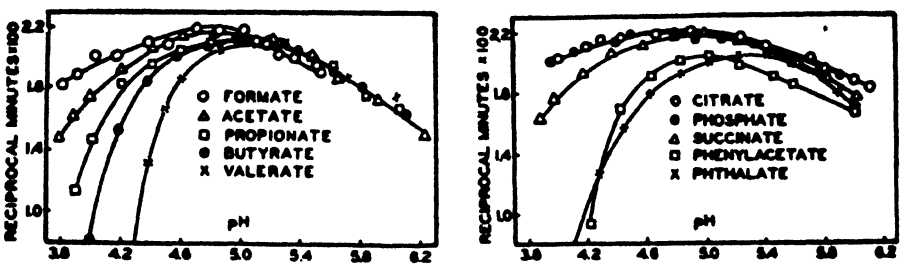

FIG. 5. Activity–pH curves for β-amylase at a constant ionic strength of 0.05 and different buffer salts. Index of activity, reciprocal of time for the reduction of 2 ml. of 0.05*N* iodine.[141]

Osborne[145] in 1895 stated that the amylase activity of barley accompanies the albumin fraction of the proteins. These experiments have been repeated with modern methods by Danielsson and Sandegren,[146] who found that β-amylase and, in the case of malt, both α- and β-amylase, accumulate in the albumin fraction. The activity increased parallel to the purity of the albumin. The effect of the purification is seen in Table II. The purified

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[136] O. Holmbergh, *Svensk Kem. Tid.* **49**, 252 (1937).
[137] E. Waldschmidt-Leitz and A. Purr, *Naturwissenschaften* **20**, 254 (1932).
[138] A. Purr, *Biochem. J.* **28**, 1141 (1934).
[139] O. Holmbergh, *Biochem. Z.* **258**, 134 (1933); **266**, 203 (1933).
[140] K. Linderstrøm-Lang and C. Engel, *Enzymologia* **3**, 138 (1937).
[141] G. A. Ballou and J. M. Luck, *J. Biol. Chem.* **139**, 233 (1941).
[142] C. E. Weill and M. L. Caldwell, *J. Am. Chem. Soc.* **67**, 212, 214 (1945).
[143] H. C. Sherman, M. L. Caldwell, and S. E. Doebbeling, *J. Biol. Chem.* **104**, 501 (1934).
[144] R. S. Potter, *J. Soc. Chem. Ind. London* **59**, 45 (1940).
[145] T. B. Osborne, *J. Am. Chem. Soc.* **17**, 587 (1895).
[146] C.-E. Danielsson and E. Sandegren, *Acta Chem. Scand.* **1**, 917 (1947).

albumin solution can be concentrated by ultrafiltration, cooled to −16° and dried in a vacuum over anhydrous calcium sulfate to a stable amorphous powder. The preparation is readily soluble in water and contains about 11% nitrogen. In the ultracentrifuge the albumin fraction behaves like a single substance with sedimentation constant $s_{20} = 4.6S$. This value was found to be independent of divergences in the scheme of preparation. Electrophoresis in the Tiselius-Svensson apparatus showed that the two enzymes have the same mobility over most of the pH range 4.4 to 8.2. At pH 8 and above small differences appear. The isoelectric point of both enzymes is 5.75, coinciding approximately with the activity optimum. The almost identical behavior of the two enzymes (and presumably the enzymatically inactive barley albumin) is somewhat surprising since they

TABLE II

AMYLASE ACTIVITY OF BARLEY AND MALT ALBUMIN

Source	Dry substance, %	Saccharifying power[147]	Dextrinizing power[125]
Malt from six-row barley	93	680	770
Barley albumin solution			
1	1.30	5,400	0
2	0.82	7,800	0
Malt albumin solution			
1	1.30	12,600	12,000
2	1.27	13,400	15,400
3	1.15	20,500	25,600
4	0.85	32,600	37,500
5	0.72	45,600	51,400
6	0.55	59,800	67,100
7	0.52	63,700	60,400

have very different heat stabilities and can be easily separated by adsorption.

b. Wheat β-Amylase

Wheat is rich in β-amylase[148] and the enzyme content is of the same magnitude as in barley. The effect of wheat variety and environment on the amylase content has been studied by Kneen *et al.* and many others.[139,149] In the early stages of ripening both β- and α-amylase are said to be present, but the α-amylase vanishes during ripening and is not present in the ripe kernel.[150,151] It appears again during germina-

[147] W. Windisch and P. Kolbach, *Wochschr. Brau.* **42**, 139 (1925).

[148] F. W. Geddes, *Advances in Enzymol.* **6**, 415 (1946); J. E. Anderson, Enzymes and Their Role in Wheat Technology; Interscience, New York, 1946. C. H. Bailey, The Constituents of Wheat and Wheat Products; Reinhold, New York, 1944.

[149] E. Kneen, B. S. Miller, and R. M. Sandstedt, *Cereal Chem.* **19**, 11 (1942).

[150] P. S. Ugrümow, *Biochem. Z.* **282**, 74 (1935).

[151] S. Schwimmer, *Cereal Chem.* **24**, 167 (1947).

tion. The amount of β-amylase increases during a short period of the ripening and then decreases again, because the enzyme is transformed into the inactive, "latent" form.[151] As to the distribution of β-amylase in the wheat grain Engel[152] found no enzyme in the aleuron cells but much in the subaleuronic layer. The amylase content of the endosperm is fairly low, increasing outward. There seems to be no correlation between the number of mitochondria and amount of amylase (or of esterase or protease).[153]

Wheat amylase may be extracted and treated as the barley enzyme.[154] During germination α-amylase appears just as in the case of barley.[155] The pH curves of the two wheat amylases are almost identical with the corresponding curves of the barley enzymes.[141,156]

The amylases are, of course, important constituents of wheat flour, playing a role for instance in bread making.

c. β-Amylase in Other Cereals

The β-amylase content of rye is of the same magnitude as that of barley and wheat.[157] α-Amylase appears during germination. The pH optimum of rye β-amylase was found[158] to be about 4.0, a little to the acid side of the β-amylase optimum generally found. The two amylases in oats are very similar to the corresponding enzymes in the cereals mentioned above.[159]

Maize, rice,[160] and sorghum have a very low amylase content. Even sorghum malt contains a small amount of β-amylase together with larger amounts of α-amylase. The enzymes are similar to those of barley malt.[161]

d. β-Amylase in Soybeans

Soybean seeds are a good source for β-amylase. The enzyme is completely water soluble and only traces of α-amylase are present.[162,163] Data on the purification of soybean amylase, its inactivation, *etc.* have been published by Newton *et al.*[162,164]

e. β-Amylase of Ipomoea batatas

Sweet potatoes are very rich in amylase and the enzyme is a β-amylase practically free from other amylases. The enzyme has been successfully purified and crystallized by Balls *et al.*[165]

[152] C. Engel, *Biochim. et Biophys. Acta* **1**, 42 (1947).
[153] C. Engel and L. H. Bretschneider, *Biochim. et Biophys. Acta* **1**, 357 (1947).
[154] M. J. Blish, R. M. Sandstedt, and D. K. Meecham, *Cereal Chem.* **14**, 328 (1937).
[155] J. S. Andrews and C. H. Bailey, *Cereal Chem.* **11**, 551 (1934). F. C. Hildebrand and G. M. Burkert, *ibid.* **19**, 27 (1942). E. Kneen, *ibid.* **21**, 304 (1944).
[156] T. Stenstam, C. O. Björling, and E. Ohlson, *Z. physiol. Chem.* **226**, 265 (1934).
[157] D. A. Coleman, S. R. Snider, and H. B. Dixon, *Cereal Chem.* **11**, 523 (1934).
[158] E. Ohlson and C. E. Uddenberg, *Z. physiol. Chem.* **221**, 165 (1933).
[159] E. Ohlson and O. Edfeldt, *Z. physiol. Chem.* **221**, 174 (1933).
[160] K. V. Giri and A. Sreenivasan, *Biochem. Z.* **289**, 155 (1937); **296**, 428 (1938).
[161] E. Kneen, *Cereal Chem.* **22**, 112 (1945).
[162] J. M. Newton and N. M. Naylor, *Cereal Chem.* **16**, 71 (1939).
[163] S. Laufer, H. Tauber, and C. F. Davis, *Cereal Chem.* **21**, 267 (1944).
[164] V. D. Martin and J. M. Newton, *Cereal Chem.* **15**, 456 (1938). J. M. Newton, R. M. Hixon, and N. M. Naylor, *ibid.* **20**, 23 (1943).
[165] A. K. Balls, R. R. Thompson, and M. K. Walden, *J. Biol. Chem.* **163**, 571 (1946); **173**, 9 (1948).

The washed and coarsely ground tubers are squeezed dry in a hydraulic press. The juice which contains practically all the enzyme is heated to 60–65°, cooled, and stored for 4 days at 3–4°. It is then treated with enough lead acetate to precipitate about a quarter of the enzyme. The filtrate is precipitated by ammonium sulfate at 0.7 saturation and the precipitate collected as a paste. 250 g. of this paste is dissolved and refractionated with ammonium sulfate. The final precipitate is dialyzed in cellophane under toluene for 2 days, diluted to 200 ml., and filtered. It is made 0.2 saturated with ammonium sulfate, the pH is brought to 5.0, and any precipitate is filtered off. The solution is then brought, at a temperature of 8–10°, to pH 3.25 to 3.30 and centrifuged. The supernatant is brought to 0.25 saturation with ammonium sulfate and to pH 3.6 to 3.7. Seed crystals are added, the mixture is stirred at room temperature, and ammonium sulfate solution is slowly added to 0.4 saturation. The crystals formed are easily separated from amorphous material owing to their high density. The crystals are dissolved in water and recrystallized at pH 3.6 by addition of ammonium sulfate.

Slowly formed crystals are tetragonal prisms capped by pyramids. They are free from α-amylase, maltase, and phosphatase (the latter occurs in great amounts in the raw material).

There is no indication that the pure enzyme is a metal protein or contains any light-absorbing prosthetic group. The protein contains 15.1% nitrogen, 0.83% amino nitrogen, and 1.16% amide nitrogen. The tyrosine content (7%) is higher than usual; the cysteine content is low. The nitroprusside test is negative even after reduction with cyanide; in the presence of guanidine it is positive but is not increased by hydrogen cyanide. The heat-coagulated protein also gives a positive test. It is suggested that all sulfhydryl and disulfide groups are masked and that a large proportion of the sulfur exists as sulfhydryl.

The enzyme has a broad pH optimum. In acetate and citrate buffers the activity is rather constant in the pH range 4–5. The enzyme is inactivated by ascorbic acid.[166]

2. Amylokinase

Waldschmidt-Leitz and Purr[167] in 1931 reported the existence in germinating barley of a natural activator, called amylokinase, which was said to activate both plant and animal amylases. Later on the same authors[168] found that the reported action on animal amylases was due to contaminating salts. Weidenhagen[169] found no activation of partly purified malt enzymes and suggested that the observed action on crude preparations was due to removal of inhibiting substances. Hills and Bailey[170] could not confirm the existence of an amylokinase in green malt. They point out that papain increases the activity of barley extracts, which as mentioned above is caused by proteolytic release of the "latent" amylase.

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[166] P. Seshagirirao and K. V. Giri, *Proc. Indian Acad. Sci.* **16B**, 190 (1942).
[167] E. Waldschmidt-Leitz and A. Purr, *Z. physiol. Chem.* **203**, 117 (1931).
[168] E. Waldschmidt-Leitz and A. Purr, *Z. physiol. Chem.* **213**, 63 (1932).
[169] R. Weidenhagen, *Z. Ver. deut. Zucker-Ind.* **83**, 505 (1933).
[170] C. H. Hills and C. H. Bailey, *Cereal Chem.* **15**, 273 (1938).

3. Amylase Complement

Pringsheim *et al.*[171] observed that limit dextrin preparations, isolated after degradation of starch with amylases, were digested by mixtures of the amylases with yeast and many other materials, whereas the amylases alone had only a slight action. They claimed the existence of an "amylase complement," which was supposed somehow to enable the amylases to saccharify the otherwise almost stable limit dextrins. Several other authors,[172,173] however, were unable to confirm these results, and it seems rather superfluous at the present time to discuss this question again. Analytical errors may have played a role in certain of Pringsheim's experiments, and the positive effects, which were sometimes observed also by other investigators, are probably due either to increased stability of the amylases or to the presence of phosphorylases. The complement question may have something to do with the phenomena observed[173] when living yeast is present in amylase–substrate mixtures (*vide infra*).

4. Amylases and Living Yeasts

It is an old observation made in the fermentation industries that the yield of alcohol from starch conversion mixtures is considerably higher when the yeast acts in presence of active amylase than when the yeast acts alone. An explanation would be that the removal of the fermentable substances from the conversion mixture facilitates the slow saccharification of the limit dextrins. However this is probably of secondary importance, since isolated limit dextrins are only extremely slowly attacked by the amylases. Another explanation would be that the yeast or substances originating from the yeast activate or stabilize the amylases, but no precise information on this point is available. The problem is of great interest for the alcohol industries but only a few attempts have been made to attack it from a scientific point of view. Hopkins *et al.*[173] and Pigman[174] have proved that yeast acting on starch in the presence of different amylases in several cases yields an amount of alcohol which corresponds to a tolerably complete conversion of the starch into fermentable substances. According to Pigman this result is obtained with cereal α-amylases and fungal amylases, but not with β-amylase, animal amylases, or bacterial amylases.

Kneen and Spoerl[174a] assume the existence in malt of a third carbohydrase, "limit dextrinase," converting unfermentable dextrins to fermentable sugar.

A fact which may possibly be connected with these phenomena is that starch and most dextrins, including limit dextrins, are readily fermented by dried yeasts and yeast juice.[175] In these cases the phosphorolytic degradation of the carbohydrates

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[171] H. Pringsheim and K. Schmalz, *Biochem. Z.* **142**, 108 (1923). H. Pringsheim and V. Fuchs, *Ber.* **56**, 1762 (1923). H. Pringsheim and A. Beiser, *Biochem. Z.* **148**, 336 (1924). H. Pringsheim and G. Otto, *ibid.* **173**, 399 (1926). H. Pringsheim and M. Winter, *ibid.* **177**, 406 (1926). H. Pringsheim, J. Bondi, and E. Thilo, *ibid.* **197**, 143 (1928). J. Bondi, *ibid.* **203**, 88 (1928). H. Pringsheim, H. Borchart, and H. Hupfer, *ibid.* **238**, 476 (1931); **250**, 109 (1932).

[172] O. Holmbergh, *Z. physiol. Chem.* **134**, 68 (1924). B. Sullivan, *Ann. brass. dist.* **24**, 49 (1925); *Chem. Abstracts* **20**, 1997 (1926). R. Weidenhagen and A. Wolf, *Z. Ver. deut. Zucker-Ind.* **80**, 866 (1930). G. A. van Klinkenberg, *Z. physiol. Chem.* **212**, 173 (1932).

[173] R. H. Hopkins, J. W. Cope, and J. W. Green, *J. Inst. Brewing* **39**, 487 (1933).

[174] W. W. Pigman, *J. Research Natl. Bur. Standards* **33**, 105 (1944).

[174a] E. Kneen and J. M. Spoerl, *Am. Soc. Brewing Chemists, Proc.*, **1948**, 20.

[175] K. Myrbäck, B. Örtenblad, and K. Ahlborg, *Enzymologia* **3**, 210 (1937).

is probably a prerequisite of the fermentability. The fact that the same substrates are not at all attacked by living yeast is doubtlessly connected with the permeability of the living cell.

5. Endoamylases, α-Amylases

a. Animal α-Amylases

(*1*) *Salivary Amylase. Occurrence.* The presence of an amylase in human saliva was demonstrated in 1831 by Leuchs.[176] The name "ptyaline," sometimes used even today, was introduced by Berzelius.

The amylase content of human saliva is very high; the enzyme is found also in the saliva of several animals including ape, pig, guinea pig, squirrel, mouse, and rat,[177] but seems to be absent or almost absent in others as cat

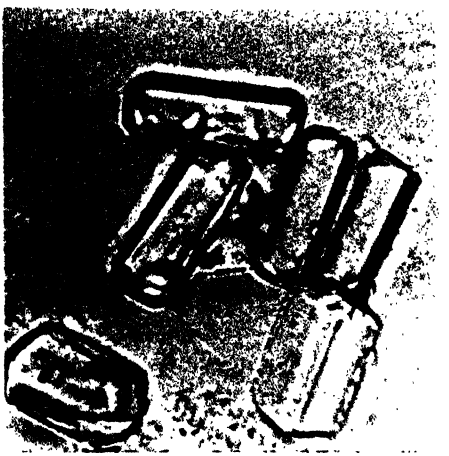

Fig. 6. Crystals of human salivary amylase ×730. This and the following micrographs have kindly been placed at the authors disposal by Dr. E. H. Fischer, Genève.

and dog,[178] sheep, goat,[179] and horse.[180] Human saliva contains about 0.33% organic material and about 0.15% sodium chloride. Many other inorganic ions are present, among them about 0.1‰ rhodanate. The amylase saliva is remarkably stable. Saliva can be stored for months in the presence of thymol.

