The CHEMISTRY *and* TECHNOLOGY *of* ENZYMES

by

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New York · JOHN WILEY & SONS, *Inc.*
London · CHAPMAN & HALL, *Limited*

SECOND PRINTING, MARCH, 1950

PRINTED IN THE UNITED STATES OF AMERICA

PREFACE

This volume deals with the *chemistry and technology of enzymes* and is an expansion of my recent book *Enzyme Technology*. I am very appreciative of the cordial reception which that book received. In accordance with the suggestion of several reviewers, I have included an extensive summary and much practical material concerning the chemistry of enzymes. This new book discusses almost all the known enzymes. The need for these additions was apparent to those interested in the technology of enzymes, since this science is obviously based on the chemistry of enzymes. To the technological part of the book, I have added a large amount of new material dealing with biosynthesis. Some pages have been replaced by more important recent work. Other sections, such as those containing the quantitative determination of enzymes, most parts on the production of enzymes, and almost all topics on general enzyme chemistry, have been placed in the first part of the book. As a result of the war effort, numerous industrially important findings have been described in the literature. Much of this work has been included in the present volume in considerable detail.

Thus, this new book contains an up-to-date review of enzyme chemistry; methods concerning the industrial production of enzymes from all known sources and their uses; the production of all the industrially important organic compounds by fermentation; and microbiological procedures for the quantitative determination of vitamins and amino acids.

I am very grateful to Dr. J. L. Stokes, of Merck and Company, for writing the section on the microbiological determination of amino acids, and to Dr. S. Redfern, of the Fleischmann Laboratories, for reading the whole manuscript and for offering many constructive suggestions.

I also wish to express my gratitude to Dr. G. L. Baker, University of Delaware; Dr. I. S. Kleiner, New York Medical College; Dr. C. C. Lindegren, Washington University; Dr. A. H. Mehler, New York University; Dr. S. Ochoa, New York University; Dr. M. A. Stahmann, University of Wisconsin; Dr. P. W. Wilson, University of Wisconsin; Dr. H. B. Woodruff, Merck and Company, who were kind enough to read parts of the manuscript and offered valuable suggestions, and to Mr. W. Goetz for going over the whole manuscript for correct chemical English.

I am also indebted to many of my colleagues who have been very cooperative in numerous ways and to the editors for giving permission to reproduce figures and tables from their journals.

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Staten Island, N. Y.

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PART I

THE CHEMISTRY OF ENZYMES

CHAPTER I

GENERAL CONSIDERATIONS

Enzymes are produced by the living cell, and no living thing can exist without them. Although animals are unable to produce the vitamins that they require for life, all living cells can produce their specific enzymes. In fact, each cell may be characterized by the type of enzymes it produces, and the origin of an impure enzyme preparation may be traced by the type of enzymes it contains. Enzyme-containing materials have been employed for practical purposes for thousands of years in baking, in preparing alcoholic beverages, and in medicaments. Pasteur demonstrated, as early as 1858, that racemic tartaric acid could be separated into dextro- and levo-tartaric acid by permitting the mixture to be fermented by a penicillium. Only the dextro form was fermented. Buchner was the first to prove that the living cell is not necessary for enzyme action. This was a very important step towards the understanding of cell-linked chemical reactions.

Recently, geneticists have shown that, within the cell, single genes control the synthesis of specific enzymes. Since enzymes are proteins which act on foods, they are, of course, part of intermediary metabolism. Although the genes, which perpetuate themselves by autocatalysis, are the actual regulators of intermediary metabolism, the enzymes are the agents that supply all living things with energy for work and for biosynthesis. These processes are usually called growth and life. Since genes determine the type of enzyme that a certain cell can synthesize, it is obvious that biosynthesis is also a gene-controlled sequence of enzymic reactions. Thus we may say: *Life is a series of catalytic reactions controlled by genes.* According to Spiegelman and Kamen (1)* these cytoplasmic self-duplicating units, the genes, compete with each other, and the result of such competitive interaction determines the enzymic composition of the cytoplasm. Inherent in this concept is changeability of the ultimate results, which depend upon the varying experimental conditions.

The animal body synthesizes many kinds of proteins from amino acids. It is not known how the pattern for these reactions is set.

* Numbers in parentheses refer to the numbered references at the end of the chapter.

Highly interesting in this connection is the synthesis of specific apoenzymes by the animal body, to which the organic crystalloid coenzymes (thiamin, riboflavin, codehydrases, etc.) must be supplied from plant sources in order to complete the specific enzyme.

Enzymes are known as biochemical catalysts. However, since there are many biochemical catalysts, a distinct differentiation must be assured between enzymes and non-enzymic catalysts. I wish to propose the following classification:

Definition of an Enzyme. Classification of Biochemical Catalysts

1. *Specific, cell-independent, biochemical catalysts or enzymes:* Catalysts which are produced by the living cell, but whose action is independent of the living cell, and which are destroyed if their solutions are heated long enough. They are all proteins. Examples: pepsin, trypsin, maltase, carboxylase, catalase, glucose oxidase.

2. *Specific, non-enzymic biochemical catalysts:* Catalysts produced by the living cell, active mainly *in vivo*. They may or may not be destroyed on being heated. Examples: genes, hormones, viruses.

Substances such as glutathione, ascorbic acid, cytochrome, and adenylic acid are not catalysts. These compounds undergo ordinary chemical changes (oxidation, reduction, etc.). However, they do take part in various enzymic reactions.

Classification of Enzymes

Enzymes may be divided into three definite classes on the basis of their chemical composition:

1. The enzyme molecule consists of protein only: pepsin, trypsin, urease, and many others.

2. The enzyme molecule contains a protein and a cation such as copper, zinc, magnesium: ascorbic acid oxidase, carbonic anhydrase, alkaline phosphatase, etc.

3. The enzyme molecule contains a protein, a non-protein low-molecular (heat-stable), organic compound, called a coenzyme or a prosthetic group. Sometimes it also contains a cation. In Table I are listed those enzymes that contain iron-porphyrin as the prosthetic group (catalase, peroxidases, and the intermediary catalyst cytochrome c). In Table II are listed the various enzymes containing flavin mononucleotides and flavin dinucleotides as their prosthetic groups. Many enzymes require diphosphothiamin (cocarboxylase) as their coenzyme; these are listed in Table III. Many others contain

nicotinamide nucleotides. Thus, diphosphopyridine nucleotide (codehydrogenase I) is required by lactic, malic, β-hydroxybutyric, glutamic, alcohol, and other dehydrogenases. Triphosphopyridine nucleotide (codehydrogenase II) is necessary for the dehydrogenation of

TABLE I

IRON-PORPHYRIN ENZYMES

Enzyme	Prosthetic Group	Remarks
Catalase	Iron-porphyrin	Decomposes hydrogen peroxide.
Peroxidase I and II; verdoperoxidase	Iron-porphyrin	Oxidize many compounds in the presence of hydrogen peroxide.
Cytochrome c (part of cytochrome c oxidase systems)	Iron-porphyrin	The cytochromes are carriers of electrons. They are not enzymes.

Robison ester, isocitric acid, phosphohexonic acid, glutamic acid, etc. Several other enzymes require different coenzymes (Table IV).

TABLE II

FLAVOPROTEIN ENZYMES

Enzyme	Coenzyme or Prosthetic Group	Substrate
Old yellow enzyme	Riboflavin phosphate	Robison ester
Cytochrome c reductase	Riboflavin phosphate	Triphosphopyridine nucleotide
l-Amino acid oxidase	Riboflavin phosphate	*l*-Amino acids, *l*-α hydroxy acids
Glycine oxidase	Riboflavin adenine dinucleotide	Glycine
d-Amino acid oxidase	Riboflavin adenine dinucleotide	*d*-Amino acids
Xanthine oxidase	Riboflavin adenine dinucleotide	Xanthine, etc.
Fumaric hydrogenase	Riboflavin adenine dinucleotide	Fumaric acid
Aldehyde oxidase	Riboflavin adenine dinucleotide	Aldehydes
Pyruvate oxidase	Riboflavin adenine dinucleotide	Pyruvic acid
Glucose oxidase	Riboflavin adenine dinucleotide	Glucose
Histamine or diamine oxidase	Flavin of unknown nature	Histamine and diamines

In addition to the enzymes in Table III, α-ketoglutaric oxidase has also been found to be a diphosphothiamin proteid.

Effect of Temperature

The velocity of enzyme action is accelerated as the temperature is increased until the optimum is attained, above which velocity decreases and enzyme activity discontinues. According to Arrhenius (2), velocity changes affected by temperature are due to two kinds of molecules

in the solution, i.e., active and inactive ones, which are in tautomeric equilibrium. This view of activated molecules has been extended by the application of the quantum theory, which identifies the tautomeric activated forms with higher quantum states of the molecules. With each 10° C. increase in temperature, there is an increase in enzymic

TABLE III

DIPHOSPHOTHIAMIN ENZYMES

Enzyme	End Product with Pyruvate as the Substrate
β-Carboxylase	Acetaldehyde and carbon dioxide
α-Ketoxidase	Acetic acid
Pyruvate mutase	Lactic and acetic acids
Enzymes catalyzing the condensation of pyruvate to	Carbohydrate Citrate Acetoacetate Succinate α-Ketoglutarate

activity of about two to three times the original. The relation between reactions at two temperatures 10° apart is called the temperature

TABLE IV

ENZYMES REQUIRING OTHER COENZYMES

Enzyme	Coenzyme	Substrate
Glyoxalase	Glutathione	Methylglyoxal
Phosphorylase	Adenylic acid	Glycogen ⇄ Cori ester
Amino acid decarboxylase	Pyridoxal orthophosphate	Amino acids
Aspartic-glutamic transaminase	Pyridoxal orthophosphate	Glutamic acid + oxalacetic acid ⇄ α-ketoglutaric acid + aspartic acid
Alanine-glutamic transaminase	Pyridoxal orthophosphate	Glutamic acid + pyruvic acid ⇄ α-ketoglutaric acid + alanine

coefficient. The estimation should be carried out at temperatures where destruction is at the minimum.

In Table V are presented a number of temperature coefficients (K_{t+10}/K_t, or Q_{10}). The temperature coefficient for enzymic reactions is less than for the same reactions catalyzed by hydrogen ions. Above 50°, enzymes in solution are rapidly inactivated. The destruction increases with an increase in temperature, and with most enzymes it is complete at 80°. Some enzymes are more resistant, for instance,

papain, bromelin (7), and the rennin of the plant *Solanum elaeagnifolium* (8). The concentration of the enzyme, as well as the presence or absence of the substrate and the duration of the experiment, however, are influential factors, and in some cases under certain conditions heat inactivation is reversible (e.g., trypsin, ribonuclease).

TABLE V

TEMPERATURE COEFFICIENTS OF A NUMBER OF ENZYME ACTIONS

Enzyme	Substrate	Temperature in Degrees Centigrade	$\frac{K_{t+10}}{K_t}$	Reference Number
Pancreatic lipase	Ethyl butyrate	0–10	1.50	[3]
		10–20	1.34	
		20–30	1.26	
Liver lipase	Ethyl butyrate	0–10	1.72	
		10–20	1.36	
		20–30	1.10	
Yeast maltase	Maltose	10–20	1.90	[4]
		20–30	1.44	
		30–40	1.28	
Malt amylase	Starch	20–30	1.96	[5]
		30–40	1.65	
Succinic oxidase	Succinate	30–40	2.0	[6]
		40–50	2.1	
		50–60	2.1	

It is of interest to note that the activation energy of most reactions catalyzed by enzymes is approximately 12,000 calories. That leads to a Q_{10} of about 2. On the other hand, activation energy for inactivation of most enzyme solutions (denaturation of protein) is 25,000 to 100,000 calories. Dry enzyme preparations can stand temperatures of 100° to 120°. Excellent reviews on the effects of temperature on enzyme kinetics have been published by Kuhn and by Sizer (9, 10).

Effect of Hydrogen Ions; Activity *p*H Curves

Activity *p*H curves represent the influence of hydrogen-ion concentration upon the relative enzyme activity. The *p*H optima, however, will change according to the condition of the experiment. This point is clearly illustrated in Fig. 1; urease is most active in the presence of 1 per cent urea and $M/8$ citrate buffer at *p*H 6.5. The *p*H optimum for urease in the presence of 2.5 per cent urea is 6.4 for acetate, 6.5 for

citrate, and 6.9 for phosphate. Urease is active from *p*H 5 to 9 in phosphate buffer, from 4 to 8.5 in citrate buffer, and 3 to 7.5 in acetate buffer (11).

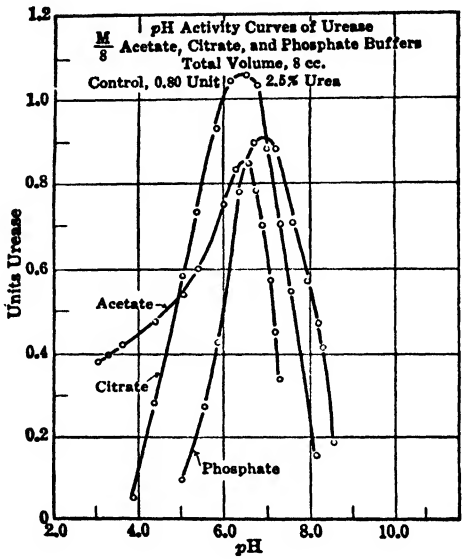

FIG. 1.

In Fig. 2 is recorded the effect of *p*H on the proteolytic activity of a 50 per cent glycerol extract of germinated soybeans, in the presence of

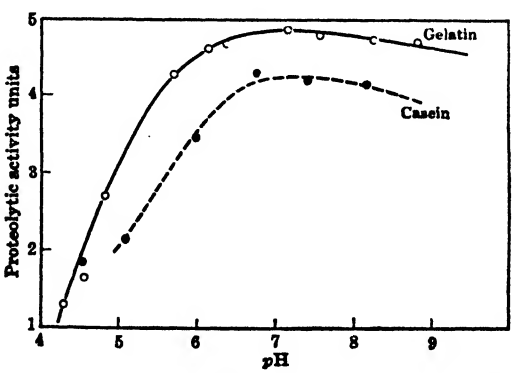

FIG. 2. Effect of *p*H on the proteolytic activity of germinated soybean extract. Proteolytic activity units: cc. of 0.05 *N* KOH per 10 grams of soybeans.

citrate buffer, using the substrate gelatin in one set of experiments and casein in the other. With increasing *p*H, the activity increased rapidly

until an optimum was attained between *p*H 6.5 and *p*H 7.0. A further increase in the *p*H caused only a slight decrease in proteolytic activity (12).

Table VI shows several examples in which the optimum *p*H of an enzyme varies with the substrate, the source of enzyme material, and the buffer employed. For other data, see individual chapters.

Specificity of Enzymes

Enzymes differ from inorganic catalysts in that the latter catalyze many reactions; i.e., hydrogen ions (of acids) will hydrolyze proteins, fats, and carbohydrates, and any ester. Hydroxyl ions behave similarly. Colloidal platinum, too, catalyzes many kinds of reactions. Enzymes, however, are more specific. Lipases do not split proteins, and proteolytic enzymes do not attack fats. Thus, their action is limited to certain types of substances. Some enzymes are absolutely specific. For instance, dipeptidase will not split a dipeptide if the amino or carboxyl group is not free (Grassmann and Dyckerhoff). Some enzymes show stereospecificity; e.g., the natural form of certain compounds is attacked much faster than the synthetic antipode. Other enzymes prefer to open certain linkages; i.e., α- and β-amylase. There are two types of maltases: true α-glucosidases and pseudo α-glucosidases, each with specific properties (Kleiner and Tauber). There are enzymes, however, which can act on a great variety of compounds (e.g., β-glucosidase), and Helferich compares such enzymes to the master key opening many locks. However. the substrate alone is not the only directive factor. Glucose, for instance, may be converted by different enzymes to gluconic acid, lactic acid, or alcohol, and pyruvic acid to acetaldehyde, acetic acid, lactic acid, and several condensation products by different diphosphothiamin enzymes (see Table III). This shows that the end product, as well as the initial structure, determines the specificity of an enzyme.

Views Concerning the Mechanism of Enzyme Action

Although enzymes are catalysts, they differ from the so-called true or ideal catalysts in various ways. After the reaction, enzymes do not remain unchanged. Soon after the action commences a certain amount of destruction takes place, even at low temperature. The reaction velocity is not always proportional to the concentration of the enzyme. In some instances enzymic catalysis can be reversed (synthesis) (pro-

TABLE VI

Variation of Optimum pH With Buffer, Type of Substrate and the Enzyme Source

Enzyme	Optimum *p*H	Authority*
Amylase (saccharifying) pancreatic	6.8	Sherman, Thomas, and Baldwin
malt	4.4–5.2	Sherman and Schlesinger
salivary, acetate buffer	5.6	Hahn and Michaelis
phosphate buffer	6.5	Hahn and Meyer
Arginase, jack-bean, depending on cation concentrations		
with Co ions	7.5–9.0	Anderson
with Mn ions	8.8	Anderson
Ascorbic acid oxidase, squash,		
phosphate-citrate buffer	5.56–5.93	Tauber, Kleiner, and Mishkind
acetate buffer	5.38–5.57	Tauber, Kleiner, and Mishkind
Asparaginase, of many sources	8.0	Busch
Invertase, yeast	4.5	Michaelis and Davidsohn
Solanum indicum	6.0	Tauber and Kleiner
Lactase, adult dog intestine	5.4–6.0	Cajori
calf intestine	5.0	Fendenberg and Hoffman
cockroach, gut	5.0–6.4	Wigglesworth
yeast	7.0	Willstätter and Oppenheimer
almond	4.2	Willstätter and Csanyi
Maltase, yeast	6.75–7.25	Willstätter and Bamann
Solanum indicum	5.5	Tauber and Kleiner
Papain, egg albumin as substrate	7.5	Greenberg and Winnick
gelatin as substrate	5.0	Willstätter and Grassmann
Pepsin, egg albumin as substrate	1.5	Sörensen
casein as substrate	1.8	Northrop
hemoglobin as substrate	2.2	Northrop
gelatin as substrate	2.2	Northrop
Phosphatase, bone	8.4	Martland and Robinson
kidney	8.8–9.2	Kay
plant sources	3.4–6.0	Kay and Lee
synthetic action	9.4	Kay
Transaminase, *B. coli*	8.5	Lichstein and Cohen
oat seedlings	8.5	Lichstein and Cohen
animal tissues	7.5	Lichstein and Cohen
Urease, crystalline; citrate buffer	6.5	Howell and Sumner
acetate buffer	6.4	Howell and Sumner
phosphate buffer	6.9	Howell and Sumner

* For further data and references, see individual chapters.

teins, organic esters, carbohydrates, etc.). In contrast to inorganic catalysts (as hydrochloric acid), only a few enzymes, and these under certain conditions, follow the course of a monomolecular reaction.

According to the theory of Bayliss (13) enzyme action is based on an adsorption process caused by the colloidal state of the enzyme; i.e., the enzyme adsorbs the substrate, and then the chemical reaction takes place at the interface. This reaction may be explained by the law of mass action; the amount of adsorbed substance, however, is the controlling factor. The Michaelis school believes that in certain reactions there is a combination between enzyme and substrate. Michaelis, too, applied the mass-action law for enzymic reactions, showing that the amount of combination between enzyme and substrate depends upon their concentrations. Northrop found that with pepsin and trypsin there is no combination between enzyme and substrate; the reaction takes place with the ionized part of the substrate. He found also that the reactions proceed (with slight deviations) according to the law of mass action, but that there is a reversible combination between the enzyme and the reaction products.

Rothen (14) found, by using systems consisting of certain polymer films and crystalline trypsin and pepsin, and the antigen bovine albumin and homologous antisera to test proteolysis, that the proteolytic enzymes may act at a distance greater than 100 Å. This would mean that no direct contact is necessary between enzyme molecules and the molecules subjected to decomposition. This would be in accordance with the suggestion of London that enzyme action may originate through a field of forces resulting from extended resonators.

KINETICS OF ENZYME REACTIONS

Enzymic reactions are influenced by many factors, the more complicated of which are not fully understood — for example, the nature of reacting or active groups in the enzyme molecule, the possibility of more than one kind of active group in the enzyme molecule, the quality and quantity of accompanying substances which may be either activators or inhibitors. These are the factors that make an enzymic reaction differ quantitatively but not qualitatively from the inorganic catalyst.

The determination of the velocity or rate of the reaction catalyzed by an enzyme gives much information concerning the nature of the enzyme reaction. The velocity is also often used as a measure of the quantity of enzyme present, as shown by Johnston and Józsa (15).

The velocity of an enzyme reaction is the instantaneous rate of change of the substrate at any given time. Many enzymatic reactions frequently exhibit over at least a part of their reaction course the kinetics of a zero-order reaction. In such a reaction the rate of change is constant and independent of the substrate concentration. This may be expressed by the following equation:

$$\text{Velocity} = \frac{dx}{dt} = k \quad \text{or} \quad x = kt$$

where x is the quantity of substrate changed in time t, and k is the velocity constant for this reaction.