Purification and Isolation. A partly purified enzyme was prepared by Myrbäck[181] by adsorption on aluminum hydroxide, elution with phosphate buffer, and dialysis. The activity was about 25 times that of saliva, calculated on dry weight basis.

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[176] E. F. Leuchs, *Poggendorff's Ann. Phys. Chem.* **22**, 623 (1831).
[177] E. W. Cohn and M. H. Brookes, *J. Biol. Chem.* **114**, 139 (1936).
[178] L. B. Mendel and F. P. Underhill, *J. Biol. Chem.* **3**, 135 (1907).
[179] C. Schwarz and K. Steinmetzer, *Fermentforsch.* **7**, 229 (1924).
[180] T. Matsuoka, *Bull. Agr. Chem. Soc. Japan* **13**; 87 (1937).
[181] K. Myrbäck, *Z. physiol. Chem.* **159**, 1 (1926).

Human salivary amylase has recently been isolated in crystalline and presumably pure form by Meyer *et al.*[182] The yield is excellent (50%; after recrystallization, 30%). (Fig. 6).

Saliva (1.5 l.) is precipitated with 48% acetone and the precipitate is rejected. A second precipitate is obtained at pH 6.3 with 70% acetone. The solution of this precipitate (350 ml.) is precipitated again in the presence of sodium acetate with 50% acetone and the precipitate rejected. The enzyme is precipitated with 70% acetone and the precipitate is dissolved in 250 ml. water. The pH is adjusted to 8.0 with ammonia and made 0.455 saturated with ammonium sulfate. The precipitate is dissolved in 80 ml. water, brought to pH 8, and made 0.40 saturated with ammonium sulfate. The solution of the precipitate in 40 ml. water is brought to pH 7 and precipitated with 52% acetone. The precipitate is discarded, 1 g. sodium acetate is added to the solution, and the amylase is precipitated with acetone to 70%. The solution of the precipitate in 15 ml. water is freed from sulfate by means of 3 g. Amberlite IR-4B and precipitated with 70% acetone. The precipitate is dissolved in 2 ml. 0.1 *N* sodium hydroxide. The enzyme crystallizes in the cold. The crystals are centrifuged off, washed with small quantities of ice-cold 30% acetone, and dissolved in a small quantity of water with enough sodium hydroxide to bring the pH to 11. The solution is then neutralized with acetic acid and filtered. Crystallization occurs as above. After three recrystallizations the mother liquor has the same degree of purity as the crystals.

Properties of the Crystalline Enzyme. In electrophoresis the preparation behaves as a single substance. It gives the ordinary protein reactions, coagulates on heating, and is irreversibly inactivated by trichloroacetic acid.[183] The protein contains 15.8% nitrogen and 0.01% phosphorus. Since the molecular weight is lower than 45,000 it must be concluded that the enzyme protein is free of phosphorus. The solubility in water at pH 8.5 (ammonia) is about 0.3%. The electrophoretic mobility at pH 10.14 is 3.75×10^{-5} cm.^{2} sec.$^{-1}$ volt^{-1}. The ultraviolet absorption spectrum has a maximum at 280 mμ and an inflection at 292 mμ. Some properties of crystalline amylases are summarized in Table III.

Salivary amylase is activated by chloride and certain other anions, and the activation depends in a characteristic manner on the pH of the solution. Since the same phenomena are met with also in the case of other animal amylases, they will be dealt with later on. The pH optimum in the presence of sodium chloride is 6.9. Apart from the ions mentioned no dialyzable activators of the enzyme are known. The crystalline enzyme does not lose any activity on dialysis provided sodium chloride is present in the activity assay.

The crystalline enzyme is extraordinarily stable[183] at room temperature in the pH range 5–11. Partly purified preparations are slowly inactivated

[182] K. H. Meyer, E. H. Fischer, P. Bernfeld, and A. Staub, *Experientia* **3**, 455 (1947). K. H. Meyer, E. H. Fischer, A. Staub, and P. Bernfeld, *Helv. Chim. Acta* **31**, 2158 (1948).

[183] P. Bernfeld, A. Staub, and E. H. Fischer, *Helv. Chim. Acta.* **31**, 2165 (1948).

by trypsin and still more slowly by papain-H_2S.[184] Pepsin destroys the enzyme completely.[185]

The action of salivary amylase on the natural substrates seems to be identical with that of pancreatic amylase. However, this does not mean that

TABLE III

PROPERTIES OF CRYSTALLINE α-AMYLASES[a]

Properties	Malt	*B. subtilis*	Swine pancreas	Human pancreas	Human saliva
Activity/mg. N[b]	1300	3600	4000	6200	6200
Per cent N	13.4	14	15.8	15.8	15.8
Per cent P	0.01	0.01	0.05	0.01	0.01
Per cent S	0.4	?	0	0	0
Sulfhydryl groups	+	−	−	−	−
Optimum pH[c]	5.3	5.3–6.8	6.9	6.9	6.9
pH stability range	4.8–8.0	4.8–8.5	7–8.5	4.8–11	4.8–11
Solubility at 2°, %					
pH 6.5			0.22	very low	very low
pH 8.5	10	6	6	0.3	0.3
pH 11				readily soluble	readily soluble
Molecular weight	59,500[d]		45,000		
Electrophoretic mobility, cm.^{2} sec.$^{-1}$ volt^{-1} 10^5					
pH 6.5			1.8		
pH 7.9	3.2	3.2	3.1	3.3	3.3
pH 10.1			3.55		3.75
Activation by NaCl	−	+	+	+	+
Saccharogenic / Dextrinogenic power[e]	9.8	9.8±0.2	9.8	9.5	9.6

[a] This table appeared in a lecture at the International Congress of Biochemistry, Cambridge, England, 1949, by Dr. E. H. Fischer, Geneva, who kindly permitted us to use it in this paper.

[b] By reductometric method used by K. H. Meyer *et al.*

[c] In the presence of optimum concentration of sodium chloride.

[d] Osmotic method by Balls and Schwimmer (p. 695).

[e] Dextrinogenic power by iodine coloration method.

all animal amylases are identical (Table III). Meyer concludes that animal amylase action on starch is identical also with that of malt α-amylase; this, however, is not quite correct[186] (page 711). Concerning the limit dextrins produced by salivary amylase, see Myrbäck *et al.*[187] Crude saliva

[184] H. Tauber and I. S. Kleiner, *J. Biol. Chem.* **105**, 411 (1934).
[185] H. Ninomiya, *J. Biochem. Japan* **31**, 421 (1940).
[186] C. S. Hanes and M. Cattle, *Proc. Roy. Soc. London* **B125**, 387 (1938).
[187] K. Myrbäck, B. Örtenblad, and W. Thorsell, *Biochem. Z.* **316**, 424 (1944).

has the same action on starch as the crystalline enzyme, no other starch-splitting enzymes being present.[187a].

(2) *Pancreatic Amylase. Occurrence.* The presence of amylase in human pancreatic juice was demonstrated in 1845 by Bouchardat and Sandras.[188] The amylase content of the pancreas is far greater than that of any other organ. The pancreas of the pig is also rich in amylase; the enzyme content of cattle, sheep, and dog pancreases is lower.

(a) *Amylase of Swine Pancreas. Purification and Isolation.* Fundamental work on this subject was carried out by Sherman and associates[189] and later on by Willstätter and pupils.[190] The latter investigators state that their most active preparations did not give any protein reactions,[89] which must be a mistake. The enzyme solutions which were tested were probably too dilute to give any color reactions. Contrary to this, Sherman *et al.* found that their best preparations, which probably had about twice the activity of the Willstätter and Waldschmidt-Leitz preparations,

FIG. 7. Crystals of pig pancreatic amylase ×220

were protein in nature. They gave the ordinary protein reactions and contained about 15% nitrogen, 1% sulfur, and 0.8% phosphorus.

In 1931 Caldwell, Bocher, and Sherman[191] announced, in a preliminary communication, the crystallization of pancreatic amylase. These results, however, were not confirmed. The amylase from pig pancreas was isolated in crystalline form in 1947 by Meyer *et al.*[192-195] (Fig. 7).

[187a] K. H. Meyer, F. Duckert and E. H. Fischer, *Helv. Chim. Acta* **33**, 207 (1950).

[188] G. Bouchardat and Sandras, *Compt. rend.* **20**, 143, 1085 (1845).

[189] H. C. Sherman and M. D. Schlesinger, *J. Am. Chem. Soc.* **33**, 1195 (1911). H. C. Sherman and M. Caldwell, *ibid.* **43**, 2469 (1921). H. C. Sherman, M. Caldwell, and M. Adams, *ibid.* **48**, 2947 (1926). H. C. Sherman, M. Caldwell, and M. Adams, *J. Biol. Chem.* **88**, 295 (1930).

[190] R. Willstätter and E. Waldschmidt-Leitz, *Z. physiol. Chem.* **125**, 132 (1923). R. Willstätter, E. Waldschmidt-Leitz, and R. Hesse, *ibid.* **126**, 143 (1923); **142**, 14 (1924).

[191] M. L. Caldwell, L. Bocher, and H. C. Sherman, *Science* **74**, 37 (1931).

[192] K. H. Meyer, E. H. Fischer, and P. Bernfeld, *Experientia* **2**, 362 (1946); **3**, 106 (1947).

A dry fat-free powder is prepared by treatment of the gland material with acetone and diethyl ether. This powder is quite stable. It is extracted with water, and impurities (most of the proteases) are removed by precipitation with 50% acetone. The crude amylase is then precipitated with 64% acetone. The solution of the active precipitate is brought to 0.325 saturation with ammonium sulfate. The solution of the material so obtained is reprecipitated with ammonium sulfate at 0.225 saturation. After repeating the fractional precipitation with acetone, inactive proteins are removed by shaking the solution repeatedly with ethanol and chloroform. The active solution is then treated with Wofatit charged with acetate to remove sulfate. After repeated precipitation with 55 and 70% acetone, the active precipitate is dissolved in a little water and freeze-dried. The powder is dissolved in 20 parts of water and the same volume of acetone is added. Crystallization takes place.

The impure solutions are very unstable; the first crystallization was possible only after stabilization of the enzyme with a boiled and filtered solution of crude enzyme. The method was later simplified. By working in a faintly alkaline medium the addition of boiled enzyme can be avoided and the enzyme is crystallized from a 5–8% solution in water at pH 6.5.

Properties of the Crystallized Enzyme. The crystallization involves a 23-fold increase in activity per mg. nitrogen. The crystals are said to be about three times as active as the best preparations of Sherman *et al.* The recrystallized product is a protein giving the normal protein reactions. It coagulates on heating the solution. It is homogeneous in electrophoresis and in the ultracentrifuge.[194] It contains 15.8% nitrogen but no phosphorus and no sulfur. It probably yields no sugar on acid hydrolysis. The molecular weight[196] is 45,000. The isolectric point lies between 5.2 and 4.6 (the enzyme is inactivated at this pH). The ultraviolet absorption spectrum has a maximum at 280 mμ and an inflection at 290 mμ (tryptophan and tyrosine, respectively).

Solutions of the pure enzyme are quite stable. The instability of impure solutions is due to the presence of a specific protease[195] which rapidly inactivates the amylase with the formation of dialyzable nitrogeneous substances. The action of this protease is inhibited by a heat-stable substance in crude enzyme solutions, hence the stabilization of the enzyme by boiled enzyme solution. The amylase is not inactivated by crystalline trypsin or crystalline lysozyme.[195]

Williams, Schlenk, and Eppright[197] found partly purified pancreatic amylase to contain 4.1 mg. inositol per gram. Lane and Williams[198] believe

[193] K. H. Meyer, E. H. Fischer, and P. Bernfeld, *Helv. Chim. Acta.* **30**, 64 (1947).
[194] K. H. Meyer, E. H. Fischer, and P. Bernfeld, *Arch. Biochem.* **14**, 149 (1947). E. H. Fischer, Doctoral Thesis. Univ. Genève, 1947. E. H. Fischer and P. Bernfeld, *Helv. Chim. Acta* **31**, 1831 (1948).
[195] E. H. Fischer and P. Bernfeld, *Helv. Chim. Acta* **31**, 1839 (1948).
[196] C.-E. Danielsson, *Nature* **160**, 899 (1947).
[197] R. J. Williams, F. Schlenk, and M. A. Eppright, *J. Am. Chem. Soc.* **66**, 896 (1944).
[198] R. L. Lane and R. J. Williams, *Arch. Biochem.* **19**, 329 (1948).

inositol to be an essential component of the enzyme since the inhibition by Gammexane is prevented competively by inositol. Fischer and Bernfeld,[199] however, deny the occurrence of inositol in pancreatic and salivary amylase and find added inositol to be without action on the activity or stability of pure pancreatic amylase, which, moreover, is not inhibited by γ-hexachloro-1,2,3,4,5,6-cyclohexane.

Little and Caldwell[200] have established that primary amino groups are essential to the enzyme action (inactivation by ketene *etc.*), whereas the hydroxyl group of tyrosine seems to have no importance. The inactivation of pancreatic amylase by nitrous acid was studied by Myrbäck[181]; the inactivation is very strongly enhanced in the presence of chloride and certain other anions, which activate the enzyme (page 691). This seems to indicate that the activation of the enzyme by the anions somehow exposes the amino groups to the destroying agent. For additional experiments on the action of inhibitors see Van-Thoai and Silhol-Bernère.[201] The reaction products at different stages of hydrolysis have been studied by Alfin and Caldwell,[202] and by Blom and Rosted.[203] The latter authors find that the reaction products at 64% hydrolysis, calculated as maltose, are approximately: one third maltose, one third trisaccharide, and one third dextrin. Since the trisaccharide, on further action of the enzyme, is hydrolyzed to glucose and a disaccharide, it seems safe to assume that the essential component is maltotriose. The limit dextrins formed from potato starch by pancreactic amylase were investigated by Myrbäck *et al.*[204]

(b) *Amylase from Human Pancreas.* This enzyme has been isolated in crystalline form by Meyer *et al.*[205] The method of isolation is nearly the same as that used for the pig pancreatic amylase. The yield is about 25% and the enrichment 25-fold with respect to the crude extract. The enzyme is a typical protein. Some of the properties of the α-amylases, isolated in pure form, are shown in Table III. The three animal enzymes are very similar in many respects. The pH optimum is the same; the action on the substrates seems to be the same; they have the same nitrogen content and contain no phosphorus or sulfur. The absorption spectra are very similar, and the differences in electrophoretic mobility scarcely exceed the experimental errors. On the other hand there are a few properties in which the enzymes of human origin differ sharply from the pig pancreas amylase. The activity per mg. enzyme is about 50% higher, the pH range of stability

[199] E. H. Fischer and P. Bernfeld, *Helv. Chim. Acta* **32**, 1146 (1949).
[200] J. E. Little and M. L. Caldwell, *J. Biol. Chem.* **142**, 585 (1942); **147**, 229 (1943).
[201] N. Van-Thoai and M. J. Silhol-Bernère, *Compt. rend.* **223**, 961 (1946). E. Ohlson, *Arch. intern. pharmacodynamie* **37**, 108 (1930).
[202] R. B. Alfin and M. L. Caldwell, *J. Am. Chem. Soc.* **70**, 2534 (1948); **71**, 128 (1949).
[203] J. Blom and C. O. Rosted, *Acta Chem. Scand.* **1**, 230 and 233 (1947).
[204] K. Myrbäck, B. Örtenblad, and K. Ahlborg, *Biochem. Z.* **307**, 49, 53 (1940).
[205] K. H. Meyer, E. H. Fischer, P. Bernfeld, and F. Duckert, *Arch. Biochem.* **18**, 203 (1948).

is much broader, and the solubilities in water at certain pH values are quite different. Meyer *et al.* therefore conclude that both human amylases are chemically identical, whereas the protein of the pig pancreas amylase is different (Fig. 8).

(3) *Amylases in Other Animal Organs.* Degradation of starch and glycogen has been demonstrated in extracts of many organs and cells, in blood, and urine. As pointed out above it may be regarded as doubtful in many cases if the degradation is really caused by amylase action and not by phosphorolytic breakdown, particularly if the degradation has only been demonstrated by an increase in reducing value. If, on the other hand, maltose has been shown to occur as the chief degradation product, this seems to offer evidence of true amylase action.