The explanation for this behavior is that enzyme reactions occur through the intermediate formation of an enzyme-substrate complex (see Michaelis-Menten theory). The velocity of the reaction depends not upon the total concentration of substrate but upon the concentration of the complex. If a large excess of substrate is present the enzyme is saturated with substrate and the concentration of the complex remains constant.

The velocity of enzyme reactions under other conditions is proportional to the concentration of the substrate present at any given time. In this case the equation for the reaction velocity is

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

where a is the concentration of substrate at the start of the reaction. If this is integrated,

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

is obtained. This type of reaction is known as a first-order reaction.

A number of enzyme reactions, particularly hydrolytic reactions, although they are actually bimolecular, belong to this order because the second reactant, water, is present in such large excess that its concentration does not change appreciably. But except under very limited conditions the first-order equation is not always completely followed. Nelson's (16) precise studies on invertase illustrate the failure of the first-order equation to hold as conditions are varied. Other examples in which it holds under certain restricted conditions are to be found in the work of von Euler (17) and Dernby (18).

The above two equations may be effectively combined as shown by

Van Slyke (19), who derived the following over-all equation

$$t = \frac{1}{K_c} \ln \frac{a}{a - x} + \frac{x}{K_d}$$

where K_c is the velocity constant for the combination of enzyme and substrate and K_d is the velocity constant for the production of x. This reaction, called by Van Slyke " two-phase," covers the course of the reaction from the presence of a high concentration of substrate to the presence of a limited quantity of substrate. When x is very small the equation reduces to the zero-order expression, and when x/K_d is negligible the equation reduces to the first-order form.

Michaelis-Menten Theory

Another approach to the problem of the relationship between the rate of an enzymic reaction and concentration of the substrate was made by Michaelis and Menten (20) in 1913. They assumed that the initial rate of hydrolysis of sucrose is proportional to the concentration of an intermediate sucrose-invertase complex. If E represents enzyme, S, substrate, and P, the products of the reaction, the proposed mechanism could be formulated as

$$E + S \rightleftarrows ES \rightarrow E + P$$

Mathematical analysis led to the equation

$$v = \frac{V(S)}{K_s + S}$$

in which v is the initial velocity of hydrolysis when the concentration of sucrose is (S); V is the maximum velocity reached at infinitely high concentrations of sucrose so that the enzyme is saturated with substrate; and K_s is the dissociation constant of the enzyme-substrate complex, ES.

Michaelis and Menten tested their derivation by assuming that the maximum observed velocity, obtained when the concentration of sucrose was 5 per cent, was equal to V. Their data are illustrated in Fig. 3; it is evident that the agreement between experimental points and the theoretical curve is satisfactory. Such curves are often called pS-activity curves since they show the relationship between the rate of reaction and the negative logarithm of the concentration of substrate.

Subsequent workers have verified, extended, and criticized the treatment of Michaelis and Menten (21, 22). Stadie and Zapp (23)

extended the theory to include the equilibrium of enzyme, coenzyme, substrate, and hydrogen ions. On the other hand, it can be readily demonstrated that the K_s calculated from the data based on the theory does not necessarily correspond to that of the substrate-enzyme complex (24). Larson (25) and Nelson and Larson (26), using phosphates and citrates, and a series of sucrose solutions having different hydrogen-ion concentrations, were unable in many experiments to find any close agreement between the experimental and theoretical pS-activity curves.

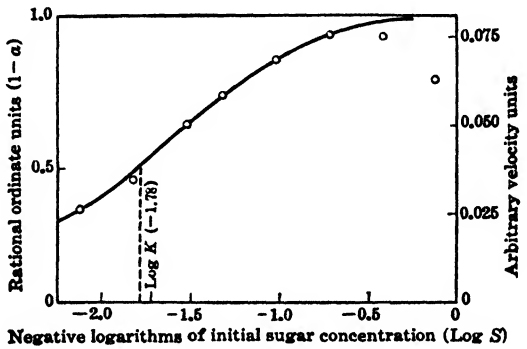

FIG. 3. Comparison of the mass-law curve with the change in activity of yeast invertase as the concentration of sucrose is varied.

Nelson and Schubert (27) explained such deviations at high concentrations of sucrose as arising from the influence of water on the velocity of the reaction. They varied the concentrations of water and sucrose by adding alcohol to the system.

It is evident from the criticism of Nelson that one of the chief difficulties in applying the theory is in determining V experimentally. If this is done by increasing the concentrations of substrate continuously, deviations arise from secondary reactions. Lineweaver and Burk (28) provided an ingenious solution of this problem by algebraic manipulation of the equation. If the reciprocal is taken of both sides, the equation reduces to

$$\frac{1}{v} = \frac{K_s}{V}\left[\frac{1}{(S)}\right] + \frac{1}{V}$$

Consideration of this form of the equation shows that, if the reciprocal of the velocity is plotted against the reciprocal of the substrate concentration, a straight line should result whose intercept estimates $1/V$, and whose slope estimates K_s/V. This means experimentally that low to moderate concentrations of the substrate can be used, since. when the

line is drawn either graphically or by the method of least squares, V and K_s are readily and accurately determined by mere extrapolation to zero.

A consequence of this modification is that it provides a method for testing whether a given reaction is governed by the assumptions of the Michaelis-Menten treatment or whether a more complicated type of reaction is concerned. If the data, when plotted as indicated, do not follow a straight line within experimental error, some other mechanism should be sought (28).

A second use that can be made of the equation is for testing the type of inhibition. In studies of the mechanism of enzyme reactions, it often is important to know how different inhibitors affect the reaction. The simplest instance that can be tested is to differentiate between an inhibitor that competes with the substrate for the enzyme (competitive), and one that combines directly with the enzyme resulting in the elimination of functional groups (non-competitive). By formulations that are primarily extensions of the Michaelis-Menten treatment, and development of their mathematical implications, the following equations are obtained:

For competitive inhibition:

$$\frac{v}{v_i} = 1 + \frac{K_s}{K_i} \frac{(I)}{K_s + (S)}$$

For non-competitive inhibition:

$$\frac{v}{v_i} = 1 + \frac{(I)}{K_i}$$

in which v_i is the velocity in the presence of inhibitor of concentration (I), K_i is the dissociation constant of the inhibitor-enzyme complex, and the other symbols have the significance previously assigned. If v/v_i is plotted against the concentration of inhibitor (I), for different values of (S), the result should be a straight line with an intercept of unity for both competitive and non-competitive inhibition. If the inhibition is competitive, the lines will have different slopes; but they will coincide if non-competitive, since (S) does not enter into the equation for this type of inhibition. Ebersole, Guttentag, and Wilson (29) and McElroy (30) discuss more involved types of inhibition and their mathematical treatment. More extensive consideration of the kinetics of enzyme reactions and detailed derivation of the mathematical equations are furnished in references 19, 24, and 31.

General Rule for Expressing Enzyme Activity. The method employed should yield results that are independent of the concentration of the enzyme solutions. With most enzymes, the amount of change produced by the enzyme is only proportional to the enzyme concentration in the early part of the reaction. The potency of an enzyme is expressed in units. The unit of activity is the amount of change or destruction of a given quantity of substrate under certain definite conditions. Thus, the specific activity (purity) of an enzyme preparation is measured by the number of activity units per gram of dry weight. Northrop uses the number of units per milligram of nitrogen.

Activators

Non-specific substances, which are necessary for the activity of an enzyme or which activate a precursor of an enzyme, are often called activators; e.g., hydrochloric acid activates pepsinogen by changing it to pepsin, and prorennin to rennin. However, the activation of pepsin is said to be an autocatalytic one. Salivary amylase is activated by sodium chloride and other salts, and arginase by manganic, cobalt, nickel, and iron, but not by other ions (32). Yeast phosphatase requires similar ions for its action (33). Leucylpeptidase is activated by magnesium and manganic ions (34). The activity of pectinesterase (pectase) at *p*H 5.7 is about 30 times greater in the presence of 0.2 *M* monovalent cations or 0.02 *M* divalent cations than in the absence of cations, but at *p*H 8.5 these concentrations of cations are without effect on the activity of the enzyme (35). Ascorbic acid oxidase, tyrosinase, and laccase require copper, and carbonic anhydrase depends on zinc for its activity. Many other enzymes require different electrolytes. The general opinion is that the cations are integral parts of the respective enzyme molecule.

Essential Groups and Their Inhibitors

The enzyme inhibitors may be divided into three groups: (A) substances that reduce the necessary concentration of the coenzymes, prosthetic groups, or the metal activators; (B) substances that compete with the substrate for the enzyme; (C) substances that destroy a necessary active group in the protein portion of the enzyme.

In the *first group* belong poisons like carbon monoxide, hydrogen sulfide, hydrogen cyanide, azide, hydroxylamine, which inhibit the iron-porphyrin enzymes; hydrogen cyanide, diethyldithiocarbamate,

which inactivates the copper-containing enzymes; and fluoride and oxalate, which inhibit the calcium-containing enzymes. Other poisons may compete with the coenzyme for the apoenzyme. Examples for this group are the inhibition of flavoproteins by atabrine (36), and the diphosphothiamin enzymes by compounds related to thiamin, such as sulfathiazole and pyrithiamin. Sevag and associates (37) found that, though sulfathiazole inhibits the carboxylase activity of whole yeast, one molecule of cocarboxylase added to the reaction mixture is capable of counteracting the inhibitory effect of 8088 to 53,400 molecules of sulfathiazole. These, and other investigators, observed that several sulfonamide drugs have a strong inhibitory action on various respiratory enzymes. A hypothesis had been postulated that sulfonamides exert their bacteriostatic action through chemical affinity for the apoenzymes of certain respiratory enzymes of the bacterial cell, and that this affinity is based on structural similarity of the drugs and the coenzymes. However, other investigators believe that the host cell metabolism may be so inhibited as to be insufficient to support the growth of the invading organism. Thus, death from poliomyelitis virus, and especially paralysis, decreased in mice subjected to thiamin (cocarboxylase) deficiency. Thiamin deficiency may be rapidly produced by feeding the homologs pyrithiamin, 2-*n*-butyl thiamin, or *o*-amino-benzyl-methyl thiazolium chloride. In chickens with riboflavin deficiency, infections with *Plasmodium lophurae* malaria are less severe than in normal controls. Here, galactoflavin or isoriboflavin may be efficient in producing a deficiency in the flavoprotein enzymes Other vitamin antagonists are pyridine-3-sulfonic acid and β-acetylpyridine for pyridoxine; desthiobiotin and biotinsulfone for biotin; phenylpantothenone and pantoyltaurine for pantothenic acid; dicumarol and salicylic acid for vitamin K. This subject has been reviewed by Frenkel (38).

In the *second group* of inhibitors belong the substrate competitors. It is believed that, though poisons of this type are not acted upon by the enzyme, they do combine with them owing to the similarity of structure and, by doing so, block "active centers," preventing access of the substrate to the enzyme (39).

In the *third group* of inhibitors are classed those that are specific for their action on well-defined chemical groupings in the enzyme molecule. These are oxidants, reductants, and reagents that combine with or act on aldehyde, keto, and phenolic radicals. The poisoning of enzymes by traces of heavy metals has been known for many years. Later, it was found that the enzymes sensitive to heavy metal ions contained sulfhydryl groups necessary for their action. Thus, enzymes with

such groups are readily oxidized by atmospheric oxygen. Traces of copper accelerate the oxidation. Mild reducing agents have a protective action upon these enzymes. The most powerful and specific sulfhydryl inhibitors are the mercaptide-forming reagents, such as trivalent organic arsenoxides and *p*-chloromercuribenzoate (40). Grant and Kinsey (41) found that *bis*-β-chloroethyl sulfide (mustard gas) is a very powerful inhibitor of urease, acting on its sulfhydryl group. Inactivation of the enzyme by 0.001 and 0.002 *M bis*-β-chloroethyl sulfide increased with increase of *p*H from 6 to 8, and also increased with decrease in phosphate concentration when the *p*H was maintained constant. Many enzymes that contain sulfhydryl groups and have been inactivated by the action of oxidants may be reactivated by the use of reducing agents.

Enzymes with Sulfhydryl Groups

The following enzymes are now known to contain sulfhydryl groups (reviewed in references 42 and 43). Acetate oxidase of yeast and bacteria, adenosinetriphosphatase of myosin, adenylpyrophosphatase of liver, alcohol oxidase of yeast, β-amylase of barley, asclepain *m* of milkweeds, asclepain *s* of milkweeds, bromelin, carbonic anhydrase, carboxylase of yeast, carboxylase of heart, cerebrosidase, cholinesterase, choline oxidase, *d*-amino acid oxidase, esterase of pancrease, ficin, glycerol oxidase of bacteria, β-hydroxy butyric oxidase, α-ketoglutaric oxidase, *l*-glutamic oxidase, glycerol dehydrogenase, lipase of pancrease, lysozyme, malate oxidase, monoamine oxidase, myokinase, oleate oxidation enzyme of *B. coli*, papain (crude and semi pure), phosphoglucomutase, phosphoglyceraldehyde oxidase, phosphorylase of potato, pyruvate condensation enzymes, pyruvate dismutase, pyruvate oxidase of liver and bacteria, succinoxidase, stearate oxidase of liver and bacteria, succinic dehydrogenase, transaminase, triosephosphate dehydrogenase, and urease.

Enzymes with Other Essential Groups

Essential phenolic (tyrosine) groups had been found in pepsin by Herriott (44), who converted these groupings with iodine into diiodotyrosin. Zeller (45) demonstrated the presence of active carbonyl groups in diamine oxidase. Sizer (43) found, by studying the effect of ketene, nitrous acid, phenyl isocyanate, and formaldehyde upon chymotrypsin, that primary amino, sulfhydryl, or disulfide groups are not essential for the activity of this enzyme, while tyrosine is an essen-

tial group. Edman (46) could not substantiate the more recent claim of Sizer that tyrosinase catalyzes the oxidation of the tyrosyl residues of chymotrypsin, trypsin, and pepsin without decreasing their proteolytic activity. Edman found that, when the main portion of the non-protein tyrosine is removed from pepsin, the capacity of the preparation to be oxidized by tyrosine is also mainly lost. Pepsin is instantly inactivated at the *p*H (7.3) of the reaction mixture, so that no conclusion as to the effect of tyrosinase on the activity of pepsin is possible. Trypsin is also very unstable at *p*H 7.3, being rapidly autolyzed during the incubation. This results in the formation of non-protein tyrosine. Edman states that, in the case of chymotrypsin, there is no conflict with the explanation suggested by Sizer. However, the small quantity of oxygen uptake might well be explained by the amount of non-protein tyrosine present in the chymotrypsin. Sizer (47) has found, by using proteinases from which traces of tyrosine had been removed, that products of protein autolysis account for only a fraction of the total action of tyrosinase, and that the residual tyrosyl groups of the proteinases were extensively oxidized by the tyrosinase.

Inhibition of Enzymes by Ionizing Radiations

The primary reaction on the radiation of water is the splitting into hydrogen atoms and hydroxyl groups. In the presence of dissolved oxygen, there is also formation of hydrogen peroxide. Ionizing radiations may then inhibit sulfhydryl enzymes by oxidation of the sulfhydryl groups by the hydroxyl group and hydrogen peroxide.

Barron and associates (48) irradiated phosphoglyceraldehyde dehydrogenase with increasing quantities of X-rays from 25 r to 500 r. The dehydrogenase was half-inhibited with 200 r and almost completely inhibited with 500 r. On the addition of glutathione, the enzyme was reactivated. Previous addition of glutathione caused less inhibition. Inhibition was also displayed with γ particles (polonium) and β (Sn^{39}) and γ (radium) radiations. Other sulfhydryl-containing enzymes, such as adenosinetriphosphatase, urease, and succinodehydrogenase, were similarly affected. However, inhibition of urease by γ radiations could not be changed by glutathione. When a mercaptide-forming compound, such as *p*-chloromercuribenzoate, was present, the urease was protected from γ radiations. Catalase reduced the inhibition of phosphoglyceraldehyde dehydrogenase by γ particles. By this device, it was found that half of the total inhibition was brought about by the hydrogen peroxide formed during irradiation. Beta radiations could be almost completely eliminated by previous addition of catalase.

Some Other Inhibitions

Inhibition of Trypsin. As has been shown, enzymes may be inhibited by a great variety of compounds. The following have been found to inhibit the activity of trypsin: serum, plasma, charcoal, unsaturated fatty acids, tryptic digests of proteins, crystalline pancreatic trypsin inhibitor, crystalline serum trypsin inhibitor, egg-white antitrypsin, cysteine, hydrogen sulfide, hydrogen cyanide, pyrophosphate, alcohol, formaldehyde, thymol, chloroform, toluol, alkaloids, glycerol, fats, sugar, asparagine, glutamic acid, glycine, leucylglycine, alanylglycine, a number of inorganic salts, lecithin, heparin, quinine salts, urea, germanin (Bayer 205), azo dyes, acridine dyes, congo red, X-rays, ultraviolet light, and bacteria (reviewed in references 42 and 49).

Reversible Inactivation of Trypsin and Pepsin. In the following, two examples of reversible inactivation of enzymes will be given. Here, the *p*H and temperature are the influential factors. Kunitz and Northrop (50) inactivated crystalline trypsin solutions at various *p*H's and the temperatures below 37°. They found that trypsin may be reversibly or irreversibly inactivated. The reversible inactivation occurs whenever the reversible denaturization of the protein is brought about. The denatured protein is in equilibrium with the native protein. An increase in temperature or alkalinity shifts the equilibrium towards the denatured side. Below *p*H 2.0 trypsin protein is changed into an inactive state, which may be irreversibly denatured by heat. This is a unimolecular reaction. The velocity increases with acidity.

Trypsin protein is slowly hydrolyzed between *p*H 2.0 and 9.0. This reaction is bimolecular, and the inactivation increases with increase in *p*H to 10.0, when it decreases. At *p*H 2.3, there is maximum stability. At *p*H 13.0, there is also formation of inactive protein; this reaction is unimolecular. With increasing *p*H, the velocity increases. From *p*H 9.0 to 12.0, some of the protein is hydrolyzed and some inactive protein is formed. At *p*H 13.0, inactivation is at the minimum. Decrease in activity was always proportional to decrease in tryptic protein. The rapid inactivation at higher temperature or in alkaline solutions is reversible for a short period only. The longer the solutions stand, the greater the loss in activity and irreversibility. Figure 4 shows inactivation at various *p*H's at 30° C. and 0° C.

Pavlov and Parastschuk (51) found that alkali-inactivated pepsin may be reactivated when kept in a slightly acid solution for several hours. This has been confirmed by Northrop (52), using solutions of crystalline pepsin. He showed that pepsin solutions which have been completely denatured and inactivated by alkali (*p*H 10.5) could be

reactivated by adjusting the *p*H to 5.4 and allowing to stand for 24 hours at 22° C. The reactivated material was recrystallized and had the same peptic activity as the original pepsin.

For studies concerning inhibition kinetics, the publications by Michaelis and Menten (20) and by Moelwyn-Hughes (53) should be consulted.

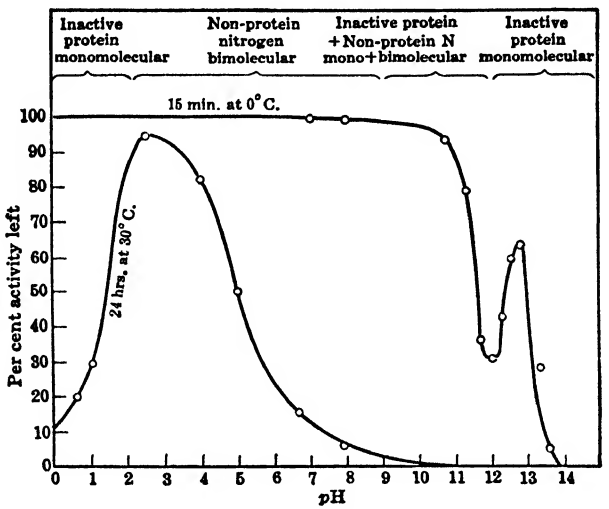

FIG. 4. Loss in tryptic activity at various *p*H's.

Antienzymes. Another type of inhibitors is the antienzymes. They are specific inhibitors normally produced by plant and animal cells. Very powerful antienzymes may be produced by immunological means. However, some enzymes (urease) are very toxic to animals, and the animals must be permitted to build up a proper defense (antienzymes) against the enzymes' toxicity. Sevag (54) has recently written an excellent monograph concerning such problems.

PREPARATION OF ENZYMES

The method to be followed in the preparation of a given enzyme must depend on the specific use to which the product will be put. To demonstrate the presence of most enzymes in a biological source, it is only necessary to suspend the tissue in a small amount of water and extract it by breaking up the tissue with a glass rod. From most tissues, an active juice may be obtained by light pressing. Bacterial cells (from a culture) may be collected by centrifuging. When suspended in water

they show the activity of many enzymes. For more serious work, it is necessary to rupture the cell that contains the enzyme. Kalnitsky and Werkman (55) use a method in which the mass of bacterial cells is ground with powdered Pyrex glass and the ground cell-glass mixture is extracted with a suitable buffer. Stumpf and Green (56) found that cell suspensions of *Proteus vulgaris* were rapidly disintegrated by exposure to ultra sound, which was generated by a crystal-controlled oscillator operating at 1000 volts and putting out 500 watts. This enzyme material was employed as a source of *l*-amino acid oxidase. Other physical methods that are employed in the purification of enzymes are dialysis, electrodialysis, pervaporation, electrophoresis, ultracentrifugation, and chromatography (57).

Since enzymes are proteins, they may be adsorbed by many substances, especially colloids. The adsorption method was employed by early investigators. It is based on the separation of a given enzyme or group of enzymes from extracts by adsorption on kaolin, tannin, charcoal, etc., or on a suitable colloid, such as certain hydroxides of aluminum, and the subsequent elution (freeing) of the enzymes from the adsorbent by weak alkalis, weak acids, or by phosphates. Cotton exerts selective adsorption toward enzymes (58). Here, no contamination of the enzyme material takes place. Butler (59) found that solid yeast nucleic acid is a powerful adsorbent of malt amylases. The enzymes (α- and β-amylase) may be removed from the nucleate complex by dissolving it in a small volume of phosphate buffer of *p*H 6.0. McColloch and Kertesz (60) employed an ion exchange resin for the complete removal of pectin methylesterase from commercial pectinase. Here the resin acts as the adsorbent.

For the extraction of biological material, dilute solutions of alcohol, acetone, dioxane, glycerol, and acids, alkalis, and salts may be used. For many industrial uses, it is not necessary that the enzyme product be pure. For example, barley malt, an important source of amylases, is employed without being treated in any manner, and defatted, dry pancreas glands, after slight pretreatment (activation, drying), are a good source of trypsin and amylase. The latex of the papaya fruit is merely treated with sulfite to activate it; after it is dried, it is ready for commercial use. For most purposes, it is desirable that the extraction procedure be an efficient one and that the yield of the final product be high. These results may be accomplished by selecting the best extraction method and proper cultivation procedures, by excluding contamination, and by rapid drying in vacuum at low pressure. Saturated solutions of neutral salts or organic solvents (alcohol, acetone, dioxane) may be employed for the precipitation of enzymes, provided that the activity of a given enzyme is not impaired by the procedure. Often, it

may be necessary to destroy cellular matter. Thus, for the preparation of yeast invertase, plasmolyzing agents such as ether, chloroform, ethyl acetate, sodium chloride, or sucrose are used in order to liberate the enzyme. However, some enzymes have not yet been separated from the insoluble cell membrane. Many highly active enzyme preparations have been obtained by some of the methods just mentioned. Detailed descriptions may be found in the chapter "Production of Enzymes" and in the various other chapters.

A very useful book describing the preparation of microbiological and other tissues for metabolic studies and the necessary manometric techniques has been published by Umbreit, Burris, and Stauffer (61).

Crystallization of Enzymes. The purification of enzymes by crystallization had been initiated by Sumner in 1926, while studying the enzyme urease. Three years later, Northrop announced the crystallization of pepsin. This was followed by the crystallization of other proteinases by Northrop and Kunitz. Many other enzymes were isolated in this way by other investigators. A few of the crystalline enzymes are shown in Fig. 5.

The development of methods for the crystallization of enzymes has given an immense impetus to enzyme chemistry. This will be readily acknowledged by all who acquaint themselves with the present advances in this field of biochemistry. Dr. James B. Sumner and Dr. John H. Northrop were awarded part of the 1946 Nobel Prize for chemistry in recognition of their contributions to enzyme chemistry, and Carl F. Cori and his wife Gerty Cori received part of the 1947 Nobel Prize in medicine for discoveries concerning the catalytic metabolism of glycogen.

Synthesis by Enzymes. Under certain well-controlled conditions, reactions for a series of enzymes may be reversed. Thus a variety of esters, glucosides, and nucleosides (62) may be obtained by enzyme action. Several carbohydrates are synthesized; others are phosphorylated by specific enzymes. Polypeptides are readily formed from simpler compounds by proteinases (63). New types of proteins are obtained when pepsin and trypsin are allowed to autolyze at *p*H 1.6 at 55° C. and when the end products are exposed to the action of pepsin at *p*H 4.0. The viscous proteins that form are called plastein. However, these synthetic proteins do not resemble the original enzyme proteins since they do not possess proteolytic activity (64). Much of the work concerning synthesis by enzymes *in vitro* will be described in the present volume.

Antiseptics for Enzymes. Enzymes, being proteins, are exposed to the destructive influence of microorganisms. Toluol, chloroform, sodium fluoride, ammonium bifluoride, formaldehyde, phenol, thymol,

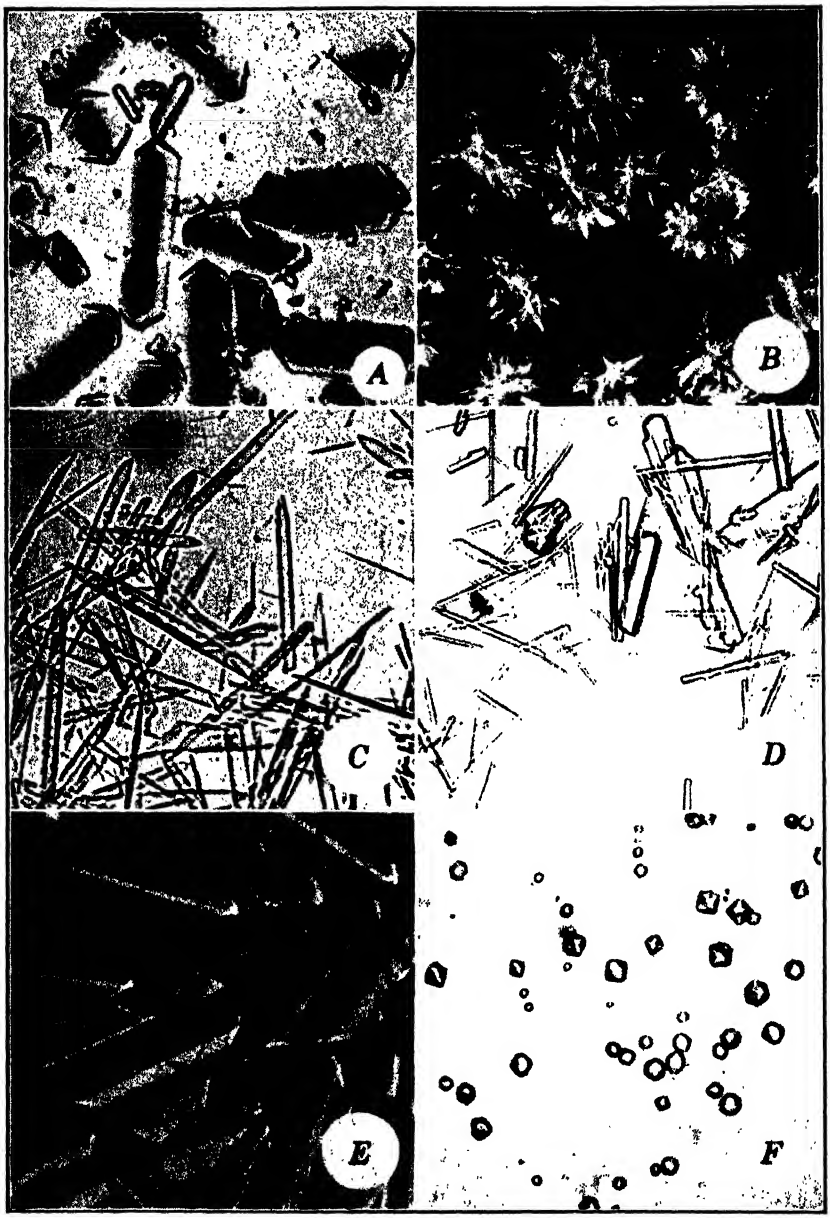

FIG. 5. Some crystalline enzymes.

A, Beef liver catalase (from Sumner, J. B., and Dounce, A. L.: *J. Biol. Chem.*, 121, 417, 1937).
B, Beef pancreas ribonuclease (from Kunitz, M.: *J. Gen. Physiol.*, 20, 15, 1940).
C, Swine stomach pepsin (from Northrop, J. H.: *J. Gen. Physiol.*, 30, 177, 1946).
D, Yeast hexokinase (from Kunitz, M., and McDonald, R.: *J. Gen. Physiol.*, 29, 393, 1946).
E, Swine pancreas α-amylase (from Meyer, K. H., Fischer, E. H., and Bernfeld, P.: *Experientia*, 3, 106, 1947).
F, Rabbit muscle aldolase (from Cori, C. F.: unpublished).

and other phenol derivatives are employed for the preservation of enzyme solutions. For ordinary purposes, toluol is most suitable and least destructive. It can be separated by filtration from the enzyme solutions. In any event, it is best to keep liquid enzyme preparations in a refrigerator when not in use. Brasch and Huber (65) have described an apparatus which employs ultrashort penetrating electrons for the sterilization of foods, enzymes, yeast, antibiotics, etc. Most dry enzyme products retain their activity for many years. However, they should be sterile, or almost sterile, when processed for future use.

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CHAPTER II

ESTERASES

The enzymic ester hydrolysis or ester synthesis proceeds according to the equation

$$R{\cdot}COOR' + H_2O \rightleftarrows R{\cdot}COOH + R'OH$$

If in the above equation R·COOH is a higher fatty acid and R'OH is glycerol, the enzyme responsible for the hydrolysis is lipase, e.g., pancreatic lipase, gastric lipase, ricinus lipase. If, however, R·COOH is any other organic acid, or if the acid is an inorganic one and R'OH a simple alcohol (aliphatic or aromatic) or a carbohydrate, then the reaction is catalyzed by an esterase, e.g., liver esterase, sulfatase, phosphatase, etc. While the specificity of lipases and certain esterases is not absolute, the presence of an asymmetric carbon atom in the alcohol or acid radical has a pronounced influence on the activity of these enzymes.

Esterases exert a selective action on a racemic mixture. This action is called stereospecificity. For instance, pig-liver esterase added to *dl*-mandelic acid esters would hydrolyze the *d*-form first. If, however, another *dl* ester was used, the *l* form was first hydrolyzed. Pancreatic (pig) lipase hydrolyzed the *d* form first when added to the racemic mixture of *dl*-mandelic ester, but when the *d* form and *l* form were split separately, the *l* was hydrolyzed faster (1). Experiments of this sort are unlimited, since the number of esters is great. An extensive review of this subject has been given by Rona and Ammon (2).

PANCREATIC LIPASE

Pancreatic lipase may be obtained either directly from the pancreas gland or from the pancreatic juice. In accordance with the classification above, pancreatic lipase hydrolyzes glycerides of the higher fatty acids but does not readily attack lower esters. Claude Bernard was the first to show (in 1856) that pancreatic juice has lipolytic activity. It was believed that the pancreatic juice contained two lipolytic enzymes: i.e., a " fat- " (lipase) and a " low ester- " (esterase) splitting enzyme. Later, it was definitely established that there was only

one such enzyme, the fat-hydrolyzing lipase present in the pancreas. This lipase hydrolyzes true fats very rapidly.

Preparation. Willstätter and Waldschmidt-Leitz (3) obtained active lipase preparations by extracting, with water and glycerol, pig pancreas that had been dehydrated and defatted with acetone and ether. Glick and King (4) employed 10 per cent sodium chloride for the extraction of lipase from dry pancreatic tissue. Their method was more suitable for concentrating the enzyme than that of any of the earlier investigators. The lipase could be quantitatively removed from the extract on saturation with magnesium sulfate.

Kinetics of Pancreatic Lipase. The enzyme does not require a specific coenzyme. Activation is brought about mainly by the buffering capacity of the added substances. Certain concentrations of sodium taurocholate accelerate, while greater concentrations inhibit, pancreatic and liver lipase (5). It is impossible to designate any specific optimum *p*H for pancreatic lipase and a few other enzymes. Their optimum *p*H depends strongly upon the nature of the enzyme preparation, the rate of hydrolysis, the buffer, and the substrate. Schonheyder and Volqvartz (6) found that the optimum *p*H for pig's pancreatic lipase, prepared according to the method of Willstätter and Waldschmidt-Leitz, increased from 7 to 8.8 with increasing number of carbon atoms in the fatty acid component of the triglyceride. The substrates examined were triacetin, tripropionin, tributyrin, tricaproin, tricaprin, trilaurin, trimyristin, tristearin, and triolein. The addition of calcium chloride does not alter the optimum *p*H but activates the hydrolysis in alkaline and in acid medium. This is brought about by the strong inhibition of pancreatic lipase activity by the free higher fatty acids. The strong activation by calcium chloride results from the removal of such acids as insoluble soaps. The Danish workers found that during the first phase of the reaction the hydrolysis curve was linear for all the substrates they examined, provided that suitable buffers were employed and that lipase activity was determined by adding continuously and dropwise 0.1 *N* sodium hydroxide in order to maintain an almost constant *p*H. In some cases, in the alkaline *p*H range, corrections had to be made for spontaneous hydrolysis.

A critical review concerning the kinetics of pig pancreatic lipase has been published by Schonheyder and Volqvartz (7). These investigators found that the affinity of pig pancreatic lipase for triacetin, tripropionin, tributyrin, trivalerin, and tricaproin, in homogeneous solution is extremely low. There is a direct proportionality between substrate concentration and the initial velocity of enzymic action, when the enzyme concentration is the same.

GASTRIC LIPASE

This enzyme does not hydrolyze fats readily, unless they are emulsified, like the fat of the cow's milk and that of the egg yolk.

According to Willstätter and associates (8), the *p*H optimum of gastric lipase differs with each mammal. It is at 6.3 for the dog, 5.5 for the cat, 6.3 for the rabbit, 8.6 for the horse, and 7.9 for the hog. These values were obtained by using crude extracts of dry gastric mucosa. Schonheyder and Volqvartz (9) found that the optimum *p*H for gastric lipase of man (without $CaCl_2$) with triglycerides of propionic, butyric, and caproic acids is 5.5 to 5.8 but shifts to the alkaline side with increasing number of carbon atoms (for triglycerides of capric, lauric, stearic it is 7.2 to 7.9). Upon the addition of calcium chloride to the system, the lipase hydrolysis of trilaurin or tristearin at *p*H < 7.0 was activated, and the optimum *p*H was shifted 1.5 to 2.0 *p*H units to the acid side. Tributyrin was split by gastric lipase with the greatest initial velocity, but the activities toward solid triglycerides was very small. In an acid medium, gastric lipase was very stable at 40°. *In vivo* experiments showed that only lower triglycerides were appreciably split during the test period (25 minutes). In children, there was a tendency to split cow butter more rapidly than woman's milk fat.

RICINUS LIPASE

Ricinus lipase was first described in 1890 by Green (10) in the germinating seeds of the castor bean (*Ricinus communis*). Green found that this plant enzyme is a typical lipase, hydrolyzing true fats, similar to pancreatic lipase. Green's findings have been confirmed many times. Lower esters are hardly attacked by this lipase (11). Its optimum *p*H is between 4.7 and 5.0 (12), varying slightly with the buffer employed. Longenecker and Haley (13) prepared ricinus lipase of good activity by defatting the dehulled castor beans with petroleum ether at 34° C. After grinding, the material was ready for use. A method for the preparation of a highly active castor-bean lipase has been published by Takamija (14).

Longenecker and Haley (15) found that ricinus lipase showed no specificity in its attack on glyceride molecules containing carbon chains of different length. The number of moles of glyceride hydrolyzed was taken as a basis.

The castor bean contains toxic proteins. The shells must be handled with rubber gloves, and when working with dry enzyme preparations the powder should not be inhaled.

Method for the Estimation of Lipolytic Activity (16, 17)

1. The Substrate. In this test, 0.214 gram benzyl butyrate (b.p. 108–110° C. at 9 mm.) per 30 cc. of digestion mixture is employed; or 0.5 gram of olive oil may be used instead of the benzyl butyrate. With benzyl butyrate as the substrate, the speed of hydrolysis, at 40° C. with various quantities of enzyme (pancreatic lipase), is proportional to the amount of enzyme added (zero-order reaction).

2. Ox Bile Glycerol. To dried bile an equal weight of water is added, and the mixture is heated for 1 to 2 hours at 15 pounds pressure. Then, 10 cc. of glycerol is added for each gram of dried bile, and the mixture is allowed to dissolve on the steam bath. Several hours may be necessary for this. The bile-glycerol solution may be stored in a refrigerator and warmed up whenever it is used.

Procedure. In a 125-cc. glass-stoppered bottle is placed 0.214 gram of benzyl butyrate. To this is added 5 cc. of bile-glycerol solution, maintained at 60–70° C. for convenience in measuring. Some glass beads are added, and the bottle is placed in boiling water and shaken until the substrate is completely dissolved. Then the bottle is cooled under the cold-water tap while the contents are being shaken continuously. To the emulsion are then added 10 cc. of 0.5 *M* ammonium chloride-ammonia buffer at *p*H 8.0, 100 milligrams of calcium chloride (in water solution), and 0.25 cc. of 3 per cent phenolphthalein solution (or other indicator). Sufficient water is introduced to make a total volume of 30 cc. after the enzyme solution has been added. The substrate mixture is allowed to stand in a water bath at 40° C. until it reaches the temperature of the bath. Now the enzyme solution is introduced, and the contents of the bottle are well mixed, but no froth should form. A 5-cc. sample of the digest is pipetted into 75 cc. of a mixture of 9 volumes of alcohol and 1 volume of ether, and titrated with 0.1 *N* alcoholic potassium hydroxide, using phenolphthalein, *o*-cresolphthalein, or thymolphthalein as an indicator.

Occasionally, small amounts of 5 *N* ammonium hydroxide are added during lipolysis to maintain a fairly constant *p*H of the digestion mixture. Willstätter and Waldschmidt-Leitz (18) have shown that in strong alcohol ammonia has a negligible effect on basic indicators. Aliquots may be removed from the digest at desired intervals from 1 to 24 hours.

LIVER ESTERASE

Liver esterase differs from pancreatic lipase, since it is a typical ester- (not fat-) splitting enzyme. It readily hydrolyzes esters of

simple alcohols, ethyl acetate, and ethyl butyrate. The optimum of liver esterase is between *p*H 6.7 and 8.2, depending upon the buffer, the substrate, and the source of the enzyme (19). Ruffo (20) found that dialyzed pig liver esterase was activated by traces of copper but not by other heavy metals. The ash of the dialyzate activated slightly better. Long dialysis, however, removed additional substances, which prevented reactivation by copper. Matlack and Tucker (21) reported that muscle tissues of the pig, cow, sheep, and fish contain a typical esterase, acting on esters of lower fatty acids. This esterase could not be liberated from the muscle proteins.

Preparation. All kinds of procedures have been tried for the purification of liver esterase, without much success as to the concentration of the enzyme. Pierce (22) obtained a liver esterase preparation by dialysis of a liver tissue extract and precipitation with an equal volume of saturated ammonium sulfate. The precipitate had considerable activity. Full saturation of the filtrate with ammonium sulfate yielded an inactive filtrate and an active precipitate. The precipitate was dialyzed until free of sulfate ions. The remaining solution was highly active. Kraut and Rübenbauer (23) state that they obtained by dialysis and adsorption a protein-free active liver esterase. This finding, however, is not in accord with present views concerning enzymes. See also reference 24.

SYM'S METHOD FOR THE ENZYMIC SYNTHESIS OF ESTERS

Sym (25) developed a practical and excellent method for the enzymic synthesis of esters. He found that the sodium salts of bile acids and sodium oleate respectively, which are able to form water-soluble addition compounds with fatty acids, cholesterol, aromatic hydrocarbons, etc., have an exceedingly great activating effect on enzymic ester synthesis. The synthesis, however, must be carried out in a medium of carbon tetrachloride or benzene, which are good solvents for the esters produced. The degree of esterification may be determined (*a*) by titrating the concentration of acid with 0.1 *N* sodium hydroxide, (*b*) by measuring the alcohol concentration, or (*c*) by measuring the ester formation. The esters may be readily isolated, owing to their solubility in the organic solvent employed.

Preparation of Enzyme Material. Pancreas glands are finely ground in a meat chopper, twice extracted with 5 times their weight of acetone, and dried in the air. The dried tissue is chopped once more and is sifted. The siftings contain 8 per cent of water and are ready for use.

Synthesis of Butylbenzoate. To 25 cc. of carbon tetrachloride, containing 0.45 *M* benzoic acid and 0.5 *M* butyl alcohol, 2 grams of pancreas powder and 1 cc. of a 30 per cent solution of bile salts (sodium salts of bile acids prepared from ox bile) were added. The mixture was shaken for 24 hours in a water thermostat at 37°. After 24 hours, the concentration of the ester formed was 0.31 *M*, and after 48 hours it was 0.39 *M*. In a parallel experiment in which the bile salt solution was substituted for 1 cc. of water, the ester formation was 16 times slower. Similar results may be obtained by using benzene instead of carbon tetrachloride.

Synthesis of Cholesterol Ester. To 12.5 cc. of carbon tetrachloride, containing 0.5 *M* of butyric acid, 1 gram of pancreas powder and 1 cc. of a 15 per cent bile salt solution were added. After 24 hours at 37°, the concentration of ester formed was 0.4 *M*. No ester formation took place when 1 cc. of water was used instead of 1 cc. of bile salt solution.