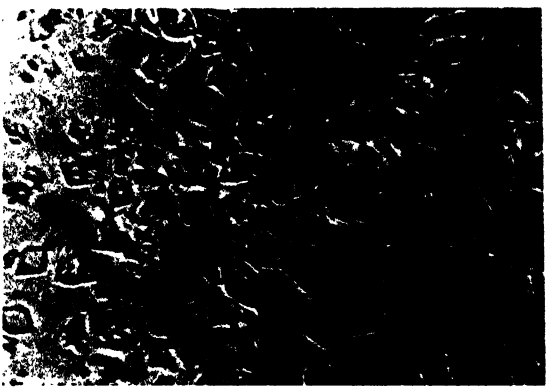

FIG. 8. Human pancreatic amylase ×730

(a) *Blood amylase.* This enzyme has been extensively studied and special methods have been worked out for its determination.[206,207] Schlesinger[207a] introduced the idea that the blood amylase originates from the pancreas, and later investigations have shown that in any case occlusion of the pancreatic duct or inflammatory condition of the organ increases the amylase activity of the blood. On the other hand, in depancreatized dogs, the amylase content of the blood is lowered during the first few days after operation but returns to the normal or above it if the diabetes is controlled by insulin.[208]

A connection between blood amylase and the diabetic condition has been sought by many authors. Myers *et al.*[208, 209] found the blood amylase value in diabetic pa-

[206] V. C. Myers and E. Reid, *J. Biol. Chem.* **99**, 595 (1933). W. R. Thompson, R. Tennant, and C. H. Wies, *ibid.* **108**, 85 (1935).

[207] V. C. Myers, A. H. Free, and E. E. Rosinski, *J. Biol. Chem.* **154**, 39 (1944).

[207a] A. Schlesinger, *Deut. med. Wochschr.* **34**, 503 (1908).

[208] E. Reid and V. C. Myers, *J. Biol. Chem.* **99**, 607 (1933).

[209] V. C. Myers and J. A. Killian, *J. Biol. Chem.* **29**, 179 (1917). E. Reid, J. P. Quigley, and V. C. Myers, *ibid.* **99**, 615 (1933).

tients not treated with insulin to be higher than normal, but normal in the insulin-treated patient. In normal dogs insulin was found to decrease the blood amylase values but to increase those of the liver. Myers *et al.* suggest that the blood enzyme, which is thought to have no physiological role in itself, is recalled by insulin into the liver, where it functions in the carbohydrate metabolism. These inferences have, however, been criticized by Somogyi,[210] who, from a statistical treatment of a large number of blood amylase determinations in normal and diabetic subjects, concludes that in the majority of cases the diabetic patients show lowered blood amylase values, which is taken as a sign of fatty liver or other disturbances of the liver function. The depression of the blood amylase values is roughly parallel to the severity of the diabetes, very low values being observed in *coma*.[207] Hecht[211] found no difference in the amylase content of blood and urine of normal and diabetic rats.

The products produced from starch by blood amylase have been studied by Somogyi.[212] The decrease in the blood sugar level observed after injection of certain amylase preparations probably has nothing to do with the amylase.[213]

(b) *Urine Amylase.* Human urine regularly contains amylase; the amount depends on the permeability of the renal tissues. The amount is increased if the pancreatic duct is blocked,[214] but decreased after extirpation of the pancreas or in diabetes.[215] The action of urine amylase on starch has been investigated in detail by Somogyi.[216] The primary products are reducing dextrins; later on maltose and finally also glucose appear. Glucose is formed from low-molecular reducing dextrins, not from maltose. This type of action seems to coincide with that of the animal amylases in general.

(c) *Muscle Amylase.* A glycogenolytic enzyme in muscle has been known for a long time. It is active also in starch degradation and has been designated an amylase. In reality most of the observed effects have probably been caused by phosphorolytic degradation of the polysaccharides and subsequent transformation of the glucose-1-phosphate into reducing substances. However, as pointed out by Mystkowski[217] and Willstätter,[218] there is in muscle, in addition to the phosphorolytic system, an amylatic one as well. The amount of amylase is small, and the enzyme plays no role in the glycogenolytic cycle of the muscle.

Muscle amylase is inactive except in the presence of chloride ions and thus resembles the other animal amylases in this respect.

[210] M. Somogyi, *J. Biol. Chem.* **134**, 315 (1940).
[211] E. Hecht, *Biochim. et Biophys. Acta* **1**, 425 (1947).
[212] M. Somogyi, *Proc. Soc. Exptl. Biol. Med.* **29**, 1126 (1932).
[213] H. E. C. Wilson and F. Strieck, *Biochem. Z.* **251**, 199 (1932). W. Deichmann-Grübler and V. C. Myers, *ibid.* **288**, 149 (1936).
[214] J. Wohlgemuth, *Z. Urol.* **5**, 801 (1911).
[215] A. Rosenthal, *Deut. med. Wochschr.* **37**, 923 (1911). E. Marino, *Deut. Arch. klin. Med.* **103**, 325 (1911).
[216] M. Somogyi, *J. Biol. Chem.* **134**, 301 (1940).
[217] E. M. Mystkowski, *Enzymologia* **2**, 152 (1937–1938).
[218] R. Willstätter and M. Rohdewald, *Compt. rend. lab. Carlsberg Sér. chim.* **22**, 553 (1938).

Barbour[219] stated that the main product of muscle enzyme action is a trisaccharide. His experiments were, however, criticized by Carruthers *et al.*[220] and by Gray,[221] whose data suggest that maltose is formed in great amounts. It may be that the trisaccharide observed by Barbour was maltotriose, which is formed by α-amylase (in moderate amounts) as an intermediary product; in any case the isolation of maltose seems to prove that the observed action is really a hydrolysis. Since the extracts contain maltase, glucose appears as the final product if maltase action is not inhibited, *e.g.*, by glycerol.

(d) *Liver Amylase.* The blood-free liver contains an amylolytic system. The disappearance of the color reaction with iodine at an apparent yield of 15% maltose in experiments with starch and liver preparations, and the fact that the reducing reaction products display a mutarotation downward[222] characterize the enzyme as an α-amylase. The enzyme is activated by chloride ions in the same way as other animal amylases.[223] Maltose is the main product.[224]

A dry, stable preparation is obtained if the blood-free organ is minced, defatted, and dried with acetone and diethyl ether.[219] Active solutions may be prepared by extraction of this powder with water or glycerol. The pH optimum is 6.9 in the presence of sodium chloride (0.01 to 0.5 *M*). Holmbergh[223] found that iodides depress the saccharifying activity of the enzyme but activate the dextrinization (disappearance of the iodine reaction). This result is not clearly understood; it may be that phosphorylases have been present.

(e) *Miscellaneous.* The distribution of amylase (and certain other enzymes) in the duodenum and the ileum of the rat has been studied by van Genderen and Engel.[225]

Concerning degradation of starch and glycogen by other organs the reader is referred to the survey given by Purr.[106] Generally it is impossible to decide whether the observed action is due to amylases, phosphorylases, or both.

The amylase, which occurs regularly in honey has been shown to originate from the bee.[226]

Holter and Doyle[227] have investigated the localization of the amylase in amoebas.

(4) *Activation of Animal Amylases by Certain Ions.* It has been known for a long time that certain anions, especially the chloride ion, have a powerful activating effect on animal amylases. Dialysis of enzyme solutions decreases the activity strongly, but in many cases almost complete regeneration is obtained by addition of sodium chloride or other soluble chlorides.

[219] A. D. Barbour, *J. Biol. Chem.* **85**, 29 (1929).
[220] A. Carruthers and W. Lee, *J. Biol. Chem.* **108**, 525 (1935). A. Carruthers, *ibid.* **108**, 535 (1935).
[221] C. H. Gray, *Nature* **135**, 1002 (1935).
[222] L. Hollander, *Science* **79**, 17 (1934).
[223] O. Holmbergh, *Z. physiol. Chem.* **134**, 68 (1924).
[224] G. E. Glock, *Biochem. J.* **32**, 235 (1938).
[225] H. van Genderen and C. Engel, *Enzymologia* **5**, 71 (1938–1939).
[226] R. Ammon, *Biochem. Z.* **319**, 295 (1949).
[227] H. Holter and W. L. Doyle, *Compt. rend. trav. lab. Carlsberg Sér. chim.* **22**, 219 (1938).

It does not seem possible at the moment to decide if the salt-free amylases are completely inactive or if they have a certain low activity. Earlier it was assumed by most authors that salt-free amylases are quite inactive. Norris[228] found for instance that an activation of about 400% was obtainable and concluded that the salt-free enzyme should have no activity at all. These older investigations, however, were mostly carried out regardless of the acidity of the test solution. Michaelis and Pechstein[229] reinvestigated the question and determined the action of various salts over the whole pH range of the enzyme. The Wohlgemuth colorimetric method was used for the activity determination. The authors never found the enzyme quite inactive in absence of salts but assume that the salt-free enzyme is inactive and postulate combinations of enzyme and the activating ions as the active molecules. The pH optimum was found to vary with the anion used. The "combinations" with acetate, sulfate, and phosphate had their maximum activity at pH 6.1 to 6.2, the combination with chloride at 6.7 and the combination with the nitrate ion at pH 6.9. According to Michaelis and Pechstein the action of the ions is explained by: (*1*) the affinity of the various ions for the enzyme, (*2*) the affinity of the enzyme–salt combinations for the substrate, and (*3*) the isoelectric point of the active combinations, which is taken as identical with the pH optimum. Myrbäck,[181] using the reductometric method of assay, found that purified preparations of salivary and pancreatic amylase were not quite inactive in the absence of salts. At pH 6.8 to 6.9, the optimum of the enzyme when activated with enough sodium chloride, the "salt-free" enzyme had an activity of about 25% of that of the activated enzyme. The enzyme preparation was obtained by adsorption on aluminum hydroxide, washed repeatedly with distilled water, eluted with pure disodium phosphate solution, and dialyzed against redistilled water. The enzyme had no action at all on ordinary starch paste; this was however due to the faintly acid reaction of the paste. If the pH was adjusted to 6.9 with sodium hydroxide the activity was about 25% of that of the activated enzyme. The same value was obtained with dialyzed starch solution. Nevertheless it is perhaps not quite excluded that the reaction mixture may have contained traces of chlorides. Better results would be expected with the crystalline enzyme; Bernfeld[183] states, however that the activity of salt-free pancreatic amylase is about 15% of the maximum value in the presence of sodium chloride.

In any case it is evident, as already mentioned, that the activation of the amylases with chloride (and other ions) is dependent on the pH. The fully activated enzyme, in the presence of 0.01*M* or greater sodium chloride has its optimum at pH 6.8 to 6.9. If the chloride concentration is lower, the optimum is found in more acid solution, and the "salt-free" enzyme has

[228] R. V. Norris, *Biochem. J.* **7**, 622 (1913).
[229] L. Michaelis and H. Pechstein, *Biochem. Z.* **59**, 77 (1914).

its optimum at about 6.0 (Fig. 9). Phosphate, acetate, and sulfate were found to have no specific action on amylase; the affinity, if any, of these ions to the enzyme must be extremely low. Moderate concentrations of sulfate for instance do not depress the activity of the chloride amylase.[181,228]

Chloride ion has an extremely powerful action; half the maximum activity is found in about 0.0005*M* sodium chloride. Somewhat less active are bromide ions. The optimum of the bromide amylase is at pH 6.8. Less active are iodide ions but the optimum is still about 6.8; the enzyme is strongly activated in the pH range 6–8 but the action of the salt-free enzyme is depressed in more acid solution. Fluoride ions seem to have no

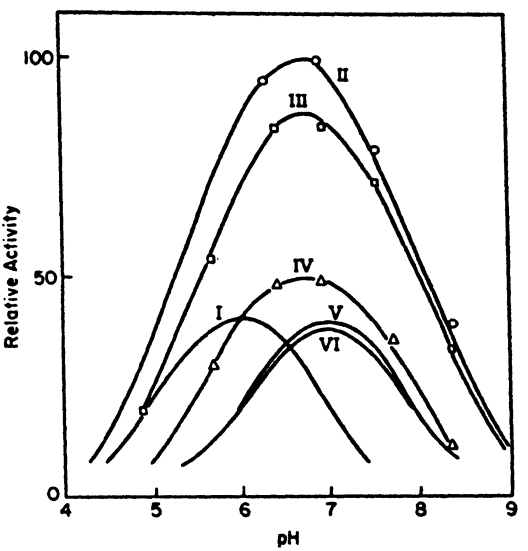

FIG. 9. Relative activity of the different salt amylases at different pH values.[181] (I) salt free (?) enzyme. Optimum concentration of chloride (II), bromide (III), iodide (IV), nitrate (V), and perchlorate (VI).

action and hydrogen cyanide inhibits strongly in 0.1*M* solution even if the pH is kept at the optimal value. Rhodanate seems to have no specific action.

The action of the nitrate ion can be described (Fig. 9) as nothing but a displacement of the pH curve. Practically the same action is shared by chlorate ions. The optimum pH is about 7.0, but the activity at this point is about the same as that of the salt-free enzyme at pH 6. On the acid side of pH 6.5 nitrate and chlorate have an inhibiting action; on the alkaline side of the same pH they activate. The different maximal activities of the various salt amylases could not be explained as due to different affinity for the substrate. The kinetics of starch hydrolysis was found to be the same for chloride and nitrate amylase; the ratio between the reaction rates as

measured by the increase in reduction value on one hand and by the Wohlgemuth method on the other was also found to be the same.

If it is assumed, as has been done by Michaelis and Pechstein,[229] that the isoelectric form of the enzyme is the active one, the action of the ions can be explained on the assumption that the dissociation constants, K_a and K_b, are different for the different enzyme–salt combinations. The combinations with halogen ions would then have the following approximate values:

Amylase	pK_a	pK_b
Chloride	8.1	8.6
Bromide	8.0	8.4
Iodide	7.4	7.8
Salt-free	6.3	8.4

It is emphasized that these values are, *pro tempore*, fictive values, calculated on the basis of certain assumptions. It will be remembered that the isoelectric point of pure (salt-free?) pancreatic amylase[196] is at about pH 5.0.

The activation of animal amylases by salts has nothing to do with the well known action of salts on the solubility of globulins.

Barmenkow[230] advanced the theory that the salt-free amylases are inactive compounds of the enzymes with mucins. The salt activation is thought to be due to liberation of the enzymes from such compounds. In view of the results obtained with the crystalline amylases, this possibility seems rather remote.

b. Cereal α-Amylase (Malt α-Amylase)

Ripe, nongerminated cereals, as indicated previously, generally contain only traces of α-amylase, but during germination of the grains large amounts of the enzyme are formed or set free from an inactive complex. Little is known about the mechanism of this reaction. (On the influence of heavy water, see Caldwell and Doebbeling.[231]) The α-amylase is said to be excreted by the *scutellum*. The β-amylase content does not increase very much during germination but the "latent" β-amylase is set free so that in barley malt, which is the generally used raw material, both enzymes are completely water soluble.

Solutions of "malt amylase" may be obtained by short extraction of ground malt with cold water in the presence of toluene or thymol. Small amounts of calcium salts have a stabilizing influence on malt α-amylase and may conveniently be added. The best method of freeing the α-amylase from the accompanying β-amylase seems to be the one originally indicated

[230] J. P. Barmenkow, *Biokhimiya* **4**, 160 (1939).
[231] M. L. Caldwell and S. E. Doebbeling, *J. Biol. Chem.* **123**, 479 (1938).

by Ohlsson.[95,96,102] Slight modifications have been introduced by several authors.[232] Kneen *et al.*[233] recommend the following procedure: ground barley or wheat malt is extracted for 60 minutes with 5 parts of a solution containing 2 mg. calcium chloride per ml. The extract is brought to a pH of about 6 and held for 15 minutes at 70° and filtered. Schwimmer and Balls[234] have succeeded in crystallizing α-amylase.

A concentrated malt extract is heated as indicated above. The filtrate is made 0.45 saturated with ammonium sulfate at pH 5.6 to 6. The precipitate is washed with 0.33-saturated ammonium sulfate solution saturated with calcium sulfate and dissolved in 5–10 times its original volume of 40% ethanol at 5°. Calcium sulfate is filtered off and the enzyme is absorbed[235] on starch mixed with Filter-Cel in a column.[234, 235] The starch is washed with 40% alcohol and the amylase brought into solution with water saturated with calcium sulfate. The enzyme is precipitated with 0.66-saturated ammonium sulfate and extracted from the precipitate with water saturated with calcium sulfate. The solution containing about 2 mg. of protein nitrogen per ml. is brought to pH 6 and solid ammonium sulfate is added, whereupon crystals separate, generally between 0.25 and 0.28 saturation.

The crystals are hexagonal prisms capped by pyramids and are readily soluble in 40% alcohol. Twice recrystallized substance contained 13.4% nitrogen, 0.01% phosphorus, and 0.035% iron. The activity was 67 times that of the crude extract. It is not possible at the moment to compare the activity of the crystals with that of the "malt albumin" preparations of Danielsson and Sandegren[146] (Table II). Their purest preparation had an α-amylase activity about 78 times that of the malt.