Sym and associates have synthesized a number of esters by this method, and Rona, Ammon, and Fischgold (26) synthesized wax (cetylpalmitate) by it.

TANNASE

The enzymic hydrolysis of tannin was described by Scheele in 1786. Powerful preparations have been obtained from the mold *Aspergillus niger* (27). Freudenberg (28) has obtained valuable information concerning the structure of various tannins by studying the action of tannase. Dyckerhoff and Armbruster (29) have prepared tannase solutions free from esterases by selective destruction of the esterases at an alkaline *p*H. In contrast to other esterases, tannase hydrolyzes only those esters that have an acid component containing at least two phenolic hydroxyl groups. None, however, can be *ortho* to the carboxyl group. The alcohol group does not affect the specificity. A direct combination of the ester carboxyl to the oxidized benzene ring is necessary for an ester to be hydrolyzable by tannase. Tannase also hydrolyzes *m*-digallic acid, gallotannin, and chebullinic acid.

Tóth and Bársony (30) separated tannase, esterase, and β-glucosidase from each other by the chromatographic method. The proportion of the hydrolyzing effect on methyl gallate, tannin, and *m*-digallic acid was very much changed during the adsorption of an extract of *Aspergillus niger*. The simple esters of gallic acid and depsides were not broken down by the same enzyme system. Glucogallin was hydrolyzed by both β-glucosidase and tannase.

SULFATASES

These enzymes hydrolyze ethereal sulfates according to the general equation:

$$ROSO_3K + H_2O = ROH + KHSO_4$$

Fromageot (31) divided sulfatases into four groups:

Phenolsulfatase acting on phenyl sulfate.
Glucosulfatase acting on sulfuric esters of sugars.
Chondrosulfatase acting on chondroitin sulfuric acid.
Myrosulfatase acting on sinigrin.

Phenolsulfatase occurs in the mold *Aspergillus oryzae* and in a large number of vertebrates and invertebrates. Glucosulfatase is found in many species of molluses and in other invertebrates. Chondrosulfatase occurs in a number of species of bacteria. Myrosulfatase may be prepared from horseradish root and from mustard seeds. Myrosulfate, glucose sulfate, and potassium myronate (33). Myrosulfatase esterases. One, myrosulfatase, splits off $KHSO_4$ from sinigrin. The other, called thioglucosidase, liberates glucose from this glucoside (32). Bacterial sulfatase hydrolyzes chondroitin sulfuric acid, saccharose sulfate, glucose sulfate, and potassium myronate (33). Myrosulfatase of horseradish and mustard seed acts only on the natural glucosides, such as sinigrin of the mustard plant. All sulfatases liberate $KHSO_4$ from their substrates. By estimating the bisulfate that is split off, or by determining the liberated ROH, the activity of the sulfatase may be calculated.

Lecithinase A. The venom of snakes and scorpions, bee stings, the pancreas, blood, and rice hulls contain an enzyme that converts lecithin into lysolecithin, and cephalin into lysocephalin. In both reactions an unsaturated fatty acid is split off (34). Cerebrosides, sphingomyelins, acetal phospholipids, and lysophospholipids are not attacked (35). This is a very toxic enzyme.

Lecithinase B. This enzyme is present in heart, liver, spleen, pancreas, and brain tissue. It removes the remaining fatty acids from lysolecithin or all the fatty acids from lecithin. The end product is the glycerophosphoric ester of cholin, resulting in the detoxification of the hemolytic lysolecithin (36).

CHLOROPHYLLASE

Chlorophyllase is present in all plant tissues that contain chorophyll. The quantity of enzyme varies considerably in various plant species, however, and is highest during May and September, when chlorophyll is formed and decomposed respectively. Excellent sources of chlorophyllase are *Datura stramonium, Heracleum spondylium, Galeopsis tetrahit, Strochys silvatica,* and *Lamium maculatum* (37). This enzyme splits the alcohol phytol $C_{20}H_{39}OH$ from chlorophyll A or chlorophyll B and exchanges it with the methyl or ethyl radical, depending upon whether the enzyme digest contained methyl or ethyl alcohol. The green crystalline substance that forms is methyl or ethyl chlorophyllide. Fats or waxes are not hydrolyzed by chlorophyllase. The enzyme becomes inactive when freed of inorganic salts. It becomes fully active on the addition of calcium chloride (38). The action of chlorophyllase may be readily demonstrated by placing a slice of a leaf on a microscope slide, adding a drop of alcohol, and covering it with a cover slide. After a short while, depending on the quantity of chlorophyllase present, three-sided and six-sided dark-green crystals of ethyl chlorophyllide may be seen (37). This enzyme has been extensively reviewed by Lambrecht (39).

PHOSPHATASES

Phosphatases play an important role in bone formation, muscle metabolism, lactation, and alcoholic fermentation. They hydrolyze phospholipids, phosphoproteins, phosphocreatine, phosphoarginine, phosphoric esters of carbohydrates, phosphoglyceric acids, glycerophosphoric acids, and nucleic acids.

The following tentative classification has been suggested by Folley and Kay (40).

I. Phosphomonoesterases. These esterases are found in various plant and animal tissues and are abundant especially in mammalian tissues. They readily split all monoesters of orthophosphoric acid but not disubstituted esters. Phosphomonoesterases, the most studied phosphatases, may be grouped into four classes. (*a*) The " alkaline phosphatase " of bone, kidney, and intestine is best known. It has an optimum *p*H of 9 to 10 (41), which is the most alkaline *p*H yet observed for an enzyme. Kay (41) believes that this phosphatase may be identical with nucleotidase. This enzyme splits sodium β-glycerophosphate more readily than the α-salt. (*b*) A second phosphatase has been separated from the alkaline one, using extracts of certain mammalian

organs, by selective hydrogen-ion inactivation (42). The *p*H activity curve shows two peaks, one at *p*H 9.0 and one at *p*H 5.0, with the maximum at 9.0. (*c*) The third type of phosphomonoesterase has an optimum *p*H at 3.0 to 4.0. This enzyme is found in *Aspergillus oryzae* (43). (*d*) The fourth type of phosphomonoesterase has an optimum *p*H at 6.5 and is present in red blood cells (44).

II. Phosphodiesterases. This group of enzymes splits only one of the two linkages in a diesterified orthophosphoric acid, and further action of a phosphomonoesterase is necessary for complete hydrolysis of the diester. Rice bran and snake venom are good sources of phosphodiesterases (45). Triesters of phosphoric acid are not hydrolyzed by these phosphatases.

III. Pyrophosphatases. This group hydrolyzes salts and symmetrical diesters of pyrophosphoric acid, such as sodium pyrophosphate and phenyl pyrophosphate.

IV. Metaphosphatase. This enzyme splits salts of metaphosphoric acid, such as sodium metaphosphate.

V. Phosphoamidase splits N-substituted amido phosphoric acids, such as phosphocreatine.

VI. Unclassified phosphatases.

a. Lecithinase, splits lecithin, cephalin.

b. Phytase, splits phytin.

c. Adenylpyrophosphatase, splits adenosinetriphosphate.

d. Hexosediphosphatase, splits fructose-1,6-diphosphate.

MAMMALIAN PHOSPHATASES

Differentiation of the "Alkaline" Phosphatases (Phosphomonoesterases and Pyrophosphatases). Roche and associates (46) found that nearly all animal organs contain a phosphomonoesterase with an optimum *p*H at 9.0 to 9.5, and a pyrophosphatase with an optimum *p*H at 7.8 to 8.2. Both groups of enzymes were activated by magnesium ions. Cysteine in concentrations above 0.0005 *M* strongly inhibited the phosphomonoesterase of all organs, inhibited the pyrophosphatase of intestine and kidney to some extent, and had no effect on liver pyrophosphatase. Oxalate and fluoride had no effect on any of the pyrophosphatases at *p*H 7.8. Oxalate at 0.0001 to 0.01 *M* concentration strongly inhibited the phosphomonoesterase of bone and white corpuscles, slightly inhibited the phosphomonoesterase of liver and kidney, and had no effect on phosphomonoesterase of intestine. Sodium fluoride slightly inhibited the phosphomonoesterase of liver and white corpuscles and did not affect the phosphomonoesterase of bone, kidney,

or intestine. Bile salts in concentrations of 0.002 to 0.1 *M* strongly inhibited the phosphomonoesterase of liver, bone, and kidney and the pyrophosphatase of kidney. They did not inhibit the phosphomonoesterase of intestine or the pyrophosphatase of intestine and bone, and they activated liver pyrophosphatase.

Phosphatases, like a number of other enzymes, are activated by very low concentrations of α-amino acids and are inhibited by higher concentrations. Bodansky (47) observed that the inhibition of phosphatases (bone and intestinal) by glycine depended to a very considerable extent upon the availability of the carboxyl and amino groups of glycine. Esterification of the carboxyl groups, or introduction of methyl groups into the amino radical, causes a marked decrease in inhibition. This paper contains a study concerning the mechanism of inhibition of phosphatase activity by glycine.

Gould (48) reported that preparations of the alkaline phosphatases of intestine, bone, and kidney were inactivated or inhibited by ketene, phenylisocyanate, nitrous acid, and formic acid. This indicates that a free amino group and a free phenolic hydroxyl group, possibly lysine, is required for full phosphatase activity. It does not exclude, however, the possibility that other groups may be necessary for complete phosphatase activity.

According to Van Thoai, Roche, and Roger (49) the dialysis of alkaline phosphatase resulted in partial dissociation, leading to the elimination of a metallic constituent and the rupture of intramolecular bonds of the protein apoenzyme. The reversibility of the process required first that the apoenzyme be saturated with metal. Different metal cations served for this purpose, each having its particular optimum concentration. The metal probably did not participate directly in the catalytic activity. Metallic complexes of alanine participated in the reaction of the alkaline phosphatase.

Crystallization of Alkaline Phosphatase of Kidney. Van Thoai and coworkers (50) obtained alkaline phosphomonoesterase in crystalline form by subjecting a beef-kidney extract to fractional precipitation with acetone at 0° C. At an acetone concentration of 38 to 50 per cent, a precipitate was obtained. When dissolved in a small quantity of water and kept at 6° C., it formed needle-shaped crystals. The crystals were readily soluble in very dilute alkali. From this solution, the enzyme was precipitated with acetone. It had a very high phosphomonoesterase activity at *p*H 9.2 when sodium β-glycerophosphate was the substrate and a trace of ionic magnesium was added. Pyrophosphatase activity at *p*H 8.6 was very slight.

Method for the Determination of Alkaline Phosphatase. Bessey, Lowry, and Brock (51) described a method based on the incubation

of serum alkaline phosphatase with the colorless compound *p*-nitrophenyl phosphate. On splitting off the phosphate group, the yellow salt of *p*-nitrophenol is liberated. This acts as a measure of activity. The method may be used to determine both acid and alkaline phosphatases.

The Acid-Phosphomonoesterase of Erythrocytes. Paget and Vittu (52) found that in most samples of human erythrocytes there is only one *p*H optimum for the acid phosphatase system. This is usually at 5.6 but in some samples it is at *p*H 5.4 or 5.2. A few specimens, however, show a second *p*H optimum between *p*H 4.6 and 4.8. The activity toward α-glycerophosphate is much more sensitive to changes in *p*H than the activity toward β-glycerophosphate. These authors presume that two phosphomonoesterases are present. The enzyme system from corpuscles of normal adults, at *p*H 5.6, hydrolyzes α-glycerophosphate about 8 times as fast as β-glycerophosphate. For children, the corresponding activity ration is approximately 6 to 1. There is no correlation between the acid phosphatase activity of the corpuscles and that of serum. Physiological concentrations of sulfanilamide inhibit the action of the corpuscle enzyme system 30 to 35 per cent at *p*H 4.2 to 4.6 and 10 per cent at *p*H 5.6.

Acid Phosphatase of Liver. Roche and Baudoin (53) found that the liver phosphomonoesterase having maximum activity at *p*H 5.5 hydrolyzed sodium-β-glycerophosphate faster than α-glycerophosphate. It was inhibited by fluoride and bile salts but not by thiol compounds or amino acids. It was slightly activated by ascorbic acid and by magnesium ions. The accompanying pyrophosphatase may be separated from the phosphomonoesterase by adsorption on kaolin at *p*H 4.5 and elution with 1 per cent ammonium hydroxide. This hepatic pyrophosphatase did not act on glycerophosphates. Glycerophosphates did not inhibit its action, but inorganic phosphates did. It is strongly activated by magnesium ions, inhibited by sodium fluoride and by sodium cholate, and not affected by oxalate, thiol compounds, or amino acids.

Roche, Van Thoai, and Michel-Lila (54) found that the apoenzyme of liver acid phosphomonoesterase combined with a coenzyme prepared from bottom yeast and resulted in a hepatic phosphatase. These experiments indicated that all isodynamic acid phosphomonoesterases possessed the same organic coenzyme, and that the specificities, *p*H optima, and activation behaviors depended on the protein constituents (apoenzyme) and metals with which they combined.

Other Mammalian Phosphatases. Guinea-pig bone marrow and polynuclear leucocytes contain three phosphomonoesterases with *p*H optima at 4.0, 5.6, and 9.0 to 9.5; the lymphatic ganglions contain three

with *p*H optima at 3.7, 5.8, and 9.0 to 9.5; and rabbit blood platelets contain only one phosphatase with a *p*H optimum at 5.0. All these acid phosphatases are more active on β- than on α-glycerophosphate. The reverse is true of the alkaline phosphatases (55).

The Use of Phosphatase Tests in Forensic Medicine. Riisfeldt (56) applied the acid phosphatase method of Gutman to the demonstration of seminal spots in forensic medicine. The basis of the test is the fact that no other body fluid contains as much acid phosphatase as semen, the average being 2600 units per cubic centimeter. Riisfeldt found that, whereas the acid phosphatase content in no sample of semen was lower than 400 units per cubic centimeter, other body fluids never showed a higher value than 10 units per cubic centimeter. This article contains a large number of experimental data.

Phosphatases in diseases have been reviewed by Jaffe and Bodansky (57) and by Greenstein (58).

PLANT PHOSPHATASES

A " very acid " phosphomonoesterase similar to the acid phosphomonoesterase of animal tissues occurs in green and other plant tissues. Its natural inhibition is general. It is, as a rule, inhibited by magnesium ions, but not in the presence of emulsin (59).

Van Thoai, Roche, and Roger (60) showed that the acid phosphomonoesterase prepared from the autolyzed mold *Boletus edulis* was active on β-glycerophosphate at *p*H 3.8 to 5.6, with maximum activity at *p*H 4.7. It was almost completely inactivated by sodium fluoride, 0.005 *M*, and the activity was not restored by the addition of calcium or magnesium acetate. The activity was completely restored by prolonged dialysis at 37° against distilled water without subsequent addition of magnesium or calcium salt. This shows that fluorine forms a dissociable complex with the enzyme-magnesium or -calcium system.

Courtois and Bossard (61) observed that in acid media (*p*H 4 to 6) Na_2MoO_4 strongly inhibited the action of the phosphatases of sweet and bitter almonds, yeast, *Aspergillus niger*, and erythrocytes on β-glycerophosphate and slightly inhibited the action of the phosphatases of calf bone and human kidney. Removal of the molybdate by dialysis restored the activity. Molybdate did not inhibit the action of β-glucosidase (from almonds), yeast invertase, barley amylase, and pepsin.

Yeast Phosphatases. According to Schäffner and Krumey (62), yeast contains several phosphatases, the presence of which can be

demonstrated by various methods. "Top-yeast phosphatase" is active optimally at *p*H 4 and hydrolyzes most phosphoric esters. It is not activated by magnesium ions and is 50 per cent inactivated at *p*H 7.6 in 5 minutes. It is not removed from yeast by glycerol but is present in maceration juice. The alkali-inactivated enzyme is not reactivated by hydrogen sulfide, hydrocyanic acid, potassium bromate, cysteine, ascorbic acid, or iodoacetate, nor is the original enzyme affected by these substances. A magnesium-activated α-glycerophosphatase, with an optimal activity at *p*H 6.4, is another specific enzyme. It is extracted by water or glycerol and is destroyed by heating to 50° C. for 30 minutes. The heated preparation contains an "alkaline phosphatase" with an alkaline optimum *p*H. Magnesium does not activate this enzyme but shifts the optimum *p*H to a more acid point. Metaphosphate, triphosphate, and pyrophosphate are not affected except when magnesium is added to the pyrophosphate. The optimum *p*H is then 7, indicating the presence of another phosphatase.

Yeast also contains a pyrophosphatase which acts like adenylpyrophosphatase. This enzyme splits off one molecule of orthophosphoric acid from cocarboxylase and is inhibited by thiamin (63).

Bailey and Webb (64) published interesting data concerning yeast pyrophosphatase. The enzyme was prepared from baker's yeast autolyzate. It was purified by a series of fractional precipitations with ammonium sulfate and fractional adsorptions on calcium phosphate gel, followed by elution with strong salt solutions. By this procedure, a 133-fold concentration was attained. The enzyme showed a single boundary for a period of 3 hours at *p*H 6 in electrophoresis tests. It was activated by magnesium ions, and it specifically catalyzed the reaction $HP_2O_7^{=} + H_2O \rightarrow 2HPO_4^{=} + H^+$. Neither α-glycerophosphate, hexosediphosphate, $NaPO_3$, Na_3PO_4, adenosine triphosphate, nor potassium diphenylpyrophosphate and flavin adenine dinucleotide were attacked. The optimum *p*H for the hydrolysis of inorganic pyrophosphate was at 6.9 to 7.0. The activation of magnesium ions was antagonized by calcium ions. The most powerful inhibitors were sodium fluoride, cupric ions, and iodoacetate. Cyanide had no effect. These results show that pyrophosphatase is a sulfhydryl enzyme.

Phosphomonoesterases of Bacteria. Paget and Vittu (65) observed two *p*H optima, one at 3.6 and one at 6.6, for the phosphomonoesterase of *Escherichia coli*. β-Glycerophosphate was more readily split than the α-isomer. Magnesium ions had a variable activating action. *p*-Aminophenylsulfamide was a weak inhibitor. Both *Staphylococcus aureus* and *S. albus* phosphomonoesterase had one optimum *p*H (6 to 7). This enzyme was activated by magnesium ions

and hydrolyzed α-glycerophosphate more readily than the β form. It was strongly inhibited by *p*-aminophenylsulfamide.

ADENOSINETRIPHOSPHATASE (ADENYLPYROPHOSPHATASE)

Adenosinetriphosphate is one of the most important constituents of muscle tissue. A large number of chemical reactions may be inhibited, yet muscular contractions will continue as long as adenosinetriphosphate is present. This substance is continuously regenerated at the expense of phosphocreatine. Adenosinetriphosphate is the last link in a long series of reactions which transform into mechanical work the chemical energy stored in carbohydrates. The catalyst responsible for this transfer of energy is the enzyme adenosinetriphosphatase. Whereas the hydrolysis of simple esters of phosphoric acid by phosphatase is accompanied by the release of only small quantities of free energy, the hydrolysis of the terminal phosphate bond of adenosinetriphosphate, with the formation of adenosinediphosphate and orthophosphoric acid, is accompanied by the liberation of a large amount of free energy. Lipmann (66) calls the pyrophosphate bonds of adenosinetriphosphate energy-rich phosphate bonds, and he uses the symbol ~ph to indicate such type of linkage. He calls the phosphoric ester bonds energy-poor. The enzymic splitting of adenosinetriphosphate furnishes immediate energy for muscular contraction and probably for other kind of cellular work, including certain reactions in plants and bacteria (67).

Lohmann (68) has shown that muscle tissue is capable of removing two molecules of orthophosphoric acid from adenosinetriphosphate. Washed muscle tissue hydrolyzed only one phosphate group, and the addition of magnesium restored it to full activity.

Engelhardt and Lyubimowa (69, 70) believed that the adenosinetriphosphatase action is a property of myosin and that this enzyme splits off one orthophosphate group from adenosinetriphosphate, the second phosphate being removed by a water-soluble enzyme. Other investigators confirmed the contention that the protein myosin, constituting 57 to 70 per cent of the total protein of muscle tissue, is identical with the enzyme adenosinetriphosphatase, and that this protein is responsible for the contractile and elastic action of muscle (71, 72).

Szent-Györgyi and his collaborators (73) prepared myosin in crystalline form. These workers state that twice-recrystallized myosin had the same enzyme activity as impure myosin, and they conclude " This seems to prove definitely that myosin is the enzyme itself." However, a number of other investigators do not hold with this view. Bailey

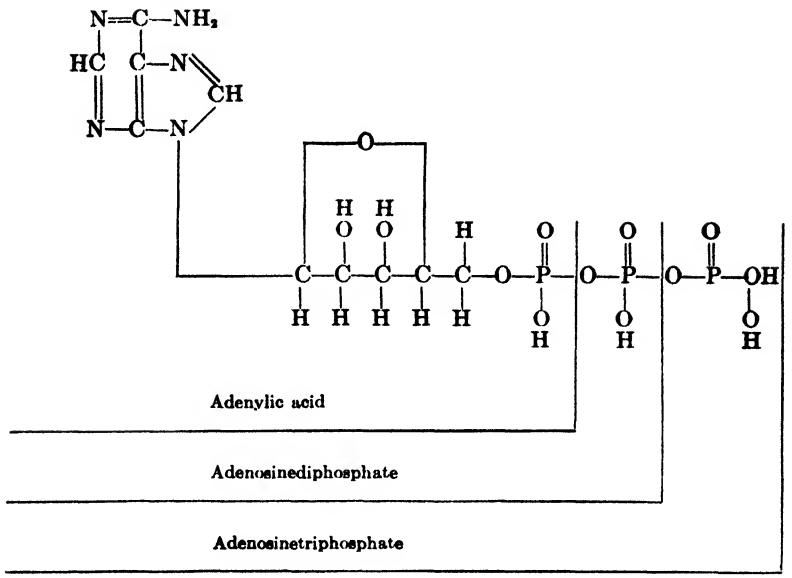

FIG. 6.

(74) found that the usual myosin preparation is not a single protein but contains a small quantity of a very active adenosinetriphosphatase, which is difficult to remove. Schramm and Weber (75) claim that myosin can be separated into four proteins possessing different sedimentation constants.

From potatoes, Kalckar (76) prepared water-soluble adenosinetriphosphatase 20 times as active as myosin per milligram protein. When myosin was precipitated as a result of the addition of the potato enzyme solution, the enzyme became adsorbed on the myosin.

Separation of Adenosinetriphosphatase from Myosin. Price and Cori (77) have been able to separate myosin from adenosinetriphosphatase. Myosin prepared from rabbit muscle was precipitated 3 times at 5° C., dissolved in Weber's solution (0.6 *M* sodium chloride, 0.01 *M* sodium carbonate, 0.04 *M* sodium bicarbonate), and dialyzed for 3 hours against 0.5 saturated ammonium sulfate at *p*H 5.2. The precipitate was stirred with a solution containing 0.03 *M* adenosinetriphosphate of *p*H 7.4 and precipitated by 4 volumes of acetone. The gummy precipitate was ground with 0.1 *M* potassium chloride and dialyzed in water for 10 hours to give a solution containing adenosinetriphosphatase 30 times more active per milligram of protein than myosin. By Tiselius diagrams, one main component comprised 85 per cent of the total protein. The activity of the enzyme was increased

by creatine, half maximal effect at 1.5×10^{-3} *M*, but was not affected by calcium until the enzyme was readsorbed on myosin. Rat muscle contained an enzyme with similar properties.

The Specificity of Adenosinetriphosphatase. Of a large number of phosphoric acid esters tested, only adenosinetriphosphate, inosinetriphosphate, and inorganic triphosphate can be hydrolyzed by this enzyme (78). Purified myosin, reprecipitated several times, splits only the terminal phosphate bond. This affords a method for the isolation of adenosinediphosphate and inosinediphosphate (79). The activity of this enzyme is markedly increased on the addition of calcium ions. Liver and other tissues also contain an adenosinetriphosphatase whose action is several times increased by the addition of calcium (80). Magnesium ions, however, strongly inhibit "myosin" activity (81). Myosin is most active at *p*H 9.0, with a second but much lower optimum at *p*H 6.3 (81). This enzyme is completely destroyed if kept for 10 minutes at 37° C. The inactivation may be prevented by the presence of adenosinetriphosphate or adenosinediphosphate (69).

PHYTASE

Phytase hydrolyzes phytin or phytic acid (inositolhexaphosphate) and its derivatives into inositol and orthophosphoric acid. The enzyme is present in grains, especially in the pericarp. The best sources are the bran of wheat, barley, and rice. Considerable quantities have been found in kidneys, *Aspergillus oryzae,* barley malt, and alfalfa. Hay (82) found that between 85 and 100 per cent of the total phosphorus of wheat bran is present as phytate. He postulated that phytase provides the germinating plant with soluble phosphate.

Fleury and Courtois (83) showed that, although many phosphatases will not hydrolyze phytin, no enzymic preparation hydrolyzing phytin has been described that will not also hydrolyze other phosphoric acid esters such as glycerophosphate. These authors found that preparations of wheat bran were able to hydrolyze phytin more rapidly than glycerophosphate. The activity could be fractionated, yielding a preparation hydrolyzing glycerophosphate, but was inactive for phytin, and another preparation was obtained, hydrolyzing both substrates. The last preparation hydrolyzed phytin more rapidly than glycerophosphate, but the activity could not be separated by further purification. Inactivation rates by various methods indicate that a single enzyme is involved. The name phytophosphatase was suggested for those enzymes hydrolyzing both inositolhexaphosphates and other phosphoric acid esters.

From alfalfa, Martin and Doty (84) obtained a phytase preparation which was 200 times more active than the plant itself. The aqueous extract of the alfalfa was precipitated with 3 *M* ammonium sulfate or by adding ethyl alcohol to 90 per cent concentration. The enzyme had an optimum *p*H at 4.6 to 5.4, depending on the nature and concentration of buffer. All preparations exhibited strong phosphomonoesterase activity for sodium glycerophosphate, but the ratio of activities toward phytin and glycerophosphate could be changed greatly, indicating that the two activities present in alfalfa were due to two different enzymes. When acting on phytin, the rate of hydrolysis by the phytase was nearly independent of the substrate concentration (zero-order reaction).

CHOLINESTERASE

The pharmacological action of acetylcholine was discovered by Otto Loewi. Acetylcholine has marked physiological effects when introduced intravenously into an animal. Acetylcholine takes part in the humoral transmission of the effects of nervous stimulation in cholinergic systems. Acetylcholine is hydrolyzed during stimulation of the vagus, the motor nerves to skeletal muscle, the preganglionic fibers of the sympathetic nervous system, and probably when impulses cross the synapses of the spinal cord (85).

The Specificity of Cholinesterase. The evidence appears to indicate that there are two distinct types of esterases capable of hydrolyzing acetylcholine. Thus, Mendel and coworkers (86) have demonstrated that the specific or "true" cholinesterase, which is present in the nervous system and red blood cells, is unable to hydrolyze benzoylcholine. The non-specific cholinesterase of blood serum and certain glands readily splits the ester just mentioned. The specific cholinesterase, but not the non-specific esterase, hydrolyzes acetyl-β-methylcholine. However, both these enzymes split acetylcholine more efficiently than their specific substrates. Sawyer (87) observed that a powerful esterase is present in guinea-pig and rabbit liver, which hydrolyzes benzoylcholine but does not act on acetylcholine. He named this enzyme benzoylcholine esterase, and he cautions against the acceptance of benzoylcholine hydrolysis as an absolute measure of pseudocholinesterase action. Sawyer suggests that pseudo or non-specific cholinesterase assays should be made, as before, in conjunction with activity tests on both acetylcholine and acetyl-β-methylcholine (Merck's Mecholyl).

Nachmansohn and Rothenberg (88) have recently reviewed the

question concerning the specificity of the cholinesterase of the nerve and other tissues of several warm-blooded and cold-blooded animals. They found that the main characteristic of nerve cholinesterase is that no other ester is split at a higher rate than acetylcholine. If butyrylcholine is the substrate, the rate of hydrolysis is much lower, or it may be zero. Carbaminylcholine and benzoylcholine are not split. Esters of simple alcohols are hydrolyzed either very slowly or not at all. The other esterases, representing a variety of enzymes, show definite differences and may be readily distinguished. These enzymes do not split acetylcholine at the highest rate. The esterase of human serum is unspecific, and the esterase in the red blood cells is specific for acetylcholine. These findings confirm those of other investigators. Heart muscle also contains a specific cholinesterase. Nachmansohn and Rothenberg conclude, "The esterase in all nerve tissues is either exclusively or predominantly cholinesterase." The views of Augustinson (89) are similar.

Inhibitors of Cholinesterase. Eserine (physostigmine) in quantities as small as 1 gamma per 2 cc. of reaction mixture completely stops cholinesterase activity. Other esterases require much larger quantities. Cholinesterase is also inhibited, but to a lesser extent, by compounds such as ergotamine and muscarine. All these inhibitions are removed on dialysis.

Koelle (90) found that eserine, by combining reversibly with rat-brain cholinesterase, protects the enzyme against irreversible inactivation by diisopropyl fluorophosphate (DFP) *in vitro*. The degree of protection varies directly with the concentration of eserine. This may explain how eserine protects cats against DFP poisoning. Of 19 other anticholinesterase drugs similarly tested, prostigmine and carbamylcholine afforded marked protection. Pilocarpine, nicotine, atropine, choline, procaine, and morphine afforded relatively slight protection. Methylene blue, strychnine, atebrin, quinine, sodium fluoride, thiamin, cysteine, sodium *p*-aminobenzoate, acetyl-β-methylcholine, acetylcholine, and curare (intocostrin) gave no protection. The protective property does not appear to depend on the potency of anticholinesterase activity alone but also on the ability of the compound to compete with DFP for a specific active group of the cholinesterase molecule.

Crystalline Cholinesterase. Bader, Schütz, and Stacy (91) described a method for the preparation of a crystalline serum mucoprotein having cholinesterase activity. The starting material was horse serum, and the procedure involved adsorption on foam. Barnard (92) believes that cholinesterase may be an iron-containing enzyme.

Enzymic Acetylcholine Synthesis — Cholineacetylase

The enzymic synthesis of acetylcholine was reported in 1925 by Abderhalden and Paffrath (93) using an extract of the intestinal mucosa, and in 1934 by Ammon and Kwiatkowski (94) employing blood serum. It is now well known that nerve tissues synthesize acetylcholine *in vitro*, and several groups of investigators have furnished evidence to show that the acetylcholine synthesis is brought about by a specific enzyme (85). Nachmansohn and Machado (95) have extracted from brain tissue of the rat and from the electric organ of *Electrophorus electricus* an enzyme, cholinacetylase, which synthesizes acetylcholine. This enzyme has been extensively studied by Nachmansohn and John (96), and many interesting observations have been made. Their reaction mixture contains in final concentrations: choline 0.0025 *M*, acetate 0.02 *M*, fluoride 0.02 *M*, physostigmine 0.0017 *M*. The source of enzyme is a cell-free brain extract of young rats or of guinea pigs. Acetylcholine is formed under strict anaerobic conditions in the presence of adenosinetriphosphate. The enzyme requires 0.08 *M* potassium ions, which is close to that found in mammalian brain. The amount of acetylcholine formed is 100 to 150 gammas per gram per hour. Two hours of dialysis removes 85 per cent of the original enzyme activity. Potassium, citric acid, and *l*(+) glumatic acid restore part of choline acetylase activity. Cysteine reactivates the greater part of the enzyme. Cholinesterase is almost completely inactivated by acetone, but cholinacetylase is not. In this manner, the two enzymes may be separated. The work of Nachmansohn and associates concerning cholinacetylase had been confirmed and expanded by Feldberg and Mann (97).

Minz (98) reported that the dorsal muscle of the leech, in the presence of air, cocarboxylase, choline, and eserine, rapidly forms a substance which causes muscle to contract. According to the author, cocarboxylase has an important function in the formation of acetylcholine.

Lipton and Barron (99) found that the brain-enzyme system, which anaerobically synthesizes acetylcholine, is water soluble and can be extracted from acetone-dried preparations. For activity of this enzyme system, the presence of choline, a suitable substrate, potassium, adenosinetriphosphate, and a coenzyme present in boiled aqueous extracts of brewer's yeast or animal tissues is required. The suitable substrates are citrate, *cis*-aconitate, and acetoacetate. Anaerobic synthesis of acetylcholine consists of two different reactions. One is the formation of " active " acetate from the anaerobic breakdown of citrate

or acetate. The second is the acetylation of choline by the " active " acetate. It is not known which of the two reactions requires the coenzymes (see also reference 100).

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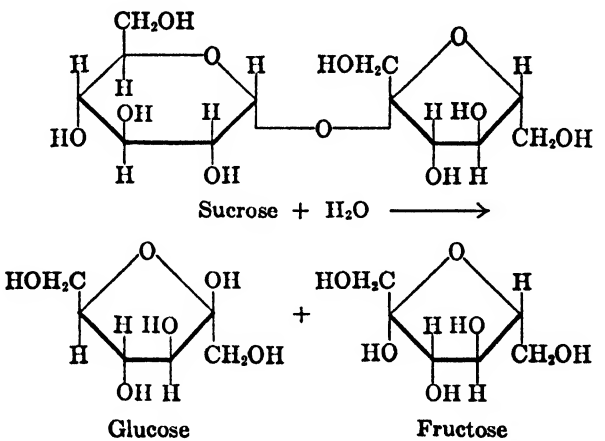
CHAPTER III

CARBOHYDRASES

INVERTASE (BETA-FRUCTOFURANOSIDASE, SUCRASE, SACCHARASE)

Invertase is widely found in nature. The best source is yeast, from which it may be prepared in fairly pure state. Dry invertase is quite stable, and its substrate, sucrose, is inexpensive. The end products of sucrose hydrolysis are easily determined. For these reasons, invertase has been extensively studied by many chemists. The name invertase has been coined because the sucrose solution, which is at first dextrorotatory, becomes levorotatory as a result of the formation of glucose and fructose. From one mole of sucrose is formed one mole each of glucose and fructose:



HYDROLYSIS OF SUCROSE BY INVERTASE

The Specificity of Invertase

Yeast invertase is a β-*h*-fructosidase (or β-*d*-fructofuranosidase) or simply a fructosidase. It removes the fructose residue (having a β configuration and the 2,4 oxygen ring) of the substrate. Mold invertase, however, is an α-*n*-glucosidase, which splits off the glucose end of the substrate. For example, in the trisaccharide raffinose, which is fructosylglucosylgalactoside, the glucose is blocked by the glucosidic

linkage with galactose. This saccharide is not hydrolyzed by mold invertase but is split into fructose and melibiose (galactosylglucose) by yeast invertase (1). Sucrose, being a glucoside built of the two monosaccharides glucose and fructose, is, of course, not suitable for demonstrating the specificity of the two enzymes. The trisaccharide gentianose (sucrose-glucoside) and the tetrasaccharide stachyose (sucrose-digalactoside) are also hydrolyzed by yeast fructosidase, since they are *β-d*-fructofuranosides.

Adams, Richtmyer, and Hudson (2), using several highly purified invertase preparations from brewers' and bakers' yeasts, verified the earlier findings of others, that yeast invertase splits the sucrose union in both sucrose and raffinose. However, activity ratios and optimum *p*H values suggest that inulin is split by a specific inulase, and not by the yeast invertase (*β-d*-fructofuranosidase) as postulated by Weidenhagen. Thus, one of the enzyme preparations had only one-tenth the inulase activity of the other two, whereas their invertase activity was not much different. These invertase preparations did not hydrolyze melizitose (glucose-fructose-glucose) and isosucrose.

The purest invertase preparations, from brewers' yeast, contained a small quantity of beta-*d*-glucosidase which hydrolyzed amygdalin, gentiobiose, and *β*-phenyl-*d*-glucoside, but not cellobiose or lactose. Both brewers' and bakers' yeasts contain small quantities of a new enzyme, a *β-d-mannosidase,* which hydrolyzed *β*-phenyl-*d*-mannoside. Weidenhagen (3) denies the existence of a specific invertase, maltase, or *α*-methylglucosidase. Much interest had been aroused in his theory, but it has not found general acceptance.

Yeast as an Invertase Source. Most bakers', brewers', and distillers' yeasts contain large quantities of invertase, and there appears to be some even in lactose-fermenting yeasts. *Saccharomyces albicans* and some varieties of *Saccharomyces apiculatus, Hanseniaspora,* and *Torula* do not contain this enzyme. The invertase-free yeasts have been employed to remove, by fermentation, monosaccharides from molasses used in sucrose production (4). Other invertase-containing yeasts are *Saccharomycoides ludwigii, Saccharomyces exiguus,* and *Zygosaccharomyces marxianus.* These yeasts, however, do not contain maltase and are unable to ferment maltose. These facts indicate that invertase and maltase are two specific enzymes.

The Distribution of Invertase in Nature

The invertases of other microorganisms than yeast have not been exhaustively studied. A variety of molds, such as *Aspergilli, Mucors,* and *Penicillia* are able to produce invertase (5, 6).

Kertesz (7) reported that *Aspergillus niger* is unable to produce invertase unless grown on a culture medium which contained sucrose or raffinose. The invertase formed was proportional to the amount of sucrose in the culture, provided that the sucrose concentration was not over 30 per cent. The omission of potassium and magnesium from the medium resulted in a considerable increase in the formation of invertase by this mold (8). Many bacteria of the lactic acid and acetic acid group produce invertase, but not all strains (9). They are not, however, very good sources of this enzyme. Small amounts of invertase are also present in most higher plants. For instance, invertase occurs in the stem and leaves but not in the roots of sugar beets. This is explained by the interaction of invertase with respiration of the plant. It supplies the plant with monosaccharides during anaerobic conditions (10).

The invertase of the honeybee has been frequently studied, because of its function in the production of honey (11, 12). This enzyme is said to differ from yeast in its inability to hydrolyze raffinose and in having an optimum *p*H at 5.5 to 6.3, instead of at 4.8 to 5.8 like the yeast enzyme. Invertase has also been found in Crustacea and in many other lower animals (13). Various tissues of higher animals were reported to contain invertase. Ingested sucrose, however, is split by the intestinal microbes, and parenterally introduced sucrose is eliminated by the kidneys as a foreign substance.

Preparation of Yeast Invertase

Various procedures have been tried for the preparation of invertase. Since the enzyme is located in the interior of the yeast cell, the cell wall must be ruptured (plasmolized) in order to liberate it. The yeast may be allowed to autolyze at room temperature until the yeast cells die and are partially liquefied. This is a very slow procedure. It may be speeded up by the addition of toluene, so that within a short time liquefaction is complete. Various authors have used benzene, xylene, carbon tetrachloride, amyl alcohol, ethyl acetate, chloroform, sucrose, or sodium chloride as the plasmolyzing agent.

Lutz and Nelson (14) obtained a highly active invertase from yeast by a method based to a certain extent on several earlier procedures (autolysis, precipitation by alcohol, kaolin adsorption, elution from kaolin with secondary ammonium phosphate, dialysis of the eluate, adsorption of this on alumina, elution with secondary sodium phosphate, ammonium sulfate treatment, dialysis). This invertase showed protein and yeast gum tests (15).

Sizer (16) has prepared invertase by the adsorption-elution method

of Lutz and Nelson and found that by this procedure a 500-fold increase in activity above that of the original toluene autolyzate may be obtained. More recently, Adams and Hudson (17) described a simple method in which bentonite is used as an adsorbent. These invertase preparations were of high and constant activity, and purification was as effective as when more involved procedures, applying fractional adsorption, were employed. The final preparations consisted mainly of protein and small amounts of carbohydrates.

The Preparation of Commercial Yeast Invertase

Grassmann and Peters (18) described the following method for the preparation of invertase from yeast. Two kilograms of yeast are treated with ethyl acetate for 1 hour at 40° C. The separated yeast juice is removed by centrifuging. The solid residue containing 70 per cent of the invertase is twice washed with ether and dried at room temperature. The yield is 315 grams.

To obtain the invertase in soluble state, the insoluble preparation must be treated with proteolytic or certain amylolytic enzymes. A sample of 9.84 grams of dry preparation, when treated with 150 milligrams of activated papain, released 83 per cent of the invertase in soluble form. Mold enzymes, such as those of *Aspergillus oryzae* or malt diastase, had a similar effect. Animal amylase, however, did not liberate the invertase.

Neuberg (19) precipitates the invertase from its solution in the form of an adsorption complex, using calcium phosphate prepared from $CaCl_2$ and Na_2HPO_4. The precipitate is washed free from the excessive salts and dried at low temperature. The dry powder is said to be highly active. Acid salts, such as potassium acid phosphate or tartrate, or tartaric, mucic, or citric acid, serve as stabilizers of the dry invertase. The patent literature concerning the technical preparation of invertase has been reviewed by Neuberg and Roberts (20).

Yeast Invertase Values. Sumner and Howell (21) reported the following invertase values for a series of products: Fleischmann's yeast, 994; Nectar Brewing Company yeast, 830; Difco invertase, 18,300; takadiastase, 48.

The Industrial Application of Invertase. The enzyme invertase is employed in the candy industry to make soft cream centers by inversion of the fondant after the center has been coated. Sucrose forms crystals readily. Invert sugar, however, as produced by invertase action from sucrose, remains liquid even in higher concentrations.

Owing to the desirable properties of invert sugar, invertase is em-

ployed in the manufacture of cane and sorghum syrups. A British patent (22) suggests the simultaneous inversion and decolorization of sucrose liquors by passing them through granular bone charcoal on which invertase has been adsorbed.

Kinetics. It may be seen from Fig. 7 that the optimal zone for the hydrolysis of sucrose by purified yeast invertase is at 4.8 to 5.8, although the decrease in activity on the acid side of the optimum is very small, even at *p*H 2.8 (2). There is close parallelism for sucrose and raffinose throughout the *p*H range. There is a marked difference between the *p*H activity curve for inulin and the curves for sucrose and raffinose. The optimum *p*H for the hydrolysis of inulin by the yeast invertase preparation is at 3.2 and 4.0. This indicates that yeast inulase is a specific enzyme, not identical with β-fructofuranosidase, which acts on sucrose and raffinose.