Amylase crystals could not be obtained at temperatures below 25°; the solubility of the enzyme seems to increase with falling temperature. The solubility of the crystalline enzyme in 0.5-saturated ammonium sulfate solution at pH 5.95 was determined in the presence of increasing amounts of the crystals. The solubility curve obtained shows the presence of 95–97% of one protein component.

The molecular weight calculated from osmotic pressure measurements is 59,500 ± 900. The ultraviolet absorption spectrum has the usual form a maximum at 280 mμ and a second maximum (or inflection point) at 290 mμ. Qualitative tests for inositol were negative. No inhibition was caused by a mixture of isomers of hexachlorocyclohexane.

A highly important fact is that the action of the crystalline enzyme on starch seems to be identical with that observed with partly purified enzyme preparations. When the percentage of glucosidic bonds hydrolyzed was

[232] W. J. Olson, B. A. Burkhart, and A. D. Dickson, *Cereal Chem.* **20**, 126 (1943)
[233] E. Kneen, R. M. Sandstedt, and C. M. Hollenbeck, *Cereal Chem.* **20**, 399 (1943)
[234] S. Schwimmer, *Cereal Chem.* **24**, 315 (1947). S. Schwimmer and A. K. Balls *J. Biol. Chem.* **176**, 465 (1948); **179**, 1063 (1949).
[235] O. Holmbergh, *Arkiv Kemi Mineral. Geol.* **11B**, No. 4 (1932).
[236] S. Schwimmer, Rapport du VII[e] Congrès International des Industries agricoles Paris, 1948. Vol. 1, Question 6-D.

plotted against reaction time, Schwimmer and Balls obtained a curve almost identical with the curve I in Fig. 15. Thus, the action of the crystalline enzyme, like that of less pure preparations, is composed of two phases: one initial, rapid phase, the dextrinization, comprizing the hydrolysis of about 17% of the glucosidic linkages of starch, and after that a slow saccharification phase (*vide infra*).

The pH optimum of the enzyme is about 5.0. The activity–pH curves published by different authors vary somewhat, doubtlessly depending on the methods of assay used. In any case the differences most probably do not indicate that the malt α-amylase should be, in reality, a mixture of different enzymes. Malt α-amylase is very unstable in acid solution. It is, for example, rapidly inactivated at pH 3.6. and the experimentally determined activity values at this pH must be strongly dependent on the method of assay. Myrbäck and Frostell[237] determined the activity by the

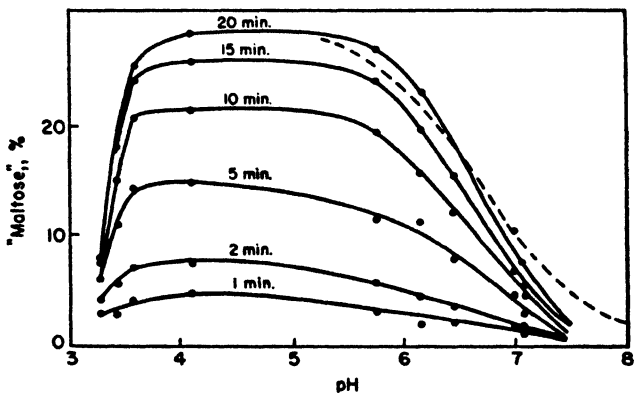

FIG. 10. pH curves of malt α-amylase.[000] Dotted curve is theoretical dissociation curve of an acid with K_a $10^{-6.7}$.

reductometric method and found, for different reaction times, the curves in Fig. 10. The alkaline branches of these curves are independent of the reaction time because no inactivation occurs in this region. This curve has approximately the form of a dissociation curve (dotted line). The acid branches of the curves on the other hand have quite another form, depending strongly on the reaction time. Thus it can be concluded that this part of the pH curve is mainly due to the instability of the enzyme in acid solution. Nevertheless it can be assumed that the acid branch of the curve depends on the dissociation of the amino groups of the enzyme. The fact that the substrate and other saccharides to which the enzyme has an affinity protects it in acid solution seems to support the assumption that primary amino groups are essential for the combination of the enzyme with the substrate.[181]

[237] K. Myrbäck and K. Frostell, *Arkiv Kemi Mineral. Geol.* **24A**, No. 11 (1947).

Malt α-amylase is not activated by chloride ions. The enzyme–substrate relationships are in agreement with the Michaelis-Menten theory.[238] Concerning the products of the action of malt α-amylase on starch the reader is referred to Myrbäck's review[239] and the literature cited there.

c. Amylase in Yeast

The question of the amylolytic properties of yeasts has been extensively and adequately reviewed by Smits van Waesberghe.[240] Sixty-four species of yeasts of widely different genera were tested with various methods; the majority was capable of enzymatic degradation of starch. *Saccharomyces fragilis* Jörgensen was investigated in detail; glucose was the sole product of the enzymatic action. The general conclusion is that the degradation is due, not to amylases, but to phosphorylases. Schäffner and Specht[241] likewise state that the so-called "phosphate-requiring amylase" of yeast is a phosphorylase.

The question of starch-degrading enzymes in yeast has acquired renewed interest since Meyer *et al.*[242] found that β-amylase limit dextrin (amylodextrin) is altered by a yeast enzyme in such a way that it can be further attacked by the β-amylase. (These experiments strongly support the irregular formulas of amylopectin and glycogen proposed by Meyer, Fig. 1.) The yeast enzyme evidently must be capable of removing "anomalous" end groups of the dextrin (page 710). The nature of the yeast enzyme has been discussed by Meyer and by Bernfeld. It seems probable at the present time that it is identical with the "isophosphorylase"[243] capable of breaking and synthesizing 1,6-α-glucosidic linkages. Following the removal of the anomalous end groups by isophosphorylase, action of the phosphorylase in yeast on the 1,4 linkages of the substrate may occur.

Starch and dextrins, even limit dextrins, are readily fermented by dried yeasts and yeast juices. As mentioned[176] above, this fermentation must be rendered possible by a preceding phosphorolytic degradation of the saccharides.

d. Amylase in Molds

Many molds are extremely rich in amylase and important commercial amylase preparations originate from molds. Mold amylases have acquired great industrial importance as in the so-called "amylo-procedures" in the breweries, and as saccharifying agents in the alcohol industry and in the manufacture of enzyme-converted sirups. A survey of the industrial use of "mold bran" has been given by

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[238] C. S. Hanes, *Biochem. J.* **26**, 1406 (1931).
[239] K. Myrbäck, *Advances in Carbohydrate Chem.* **3**, 251 (1948).
[240] F. A. M. J. Smits van Waesberghe, Doctoral Thesis, Delft, 1941.
[241] A. Schäffner and H. Specht, *Naturwissenschaften* **26**, 494 (1938).
[242] K. H. Meyer and P. Bernfeld, *Helv. Chim. Acta* **23**, 875 (1940); **24**, 1400 (1941); **25**, 399 (1942).
[243] P. Bernfeld and A. Meutémédian, *Nature* **162**, 297 (1948); **162**, 618 (1948); *Helv. Chim. Acta* **31**, 1724 (1948); **31**, 1735 (1948).

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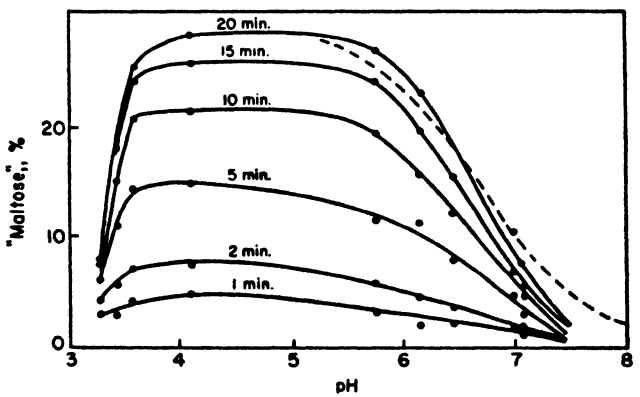

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[242] K. H. Meyer and P. Bernfeld, *Helv. Chim. Acta* **23**, 875 (1940); **24**, 1400 (1941); **25**, 399 (1942).
[243] P. Bernfeld and A. Meutémédian, *Nature* **162**, 297 (1948); **162**, 618 (1948); *Helv. Chim. Acta* **31**, 1724 (1948); **31**, 1735 (1948).

Underkofler *et al.*[244] Methods for precipitation of amylase from mold bran extracts have been described by Gates and Kneen.[245]

Typical mold amylase is the one originating from *Aspergillus oryzae* and known as taka-diastase. The crude preparations contain a great variety of enzymes besides amylase, such as proteases, phosphatases, maltase, etc. In crude preparations carbohydrases are sometimes present which are capable of hydrolyzing 1,6-α-glucosidic linkages, and the presence of these enzymes together with the maltase explains the fact that a practically complete conversion of starch to glucose is possible when using large amounts of crude taka-diastase preparations.[246] On the other hand taka-diastase (or certain preparations thereof) evidently hydrolyzes the 1,6-α-glucosidic linkage much more slowly than the normal 1,4-linkage. Myrbäck and Ahlborg[118] isolated isomaltose (6-α-glucosidoglucose) from the reaction products and determined its constitution by means of the methylation technique. Montgomery, Weakley, and Hilbert[119] found the chief reaction products to be glucose and isomaltose; the latter sugar was isolated in pure crystalline form.

The amylase in taka-diastase, conveniently called taka-amylase, has been purified considerably by Caldwell *et al.*[247] The purified preparations have no maltase action and there is no indication of the presence of more than one amylase. The action on starch is a typical α-amylase action. Reducing dextrins, maltose, and small amounts of glucose are formed in the early stages. Later on the amounts of dextrins decrease with the formation of maltose and some glucose. There is no evidence of a sharp "limit of saccharification" as in the case of β-amylase. The presence, in mold enzymes, of a "glucogenic system" producing glucose from higher saccharides, has been stressed by Corman and Langlykke.[248] The action of the taka-amylase seems on the whole to be rather similar to that of the animal amylases. (Concerning the limit dextrins, see Waldschmidt-Leitz and Purr.)[167]

The pH optimum of taka-amylase is 5.0 to 5.5. The nature of the buffer has no great influence on the position of the optimum but at the sides thereof differences may be observed.[249]

Ohlsson *et al.*[250] found that after heat inactivation of (crude) taka-amylase a certain reactivation may occur if the inactive solution is stored. Inactivation and reactivation depend on the pH of the solution. The reactivation has its optimum at pH 7. Inactivation of the enzyme by acids has been studied by Akabori *et al.*[251]

[244] L. A. Underkofler, G. M. Severson, K. J. Goering, and L. M. Christensen, *Cereal Chem.* **24**, 1 (1947).

[245] R. L. Gates and E. Kneen, *Cereal Chem.* **25**, 1 (1948).

[246] H. Weinmann, *J. Biol. Chem.* **164**, 7 (1946).

[247] M. L. Caldwell and S. E. Doebbeling, *J. Am. Chem. Soc.* **59**, 1835 (1937). M. L. Caldwell, R. M. Chester, A. H. Doebbeling, and G. Volz, *J. Biol. Chem.* **161**, 361 (1945). G. Volz and M. L. Caldwell, *ibid.* **171**, 667 (1947).

[248] J. Corman and A. F. Langlykke, *Cereal Chem.* **25**, 190 (1948).

[249] E. Ohlson, *Arch. intern. pharmacodynamie* **37**, 98 (1930). G. A. Ballou and J. M. Luck, *J. Biol. Chem.* **135**, 111 (1940).

[250] E. Ohlson and T. Swaetichin, *Bull. soc. chim. biol.* **11**, 333 (1929). E. Ohlson and O. Edfeldt, *ibid.* **15**, 470 (1933).

A spontaneous reactivation is observed also in this case. The taka-amylase is not inactivated by trypsin.

The amylase of *Rhizopus japonicus* has been investigated by Leopold and Starbanow.[252] Liquefaction of starch and dextrinization have an optimum at pH 5.0, but the saccharification optimum is at 4.0, which is taken as an indication of the presence of a second *amylase*, possibly β-amylase.

Endomyces fibuliger, belonging to the Fungi Imperfecti can, under certain conditions, produce an extracellular amylase.[253]

e. Bacterial α-Amylases

Degradation of starch by bacteria was observed as early as in 1819 by de Saussure.[254] Reviews on the occurrence of starch-splitting enzymes in bacteria are given by Thaysen and Galloway[255] and by Smits van Waesberghe.[240]

Peltier and Beckord[256] investigated more than 1000 bacterial isolates; 265 hydrolyzed starch and of these 37 had a high dextrinizing ability. Ropy bread and, to a minor degree, air dust are good sources of starch-splitting organisms, which belong to the *Bacillus subtilis* group. Kneen and Beckord[257] determined the quantity and quality of amylase in a large number of bacterial isolates. They classify the bacterial amylases in the following four groups: (*1*) saccharifying amylase from bacteria of the *B. subtilis* type, (*2*) α-amylase from bacteria of the *B. subtilis* type, (*3*) amylase from bacteria of the *B. polymyxa* type, and (*4*) amylase from *B. macerans*. Since the last-mentioned enzyme differs sharply from all other known amylases, it will be dealt with in a special section (Sect. VII).

(1) *Saccharifying Bacillus subtilis Amylase.* The enzymes belonging to this group are inhibited by the amylase inhibitor present in wheat; whereas the amylases of groups *2* to *4* are not.

Beckord, Kneen, and Lewis[258] found that a bacterial isolate belonging to the *B. subtilis* group, when cultivated on wheat bran moistened with phosphate buffer, developed an amylase differing in qualities from the commercial preparations. The enzyme (or system of enzymes) dextrinizes starch and is, or contains, an α-amylase, but the saccharifying capacity, compared to the dextrinizing power, is considerably higher than that of commercial bacterial amylases or malt α-amylase. The action on starch is rather similar to that of malt extract (α- and β-amylase) or the animal α-amylases.

[251] S. Akabori, S. Hayasi, and K. Kasimoto, *J. Chem. Soc. Japan* **61**, 1035 (1940). S. Akabori and K. Okahara, *ibid.* **12**, 55 (1937).

[252] H. Leopold and M. P. Starbanow, *Biochem. Z.* **314**, 232 (1943).

[253] L. J. Wickerham, L. B. Lockwood, O. G. Pettijohn, and G. E. Ward, *J. Bact.* **48**, 413 (1944).

[254] T. de Saussure, *Ann. chim. et phys.* **11**, 379 (1819).

[255] A. C. Thaysen and L. D. Galloway, The Microbiology of Starch and Sugars. Oxford Univ. Press, 1930.

[256] G. L. Peltier and L. D. Beckord, *J. Bact.* **50**, 711 (1945).

[257] E. Kneen and L. D. Beckord, *Arch. Biochem.* **10**, 41 (1946).

[258] L. D. Beckord, E. Kneen, and K. H. Lewis, *Ind. Eng. Chem.* **37**, 692 (1945).

The pH optimum of the dextrinization is 7.0 to 7.6; the saccharification has its optimum at pH 6.6 to 7.0. The enzyme is unusually stable at high temperatures and the stability is increased by calcium ions.

(2) *Bacillus subtilis α-Amylase.* The commercial preparations of bacterial amylase (Rapidase, Superclastase, Biolase, Liquefase, *etc.*) belong in this group. Such preparations, presumably originating from bacilli of the *subtilis-mesentericus* group have been investigated by several authors.

Hopkins *et al.*[259] found the amylase from *B. subtilis* to be very similar to malt α-amylase. The pH optimum is 6.5 to 7. The enzyme has a high heat stability. The reaction product at about 16% hydrolysis is a rather inhomogeneous mixture of dextrins ("α-dextrins"), containing saccharides of chain lengths between 3 and about 13. Hexasaccharides, at this stage of hydrolysis, constituted about 15% of the mixture. However, if the hydrolysis was carried further to about 21%, the more complex dextrins are broken down to compounds of about 6 glucose units. Smits van Waesberghe[240] states that the final, main product of the action of Superclastase on starch is maltose, but small amounts of glucose are formed as a primary product (not from maltose). In the early stages of hydrolysis a fermentable saccharide is present which has a higher *dextro* rotation than maltose. This saccharide disappears in the later phase of the conversion. It seems permissible to assume that this saccharide is maltotriose, which is slowly split into maltose and glucose by some (or possibly all) α-amylases.

Di Carlo and Redfern[260] have purified the amylase of Liquefase, a commercial preparation from *B. subtilis*. About 900-fold purification was attained by fractional precipitation with ethanol, ammonium sulfate, and acetone. The purified product contained 12% nitrogen. It is destroyed by ficin, which is regarded as evidence of its protein nature. A small amount of inositol found in the preparations is considered an impurity.