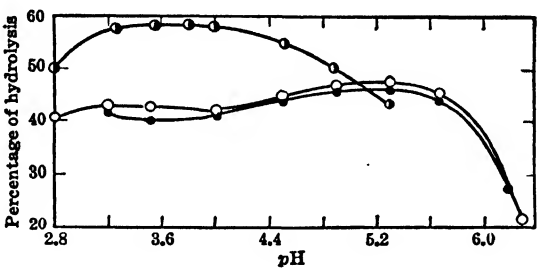


FIG. 7. *p*H-Activity curves for the hydrolysis of sucrose, raffinose, and inulin by bakers' yeast invertase preparation: ○, sucrose; ●, raffinose; ◑, inulin.

Fifty cubic centimeters of reaction mixture contained 2.5 grams of the respective substrates, 5 cc. of *M* acetic acid to maintain a *p*H of 2.8, or 5 cc. of mixtures of 0.2 *M* acetic acid and 0.2 *M* sodium hydroxide to furnish *p*H values between 3.2 and 6.3, or 12.5 cc. of mixtures of 0.2 *M* primary and secondary sodium phosphates when a higher *p*H was desired.

Invertase is most active in the presence of 5 to 10 per cent of sucrose. The inversion of sucrose by invertase follows very closely a first-order reaction. The purity of the invertase preparation has no effect on the kinetics of the enzyme. The rate of inversion increases with increase in temperature and is 3.5 times greater at 45° C. than at 35° (23). The kinetics of invertase action has been extensively discussed by Nelson (24) and by Weidenhagen (25).

Estimation of Invertase Activity. The time necessary to bring the rotation of a sucrose solution to 0 is estimated with the aid of the polariscope, and is called the "time value." Thus, according to

Willstätter and Kuhn (26), the enzyme preparation has the potency of 1 invertase unit when 50 milligrams have a time value of 1, using 25 cc. of 16 per cent sucrose at 15.5°. This means that the purity of an invertase preparation is expressed by the enzyme value, or number of units in 50 milligrams of enzyme preparation. The unit of Euler and Josephson (27) is the *Inversionsfähigkeit* (power of inversion), where $If = (k \times \text{grams of substrate}) \div \text{grams of enzyme}$. Sumner and Howell (28, 29) propose the dinitrosalicylic acid method for reducing sugars for the estimation of invertase activity, which would be much faster and simpler than the earlier methods. They use sucrose concentrations of 5 to 10 per cent to obtain maximum velocity of invertase activity (30). In the digest of Sumner and Howell, no more sucrase is used than is necessary to obtain 10 milligrams of invert sugar by hydrolysis of 6 cc. of 5.4 per cent sucrose in 5 minutes at 20°. Under these conditions, they found that the velocity is only 1 per cent less than it is at 0 time. The reaction is stopped by the addition of 5 cc. of approximately 0.1 *N* NaOH, and the invert sugar is determined colorimetrically. By this method, the sucrose units are expressed in terms of milligrams of invert sugar produced in 5 minutes at 20°, at *p*H 4.5 (*N* acetate buffer). Other colorimetric methods may also be used.

Inhibitors of Invertase

Crude yeast invertase solutions are very stable. Purification gradually lowers the enzyme's resistance, even when kept at a low temperature. Concentrated solutions are less sensitive than dilute ones. Any step of purification results in some destruction of enzyme activity. Invertase solutions are most resistant at *p*H 4 to 5. Dry invertase preparations may be kept for many years without loss of activity. Purified invertase solutions are most rapidly destroyed at an alkaline *p*H. The yeast cell, however, offers adequate protection against an alkaline medium. Thus, sucrose may be hydrolyzed and fermented at a *p*H of about 8 in the presence of Na_2SO_3, MgO, Na_2HPO_4, NH_4OH, and Na_2CO_3, forming glycerin and acetaldehyde, or their products of dismutation (31).

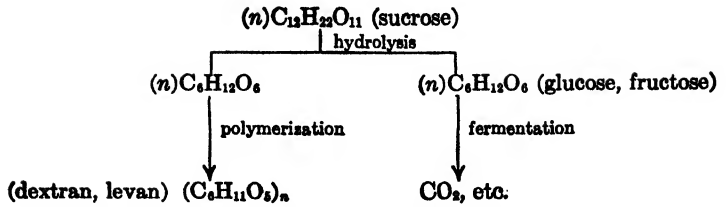
Crude yeast invertase is not very sensitive to ethyl alcohol. Highly purified invertase, however, is rapidly destroyed by alcohol. Filtrates of autolyzed yeast, which have been maintained at an acid *p*H, are most suitable for precipitation with alcohol. In order to avoid destruction of the invertase, it is best to adjust the autolyzates to *p*H 4.5 to 4.8 just before the alcohol is added. Working at low temperatures is very desirable. An equal volume of alcohol precipitates almost all the

invertase present in the autolyzate. By adding between 20 and 40 per cent alcohol at first, some of the impurities may be removed with very little loss of activity (32). Purified invertase is irreversibly destroyed by alcohol concentrations over 20 per cent, with a maximum toxicity at 50 per cent concentration. However, no destruction of the enzyme occurs below 20 and above 80 per cent concentration of alcohol (33). Sucrose protects the invertase from the toxicity of alcohol. Six per cent sucrose reduces the inactivation by 50 per cent alcohol concentration to 1 per cent of the original activity of the enzyme. Crude but not pure invertase is reversibly inactivated by mercuric and silver salts, but on dialysis or removal of the heavy metals the activity is fully regained. This kind of inactivation is considerably reduced in the presence of sucrose (34). Iodine, at room temperature, inactivates only partially at first. This fractional type of inactivation is instantaneous, amounting to 40 per cent of the total invertase. This is followed by a further but slow inactivation. The phenomenon has been extensively studied by Myrbäck and his coworkers (35). It is believed that an iodine-invertase compound is formed, having about half the activity of the original invertase. It does not affect the active grouping of the enzyme molecule, since, on addition of an excess of sodium thiosulfate, much of the activity may be recovered. At low temperatures, toluene or thymol does not inhibit invertase; at 30° C., however, the inhibition by toluene is very considerable (2).

Sizer (16) studied the effect of a series of oxidants and reductants on the activity of purified yeast invertase. Oxidation inhibits the enzyme irreversibly and acts on the enzyme rather than the substrate. The action of oxidation-reduction potentials on invertase are independent of the purity of the enzyme. Chase and colloborators (36) found that invertase that had been partially inactivated at 50° C. regained its activity completely upon being cooled to 21°.

Polymerization of Sucrose by Bacteria and by Bacterial Enzymes

Certain bacteria have the ability to convert sucrose to the polysaccharide levan; others change it to the polysaccharide dextran. In addition to these polymerizations, however, a simultaneous hydrolysis into hexoses takes place and one-half of the disaccharide molecule is fermented to the usual end products. Phosphate ions do not appear to interact in these reactions, and monosaccharides are not polymerized in this manner (37). Leibowitz and Hestrin proposed the following scheme for the polymerization-fermentation reaction:



This scheme has been criticized by Doudoroff and O'Neal (38).

SYNTHESIS OF LEVAN BY BACTERIA

The fructosan levan is a white, amorphous powder with a melting point at about 200° C. and an optical rotation of $[\alpha]_D^{22} = -46.1°$. The polysaccharide is sparingly soluble in water, soluble in glycol and glycerol, and insoluble in ethyl alcohol and methyl alcohol. It does not reduce Fehling's solution (39).

Levan may be prepared by the action of *B. mesentericus, B. subtilis, B. polymyxa, Aerobacter levanicum,* and other bacteria on sucrose solutions. When one of these bacteria is grown in a fluid medium containing sucrose, the solution turns opalescent and viscous. These properties are associated with the formation of levan.

For instance, the polysaccharide may be readily prepared by inoculating *B. subtilis* into sterile 0.25 per cent solution of peptone. The peptone is in phosphate buffer contained within a cellophane bag, which is suspended in a large volume of 10 per cent sucrose in 0.25 per cent peptone. After 4 days at 30° C., the levan concentration in the internal phase reaches 12.5 per cent. Outside the cellophane container, however, none of the fructosan is formed (40, 41). The bacterial cells may be removed by centrifuging and the levan precipitated with 75 per cent ethyl alcohol.

SYNTHESIS OF LEVAN BY LEVANSUCRASE

Hestrin and Shapiro (42) obtained levansucrase, the enzyme responsible for levan synthesis, in a cell-free state. They ascribe to the enzyme (of *Aerobacter levanicum*) the following hydrolytic and polymeric actions:

$$(n+m)\text{sucrose} + (n)H_2O \rightarrow (n+m)\text{glucose} + (n)\text{fructose} + [\text{levan}]$$

$$(n+m)\text{raffinose} + (n)H_2O \rightarrow (n+m)\text{melibiose} + (n)\text{fructose} + [\text{levan}]$$

where *n* and *m* are moles of substrate converted into levan and aldose, and into fructose and aldose, respectively; where [levan] represents the levan formed from *m* fructosidic residues of substrate; and where the above reaction products represent the total amount of substrate acted upon by the enzyme. Phosphate, adenylic acid, levan itself, and the dialyzable components of the enzyme preparation are not essential components of the levan-forming enzyme system. Reversal of the enzyme reaction, which leads to the synthesis of levan, has not been demonstrated, but its possible existence has not been excluded. Although living cells of *Aerobacter levanicum* ferment levan, the levansucrase preparation obtained from them contained no levan hydrolase.

Doudoroff and O'Neal (38) reported on the preparation, from *Bacillus subtilis*, of a concentrate that, in addition to the levan-synthesizing enzyme, also contained a hydrolytic enzyme that attacked levan.

Enzymic Synthesis of Dextran by Dextransucrase

From cells of *Leuconostoc mesenteroides* and related organisms, soluble enzyme solutions may be prepared that convert sucrose to dextran. In addition to this polysaccharide, however, free hexoses are also formed (43, 44). Dextran is a water-soluble colloidal glucosan of high viscosity, strongly dextrorotatory, and is precipitated by alcohol. Dextran may be readily produced in large quantities on various crude, sucrose-containing media, or by employing the following medium (45):

Sucrose	150 grams
Tryptone	10 grams
Yeast extract	1 gram
Dipotassium phosphate	1 gram
Water	1000 cc.

The sterilized medium is inoculated with a culture of *L. mesenteroides* and incubated at 30° C. At the end of 48 hours, the solution becomes so viscous that it appears like a solid mass of jelly.

MALTASE (α-*d*-GLUCOSIDASE)

Maltase is found in almost all plant and animal tissues. Yeast and molds are very good sources of maltase. The substrates of this enzyme are maltose and α-glucosides. Their optimum *p*H is close to 7.0, varying slightly according to the source and buffers employed.

There are two classes of maltases: (*a*) true α-glucosidases and (*b*) pseudo α-*d*-glucosidases. The first group splits maltose and all α-glu-

cosides. The second group hydrolyzes maltose and those α-*d*-glucosides that are easily split (46).

Separation of Yeast Maltase from Sucrase. Michaelis and Rona (47) were able to separate yeast maltase and sucrase by treating yeast autolysate with kaolin. The kaolin adsorbed the maltase, the sucrase remaining in solution. Willstätter and Bamann (48) confirmed this but suggested that certain hydroxides of aluminum are better adsorbents, since they are not as destructive to the maltase as the kaolin is.

Preparation of Yeast Maltase. Eighty-eight grams of bottom yeast is washed, pressed, and plasmolyzed with 40 cc. of toluene. One hundred thirty-two grams of water is added, and the mixture is neutralized with ammonium hydroxide. After 24 hours it is filtered; the filtrate contains the maltase. The method for bakers' yeast is as follows: 170 grams of this yeast is allowed to autolyze for 10 minutes with 80 cc. of toluene. After the addition of 130 cc. of water, the autolyzed yeast is neutralized with ammonium hydroxide over a period of several minutes, extracted for 24 hours, and filtered. The filtrate (200 to 260 cc.) contains invertase in addition to maltase. It may be kept under toluene (49).

Preparation of Invertase-Free Mold Maltase. The principle of this method, as suggested by Feigenbaum (50), is the lability of invertase to reducing agents such as $Na_2S_2O_4$ and its concentration by a new dialysis procedure.

Two grams of commercial mold diastase is dissolved in 15 cc. of distilled water. The solution is placed in a cellophane bag, covered with toluene, and dialyzed at room temperature for one day against running tap water, and one day against distilled water which is changed several times, until the solution is free of reducing substances. The dialyzed enzyme solution (about 30 cc.) is filtered and mixed with 0.3 gram of sodium hydrosulfite. After 24 hours at room temperature, the hydrosulfite is removed by dialysis for one day, against running tap water and one day against distilled water which is changed several times.

The dialyzed solution, showing only maltase activity, becomes highly diluted. It may be concentrated by dialyzing it against 96 per cent ethyl alcohol. According to this newly discovered method of the Palestinian worker, 40 cc. of the maltase solution may be concentrated in 6 hours to about 3 cc. when ethyl alcohol is used as the "outside solution." From the concentrated solution, the maltase may be obtained by precipitation with an equal volume of absolute alcohol, centrifugation, and drying over sulfuric acid in the desiccator. The maltase prepared in this manner includes all the maltase activity of the original preparation but is free of invertase activity. This con-

firms the theory that mold maltase and mold invertase are two distinct enzymes. The present author has found this procedure for the concentration and purification of other enzymes to be very applicable.

Kinetics. The kinetics of maltose hydrolysis varies with the source of the maltase. The reaction proceeds more slowly than the monomolecular one. For the estimation of maltase activity the method by Tauber and Kleiner (51) may be used.

Synthetic Action of Maltase. The following is a simple method for the synthesis of α-methylglucoside from methyl alcohol and glucose (52). In a 10-liter flask place 1800 grams of pure methyl alcohol and 500 grams of glucose dissolved in 4 liters of distilled water. Mix. Add 3 liters of filtered yeast macerate (to contain 10 per cent dry bottom yeast). Mix. Dilute to 10 liters. Allow to stand until the initial rotation of +5° 18′ goes up to +11°. Bourquelot has obtained a number of similar compounds by this method.

MELIBIASE (α-d-GALACTOSIDASE)

This enzyme hydrolyzes α-*d*-galactosides, such as melibiose (galactosidoglucose), raffinose (galactosidoglucosidofructoside), and β-*l*-arabinosides.

It is present in bottom or lager yeast, in *Aspergillus oryzae,* and in many higher plants. Testing for melibiase activity may serve to distinguish between ale and lager yeast, since ale yeast does not contain melibiase (53).

LACTASE (β-d-GALACTOSIDASE)

Lactase (β-*d*-galactosidase) hydrolyzes β-galactosides. One of its substrates, β-lactose, is hydrolyzed to β-*d*-galactose and β-*d*-glucose. It also splits β-*l*-arabinosides and heptosides with β-*d*-galactose configuration (Pigman). Röhmann and Lappe (54) found it to be present in the young mammal's small intestine. According to Porcher (55) it may be prepared from the intestine of the fetus of various animals. Foa (56) stated that the adult mammal's intestinal mucosa contains no or very little lactase. No lactase could be found in the cow's mammary glands by Bradley (57) or by Kleiner and Tauber (58). A lactose-synthesizing action of rabbit's mammary gland tissue has been observed by Michlin and Lewitow (59), and by Grant (60). Cajori (61) examined the intestinal mucosa, which he stripped from the duodenum or jejunum of adult dogs, for lactase action. He used the ground fresh tissue as well as the extracts, and he also tested the

intestinal juice from dogs with Thiry loops and with extracts of the fresh dog liver. Maximum activity was obtained with fresh, finely ground tissues. Liver showed slight activity. The juice from a Thiry loop of the colon did not exhibit lactase activity. These experiments show that lactose is hydrolyzed before it is absorbed. The slight activity of the aqueous extracts and the succus entericus indicates that this enzyme is intimately bound to the mucosal cells. Cajori (62) has also shown that the succus entericus, as a digestive fluid, is of only minor importance, since several enzymes are present only in traces. The major enzymic action results from direct contact with the mucosa or intracellularly.

The digestive tract of *Helix pomatia* has the ability to break down lactose (63). Lactase also occurs in almonds. Hofmann (64) obtained enzyme preparations from *B. coli* and *B. delbruckii,* which contained *β-d*-galactosidase free of *β-d*-glucosidase. The almond *β-d*-galactosidase, however, could not be freed of *β-d*-glucosidase. Lactose yeasts, kafir grains, and certain molds also contain lactase.

Optimum Activity. Dog intestinal lactase has optimum activity at *p*H 5.4 to 6.0 (52); calf intestinal lactase has its optimum at *p*H of 5 (65); and the gut lactase of the cockroach, at 5.0 to 6.4 (66). Yeast lactase, however, has its optimum at *p*H 7.0 (67); almond lactase, at *p*H 4.2.

Reaction Course. Armstrong (68) found that hydrolysis of lactose by the lactase of the kafir grains follows at first the course of a monomolecular reaction and later decreases. Similar results were obtained by Willstätter and Oppenheimer with yeast lactase, and by Cajori with the intestinal lactase of the adult dog. For estimation of lactase activity, the method of Tauber and Kleiner (51) may be used. (See also Cajori (61).)

β-GLUCURONIDASE

Many organic compounds that contain a hydroxyl or carboxyl group and that are foreign to the animal organism are conjugated to glucuronic acids. The main site of these syntheses is the liver. Fishman (69) has shown that the enzyme β-glucuronidase, which hydrolyzes glucuronides, is also present in good concentration in the spleen, ovary, uterus, and kidney. Fishman and Fishman (70) found that, when mice were fed menthol or borneol for a few days, the glucuronidase content of liver, kidney, and spleen increased, whereas that in the uterus remained constant and that in the ovary decreased. When estrogenic substances were fed, the uterine glucuronidase increased but

the liver enzyme did not change. Mills (71), who studied the glucuronidase content of animal tissues, found that the role of the spleen in the metabolism of conjugated glucuronic acids is to break down rather than synthesize these acids. In the rabbit, 95 per cent of the total glucuronidase is contained in the liver and only 1.5 per cent in the spleen. In the rat, however, the liver contains 60 per cent and the spleen 25 per cent of the enzyme. The kidney contains only 2 to 5 per cent of the total glucuronidase in these animals. Mills concludes that the importance of spleen glucuronidase may vary considerably from species to species.

Preparation. Fishman and Talalay (72) have published the following simple and rapid method for the preparation of β-glucuronidase.

Six rats are killed by a blow on the head, and blood is permitted to drain from the carotids. The livers, kidneys, and spleens are rapidly dissected out. These are pooled separately and are homogenized with 100 cc. of cold water in a Waring blender or in a glass homogenizer, and the homogenate is strained through cheesecloth (Step 1). The mixture is acidified to *p*H 5.0 with 1.0 *N* acetic acid and is kept at 38° for 30 minutes. The proteins that flocculate out are removed by rapid centrifugation (20 minutes at 3500 r.p.m.), and the supernatant is separated and preserved. To this fraction is added an equal volume of saturated ammonium sulfate. The mixture is then centrifuged for 30 minutes. The supernatant is removed by suction and discarded. The residuent is dissolved in 20 cc. or more of water (Step 2), an equal volume of saturated ammonium sulfate solution is added, and the resultant precipitate is centrifuged off and taken up in a small volume of water (Step 3).

The most active fraction obtained was " Step 3 " in the case of liver and kidney, and " Step 2 " in the case of spleen. The spleen preparation was 5 to 7 times more active than those obtained from the other two organs.

β-GLUCOSIDASE (EMULSIN, CELLOBIASE, GENTIOBIASE, PRUNASE)

β-Glucosidase is present in almonds, in malt, in other higher plants, in certain yeasts, molds, and bacteria, and in the digestive juice of snails (*Helix pomatia*). The best source is sweet almonds.

Specificity of β-Glucosidase. This enzyme hydrolyzes β-*d*-glucosides, β-*d*-xylosides, cellobiose (4-glucose-β-glucoside), gentiobiose (6-glucose-β-glucoside), and perhaps β-*d*-glucuronides and certain heptosides. This subject is reviewed in references 73 and 74. Fre-

quently used substrates are cellobiose, amygdalin (benzaldehyde cyanhydringentiobioside), and such simple β-glucosides as arbutin, saligrin, phlorizin, and vicin. Not all β-*d*-glucosides are hydrolyzed equally fast by the β-*d*-glucosidase. Some are split only with difficulty, requiring much more time than others. This " relative specificity " is influenced by the aglucon of the glucoside. Thus, vanillin-β-*d*-glucoside is split 400 times faster by sweet almond β-*d*-glucosidase than methyl glucoside. Orthocresol glucoside is hydrolyzed more than 10 times faster by this enzyme than the phenol glucoside. Numerous other examples may be found in the review by Helferich.

Activators and Inhibitors. β-Glucosidase of almonds is not influenced by glutathione, hydrogen sulfide, or hydrocyanic acid. Sulfides such as silver sulfide have a stabilizing action, whereas soluble silver, copper, and mercuric salts are toxic. Some oxidizing agents such as ozone and osmium tetrachloride are very destructive (74). Ammonium perchlorate and potassium thiocyanate, however, have a powerful activating effect. Less pronounced activation has been observed with a series of neutral salts (75).

Preparation of β-Glucosidase. Tauber (76) has described the following practical method for the preparation of crude emulsin.

Three hundred grams of sweet almonds is suspended in water at room temperature for 20 minutes. The almonds are skinned mechanically, dried in the air, ground, and some of the fat is pressed out, using a cheesecloth. The remainder of the oil is extracted with three 300-cc. portions of ether; 110 grams of defatted meal is obtained. This meal is extracted with 800 cc. of 33 per cent ethyl alcohol by stirring for 6 minutes, after which it is filtered through filter paper. About 500 cc. of filtrate is obtained. To this, 500 cc. of 95 per cent ethyl alcohol is added. The precipitate is centrifuged off and dried over sulfuric acid at room temperature. The yield is 2.5 grams of dry emulsin. To aid in solution of the dry emulsin, a few drops of dilute sodium hydroxide solution may be used. The enzyme solution must be immediately adjusted to *p*H 7.0. The preparation is, of course, a mixture of several enzymes, including β-glucosidase. Helferich and associates published several procedures for the purification of sweet almond β-glucosidase.

Enzymic Synthesis of β-Methyl-*d*-Glucoside With the Aid of Emulsin (77)

By the following procedure, methyl-*d*-glucoside may be synthesized from glucose and methyl alcohol by almond emulsin, with excellent yield, in slightly aqueous dioxane medium. Two grams of finely

powdered almond emulsin is added to a mixture containing 40 parts of a 20 per cent glucose solution, 120 parts pure commercial methyl alcohol, and 400 parts redistilled pure commercial dioxane. The glucose gradually disappears from the reaction mixture.

Van Thoai, Roche, and Danzas (78) found that aqueous extracts of sweet almond contain a natural activator for the enzymic synthesis of β-methylglucoside. The activator is dialyzable, subject to autolysis, and destroyed when heated at 100° C. for 15 minutes. It was obtained in purified form by alcohol precipitation of dialyzates of extracts that were previously treated with lead acetate. This activator of emulsin does not activate phosphatases.

Trehalase. This enzyme is produced by yeast, molds, and bacteria. It is probably an α-glucosidase. The specificity of trehalase is not well defined, since the configuration of the disaccharide (glucosidoglucoside) trehalose is not yet known, nor has trehalose been obtained synthetically or enzymatically *in vitro*. However, attempts at the synthesis of the substrate of trehalase have not been lacking (79). Trehalose is a metabolic product of top yeast, molds, and certain bacteria (80). Trehalose is not split by maltase, an α-glucosidase (81).

Myrbäck and Ortenblad (82) found that a variety of yeasts fermented trehalose. In fresh pressed yeast the optimum *p*H for the trehalose hydrolysis was between 5 and 6. The autolyzed juice from pressed yeast contained trehalase, which possesses greater stability than the yeast maltase. Fermentation experiments showed that trehalose was acted upon only to the extent to which it was hydrolyzed by trehalase to glucose. Cozymase was necessary; without it, the fermentation was slight and soon ceased.

Bloch and Süllman (83) prepared an extract of *Bacillus tuberculosis* that was almost free of bacterial cells. It readily hydrolyzed trehalose but was inert to sucrose and raffinose. The enzyme was active over a wide *p*H range, with the optimum at 4.9.

AMYLASES (DIASTASES)

Amylases are found in saliva, pancreatic juice, blood cells, blood serum, liver, other organs, and many plants, such as seeds, grains, molds, and bacteria. Amylases break down starches and glycogen. Under favorable conditions, the end product of hydrolysis is maltose. The breaking down of starch by amylase, however, is much more complicated than this statement implies. The fact that the structure of the starch molecule is unknown complicates the problem, and, as with

proteins and other unknown substrates, enzyme action is used as an aid in structure studies.

If a small amount of amylase is added to starch paste, decrease in viscosity, disappearance of the characteristic blue color with iodine, and formation of reducing sugar (maltose) result. The formation of maltose may be followed by measuring the increase in reducing power. These three changes, however, do not always take place in the same order or at the same rates. Sometimes there is a rapid liquefaction, paralleled by a slow maltose formation; at other times, there is a very rapid formation of maltose but slow disappearance of products that give a blue color with iodine. Because of these irregularities, it has been assumed that there must be several amylases.

The properties of malt amylase, because of its importance in the industry, have been known for many years. As early as 1878, Märcker noted that malt amylase must be a mixture of two components. His observation had been confirmed by many investigators.

Starch and Glycogen, the Substrates of Amylases

Starches. Amylases (α or β) act only very slowly on raw starches. Cooked starches, however, are readily hydrolyzed. Results obtained with native starches, which are heterogeneous mixtures of varying composition, are not reproducible. Because of this, a soluble starch product, made soluble by chemical treatment, according to Lintner (Merck), is used in research and control work.

During the past one hundred years, many papers have appeared dealing with the action of amylases, yet nothing is definitely known in regard to their mechanism. The reason for this is our limited knowledge concerning the structure of starches and glycogens. These polysaccharides differ considerably according to their biological origin, the differences being chemical as well as physical. A given starch is a mixture of 75 to 80 per cent amylopectin, the remaining portion being amylose. The general opinion is that amyloses are the straight-chain components of starches containing glucose units bound by α-1,4-glucosidic linkages. The straight chains contain 100 to 700 glucose residues (84, 85). According to Meyer (84, 85), amylopectins are built of branched chains containing large branched molecules made up of 15 to 18 glucose units. Most of these glucose units are linked by α-1,4-glucosidic bonds having branches formed by α-1,6-glucosidic linkages. Amylopectins consist of 500 to 2000 or more glucose units. Additional details concerning this theory are given by Meyer (84, 85). Haworth, Kitchen, and Peat (86), however, state, " It is not necessary

to postulate a complex, highly ramified structure for amylopectin, such as that proposed by Meyer, to explain the facts of amylosis." In any event, it is not definitely known whether any one starch may contain more than two molecular species. Myrbäck and associates (87), who have examined the products of amylolysis of a wide variety of starches, concluded that they apparently are of the same, or very nearly the same, constitution.

Glucose, however, is not the only constituent of starches. When the various starches are purified by non-hydrolytic means at low temperatures, and then are completely hydrolyzed by dilute hydrochloric acid, appreciable quantities of phosphates, fatty acids, nitrogenous substances, silica, and other materials may be recovered. Thus, potato and wheat starch yield large quantities of phosphate; corn yields fatty acids, amino acids, and occasionally silica; rice and wheat yield nitrogenous substances. Other starch-containing plants yield small amounts of magnesium, iron, sulfate, etc. (88).

Glycogens. These polysaccharides occur in almost all animal cells and in yeast, which at certain metabolic phases contains large amounts. Considerable quantities are present in the liver and in mussels (*Anodonta*). Glycogens yield glucose on hydrolysis. These polysaccharides give a red-brown, or red-violet color test with iodine-potassium iodide solution. Glycogens consist of highly branched amylopectin molecules, in which the glucose residues are united by α-1,4- and α-1,6-glucosidic linkages of very high molecular weight. Glycogens are much more stable compounds than starches. Both polysaccharides are hydrolyzed by amylases.

In most enzymic starch structure studies, only α- and β-amylase were included. It is evident that the phosphorylases, phosphatases, and dextrinases cannot be omitted from serious studies.

General Properties of Amylases

α-Amylase. The dextrinogenic or α-amylase of malted barley and other germinated grains, certain molds, and the pancreas, saliva, and some bacteria cause marked decrease of the viscosity of starch pastes. The final hydrolysis products give no color with iodine-potassium iodide solution. α-Amylase produces dextrinlike compounds and very little maltose. Most of the reducing substances formed from starch by malt α-amylase are neither glucose nor maltose (89). Besides small amounts of fermentable sugars, isomaltose and other glucosans, such as tri- and tetrasaccharides, containing an α-1,6-glucosidic linkage, were reported to be among the end products of α-amylase action (90).

α-Amylase of malt splits the amylopectin of starches to dextrins of six or seven glucose units, whereas β-amylase action results in high-molecular-weight dextrins. α-Amylase apparently acts only on α-1,4 linkages, since low-molecular-weight glucosans having α-1,6-glucosidic bonds accumulate when amylopectin is the substrate (91). The liquefaction, as caused by all α-amylases-containing products, is probably brought about by α-amylase. All these enzyme preparations, however, contain phosphatases, and some investigators believe that the phosphatases have an important function in the liquefaction of starch pastes (92). It had been suggested that glucosidases may also interact in the actions of α-amylases (93).

Hanes and Cattle (94) have shown that the dextrin-producing ability of amylases is not a specific function of α-amylase. Their conclusion was based on the observation that the addition of β-amylase to a mixture of starch and α-amylase shortened the time necessary to reach the achroic point with starch from 140 to 55 minutes. This work was verified and extended by Sandstedt and associates (95), who found that, beyond a certain point, the addition of β-amylase has no effect on the rate of dextrinization.

β-Amylase. This enzyme is present in resting grains, in most seeds, in potatoes, and in similar plants. β-Amylase is called the saccharifying enzyme, since it rapidly forms maltose from a "soluble starch" solution. Under favorable conditions, the amylose fraction of starch is completely split by β-amylase (96). Amylopectin of cornstarch, however, is only partially hydrolyzed (54 per cent) by β-amylase, since this enzyme does not hydrolyze α-1,6-glucosidic linkages. There are also some glucopyranose residual substances combined by α-1,4-glucosidic bonds, as in maltose, that are not hydrolyzed completely by β-amylase (97).

Methods for Separating α- (Dextrifying) from β- (Saccharifying) Amylase of Malt

1. Ohlsson (98) and Nordh and Ohlsson (99) first showed that a separation of the two amylases is possible. The procedure depends on the heat lability of β-amylase and the acid lability of α-amylase. Most of the β-amylase may be destroyed by heating an extract of germinated grain of *p*H 6 to 7 at 70° C. for 15 minutes. Bringing another extract to 0° C. and adjusting the *p*H to 3.3 with hydrochloric acid destroys most of the α-amylase.

2. Van Klinkenberg (100) separated the two amylases by fractional precipitation with alcohol. Addition of alcohol, to make the concen-

tration of the extract 60 per cent, precipitates the α-amylase. Further addition of alcohol, to 80 per cent concentration, precipitates a mixture of α- and β-amylase. Van Klinkenberg has shown that normal wheat is the best source of β-amylase, since it contains practically no α-amylase.

3. Waldschmidt-Leitz and associates (101) adsorbed β-amylase on alumina C_γ at *p*H 3.8. All the α-amylase remained in solution free of the β component. The β form was eluted with phosphate or citrate solution.

4. Sherman, Caldwell, and Doebbeling (102) prepared β-amylase free of α-amylase by fractional precipitation of barley malt with ammonium sulfate followed by dialysis and fractional precipitation with alcohol. The saccharogenic activity of this β-amylase was higher than that of any similar enzyme ever reported.

Preparation of β-Amylase from Barley Malt

Sherman, Caldwell, and Doebbeling (102) described a method for the preparation of malt β-amylase free of α-amylase. This method has considerable educational value and should be discussed in some detail. Their procedure was to fractionate extracts of barley malt repeatedly with ammonium sulfate, followed by dialysis and fractional precipitation with alcohol. It had much higher saccharogenic activity than any previously reported malt amylase. Lüers and Sellner (103) and others (104) have also obtained highly active malt amylase. Caldwell and Doebbeling (105) have examined the various fractions for both types of activities and found that, in the early stages of ammonium sulfate fractionation, the precipitates which are formed by low alcohol concentrations are high in amyloclastic activity. These fractions had been discarded previously, because they had slight activity when judged by their maltose-forming action. It was found that these fractions have about 30 times more amyloclastic activity than the original malt extracts, and that the separation does not involve a loss in active material. This is the first time that highly active preparations of the two kinds of malt amylases have been simultaneously obtained from a single source. Both are of protein nature. Figure 8 shows the course of the hydrolysis of starch by the two types of amylase preparations. The reducing values of the digest mixture, expressed as maltose, are plotted against time. The *p*H was 4.5 (2 per cent starch and 0.01 *M* acetate at 40°), which has been found by Caldwell and Doebbeling to be the optimum *p*H for both amylases. It can be seen from Fig. 8

that the two types of amylases hydrolyze starch differently. Curve 1 of Fig. 8 represents the case in which maltose is formed rapidly at first (β-amylase), soon reaches a maximum, and then increases only slowly. Curve 2 represents the other case. Here maltose appears only slowly at first, but its formation is maintained longer (α-amylase).

The rapid maltose production in the early part of hydrolysis, as represented by curve 1, is accompanied by a slow disappearance of products which give a blue color with iodine. Thus, if the iodine test alone were used, the observer would reach the erroneous impression that the preparation is low in amylolytic activity. On the other hand, the slower maltose formation, as affected by the second type of amylase, represented by curve 2, is accompanied by the rapid disappearance of products that give a blue color reaction with iodine.

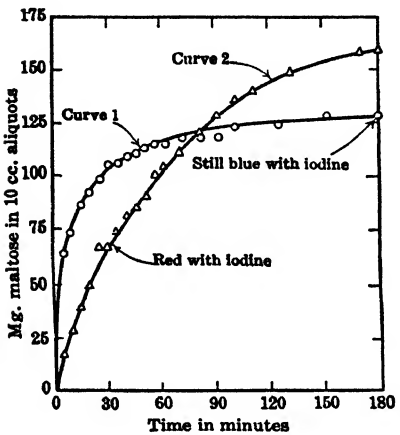


FIG. 8. Course of hydrolysis of starch by two types of malt amylase preparations.

Curve 1 represents the following results: blue color with iodine at 30 minutes and 160 milligrams of maltose per 10 cc.; nearing the red end point with iodine at 1300 minutes and 154 milligrams of maltose per 10 cc.; no color with iodine at 2700 minutes and 187 milligrams of maltose per 10 cc., which is 89 per cent of the theoretical yield of maltose. Curve 2 represents the following experiment: clear red color with iodine in 30 minutes and 65 milligrams of maltose per 10 cc.; no color with iodine in 45 minutes and 85 milligrams of maltose per 10 cc.; 209 milligrams of maltose per 10 cc. in 1300 minutes. This is the theoretical yield on maltose.

The "Starch-Liquefying Enzyme." Some amylase preparations are very active in liquefying starch. The fall in viscosity may take place before any distinct change in reducing power and iodine test occurs. It was suggested, as early as 1911, that there may be a specific liquefying amylase (106). In fact, the work of Gore and Jozsa (107) could also be understood to be in support of this view. (See Table VII.) The extracts recorded in the table were obtained by extracting 5 grams of the ground samples with 100 cc. of water at room temperature for 1 hour. If the samples showed diastatic activity, a second extract was prepared with 100 cc. of water containing 0.5 gram of sodium chloride.

TABLE VII

STARCH-LIQUEFYING AND -SACCHARIFYING POWER OF SEEDS

Seeds	Liquefying Power		Saccharifying Power	
	without NaCl	with NaCl	without NaCl °L.	with NaCl °L.
Soybeans, Mammoth Yellow	4.00	4.35	121	115
Cowpeas, black-eye	1.15	0.90	Inactive	..
Peas, Henderson's New Jubilant	0.30	1.00	Inactive	..
Beans, Scarlet Runner, pole	0.10	1.25	Inactive	..
White lupines	1.25	2.10	Inactive	..
Lentils	3.20	3.60	Inactive	..
Japanese buckwheat	0.90	1.35	Inactive	..
Kefir corn	4.35	4.60	Inactive	..
Rice, Blue Rose	2.60	3.20	Inactive	..
Field corn, New Eureka Dent	2.10	2.75	Inactive	..
Field corn, Flint Longfellow	1.65	2.10	Inactive	..
Sweet corn, Golden Bantam	1.40	2.35	Inactive	..
Sweet corn, Stowell's Evergreen	0.25	0.65	Inactive	..
Spring rye	5.75	7.80	67	76
Oats, Storm King	0.90	2.30	1	2
Barley	3.45	5.40	64	67
Malted barley	90.0	164.0	160	..
Wheat, spring, Marquis	3.40	5.15	78	96

Isolation of the Liquefying Enzyme. On the basis of earlier reports (106, 108), Waldschmidt-Leitz and Mayer (109) isolated the liquefying amylase, which they named amylophosphatase. The enzyme, together with the saccharogenic amylase, was obtained from aqueous extracts of barley by adsorption on alumina C_γ. After elution, the saccharogenic amylase was separated by repeated adsorption on kaolin. The amylophosphatase remained in solution.

The enzyme is said to produce a marked fall in viscosity of starch paste, followed by release of bound phosphorus and only a slight increase in reducing power. The typical diastatic changes could not be obtained with this enzyme even after considerable time. Kidney phosphatase showed a similar liquefying action.

Mayer and Klinga-Mayer (92) found that amylophosphatase activity paralleled the glycerophosphatase activity as the germination of the barley progressed. Thus, they conclude that these two enzymes are identical. They found that barley also contains a specific pyrophosphatase in addition to the saccharifying amylase. The amylophosphatase reached its highest activity when the embryo commenced to develop.

Hollenbeck and Blish (110) studied the dextrinizing and liquefying functions of amylases from three different sources, namely, malted wheat, *Aspergillus oryzae,* and bacterial preparation. They found that, when extracts of the three enzyme products were adjusted to equal dextrinizing activity, their liquefying power was substantially the same. Similarly, the two activities were equally affected by heat (50° to 75° C.) and by *p*H changes. Calcium ions (0.05 *N* $CaCl_2$) were found to protect both functions equally against heat destruction. They conclude that both the liquefaction and dextrinization of starch paste by amylases are brought about by α-amylase. Although these findings appear to be very conclusive, the inclusion of phosphatase tests would have greatly supported this work.

Redfern and Landis (111) have also compared the dextrinizing and liquefying activities of the α-amylase of malt, bacteria, and molds. These investigators found that the liquefaction-rate curves for equivalent α-dextrinizing amounts of the three types of enzymes were not superimposable. The α-amylases of malt, bacteria, and molds are a group of enzymes which differ quantitatively in their action on starch.

The present author feels that it has not yet been definitely shown that liquefaction is a function of α-amylases. The claim of Mayer that some phosphatases are able to liquefy starch should be further explored, since most phosphatases may be readily prepared free of α-amylase.

Kneen (112) has reported that sorghum malts contain, in addition to α- and β-amylase, another enzyme, which he called "dextrinase." This new enzyme is said to be influential in the hydrolysis of dextrins to fermentable sugars.

The Iodine Color Reaction

α-Amylase converts soluble starch into products that combine with iodine to form substances of varying red hues. When β-amylase acts on amylose, the blue iodine color is retained until about 90 per cent of the theoretical maltose has been formed. When starch is the substrate, however, the dextrin that is produced gives a blue color, and sometimes purple color, until hydrolysis has progressed to 60 per cent. A small amount of unchanged amylose, giving a blue color with iodine, masks the red color given by amylopectin and its hydrolysis product, α-amylodextrin. The iodine complexes may be readily observed by spectrophotometric procedures. The actions of the amylases on the components of starch have been discussed in a very interesting review by Hopkins (113).

Factors Influencing Amylase Activity

The activity of amylases may be influenced by many factors, such as various salts, amino acids, and lipids, by reducing and oxidizing agents, and by the usual factors that govern catalytic reactions (*p*H, temperature, reaction products, etc.). Calcium ions increase the stability of malt α-amylase and decrease the stability of malt β-amylase (114). These effects of calcium ions are not noticeable during the early part of the reaction. They become evident when loss of enzyme activity (mostly) occurs, after continued enzyme action. Specific amylase inhibitors have been isolated from grains (see "Amylase inhibitors"). β-Amylase from barley and malt requires free sulfhydryl groups for its activity (115).

The Effect of Heat on Amylases. Pure amylase solutions, like solutions of other enzymes, are less resistant to heat than crude extracts. The substrates of amylases have a stabilizing action. Barley malt α-amylase is much more resistant to heat than β-amylase of the same source. The optimum temperature of amylolysis by barley malt will depend on the duration of exposure to heat, the concentration of the substrate, the *p*H, and the ratio of α- and β-amylase in the malt. In distilleries and vinegar plants, extensive conversion (before yeasting) is not desired. Conversion is carried out in short periods, in order to preserve the amylases for action during the fermentation period, to effect slow, but complete, conversion of starch, and to get highest efficiency in fermentation. The brewers, however, destroy the amylases during conversion, so that the finished product should contain at least 35 per cent of the original unfermentable carbohydrates.