The enzyme is very stable in the pH range 5.8 to 10.8. The activity–pH curves at three different temperatures (Fig. 11) show a rather broad optimum zone between pH 5 and 7. To the alkaline side of pH 6 the three curves are similar, but in acid solution obvious differences appear. The explanation is probably the same as in the case of malt α-amylase; the acid branch of the pH curve is determined by the decrease in stability of the enzyme in acid solution. This instability is probably connected with the salt formation of the basic groups of the enzyme molecule.

No coenzyme or dialyzable prosthetic group has been found; copper and iron are not constituents of the enzyme molecule. Inactivation by specific

[259] R. H. Hopkins and D. Kulka, *J. Inst. Brewing* **48**, 170 (1942). R. H. Hopkins, D. E. Dolby, and E. G. Stopher, *ibid.* **48**, 174 (1942).

[260] F. J. Di Carlo and S. Redfern, *Arch. Biochem.* **15**, 333 (1947); **15**, 343 (1947); **17**, 1 (1948).

reagents makes it highly probable that free sulfhydryl groups and primary amino groups are essential to the enzyme action. Calcium ions increase the stability and reagents which remove these ions inactivate the enzyme presumably by denaturation of the protein.

An enzyme, presumably belonging to this group, has been isolated in the crystalline state by Meyer *et al.*[261] The commercial preparation Biolase was purified in four stages by fractional precipitation with salts and organic solvents. The material thus obtained was dissolved in a minimum of water. The solution crystallized in the cold. Recrystallization is effected by dissolving the crystals in water and ammonia at pH 7.8. The solution is then brought to pH 5.6 with acetic acid. The yield of the first crystalline product is about

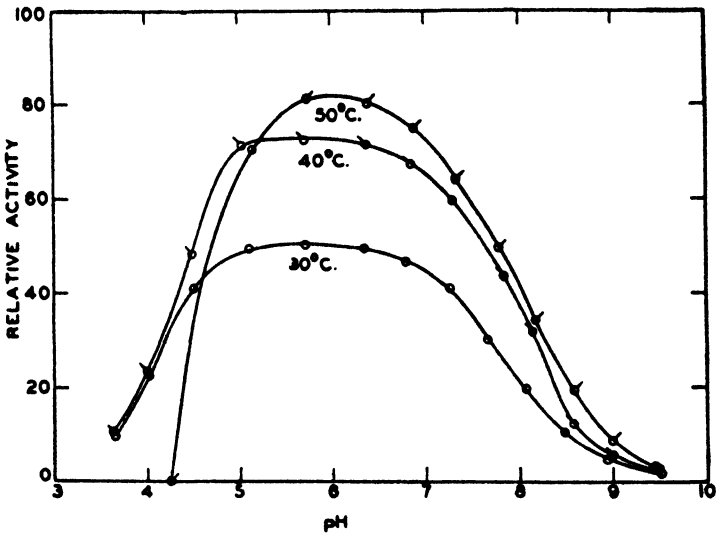

FIG. 11. Activity–pH curves of bacterial α-amylase at three different temperatures[260]

12% of the raw material. The activity does not increase on further recrystallization. The substance is electrophoretically homogeneous and gives the ordinary protein reactions. The enzyme is not identical chemically with pancreatic amylase but is believed to have much the same action on starch. It is activated by chloride ions. (Fig. 12).

The amylase from *B. mesentericus* has been studied by Minagawa[262] and Akiya.[263]

(3) *Bacillus polymyxa α-Amylase. Bacillus polymyxa* develops amylase abundantly on a peptone–starch medium at room temperature.[264] The en-

[261] K. H. Meyer, M. Fuld, and P. Bernfeld, *Experientia* **3**, 411 (1947).
[262] T. Minagawa, *J. Agr. Chem. Soc. Japan* **13**, 875 (1937).
[263] S. Akiya, *J. Pharm. Soc. Japan* **58**, 71 (1938).
[264] E. B. Tilden and C. S. Hudson, *J. Bact.* **43**, 527 (1942).

zyme is secreted into the medium. In contrast to the *B. subtilis* amylases the enzyme of *B. polymyxa* is not very stable to heat. It produces reducing dextrins and fermentable sugar but no nonreducing products of the Schardinger dextrin type.

B. polymyxa amylase has been purified to a considerable degree by Rose.[265] The enzyme was precipitated with acetone and accompanying maltase removed by adsorption on Hyflo Super Cel. The enzyme has a broad pH optimum from 6.2 to 7.5. The type of buffer has no marked influence. The reducing power of the reaction products did not exceed 80% of the theoretical yield of maltose. It may be inferred that the enzyme has an ordinary α-amylase action on starch.

FIG. 12. Crystals of B. subtilis amylase ×110

(4) *Amylase of Clostridium acetobutylicum.* Hockenhull and Herbert[266] have shown that the bacillus contains amylase and maltase as typically adaptive enzymes. The amylase is believed by the authors to produce maltose as the only (?) reaction product. The enzyme is secreted into the medium. It is not activated by chloride ions. The pH optimum is 4.8. The action of certain inhibitors is described.

IV. Amylase Inhibitors in Cereals

Kneen and Sandstedt[267] have demonstrated the occurrence of amylase inhibitors in different cereals. The inhibitor in wheat acts reversibly on

[265] D. Rose, *Arch. Biochem.* **16**, 349 (1948).
[266] D. J. D. Hockenhull and D. Herbert, *Biochem. J.* **39**, 102 (1945).
[267] E. Kneen and R. M. Sandstedt, *J. Am. Chem. Soc.* **65**, 1247 (1943).

animal and most bacterial amylases but has no action on malt amylases.[268] The authors state that the inhibitor is resistant to boiling but is destroyed on autoclaving. It is nondialyzable and is precipitated by ammonium sulfate. It is inactivated by pepsin and is probably protein in nature. The inhibitor appears in the endosperm, but not in the pericarp at the time the ripening kernel reaches full length. Kneen *et al.*[269] report that the inhibitor is destroyed by proteases and by nitrous acid, sulfite, halogens, and hydrogen peroxide. The action of certain reagents intimate that tryptophan is an essential component. The interaction between enzyme, inhibitor, and substrate is noncompetitive.

The inhibitor found in *Leoti sorghum*[268,270] acts on all amylases except those of fungi. It retains its activity after autoclaving at 15 lb. pressure for 30 min. at pH 2.5 or 10.9. In alkaline but not in acid solution it is precipitated by organic water-miscible solvents and by calcium chloride. The partially purified preparations gave positive indole tests and contains 2.6% nitrogen. The substance is supposed to be a high-molecular organic acid containing the indole group but no amino nitrogen. The inhibition seems to be due to physical adsorption. The "sisto-amylase" from buckwheat and other sources described by Chrzaszcz and Janicki[271] probably has the same mode of action.

V. Mechanism of Amylase Action[272]

1. Introduction

Starch and glycogen are generally not completely transformed into fermentable sugars by the amylases; a more or less definite limit of saccharification is reached. The apparent yield of maltose is usually 60–90%. Analysis of the reaction mixture, however, shows the presence of the following components: (*a*) Large amounts of maltose, generally 60–80% of the theoretical values. If the maltose fraction is determined by means of fermentation experiments, it may include maltotriose.[273] It should be observed that the fermentability of this sugar varies strongly with the type of yeast employed. (*b*) In some cases (with α-amylases) relatively small amounts of glucose, which may be a primary product formed from other saccharides than maltose.[274–277] The common case in which maltose is split into glucose

[268] E. Kneen and R. M. Sandstedt, *Arch. Biochem.* **9**, 235 (1946).
[269] W. Militzer, C. Ikeda, and E. Kneen, *Arch. Biochem.* **9**, 309 (1946); **9**, 321 (1946).
[270] B. S. Miller and E. Kneen, *Arch. Biochem.* **15**, 251 (1947).
[271] T. Chrzaszcz and J. Janicki, *Biochem. Z.* **260**, 354 (1933); *Biochem. J.* **28**, 296 (1934).
[272] R. H. Hopkins, *Advances in Enzymol.* **6**, 389 (1946).
[273] K. Myrbäck and E. Leissner, *Arkiv Kemi Mineral. Geol.* **17A**, No. 18 (1943).
[274] J. Baker, *J. Chem. Soc.* **81**, 1177 (1902). A. R. Ling, *J. Inst. Brewing* **9**, 446 (1903). B. F. Davis and A. R. Ling, *J. Chem. Soc.* **85**, 16 (1904). H. C. Sherman and P. W. Punnett, *J. Am. Chem. Soc.* **38**, 1877 (1916).
[275] K. Myrbäck, *Biochem. Z.* **307**, 140 (1941).

by maltase is of no interest in this connection and is not further considered here. (*c*) Nonfermentable substances called "limit dextrins" or "stable dextrins" or "residual dextrins." According to definition a stable dextrin should not be attacked at all by the amylase used in its preparation. The β-amylase limit dextrin (amylodextrin) is probably a true limit dextrin according to this definition. In the case of the α-amylases, the limit of saccharification generally is less well defined. The hydrolysis goes on for a very long time, getting slower and slower. If the experiment is interrupted at a certain time, dextrins may be isolated which for practical purposes are "stable" dextrins, but which in fact may be slowly attacked by the enzymes. The rate of this hydrolysis is, however, extremely small compared to the initial action of the enzyme on starch.

The incomplete saccharification of starch and glycogen is not due to a reverse action. The saccharification limit does not represent a dynamic equilibrium. Neither are the limit dextrins products of a synthesis from maltose or glucose, as once assumed by Pringsheim. The amount and general type of the limit dextrins varies distinctly with the starting material, whether starch, amylose, amylopectin, or glycogen, and it is quite clear that the limit dextrins are formed because of peculiarities in the constitution of the substrates. The incomplete saccharification and the formation of the limit dextrins show that starch and glycogen are not entirely built up according to the principle illustrated by the original Haworth starch formula. This was claimed in a very cautious form by Hanes[278] and at about the same time more decidedly by Myrbäck,[279] who postulated the existence in the chain molecules of "anomalies," elements of a constitution differing from that of the bulk of the polysaccharides. These anomalies were thought to constitute obstacles to the amylase action and to be the chief cause of the incomplete saccharification and the formation of the limit dextrins. It was assumed that the anomalies remain in the limit dextrins. The anomalies known at the present time are (*a*) anomalous glucosidic linkages (meaning other linkages than the 1,4-α-glucosidic linkage), and (*b*) substitution of certain glucose residues with phosphoric or other acids. The chief cause of limit dextrin formation is the occurrence of the anomalous glucosidic linkages, equivalent to ramifications of the chain molecules, the anomalous linkage being the "nucleus" of the future limit dextrin. It appears quite clear by now that the occurrence of about 4.5% of 1,6-α-glucosidic linkages in starch and about 9% in most samples of glycogen is the cause of the formation of the limit dextrins. The original number of

[278] M. Somogyi, *J. Biol. Chem.* **124**, 179 (1938).
[277] F. M. Mindell, A. L. Agnew, and M. L. Caldwell, *J. Am. Chem. Soc.* **71**, 128 (1949).
[278] C. S. Hanes, *New Phytologist* **36**, 101, 189 (1937).
[279] K. Myrbäck, *Current Sci. India* **6**, 47 (1937). K. Myrbäck and K. Ahlborg, *Svensk Kem. Tid.* **49**, 216 (1937).

these anomalous linkages in the substrates is recovered in the limit dextrins, provided these are not secondarily degraded.[280] The correctness of the theory of the anomalies as cause of the limit dextrin formation is very strong y supported by the isolation of the calculated amount of isomaltose following starch degradation with taka-amylase.[119]

The substitution of certain glucose residues in root and tuber starch with phosphoric acid is certainly an "anomaly" of the presumed kind,[281] since all phosphoric acid of potato starch is recovered in the limit dextrins,[94] provided of course that phosphatases are absent. A tetrasaccharide limit dextrin containing one phosphoric acid group was isolated by Posternak,[282] who also was able to show the formation of glucose-6-phosphate on acid hydrolysis of such phosphorus-containing limit dextrins. An ester containing 5% phosphorus was isolated by Northrop and Nelson.[283] Starch phosphorylated by means of phosphorus oxychloride, in chloroform yields a glucose monophosphoric acid on treatment with taka-amylase.[284] But the phosphorus content of the various starches is generally so low that the substitution with phosphoric acid probably plays a very subordinate role in limit dextrin formation.

On the whole we may suggest as a basic proposition that the course of amylolysis is determined: (*a*) by the occurrence in the substrate molecules of "anomalous" 1,6-α-glucosidic linkages, which are not ruptured by the amylases, and (*b*) by the fact that the amylases, although their only action is a rupture of 1,4-α-glucosidic linkages, do not attack these linkages indiscriminately. The hydrolyzability of a certain 1,4-α-glucosidic linkage depends on its situation in the substrate molecule, especially its distance from anomalies as end groups, branching points, etc.

Neither α- nor β-amylases have any action on simple glucosides,[285] nor do they hydrolyze α- or β-maltosides.[286] The fact that the simple α-maltosides are quite resistant to α-amylase should enable one to draw certain conclusions regarding the action of the enzyme on maltotriose (*vide infra*).

We must consider separately the action of the two types of amylases: (*a*) the exoamylase or saccharifying enzyme, represented by cereal β-amylase, and (*b*) the endoamylases or α-amylases, dextrinizing, or liquefying amylases. There is abundant evidence that the various types of enzymatic hydrolysis of starch and glycogen are brought about by these two types of amylases, separately or in mixture. No liquefaction of starch paste is known which is not caused by the dextrinizing α-amylases,[287] the existence of the alleged liquefying amylophosphatase being very doubtful.

[280] K. Myrbäck and K. Ahlborg, *Biochem. Z.* **307**, 69 (1940). K. Myrbäck, *J. prakt. Chem.* **162**, 29 (1943). K. Myrbäck, *Svensk Kem. Tid.* **58**, 119 (1946).

[281] T. Posternak and H. Pollaczek, *Helv. Chim. Acta* **24**, 921 (1941).

[282] T. Posternak, *Bull. soc. chim. France* (4) **27**, 507, 564 (1920); *Compt. rend.* **197**, 1157 (1933); **198**, 506 (1934); *Helv. Chim. Acta* **18**, 1351 (1935).

[283] J. H. Northrop and J. M. Nelson, *J. Am. Chem. Soc.* **38**, 472 (1916).

[284] J. Kerb, *Biochem. Z.* **100**, 3 (1919).

[285] J. Blom and B. Braae, *Enzymologia* **4**, 53 (1937).

[286] B. Helferich and S. R. Petersen, *Ber.* **68**, 790 (1935).

[287] C. M. Hollenbeck and M. J. Blish, *Cereal Chem.* **18**, 754 (1941).

It must be remembered that the dextrinizing α-amylases are, in a sense, saccharifying as well. The general difference between the two groups of enzymes is that β-amylase as an exoenzyme splits off maltose directly from the end chains of the polysaccharides, whereas the α-amylases in an initial rapid phase transform the polysaccharides into low-molecular but nonfermentable dextrins, a process identical with the so-called liquefaction. In a second, slow phase these primary dextrins (α-dextrins) are more or less completely saccharified, *i.e.*, transformed into a mixture of maltose, smaller amounts of glucose, and in most cases moderate amounts of limit dextrins containing the "anomalies" of the substrate molecules. This second phase of α-amylase action, which in the case of malt α-amylase is very much slower than the initial rapid dextrinization, is not due to contamination of the α-amylase with β-amylase but an action of the α-amylase itself.[288]

2. Mechanism of β-Amylase Action[239]

This enzyme, as already mentioned, attacks normal chain molecules (*i.e.*, those having the glucose residues united by 1,4-α-glucosidic linkages), from the nonreducing end group. The second linkage from the chain end is ruptured, giving birth to a maltose molecule. In other words, a saccharide or a nonreducing chain end like that represented in Fig. 13 is broken down in the following way: linkage 2 is hydrolyzed (*a*) if linkages 1 and 2 are

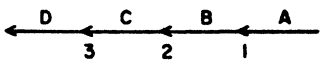

Fig. 13. Tetrasaccharide (normal α-dextrin) with four maltose linkages ("maltotetraose").

normal 1,4-α-glucosidic linkages, (*b*) if 3 is an α-glucosidic 1,4 or 1,6 linkage (3 may possibly be any glucosidic linkage whatsoever, but nothing is known about other linkages than those mentioned), and (*c*) if A, B, and C are otherwise unsubstituted. D may be engaged in a glucosidic linkage or it may carry a free pseudoaldehydic group or even a carboxyl group.[289]

If a substrate molecule contains maltose linkages exclusively (as in the original Haworth starch formula), it is completely saccharified by β-amylase. This means that unbranched amylose is saccharified to 100%, which, in its turn, means that any normal saccharide containing 4 or more glucose units and maltose linkages is hydrolyzed. The complete saccharification of amylose seems well established by now.[290] Doubts in this respect were founded on experiments in which contamination of the amylose with amylopectin or retrogradation of the amylose solution were the causes of incomplete conversion.

[288] K. Myrbäck and B. Lundberg, *Svensk Kem. Tid.* **55**, 36 (1943). K. Myrbäck and P. J. Palmcrantz, *Arkiv Kemi Mineral. Geol.* **18A**, No. 6 (1944).

[289] K. Myrbäck and K. Ahlborg, *Biochem. Z.* **311**, 213 (1942). K. Myrbäck and G. Nycander, *ibid.* **311**, 235 (1942).

It should, however, be mentioned that Peat *et al.* (First International Congress of Biochemistry, Cambridge, England, 1949), using crystalline β-amylase from sweet potato, have found a saccharification limit of native amylose at 70%, whereas the same amylose preparations were completely saccharified by soybean or barley β-amylase. These enzymes also saccharified the "limit dextrin" of sweet potato β-amylase. Amylose synthesized by crystalline muscle phosphorylase was found to be completely saccharified even by sweet potato crystalline β-amylase. If these results are not caused by the physical state of the substrate solutions (retrogradation), one would have to conclude that native amylose contains some sort of anomaly, and that the barley and soybean β-amylase contain at least two different enzymes, acting in amylose degradation.[290a]

It appears that the shortest normal saccharide to be attacked by β-amylase is the tetrasaccharide ("maltotetraose"). Maltotriose is not hydrolyzed.[273] Chain molecules with an uneven number of glucose units therefore must yield one molecule of maltotriose. In the case of amylose with several hundreds of glucose units per molecule the yield of maltotriose will be very small, but if amylose (or starch or glycogen) is primarily degraded by α-amylases to some extent, the low-molecular dextrins (α-dextrins) so formed in all probability contain considerable amounts of chains with an uneven number of glucose units (penta- and heptasaccharides, etc.). On treatment with β-amylase such products will yield fairly large amounts of maltotriose.[291]

The reducing end group of a normal saccharide chain has no active role in the attachment of the β-amylase molecule or in the enzymatic action; it can be oxidized to a carboxyl group without impairing the enzyme activity.[289] If, for instance, a normal hexasaccharide (maltohexose) is oxidized to the corresponding "-onic" acid, this is split by β-amylase into two molecules of maltose and one molecule of maltonic acid. The terminal maltose unit of the hexasaccharide is, however, split off more easily than the second, the carboxyl group (possibly also the aldehyde group in the normal saccharide) having a perceptible "anomaly" action.

Solutions of ordinary starches yield 53–60% sugar with β-amylase, the sugar being pure maltose.[128, 292] The determination of the saccharification limit is not easy since most β-amylase raw materials contain small amounts of α-amylase, which of course tend to increase the saccharification. Blom *et al.*[128] working at pH 3.6, where α-amylase is very rapidly inactivated,

[289] M. Samec and E. Waldschmidt-Leitz, *Z. physiol. Chem.* **203**, 16 (1931). G. G. Freeman and R. H. Hopkins, *Biochem. J.* **30**, 446 (1926). K. H. Meyer, P. Bernfeld, and J. Press, *Helv. Chim. Acta* **23**, 1465 (1940); **24**, 50 (1941). K. Myrbäck and W. Thorsell, *Svensk Kem. Tid.* **54**, 50 (1942). R. H. Hopkins, B. Jelinek, and L. E. Harrison, *Biochem. J.* **43**, 32 (1948). F. C. Cleveland and R. W. Kerr, *Cereal Chem.* **25**, 133 (1948). P. Bernfeld and P. Gürtler, *Helv. Chim. Acta* **31**, 106 (1948).

[290a] S. Peat, W. J. Whelan, and S. J. Pirt, *Nature* **164**, 499 (1949).

[291] K. Myrbäck and R. Lundén, *Arkiv Kemi Mineral. Geol.* **23A**, No. 7 (1946).

[292] J. L. Baker, *J. Chem. Soc.* **81**, 1177 (1902). C. S. Hanes, *Can. J. Research* **13B**, 185 (1935). G. G. Freeman and R. H. Hopkins, *Biochem. J.* **30**, 442 (1936).

found the value 53%, which they considered to be the true limit value. Hopkins *et al.*,[272, 293] however, believe 56–57% (pH 4.6) to be a truer value, since considerable inactivation of β-amylase occurs in more acid solution. Most authors give values about 60%. According to Hopkins the limit value varies somewhat with the starch employed, but retrogradation is not the cause.

If pure β-amylase degrades amylose only to 70% (Peat *et al.*, see above) it should be expected that the saccharification limit of starch is lower than 60%. Since these authors report that treatment of soybean β-amylase solution with acid at pH 3.6 causes the saccharification of amylose to stop at 70%, it might be that acid treatment destroys the hypothetical second enzyme in soybean β-amylase. This could be an explanation of the low limit of saccharification found by Blom.

Since the ordinary starches contain about 25% amylose, it should be expected that pure amylopectin would yield 50% maltose at most. Hodge, Montgomery, and Hilbert[294] found the value 55%, calculated as maltose hydrate. No differences were found between amylopectins from corn, wheat, sweet potato, potato, and tapioca. Meyer *et al.*[295] obtained 62% maltose from corn amylopectin and 59% from potato amylopectin. These values, which do seem high, are thought to support the ideas concerning the enzymatic synthesis of the polysaccharides which have been proposed by Bernfeld and Meutémédian.[243] (The problem of the synthesis had been treated earlier from a mathematical point of view and much the same ideas expressed by Sillén and Myrbäck.[296])

The saccharification of glycogen by β-amylase has been thoroughly investigated by Meyer *et al.*[297]

Different fractions of muscle glycogen gave the values 32–43% maltose; yeast glycogen gave 46–48%. Glycogen treated with acid for a short period gave 53% maltose. It is supposed that the big enzyme molecule cannot penetrate into the complicated network of the native glycogen molecules. Morris[298] found the value 45% for rabbit liver glycogen, but only 28% with corn glycogen. Meyer and Fuld,[299] however, found no difference between corn glycogen and the animal polysaccharide. Carlqvist[300] working with Hofmann-La Roche glycogen found a yield of 38–46% maltose,

[293] R. H. Hopkins, R. H. Murray, and A. R. Lockwood, *Biochem. J.* **40**, 507 (1946).

[294] J. E. Hodge, E. M. Montgomery, and G. E. Hilbert, *Cereal Chem.* **25**, 19 (1948).

[295] K. H. Meyer, P. Bernfeld, P. Rathgeb, and P. Gürtler, *Helv. Chim. Acta* **31**, 1536 (1948).

[296] L. G. Sillén and K. Myrbäck, *Svensk Kem. Tid.* **55**, 294, 354 (1943). K. Myrbäck and L. G. Sillén, *ibid.* **55**, 311 (1943); **56**, 61 (1944); *Nature* **163**, 410 (1949); *Acta Chem. Scand.* **3**, 190 (1949).

[297] K. H. Meyer and J. Press, *Helv. Chim. Acta* **24**, 50, 58 (1941). K. H. Meyer and R. Jeanloz, *ibid.* **26**, 1784 (1943). R. Jeanloz, *ibid.* **27**, 1501 (1944). K. H. Meyer, *Advances in Enzymol.* **3**, 109 (1942). K. H. Meyer, P. Bernfeld, P. Rathgeb, and P. Gürtler, *Helv. Chim. Acta* **31**, 1536 (1948).

[298] D. L. Morris, *J. Biol. Chem.* **154**, 503 (1944).

[299] K. H. Meyer and M. Fuld, *Helv. Chim. Acta* **32**, 757 (1949).

[300] B. Carlqvist, *Acta Chem. Scand.* **2**, 770 (1948).

i.e., a rupture of 19–23% of the glycosidic linkages of the substrate. After about 10% of the linkages have been ruptured, the velocity decreases considerably. Since there are, in glycogen, about 9 end chains per 100 glucose units, this fact intimates that, on the average, 1 maltose molecule is rapidly split off from each end chain, while the second or third one are loosened rather slowly.

It is to be concluded that the action of β-amylase on starch, amylopectin, or glycogen solutions, in general agreement with the theory of the anomalies, consists in (*a*) total degradation of the unbranched chains (amylose), and (*b*) saccharification of the end chains of amylopectin and glycogen.

The yield of maltose from these substrates is therefore a measure of the relative number of glucose units situated in the end chains of the polysaccharides. The values found agree rather well with those predicted by Sillén and Myrbäck[296] on the basis of a calculation of random synthesis of 1,4- and 1,6-glucosidic linkages by phosphorylase and isophosphorylase.

In addition to maltose there are formed from the branched polysaccharides practically nonreducing limit dextrins, the β-amylase limit dextrins, or simply β-dextrins. Many names for these substances appear in the literature: erythrogranulose, α-amylodextrin, etc. The β-dextrin from starch is generally said to be colored blue or purple by iodine. This is a little surprising, since amylopectin from which it originates is reported to give only a reddish color. It may be that traces of undegraded amylose, caught in the network of the amylopectin, cause the blueish color.

The fact is important that the β-dextrin from amylopectin has an end group for each 11 to 12 glucose units.[301, 302] Since the corresponding value for amylopectin is about 18 and since about 50% of the glucose residues are removed by β-amylase, one would expect β-dextrin to have 1 end group for about 9 glucose units. In any case it is quite clear that the real number of end groups is not much lower than that of the parent amylopectin molecule. The same result was obtained by Meyer and Fuld[303] in the case of glycogen.

When the end chains of amylopectin or glycogen are saccharified by β-amylase, the new end groups of the β-dextrin would conceivably be those marked A–D in Fig.14, or possibly those marked A, B, E, and F, if the enzyme is capable of liberating 1 maltose molecule from each of C and D. In the first case the β-dextrin would have the same real number of end groups as the parent molecule, but if the end groups are A, B, E, and F, the number of end groups in the β-dextrin would be about 75% of that of the parent amylopectin molecule.[304] The experimentally found end group values for amylopectin and for the corresponding β-dextrin are probably not exact enough to permit the decision between these two possibilities. If C and D are the real end groups, the specificity of the β-amylase is that presented above, but if E

[301] W. N. Haworth, E. L. Hirst, D. Kitchen, and S. Peat, *J. Chem. Soc.* **1937**, 791.
[302] K. H. Meyer, M. Wertheim, and P. Bernfeld, *Helv. Chim. Acta* **24**, 212 (1941).
[303] K. H. Meyer and M. Fuld, *Helv. Chim. Acta* **24**, 375 (1944).
[304] Not 50% as erroneously stated by Myrbäck.[296]

and F are the real end groups, linkage 2 in Fig. 13 must be split even if glucose residue C is substituted by a glucosyl unit in the 6 position.

Traces of α-amylase in β-amylase preparations may conveniently be detected by studying their action on β-dextrin, and colorimetric determinations of α-amylases may be performed with β-dextrin as a substrate.[305]

It is clear that studies of β-amylase action together with results from other fields of starch chemistry prove that amylopectin and glycogen have branched molecules. But it is not possible, on the basis of the β-amylase experiments alone to decide between the branched formulas proposed by different authors. If in the Staudinger formula the side chains are thought to have an average length of about 10 units and their average distance from each other on the principal chain is supposed to be 8–10 units, saccharification with β-amylase may be understood. The same may be said about the "laminated" formula of Haworth. Meyer[306] has pointed out that, if

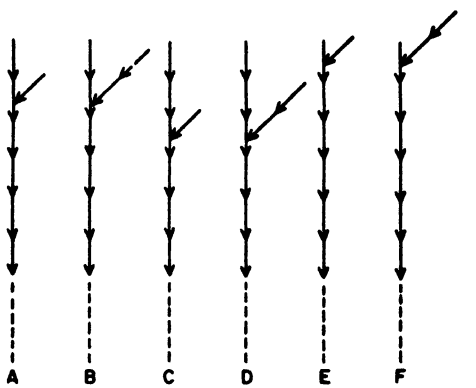

FIG. 14. End groups of β-amylase limit dextrin

amylopectin is supposed to have a formula of this kind, β-dextrin must be practically a simple chain molecule, on which only very short stubs of the side chains remain. Meyer finds that β-dextrin acetate does not have the capacity to form films of considerable solidity which would be expected for a molecule of this kind. Meyer therefore, also on this ground, prefers the irregularly branched formulas for amylopectin and glycogen which he originally founded on experiments on the action of a yeast enzyme, presumably isophosphorylase (page 697), on β-dextrin. Myrbäck[239] has pointed out that the current ideas concerning the enzymatic synthesis of polysaccharides seem to support the assumption of irregularly branched molecules similar to that proposed by Meyer.

[305] E. G. Hoskam, *Biochim. et Biophys. Acta* **1**, 419 (1947).
[306] K. H. Meyer, P. Gürtler, and P. Bernfeld, *Nature* **160**, 900 (1947).

3. Mechanism of α-Amylase Action

a. Malt α-Amylase

If the action of this enzyme on starch is determined reductometrically and the degree of hydrolysis is plotted against time, a curve like curve I in Fig. 15 is obtained.[239] The reaction evidently resolves itself into two phases with widely different velocities: an initial, rapid phase in which about 17% of the glucosidic linkages of the substrate are ruptured and a second, considerably slower phase showing no definite limit in reasonable

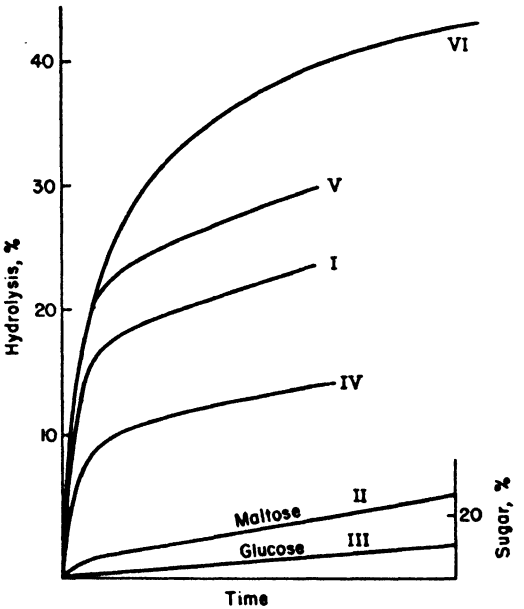

FIG. 15. (I) Starch and malt α-amylase; (II) and (III) formation of fermentable sugars; (IV) glycogen and malt α-amylase; (V) amylose and malt β-amylase; (VI) starch and animal α-amylase.

time. The initial phase is accompanied by an extremely rapid decrease in viscosity, and the products of this phase are not at all colored by iodine. These products evidently have an *average* chain length of about 6 glucose units. This initial phase of α-amylase action is the *dextrinization* of the substrate and the products we call α-dextrins. They correspond roughly to what has previously been called achroodextrins. Fermentable sugars are formed from the beginning of the reaction but in relatively small amounts[307] (curves II and III, Fig. 15). When the 17% hydrolysis stage is reached only about 10% of the substrate is fermentable with ordinary yeast; about 90%

[307] K. Myrbäck, *Biochem. Z.* **311**, 227 (1942).

is recovered as a mixture of reducing α-dextrins having an average chain length of about 8 glucose units. All these facts are in agreement with the assumption that the α-amylases are endoamylases, capable of rupturing glucosidic linkages far from end groups, not only in end chains but also between branching points of the substrate molecules.

In the second, slow phase of malt α-amylase action on starch, increasing amounts of fermentable sugars are formed; this phase is suitably called the saccharification phase. The reduction value increases steadily and may, after an extended reaction time, correspond to an apparent yield of more than 100% maltose. Fermentation experiments show that maltose is the main product, but glucose is also formed as a primary product (*i.e.*, not from maltose). The higher reduction value of the glucose is counterbalanced by the lower reduction value of the limit dextrins formed at the same time.

As mentioned before the saccharification phase is not due to contamination of the enzyme with β-amylase. Purification of the α-amylase, fractional inactivation by heating,[208] or partial adsorbtion of the enzyme on different adsorbents[210] do not alter the hydrolysis curve nor the relation between the velocities of the two phases and the time necessary to change the starch so that it is no longer colored by iodine. It is also necessary to know if the last extremely slow stages of the saccharification are really due to the α-amylase and not to other carbohydrases accidentally present in the α-amylase solution. Myrbäck and Sihlbom[208] have compared crude and purified preparations in this respect. No differences in the saccharification curve were observed and it seems possible to state that the hydrolysis curve is really characteristic of malt α-amylase.

The curve is substantially the same for all native starches investigated.[209, 209] It is the same also with soluble starches and substrates prepared from starch which by acid degradation have completely lost all paste-forming capacity. This is one proof among others that the form of the hydrolysis curve is not caused by retrogradation of the substrate. If a substrate is used which has been obtained by a very strong degradation of starch by acid, the hydrolysis curve alters its shape.[210] The relative number of linkages ruptured in the dextrinization phase (*i.e.*, with the high initial velocity) decreases (Fig. 16). If starch is degraded by acid to an average chain length of about five glucose units, no dextrinization phase is observed at all. The saccharification phase, however, remains substantially unaltered.

The explanation of this behavior of malt α-amylase toward substrates of varying chain lengths is that the enzyme is able to attack interior linkages (relatively far from end groups or other anomalies) of fairly long chains

[208] K. Myrbäck and E. Sihlbom, *Arkiv Kemi* **1**, 1 (1949).

[209] B. Örtenblad and K. Myrbäck, *Biochem. Z.* **307**, 123 (1941). K. Myrbäck and B. Örtenblad, *ibid.* **316**, 429 (1944). K. Myrbäck and B. Martelius, *ibid.* **316**, 414 (1944). K. Myrbäck and G. Stenlid, *Svensk Kem. Tid.* **54** 103, (1942). K. Myrbäck, G. Stenlid, and G. Nycander, *Biochem. Z.* **316**, 433 (1944).

[210] K. Myrbäck and W. Thorsell, *Svensk Kem. Tid.* **55**, 178 (1943).

at a much higher velocity than it attacks linkages in short-chain molecules. This, in turn, is due to the fact that malt α-amylase has a very high affinity for substrate molecules of great length but a low affinity for chain molecules shorter than a certain number of glucose units; this number should not be far from 8. The easily split linkages in the high-molecular substrates are in themselves in no way different from the majority of the 1,4-linkages in starch. The cause for their being split at a high velocity is that they are far enough removed from end groups or other anomalies. It seems that, in an endless chain molecule with 1,4-α-glucosidic linkages exclusively, the

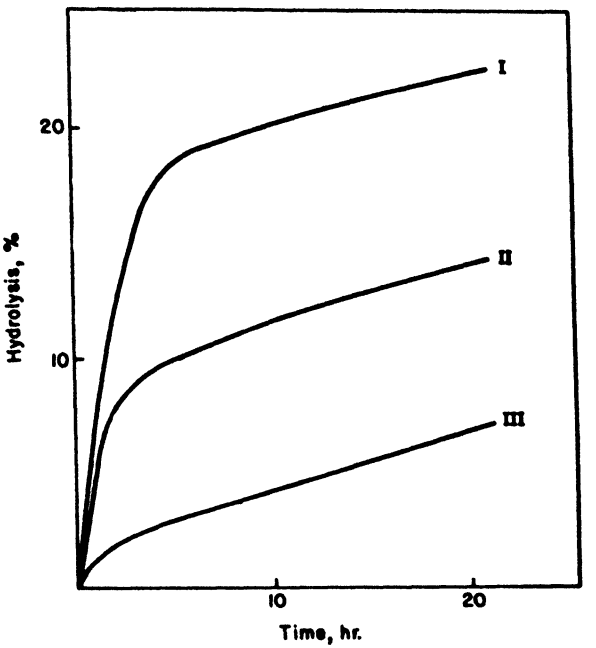

FIG. 16. Action of malt α-amylase on starch paste, pretreated with acid. (I) Untreated starch; (II) average degree of polymerization 9; (III) average degree of polymerization 5.5.

probability of being attacked by the malt α-amylase is the same for all linkages. In short chains or in chains containing anomalies as branching points, the enzyme is influenced by the end groups and anomalies in a way that decreases the enzyme–substrate affinity and reduces the velocity of hydrolysis of certain linkages (*vide infra*).

If malt α-amylase action on starch is interrupted at about 17% hydrolysis[329] and the products are fractionated after removal of the fermentable sugars, one finds that the α-dextrins are not homomolecular; however, the differences in chain length are not very great, from 4 to about 12 units

being represented. The composition will of course depend very strongly on the precise degree of hydrolysis at which the experiment was interrupted. The general nature of the α-dextrins can be ascertained to some degree by studying their degradation with β-amylase.[311] One finds that certain fractions are practically completely transformed into maltose; these α-dextrins are thought to be "normal," *i.e.*, to contain only maltose linkages. These fractions generally contain saccharides with 4–8 glucose units and the hexasaccharides seem to preponderate.

Other fractions, generally having a somewhat higher average chain length, are incompletely saccharified by β-amylase. They yield a certain amount of fermentable sugar, chiefly maltose, but nonfermentable, low-molecular limit dextrins as well. It is concluded that these α-dextrin fractions, the "anomalous α-dextrins" contain one or in some cases possibly more than one isomaltose linkage, or in other words that they contain side chains (B, Fig. 3). A molecule of this type will be only partially saccharified by β-amylase, the part enclosed in the dotted line being converted to maltose, the remainder (with the isomaltose linkage) being left as a limit dextrin. Normal α-dextrins, as E in Fig. 3, are completely saccharified and leave no limit dextrins.

It is believed that the normal unbranched α-dextrins originate from the amylose and from the relatively long end chains of amylopectin (or glycogen), and that the anomalous α-dextrins are formed from the branched "nucleus" of amylopectin or glycogen by rupture of solitary linkages between ramification points.

When malt α-amylase acts on glycogen, a curve like curve IV in Fig. 15 is observed. The dextrinization phase is shorter, obviously because there is no amylose component in glycogen and because the end chains are shorter on the average than those of amylopectin. Approximately the same curve is obtained if the enzyme acts on β-amylase limit dextrin. This is explained by the fact that in this substrate the amylose and the end chains of the amylopectin are already removed. On the other hand the interior chains of amylopectin are longer than those of glycogen. From β-dextrin, α-amylase evidently produces anomalous α-dextrins almost exclusively.

In experiments with malt α-amylase and amylose Myrbäck and Thorsell[312] found a hydrolysis curve (curve V, Fig. 15) of the same general shape as the curve observed with starch as a substrate. In contradiction to these results Bernfeld and Studer-Pécha[313] found that the rapid phase of malt α-amylase action on amylose continues to a degree of hydrolysis of 40–45%. They explain the slow increase of the reduction value after that to a slow hydrolysis of maltotriose, which probably is the correct interpretation

[311] K. Myrbäck, *Biochem. Z.* **307**, 132 (1941).
[312] K. Myrbäck and W. Thorsell, *Svensk Kem. Tid.* **54**, 50 (1942).
[313] P. Bernfeld and H. Studer-Pécha, *Helv. Chim. Acta* **30**, 1895 (1947).

of this part of the curve. The break in the curve at about 22% hydrolysis found by Myrbäck and Thorsell (Fig. 15) is ascribed by Bernfeld and Studer-Pécha to retrogradation of the substrate. However, several facts prove that this interpretation is untenable. The fact that the reaction mixture at 22% hydrolysis after boiling or treatment with alkali gives no color at all with iodine shows that no unchanged substrate is present. If during the first rapid phase of the reaction (below 22% hydrolysis) samples are removed, treated with alkali, neutralized, and used for potentiometric determination of amylose, it appears that the total amount of amylose is broken down to low-molecular dextrins before the stage of 22% hydrolysis is reached (Neumüller, unpublished). This is in good agreement with the fact that, in the experiments of Myrbäck and Thorsell, at 22% hydrolysis practically all amylose could be recovered in the form of low-molecular α-dextrins with 4–10 glucose units per molecule. These and other facts show that the curve V of Fig. 15 is not caused by retrogradation of the amylose solution. The same curve was found irrespective of the previous treatment of the solution and the same amylose solutions were saccharified to 100% by barley β-amylase. It is the authors' opinion that in the experiments of Bernfeld and Studer-Pécha the initial velocity was so high that the break in the curve at 22% hydrolysis escaped observation.

It seems permissible to conclude that in the action of malt α-amylase on amylose there is an initial rapid dextrinization period, which in this case comprises some 22% of the linkages of the substrate (compared to about 17% in the case of starch). It should be emphasized at this point that the explanation of the results mentioned does not involve any assumption of more than one kind of glucosidic linkage in amylose.

In a large-scale experiment[312] the hydrolysis of amylose by malt α-amylase was stopped at 22.4% hydrolysis. There was present 17% fermentable sugar, maltose, presumably maltotriose, but only traces of glucose. The fermentable sugar was removed and the α-dextrins fractionated. The average chain length of the six fractions isolated varied between 4 and 10 units. No high-molecular products were present. It is easy to show mathematically that this composition of the degradation products is unthinkable if it is supposed that the enzyme attacks all glucosidic linkages of the substrate with the same probability. Even the additional hypothesis of Meyer that one linkage at each end of the chain is stable or split at a greatly reduced velocity is not sufficient to explain the facts. It is necessary to assume that more than one linkage at the chain ends is more or less protected against the enzyme action. The action of the enzyme on amylose is easily understood if it is assumed, as above, that the enzyme has a high affinity for chains longer than about 8 glucose units but a much lower affinity for shorter chains. This is equivalent to the assumption that the enzyme, in order to show its maximum activity, has to arrange 6–7 glucose units of a

chain molecule in a distinct pattern on its surface, as shown schematically in Fig. 17. End groups, side chains, any anomaly in the part in question of the substrate molecule more or less disturb its arrangement on the binding area of the enzyme molecule, decrease the affinity, and decrease the velocity of hydrolysis.

In a long-chain molecule such as amylose, all linkages are attacked with about the same probability except a certain number, probably not far from six, glucosidic linkages, distributed on both chain ends.[314] These linkages will be more or less protected against the enzyme action because of the low affinity of the enzyme for the terminal parts of the chain. If the chain is very short as in the α-dextrins, both end groups at a time will probably influence the affinity or in other words the hydrolyzability of all the glucosidic linkages, and if the chain is somewhat longer, say 10–12 units, it is probable that the linkage primarily attacked by the enzyme will be close to the middle of the chain. It is then quite understandable that a long-chain molecule such as amylose, under the influence of an enzyme with this type of specificity, will eventually yield a mixture of α-dextrins in which those

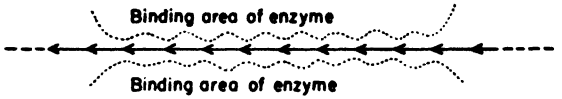

FIG. 17. Fixation of a chain molecule on the surface of the malt α-amylase molecule. About eight glucose units are arranged in a definite pattern on the binding area. This corresponds to maximum enzyme–substrate affinity.

of 6–7 units will predominate. Since both end groups are not of the same kind it is probable that the number of "protected" linkages is not the same at both ends. The problem has been treated in detail by Myrbäck and Sillén.[314]

The formation of glucose in the later stages of saccharification shows that the enzyme can hydrolyze even one terminal linkage. The velocity is, however, very low. It seems that maltotriose, which is formed in the early stages of hydrolysis by α-amylase disappears again on prolonged action of the enzyme.[308] Fig. 18 gives a schematic picture of the maltotriose molecule. Evidently one of the two linkages can be ruptured by α-amylase. Since Myrbäck and Nycander[289] found that no glucose was formed from an "-onic" acid corresponding to a hexasaccharide, whereas the saccharide itself yielded considerable amounts of glucose, it seems permissible to assume that the reducing end group of a saccharide is split off as glucose by malt α-amylase. It seems that the enzyme can attack linkage 2, but not linkage 1 (Fig. 18).

[314] K. Myrbäck and L. G. Sillén, *Svensk Kem. Tid.* **56**, 142 (1944).

When α-amylase is allowed to act on starch or amylose for a prolonged time, maltose invariably is the chief product. Since fermentable sugars are formed by hydrolysis of the α-dextrins and since in the normal α-dextrins hexasaccharides preponderate, one might ask if in a normal hexasaccharide (Fig. 19) linkages 2 and 4 are more easily ruptured than linkage 3. Myrbäck found in an experiment after a relatively short reaction time 17% glucose, 60% maltose, and 23% maltotriose.[315] However, it is not possible to decide if the maltose was split off from the hexasaccharide as such or was formed by secondary hydrolysis of maltotriose.

As already mentioned it is highly probable that, in a branched molecule, the presence of a branching or any other anomaly at a certain point renders the attachment of the enzyme more difficult and therefore decreases the affinity of the enzyme for this part of the molecule. The presence of the anomalies causes a steric hindrance; they prevent the enzyme from "getting hold" of the chain. A certain number of normal linkages in the vicinity of an anomaly will be more or less protected against the enzyme action. Therefore the end chains of amylopectin and glycogen will be primarily attacked

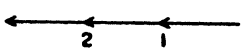

FIG. 18. Maltotriose

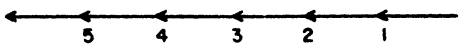

FIG. 19. Hexasaccharide with maltose linkages (normal α-dextrin, "maltohexaose")

as far as possible from the end group and from the branching point, and correspondingly the interior chains will be attacked somewhere in the middle, or possibly not attacked at all if they happen to be very short.

The differences between the theory of α-amylase action outlined here and that advocated by Meyer and associates is not very great. Meyer assumes that only one terminal linkage at each end of a normal chain is split at a strongly reduced velocity. Hence maltotriose, which has only terminal linkages, is split very slowly. However, it is a fact also that oligosaccharides containing from four to about eight glucose units and maltose linkages exclusively are hydrolyzed at a velocity which is only very small compared to that of starch or amylose. Myrbäck and Nycander[289] found that the velocity of hydrolysis of a normal hexasaccharide was only 17% of that of the hydrolysis of soluble starch. It is therefore evident that not only one terminal linkage at each end of the chain is ruptured at a reduced velocity.

b. Other α-Amylases

Malt α-amylase has an action on the substrates, especially starch, which differs somewhat from that of the other more or less well known α-amyl-

[315] K. Myrbäck, *Arch. Biochem.* **14**, 53 (1947).

ases.[315, 316] This does not mean that malt α-amylase and other α-amylases should yield substantially different reaction products; the form of the hydrolysis curve, however, is at least different. The animal amylases and most other α-amylases give curves, when the degree of hydrolysis is plotted against time, which do not deviate very much from the monomolecular form, and thus display no distinct breaks (curve VI, Fig. 15). In these cases, therefore, it is not possible to distinguish sharply between the dextrinization and the saccharification phase. On the other hand, the rapid initial decrease in viscosity of a starch paste and the loss of the color reaction with iodine characterize the action of these enzymes as primarily dextrinizing. It is also clear that in the dextrinization by these enzymes substantially the same products, the α-dextrins, are formed as in the case of malt α-amylase. As early as 1934 Waldschmidt-Leitz and Reichel[317] found that fairly large amounts of hexasaccharides could be isolated after dextrinization with pancreatic amylase. "Erythrodextrins" with higher degree of polymerization were isolated by Köhler-Hollander.[318]

Bernfeld and Fuld[319] have determined the ratio between dextrinogenic and saccharogenic power for different α-amylases and found a practically constant value (Table III). They conclude that the enzymes have the same action on starch. This is true without doubt for the dextrinization phase of the reaction; the ratios between the velocities as measured by iodine coloration, viscosity, and liberation of reducing groups are nearly constant. This means that until the "achroic" stage (stage of the α-dextrins) the reactions are practically identical. But later on, in the true saccharification phase (as defined earlier in this paper), the differences are obvious.

The mode of action of the α-amylases in general is the same; they are endoamylases and have a high affinity for normal parts of substrate molecules far from end groups and other anomalies. These more or less block the attachment of the enzyme molecules to the substrate chains. As in the case of malt α-amylase the other α-amylases have the highest affinity for such parts of the substrate molecules which are normally built over a length of at least about eight glucose units. Hence the rapid dextrinization is observed, which is, however, accompanied by formation of fermentable sugars from the beginning. The form of the hydrolysis curves shows that the rapidity of the saccharification, as measured by the increase in reducing groups, in these cases is not very much lower than that of the initial dextrinization phase. In the case of malt α-amylase on the other hand, the velocity of the saccharification is, as already mentioned, very low compared to that of the dextrinization. It is therefore to be concluded that the affinities of the other α-amylases for long-chain molecules on one hand, and for chains shorter than, for example, 8 units on the other, are not so pro-

[316] H. C. Sherman and J. C. Baker, *J. Am. Chem. Soc.* **38**, 1885 (1916).
[317] E. Waldschmidt-Leitz and M. Reichel, *Z. physiol. Chem.* **223**, 76 (1934).
[318] L. Köhler-Hollander, *Z. physiol. Chem.* **228**, 249 (1934).
[319] P. Bernfeld and M. Fuld, *Helv. Chim. Acta* **31**, 1423 (1948).

nounced as in the case of malt α-amylase. The arrangement of the substrate-binding groups on the enzyme surface is such that even comparatively short chains with, for instance, 4 or 6 glucose units can be relatively firmly bound and as a consequence thereof relatively rapidly hydrolyzed. In the case of malt α-amylase one is inclined to assume that for the "successful" attachment of a certain part of the substrate molecule on the enzyme surface so many substrate-binding groups in different positions are required that the full affinity and consequently the full enzyme activity is reached only if the enzyme has access to a normal part of a chain longer than about 8 glucose units (Fig. 17). If the available part of the chain is shorter than that, the affinity is very severely decreased probably because only a reduced number of the substrate-binding groups can exert their action.

The affinity of several α-amylases for starch has been determined by Bernfeld and Studer-Pécha[320] and the affinity constant (Michaelis-Menten constant) calculated according to Lineweaver and Burk.[321] The affinity decreases in the sequence: pancreatic, bacterial, and malt α-amylase. It must be noted that the values give no indication of the affinities for short-chain substrates. Investigations of the kinetics of starch degradation by several α-amylases have enabled Hopkins and Kulka[259] to draw certain conclusions regarding the affinity for low-grade dextrins.

The essential feature of the theory of α-amylase action outlined above is that the enzymes are thought to be endoamylases in the sense that they have an affinity for elements of substrate molecules far from end groups. Only such parts of the substrate molecules can be attacked by the enzymes which, over a length of a certain number of glucose units, have a "normal" constitution, *i.e.*, contain unsubstituted glucose residues united by 1,4-α-glucosidic linkage. It is believed that anomalous elements of constitution, as end groups, ramifications, and substitution with phosphoric acid, constitute a steric hindrance to the attachment of the enzyme to the substrate molecules. This means, for instance, that the affinity of the α-amylases to normal chain molecules decreases with the chain length of the substrate. The decrease in affinity with decreasing chain length is probably small until a certain critical chain length is reached, which in the case of malt α-amylase should be about eight glucose units, where, owing to the vicinity of the end groups (or other anomalies) a sudden and strong decrease in affinity occurs. This explains the peculiar form of the hydrolysis curve (Fig. 15). In other cases as with the animal amylases the decrease in affinity is less pronounced and may possibly occur at a different critical chain length. The velocity of the saccharification phase is therefore not very much lower than that of the dextrinization and no break occurs in the hydrolysis curve.

[320] P. Bernfeld and H. Studer-Pécha, *Helv. Chim. Acta* **30**, 1904 (1947).
[321] H. Lineweaver and D. Burk, *J. Am. Chem. Soc.* **56**, 658 (1934).

No special assumptions are made regarding a possible role for the enzyme action of the form of the substrate molecules, whether straight or, for instance, coiled to a close spiral. The idea of the helical coiling of the chain molecules with 1,4-α-glucosidic linkages was introduced by Hanes[217] and has proved extremely fruitful in several respects. It is, however, in the authors' opinion no evidence that such coiling of the substrate molecules should play an essential role in the degradation of polysaccharides by α-amylases. The phenomenon of the coiling may on the other hand be involved in the action of *Bacillus macerans* amylase.

VI. Action of Amylases on Raw Starch

Raw starch is obviously broken down in germinating seeds. It is also easily utilized in living animals. It is then somewhat surprising that most amylase preparations have extremely weak action on raw starch, so weak that it seems permissible to assume that the pure enzymes would have no action at all on absolutely undamaged starch granules. Mechanical damaging, as in ball mills etc., or treatment with certain chemicals greatly increase the susceptibility of the starch granules to amylase action.[222] The resistance of undamaged granules has been attributed to the existence of a protecting layer, thought by some to be composed of foreign matter, such as fat, protein, or hemicellulose. However, there seems to be no real proof of the existence of layers containing substances other than starch,[223] but the outermost layer of the granule may be supposed to be quite dense and more highly dehydrated than other parts of the granule.[224] Concerning the nature of the granules we shall refer here only to the comprehensive reviews given by Badenhuizen[5] and Frey-Wyssling.[6] It seems understandable that the large enzyme molecules cannot gain access to the substrate molecules in such a dense lattice structure. It seems probable that on mechanical damaging the lattice is frayed so that the chain molecules become accessible to the amylases. All authors seem to agree that the differences in susceptibility of different samples of starch is not due to granule size.

The properties of the granules are not the only determining factor; various amylases have a very different action. Stamberg and Bailey[224a] found that raw wheat starch was degraded by β-amylase only to 1%, whereas different preparations of α-amylase gave 4–10% hydrolysis. Raw wheat starch ground in a rod mill for 84 hours was easily hydrolyzed by both amylases, the rate being almost the same as with starch paste. Blish, Sandstedt, and Meecham[225] have found that wheat contains an enzymatic "raw starch factor," different from α-amylase, which accelerates the hydrolysis of raw starch.

Raw starch is utilized without difficulty by living animals. Balls and

[222] J. M. Newton, F. F. Farley, and N. M. Naylor, *Cereal Chem.* **17**, 342 (1942).
[223] C. L. Alsberg, *Proc. Exptl. Biol. Med.* **36**, 127 (1937).
[224] S. Schwimmer, *J. Biol. Chem.* **161**, 219 (1945).
[224a] O. E. Stamberg and C. H. Bailey, *Cereal Chem.* **16**, 319 (1939).
[225] M. J. Blish, R. M. Sandstedt, and D. K. Meecham, *Cereal Chem.* **14**, 605 (1937).

Schwimmer[326] therefore investigated crude pancreas extracts and mixtures thereof with other amylase preparations. A high activity was found in a mixture of pancreas extract and mold bran amylase. Schwimmer[324] finds that some enzymatic factor in addition to the recognized amylases is necessary for the rapid degradation of raw starch and believes it possible that this factor is maltase. The accelerating action should thus depend on the removal of maltose, a phenomenon which is not easy to understand but which seems to be borne out by experiments in which maltose was removed by dialysis.

Amylase action on raw starch plays an important role in bread making.

VII. Amylase of *Bacillus macerans*

Schardinger[327] found that *Bacillus macerans* when cultivated on starch-containing media produces considerable amounts of nonreducing dextrins, certain of which can readily be obtained in crystalline form. These dextrins have subsequently been called the Schardinger dextrins or cycloamyloses. Hudson *et al.*[328] as well as Freudenberg[329] were able to show that the conversion of starch into dextrins is due to the action of an enzyme, the *macerans* amylase, which is secreted by the bacillus into the culture medium.

The Schardinger Dextrins. The main constituents of the starch conversion mixture are α- and β-dextrins (α- and β-cycloamyloses). Detailed schemes of the purification have been given.[330, 331] The dextrins do not reduce Fehling solution or similar reagents. Since the molecular weight is low, about 1000, Freudenberg concluded that the Schardinger dextrins have a cyclic structure without end groups.[332] This is in accordance with methylation experiments. The methylated dextrins, on acid hydrolysis, yield more than 95% of 2,3,6-trimethylglucose. No tetramethyl- or 2,3,4-trimethylglucose are formed. Kratky and Schneidmesser[333] found, by X-ray measurements, that the α-dextrin contains 5, and the β-dextrin 6 glucose units. These values seem to be in accordance with molecular weight determinations of the methyl ethers by Freudenberg *et al.*[334] French and Rundle,[335]

[326] A. K. Balls and S. Schwimmer, *J. Biol. Chem.* **156**, 203 (1944).
[327] F. Schardinger, *Zentr. Bakt. Parasitenk.* II **29**, 188 (1911).
[328] E. B. Tilden and C. S. Hudson, *J. Am. Chem. Soc.* **61**, 2900 (1939).
[329] K. Freudenberg, E. Schaaf, G. Dumpert, and T. Ploetz, *Naturwissenschaften* **27**, 850 (1939).
[330] K. Freudenberg and R. Jacobi, *Ann.* **518**, 103 (1935).
[331] D. French, M. L. Levine, J. H. Pazur, and E. Norberg, *J. Am. Chem. Soc.* **71**, 353 (1949).
[332] K. Freudenberg, G. Blomquist, L. Ewald, and K. Soff, *Ber.* **69**, 1258 (1936). K. Freudenberg, H. Boppel, and M. Meyer-Delius, *Naturwissenschaften* **26**, 123 (1938). K. Freudenberg and M. Meyer-Delius, *Ber.* **71**, 1596 (1938).
[333] O. Kratky and B. Schneidmesser, *Ber.* **71**, 1413 (1938).
[334] K. Freudenberg and W. Rapp, *Ber.* **69**, 2041 (1936). K. Freudenberg, E. Plankenhorn, and H. Knauber, *Ann.* **558**, 1 (1947).
[335] D. French and R. E. Rundle, *J. Am. Chem. Soc.* **64**, 1561 (1942).

however, find that their X-ray measurements agree better with the assumption of 6 glucose units in the α-dextrin and 7 in the β-dextrin.

The Schardinger dextrins give characteristic compounds with iodine; the α-dextrin yields dark-purple hexagonal plates or greenish-yellow needles; the β-dextrin gives yellow prisms. The Tilden-Hudson test for the Schardinger dextrins is based on the observation of these crystals.[328] Compounds are also formed between the Schardinger dextrins and several organic solvents as tri- and tetrachloroethylene, benzene, etc. Freudenberg has presented an attractive theory on the nature of these and the iodine compounds. Data on the solubilities and other properties of the dextrins and the addition compounds are listed by French *et al.*[331]

Incomplete acid hydrolysis of the Schardinger dextrins produces a mixture of undegraded material with reducing saccharides of the maltose type.[332, 336, 336a] French *et al.*[337] have isolated a heptasaccharide, "amyloheptaose," from a hydrolyzate of the β-dextrin.

The B. macerans Amylase. The bacillus is grown on a potato medium. Enzyme formation is followed by determination of the action on starch by means of the Tilden-Hudson test or the decrease of viscosity. The culture medium is filtered through a Berkefeld candle. An approximately tenfold purification can be attained by precipitation with acetone and further purification by adsorption on aluminum hydroxide at pH 4.8 and elution with phosphate buffer at pH 7.6. In this way Hudson *et al.*[338] obtained preparations showing about 140 times the activity of the initial solution.

Action of the Enzyme. An enzyme unit may be calculated from measurement of the decrease in viscosity of a starch solution. It is not possible, however, in this way to distinguish between the *B. macerans* enzyme and ordinary α-amylases, and the determination must therefore be complemented by determination of the characteristic action, *i.e.*, the formation of the Schardinger dextrins, suitably by the Tilden-Hudson test or precipitation of the dextrins with trichloroethylene or other suitable solvents.

The pH optimum[339] of the *B. macerans* enzyme is about pH 6. At pH 4 or 8 the action is almost nil. Blinc and Samec[340] state that the dextrin formation takes place only in the pH range 5.5 to 6.5.

When the *B. macerans* enzyme acts on starch paste an initial drop in viscosity is observed. In an experiment by Myrbäck and Gjörling[339] the relative viscosity had dropped from 5.1 to 1.3 when the Schardinger dextrins began to appear. At the same time about 1% of the glucosidic linkages

[336] M. A. Swanson and C. F. Cori, *J. Biol. Chem.* **172**, 797 (1948).
[336a] K. Myrbäck and T. Järnström, *Arkiv Kemi* **1**, 129 (1949). K. Myrbäck, *ibid.* **1**, 161 (1949).
[337] D. French, M. L. Levine, and J. H. Pazur, *J. Am. Chem. Soc.* **71**, 356 (1949).
[338] E. B. Tilden, M. Adams, and C. S. Hudson, *J. Am. Chem. Soc.* **64**, 1432 (1942).
[339] K. Myrbäck and L. G. Gjörling, *Arkiv Kemi Mineral. Geol.* **20A**, No. 5 (1945).
[340] M. Blinc and M. Samec, *Z. physiol. Chem.* **282**, 149 (1947).

of the substrate had been ruptured, as judged from the increase in reduction. No fermentable sugars were present. If the viscosity is plotted against the reduction value much the same curve is obtained as in the case of malt α-amylase. This tends to show that the initial action of the *B. macerans* enzyme is about the same as in the case of the α-amylases; the polysaccharide molecules are cut up into dextrins having so low a degree of polymerization (on the average 100 glucose units or less) that their solution has an insignificant viscosity. After this initial phase or perhaps parallel to it, the Schardinger dextrins begin to appear. At this stage the reaction mixture usually gives a brownish-purple color with iodine. The yield of the dextrins increases with time, but the reduction value remains almost constant or increases slightly.[341] The maximum yield of Schardinger dextrins from starch seems to be 50–60%. This high yield precludes the possibility that the ring structures should be preformed in the starch. Evidently the rings are formed by the *B. macerans* enzyme from straight-chain molecules or parts of molecules in the substrates. It is suggested that the enzyme has the capacity to arrange the substrate chains in helical coils on its surface in such a way that when a glucosidic linkage is ruptured a "transglucosidation" occurs with the free end of the chain supposed to be in close proximity on the enzyme surface to the broken linkage. The coils are supposed to contain about six glucose units;[336a] this assumption seems the more reasonable as chain molecules of the amylose type have a tendency to assume a spiral configuration.

The yield of Schardinger dextrins depends, *ceteris paribus*, on the substrate in a characteristic manner,[342] amylose giving the highest yield of about 70% crystalline dextrins. The yield from amylopectin is considerably lower, 45–50%. Schardinger dextrins are produced also from glycogen but the yield is still lower. The enzyme seems to have no action on retrograded amylose. No dextrins are formed from β-amylase limit dextrin, and the yield from starch substrates previously subjected to acid hydrolysis decreases rapidly with increasing degree of hydrolysis. These facts support the idea that the Schardinger dextrins are formed from unbranched amylose chains and from those end chains in amylopectin, glycogen, or hydrolyzates thereof which have a sufficient length. In other words, the Schardinger dextrins are formed from the same material as is the maltose in experiments with β-amylase. The minimum length of a chain capable of being converted to a Schardinger dextrin is estimated by Kerr to be about 20 glucose units. In any case no Schardinger dextrins are formed from the

[341] W. C. McClenahan, E. B. Tilden, and C. S. Hudson, *J. Am. Chem. Soc.* **64**, 2139 (1942).

[342] E. J. Wilson, T. J. Schoch, and C. S. Hudson, *J. Am. Chem. Soc.* **65**, 1380 (1943). R. W. Kerr, *ibid.* **64**, 3044 (1942); **65**, 188 (1943).

α-dextrins with 4–10 units produced, for instance, by the action of malt α-amylase on starch.

The ratio between the yields of α- and β-dextrin varies considerably, but the cause is not known. Hudson *et al.*[341] found that when trichloroethylene or toluene was present during the digestion, the yield of the β-dextrin was strongly increased at the expense of the α-dextrin, but no explanation can be given at the present time.

The Schardinger dextrins are split into glucose by crude taka-diastase,[344] but are not hydrolyzed by the ordinary amylases and probably not by the *B. macerans* enzyme either. Nevertheless, in experiments with this enzyme and starch, it may be observed that the Schardinger dextrins formed in an early stage disappear again if the reaction time is extended over the normal. Myrbäck and Gjörling[339] found in an experiment of this kind that the dextrins were rather completely converted to maltose. The enzymatic homogeneity of the *B. macerans* amylase used was, of course, not proved, and the same may possibly be the case in the experiments of Hudson *et al.*, where the enzyme had a certain action on a pure solution of the α-dextrin; the optical rotation rose and a slight reducing action could be observed. Saccharification of the Schardinger dextrins was observed also by Kneen and Beckord.[343] French *et al.*[344] point out that, if the formation of the Schardinger dextrins is (as supposed above) an exchange of a glucosidic linkage in starch for a corresponding bond in the cyclic dextrins, the ΔF of the reaction would be so small that a reversion:

$$\text{cycloamylose} + \text{maltose} \xrightarrow[\text{enzyme}]{\textit{B. macerans}} \text{higher saccharides}$$

must be expected to occur. In experiments with α-dextrin and maltose (or certain other sugars) this reaction evidently took place, since the rotation rose considerably. The least soluble fraction of the reaction products was shown to contain open-chain saccharides with about 9 glucose units as an average. Since such saccharides should contain maltose linkages exclusively, they must be expected to be saccharified by ordinary amylases, and this is probably the explanation of experiments by Myrbäck and Willstaedt,[345] in which pure α-dextrin, mixed with maltose, was shown to be saccharified by a mixture of *B. macerans* amylase and β-amylase, whereas the single enzymes were found to be totally devoid of action.

[343] E. Kneen and L. D. Beckord, *Arch. Biochem.* **10**, 41 (1946).

[344] D. French, J. Pazur, M. L. Levine, and E. Norberg, *J. Am. Chem. Soc.* **70**, 3145 (1948); **71**, 356 (1949); E. Norberg and D. French, *ibid.* **72**, 1202 (1950); D. French, M. L. Levine, J. H. Pazur, and E. Norberg, *ibid.* **72**, 1746 (1950).

[345] K. Myrbäck and E. Willstaedt, *Acta Chem. Scand.* **3**, 91 (1949).