Pancreatic Amylase

The best sources of pancreatic amylase are hog pancreas and dog pancreas. Beef and sheep pancreas are poor sources. The pancreas gland contains only one amylase, an α-amylase. Its action on starch is similar to that of the other α- or dextrinogenic amylases. At first, it liquefies starch pastes, changing them mostly to dextrins that do not give a color with iodine. Some reducing sugars of higher molecular weight than maltose are also formed. These sugars are not fermentable by yeast (116). Pancreatic amylase action on soluble starch is fast. Reducing values increase rapidly when pancreatic amylase acts on soluble starch. This type of hydrolysis, on the basis of reduction, amounts to 50 to 55 per cent of the theoretical maltose at the achromic phase (94). Malt α-amylase, however, forms only 30 per cent of the

theoretical maltose at the achromic point. Reducing values of pancreatic amylase action, on soluble potato starch, reach the maximum when about 75 per cent of the theoretical maltose has been formed. This value, reported by various earlier investigators, has been confirmed by Blom, Bak, and Braae (117). Both pancreatic amylase and malt α-amylase rapidly form reducing dextrins from starch. These dextrins are made up, almost entirely, of 6 glucose units (91).

The Influence of Salts upon the Optimum *p*H of Amylases. Other Activators

Nasse (118) was the first to observe that animal amylases become more active in the presence of neutral salts. Later, it was found that pancreatic amylase loses its activity on dialysis, becoming active again on the addition of salts, and that malt amylase does not depend on neutral salts (119, 120). According to Haehn and Schweigart (121), potato amylase, like pancreatic amylase, also becomes reversibly inactive on dialysis. Sherman, Caldwell, and Cleaveland (122), working under carefully controlled conditions, have shown that, for the activity of malt amylase, neutral salts are not essential. Sherman, Caldwell, and Adams (123) found that the effect of neutral salts on pancreatic amylase does not depend on the purity of the enzyme but rather is a property of the enzyme. Neutral salts are necessary to the activity of pancreatic amylase. Anions, however, are far more influential than the cations. Chloride ion is the most effective, and the magnitude of activation may be indicated in the following list: NaCl, KCl, LiCl, NaBr, $NaNO_3$, $NaClO_3$, NaCNS, NaF. No effect on the activity of pancreatic amylase was shown by Na_2SO_4 and Na_2HPO_4. This is additional evidence that malt amylase and pancreatic amylase are distinctly individual enzymes.

Malt amylases have optimum *p*H at 4.3 to 4.6 in 0.01 *M* acetate buffer at 40° for the saccharogenic activity. Under similar conditions, the dextrinizing action has about the same optimum *p*H (124). Contrary to earlier findings (125, 126), Sherman, Caldwell, and Dale (127) showed that phosphate has no influence upon the activity of pancreatic amylase. Sherman, Caldwell, and Adams (123) studied the influence of salt concentrations on the optimum *p*H, and the optimal concentration of each salt at the optimum *p*H. Table VIII shows that the optimum *p*H differs as the sodium chloride concentration is increased from 0.0005 to 0.01 *M*. Above 0.01 *M*, the optimum *p*H is the same (7.1 to 7.2). With other salts, similar results were obtained, but the most favorable concentration depends upon the salt. Sodium sulfate,

however, was without influence. Thus, the optimum *p*H varied from 6.3 to 7.2. The dependence on these conditions is much more pronounced with pancreatic amylase than with other amylases. In contrast to the optimum of plant amylases, that of animal amylase is closer to the neutral point. The amylase of *Aspergillus oryzae* is most active at *p*H 5.3 to 5.5 in acetate buffer (128).

TABLE VIII

A SUMMARY OF RESULTS WITH DIFFERENT SALTS SHOWING THE INTERRELATIONSHIP BETWEEN CONCENTRATION OF SALT AND HYDROGEN-ION ACTIVITY (EXPRESSED AS *p*H) IN THEIR INFLUENCE UPON THE ACTIVITY OF PANCREATIC AMYLASE*

Concentration of salt, *M*	Most favorable hydrogen-ion activity for pancreatic amylase in the presence of different concentrations of each of the following salts, *p*H						
	NaCl	KCl	NaBr	$NaNO_3$	$NaClO_3$	NaSCN	NaF
0.0005	6.5						
.001	6.7						
.0025	6.9						
.005	7.0	7.0–7.1	.....	6.6–6.8	6.5		
.01	7.1	7.1–7.2	7.1	6.9–7.1			
.02	7.1						
.03	7.1	7.1–7.2					
.05	7.1	7.1–7.2	7.1	7.0–7.2	6.9–7.1	6.5	
.10	7.1	.....	7.1	.....	.....	6.7–6.8	6.3–6.7
.15	.....	.....	.....	.....	.....	6.7–6.8	
.20	.....	.....	7.1	7.1–7.2	6.9–7.1	6.7–6.8	6.6–6.8
.30	.....	.....	.....	.....	.....	.....	6.6–6.8

*Mixtures of acid and alkaline sodium phosphates corresponding to a total concentration of 0.01 *M* phosphate were present in all cases.

Myrbäck (129) reported that the *p*H-activity curves of pancreatic amylase are identical with those of salivary amylase, in the presence of phosphate, chloride, nitrate, and chlorate. Liver amylase is more active in the presence of NaCl (120), and dialyzed salivary amylase is completely inactive on dialyzed starch (130).

Roche, Van Thoai, and Dziri (131) found that pancreatic amylase activity on wheat starch, potato starch, and glycogen is accelerated 100 to 150 per cent by alanine, 10^{-7} *M*, or cysteine, 0.003 *M*. The simultaneous addition of magnesium sulfate, 0.001 *M*, with the amino acid, further increases the activity several fold. The action of cysteine plus $MgSO_4$ is independent of *p*H in the range 5.6 to 6.8. $ZnSO_4$ acts like $MgSO_4$ in the presence of cysteine, but not in the presence of

alanine; alone it is slightly inhibitory. The amylases of wheat and barley are inhibited by cysteine, 0.001 *M*.

Crystalline Pancreatic Amylase. In 1930 Caldwell and associates (132) announced that they obtained from buffered aqueous alcohol solution a highly purified pancreatic amylase in crystalline form. However, these investigators did not publish details concerning their method. Recently, Meyer and coworkers (133, 134) described in detail the crystallization of this α-amylase by applying an eight-step purification to a crude extract of the dry, defatted hog pancreas in a yield of 12 per cent. The enzyme is said to crystallize readily, from a 4 to 5 per cent aqueous solution, in prisms or fine needles. The enzyme is a protein containing 0.6 per cent phosphorus. The aqueous solution of the pure α-amylase is very stable. However, an impure solution containing 60 per cent impurities is rapidly destroyed at 2° C. and *p*H 6.9. In contrast to pancreatic amylase, grain β-amylase contains a free sulfhydryl group (123). Highly active amorphous pancreatic amylase preparations have been obtained by Sherman and collaborators (135).

Crystalline Salivary Amylase. Meyer and coworkers (136) have reported a method for the crystallization of α-amylase of human saliva. They employed fractional precipitation with acetone and ammonium sulfate, replacement of the sulfate ions by the acetate ions with the aid of an ion exchanger, and precipitation at an acetone concentration of 70 per cent. The precipitate was dissolved in a little water and left in the cold. Crystals formed in 48 hours containing 90 per cent of the amylase. The crystals were dissolved with the aid of 0.1 *N* ammonium hydroxide at *p*H 8.3. The solution was neutralized and continuously shaken in the cold for 48 hours. At the end of this period crystals of the amylase formed in great quantities. The yield was 25 to 30 per cent of the initial amylase of the saliva. The enzyme was found to be a protein.

Crystalline Sweet Potato β-Amylase. Balls, Thompson, and Walden (137) prepared a crystalline protein with high β-amylase activity from press juice of sweet potatoes. The press juice was heated to 60° C., cooled, and filtered after addition of lead acetate. After 0.7 saturation of the filtrate with ammonium sulfate, the resulting precipitate was dialyzed. By successive acidification of the ammonium sulfate-freed protein solution to *p*H 4.6 and 3.2, a further purification was achieved. Fractionation of the remaining material between 0.25 and 0.5 saturation with ammonium sulfate at *p*H 4.0 yielded crystals in the cold. The crystalline preparation had a slight α-amylase activity.

The Action of Barley Malt on the Components of Starch

Back and Vernon (138) studied the two components of corn starch, amylose, and amylopectin as affected by barley malt enzymes. At 63° C., amylose was rapidly and almost completely hydrolyzed to reducing sugars, whereas amylopectin-splitting was 59 per cent complete. After 15 minutes, hydrolysis progressed only very slowly. Using different amounts of malt and varying the time factor of hydrolysis in excess of 5 minutes, the optimum temperature for amylopectin hydrolysis was at 52° to 57° C., and the optimum *p*H was at 5.3 to 5.5. For 15-, 30-, and 60-minute hydrolysis periods, maximum splitting of amylopectin was shown when 15 to 20 per cent malt was employed. Larger quantities of malt gave only slightly higher conversion. Though these observations are very interesting, it should be borne in mind that the fact that barley malt contains 50 to 60 per cent starch may affect the nature of such experiments.

Mold Amylases

The enzymes produced by *Aspergilli* are very important industrially. *A. oryzae* is known to be an excellent producer of amylase and other enzymes. Different *Aspergilli* produce different amylases and a variety of different enzymes. Commercial *A. oryzae-flavus* enzyme products also contain maltase, and experiments conducted over long periods would be influenced by this enzyme. It is generally believed that the amylase of *A. oryzae-flavus* molds is an α-amylase identical with the α-amylase of barley malt. Sufficient evidence is not available for this assumption. It is true, however, that the two enzymes do have certain identical catalytic properties. Neither of the two amylases has as yet been obtained in pure state.

Caldwell and Doebbeling (139) found that the activity of the amylase of *Aspergillus oryzae* is twice as high in the presence of 0.02 to 0.10 *M* sodium chloride, when the enzyme and 1 per cent soluble potato starch solution contain 0.01 *M* acetate at *p*H 5.00. The temperature was 40° C., and the duration of the experiment was 30 minutes.

Preparation of Maltase-Free Mold Amylase. Caldwell and associates (140) prepared a maltase-free amylase of *Aspergillus oryzae* by purifying a commercial concentrate. Their method consists of fractional precipitation of aqueous extracts of the commercial product with ammonium sulfate, suspension of the precipitate in a small volume of water, dialysis to remove sulfate, concentration of the sulfate-free solution, activity tests, and repetition of the fractionation and dialysis

until no further increase in activity per milligram of total solids is noticed. In this procedure, solid ammonium sulfate was employed. The dialysis was conducted in nitrocellulose bags. The dialyzed solutions were concentrated by suspending the bags in the breeze of an electric fan. Other protein precipitants tried by these investigators gave less satisfactory results. Nor was adsorption of the amylase on alumina gel an effective procedure for the purification of *A. oryzae* amylase. The results suggest that this mold produces a single enzyme having properties similar to those of the dextrinogenic amylases of other sources.

The Question of Enzyme Adaptation in Molds. Bonnet and Bonnet (141) reported that the formation of carbohydrases does not depend on the presence in the medium of carbohydrates in the case of *A. carbonescens*. However, Le Mense and coworkers (142) found that in the case of *A. niger* NRRL 337 the addition of carbohydrates to various media increased the concentration of the dextrinizing enzyme in the medium to a considerable degree (see Table IX). The investigators of the Northern Regional Research Laboratory made a very important contribution to enzyme technology by showing that highly active amylase solutions may be obtained by submerged culture of molds. Le Mense and associates examined more than 350 mold cultures (representing *Mucor, Penicillium, Aspergillus,* and *Monilia*). Only a limited number were capable of producing α-amylase. Some had saccharogenic action; this was probably due to the maltase present.

Muscle Amylase

Petrova (143) observed that the carbohydrates in the muscle tissue of rabbits and rats are split by amylolysis and by phosphorolysis. The muscle amylase is activated by chlorides, and its action resembles that of the α type. Autolysis increases the amylolytic action.

Bacterial Amylases

The amylases of bacteria may be grouped in saccharogenic, dextrinogenic, and Schardinger dextrin-producing amylases. Strains belonging to the groups *Bacterium subtilis* and *B. mesentericus* are very important industrially. Their production is described in another chapter. *B. subtilis* amylase is most active at *p*H 6.5 to 8.0. It is very resistant to heat and is not destroyed completely even if boiled for a short time in the presence of starch (144).

Peltier and Beckord (145) have examined a few thousand bacterial isolates for amylase production. They found that 265 isolates had the

TABLE IX

THE PRODUCTION OF DEXTRINIZING ENZYME BY *Aspergillus niger NRRL 337* CULTIVATED IN VARIOUS MEDIA

Protein Source	Carbohydrate Source	Concentration of Dextrinizing Enzyme, units/ml
Corn steep liquor, 3%	None	2.2
Corn steep liquor, 3%	Glucose, 2%	8.2
Corn steep liquor, 3%	Molasses, 2%	4.6
Corn steep liquor, 3%	Corn meal, 2%	10.2
Dried tankage, 2%	None	2.1
Dried tankage, 2%	Glucose, 2%	9.3
Dried tankage, 2%	Molasses, 2%	11.5
Dried tankage, 2%	Corn meal, 2%	8.7
Soybean meal, 2%	None	7.9
Soybean meal, 2%	Glucose, 2%	7.4
Soybean meal, 2%	Molasses, 2%	8.5
Soybean meal, 2%	Corn meal, 2%	11.2
Thin stillage	None	1.7
Thin stillage	Glucose, 2%	11.5
Thin stillage	Molasses, 2%	7.9
Thin stillage	Corn meal, 2%	16.5
Thin stillage	Xylose, 2%	5.3
Thin stillage	Lactose, 2%	6.7
Thin stillage	Sucrose, 2%	11.0
Thin stillage	Maltose, 2%	14.5

Enzyme determinations were made after cultures were shaken for 5 days.

Composition of medium: protein and carbohydrate as shown plus 0.5 per cent calcium carbonate.

ability to hydrolyze starch in a starch-agar medium. Of these, however, only 71 formed enough amylase when cultured in a wheat bran-peptone-phosphate liquid medium to be of any interest. All the isolates belong to the *B. subtilis* group. Kneen and Beckord (146) studied 43 isolates of the *B. subtilis* group of the collection of Peltier and Beckord, 7 cultures of *B. polymyxa*, and 3 cultures of *B. macerans*. The amylases were classified on the basis of the relationship between two kinds of starch-decomposing action, such as dextrinization and saccharification. This resulted in a separation into four general classes with typical properties:

1. *Bacillus subtilis* (saccharifying type). These are the common aerobic spore-forming rods found on plant material. These types of bacteria vary considerably in amylase production. Some produce

none; others form very large quantities. At first, this amylase produces only dextrins from starch, followed by extensive formation of fermentable sugars. It is sensitive to a wheat inhibitor, being the only bacterial amylase that is so affected.

2. *Bacillus subtilis* (α-amylase or non-saccharifying type). These enzymes are related to the commercial type of bacterial amylases. These bacteria produce very large quantities of amylase and may be isolated from ropy bread.

3. *Bacillus polymyxa.* These organisms form an amylase, or an amylase system, having a starch-decomposing action similar to that of a barley malt extract. Large quantities of fermentable sugars form both in the dextrinization and after the dextrinization stages. The degree of conversion compares well with that given by barley malt.

4. *Bacillus macerans.* This type of amylase is typical for this organism. At first, non-reducing, non-fermentable dextrins are formed, which have been named "Schardinger dextrins." These dextrins are then converted progressively to fermentable sugars. In due time, or with proper enzyme concentration, the conversion of starch is high.

The observations of Kneen and Beckord that bacteria produce several kinds of amylase systems are very important. The earlier opinion was that all "bacterial amylases" belong to the dextrinogenic type.

Amylase of *Bacillus macerans*. *B. macerans* amylase has been extensively studied in recent years. It rapidly hydrolyzes starch to a mixture of water-soluble dextrins, from which two distinct, non-reducing, non-fermentable, readily crystallizable compounds may be obtained. These dextrins, first described by Schardinger in 1908, have been named "Schardinger dextrins." Tilden, Adams, and Hudson (147) were able to prepare this amylase in a highly active state by precipitating it from the liquid culture (Seitz filtrate of the finished culture) with acetone, dissolving the precipitate in water, adsorbing on aluminum hydroxide in the presence of acetate at *p*H 4.80, and eluting the enzyme with phosphate at *p*H 7.6. This concentrate was 140 times more active than the initial enzyme solution and was capable of converting 1000 times its weight of starch in 30 minutes at 40° C.

McClenahan and associates (148) were able to obtain the Schardinger dextrins from potato starch, by means of the *macerans* amylase, in a yield of 55 per cent. The relative proportions of the two components, α- and β-dextrin, in this product varied with different digestion conditions. β-Dextrin is stable toward *macerans* amylase at 20° C., whereas the α-dextrin is converted to a higher rotating material with slight reducing power.

Amylase of *Clostridium acetobutylicum*. Hockenhull and Herbert (149) studied the amylase and maltase present in cell-free culture filtrates of *Cl. acetobutylicum*. Such filtrates, owing to the combined action of amylase and maltase, result in 100 per cent conversion of starch to glucose. These enzymes play an important role in butanol-acetone fermentation by *Cl. acetobutylicum*, but so far they have been little investigated. Hockenhull and Herbert purified the amylase of this organism and obtained it almost free of maltase.

Comparison of Amylases of Grains and Malted Grains

Geddes and Eva (150), using a modification of the ferricyanide-reduction procedure of Hagedorn and Jensen for the estimation of diastatic activity of wheat flour, found that 80 flours varied in diastatic activity from approximately 70 to 300 units.

Kneen (151) has examined the α- and β-amylase content of ungerminated and germinated barley, wheat, rye, oats, maize, sorghum, and rice and has also reviewed the earlier literature. Germination of all these cereals produced a marked increase in starch-saccharifying and -liquefying (or dextrinizing) activity. The malts of barley, wheat, and rye contained all activities in higher degree. The malts of oats, maize, sorghum, and rice, however, showed low ratios of saccharifying to liquefying action.

Amylase of the Ungerminated Grains. In Table X, the amylase values of ungerminated grains are listed. The " free " extracts were

TABLE X

The Amylolytic Activity of Ungerminated Cereal Grains

	Saccharifying Activity		β-Amylase Activity		α-Amylase Activity	
Cereal	" Free " units	Total units	" Free " units	Total units	" Free " units	Total units
Barley	10.7	29.8	10.7	29.8	0.045	0.058
Wheat	7.5	25.1	7.5	25.1	0.050	0.063
Rye	9.1	17.8	9.1	17.8	0.089	0.111
Oats	0.7	2.4	0.7	2.4	0.262	0.297
Maize	*	Trace	*	*	0.101	0.249
Sorghum	*	Trace	*	*	0.031	0.127
Rice	*	Trace	*	*	0.075	—

*Below the sensitivity of the method used.

1-hour calcium acetate extracts; the " total " indicate 18-hour extracts with 10 per cent papain. Confirming earlier findings, Kneen's results

show that the saccharifying action of ungerminated barley, wheat, and rye was high, and that of oats, low. Ungerminated maize, sorghum, and rice had a saccharifying activity that was below the sensitivity of the method. The α-amylase activity was measurable but too low to influence saccharification. Thus, "saccharifying units" were synonymous with "β-amylase units."

Amylases of the Germinated Grains. Kneen steeped grains for 24 hours at room temperature and germinated them for 5 days at 16.5° C. The results are summarized in Table XI. Germination was coincident

TABLE XI

THE AMYLOLYTIC ACTIVITY OF GERMINATED CEREALS

Cereal	Sprout Length mm.	Saccharifying Activity		β-Amylase Activity		α-Amylase Activity	
		"Free" units	Total units	"Free" units	Total units	"Free" units	Total units
Barley	25–30	31.0	39.1	26.5	34.4	90.5	94.0
Wheat	20–30	26.4	34.4	16.5	23.7	197.3	214.7
Rye	20–30	20.1	23.6	15.4	17.6	93.2	119.8
Oats	25–35	2.2	3.0	*	*	53.1	60.3
Maize	15–20	0.24	1.2	*	*	31.1	35.6
Sorghum	10–20	1.8	1.9	*	*	73.4	75.5
Rice	2–5	0.05	0.3	*	0.2	1.4	2.3

*Below the sensitivity of the method used.

with an increase in α-amylase activity, but this enzyme action was not proportional to sprout length. Growth resulted in a release of "bound" β-amylase so that most of the β-amylase in the germinated samples was extracted without the use of papain. The cereals, maize and sorghum, that showed no measurable β-amylase action in the ungerminated state similarly exhibited none when germinated. The results show that the total β-amylase activities of these cereals remained constant during germination. It is interesting to note that oats, maize, and sorghum are typical α-amylase sources.

Sorghum Malt α-Amylase. Kneen (152) has published an extensive study concerning sorghum amylase. He germinated a series of sorghum varieties and determined the nature of the amylases present. Both these amylases were found to be similar to the corresponding enzyme components of barley malt. The sorghum α-amylase has the same optimum *p*H (4.5) as malt α-amylase. This germinated grain contained only a minute amount of β-amylase. This trace of β-amylase, however, was sufficient to influence considerably the post-dextrin-

ization saccharification of starch by sorghum malt extracts. Malted sorghum is a good source of α-amylase. It should find wide use in various industries.

Sulfite Extraction of Diastase and Recovery of Protein from Wheat. Balls and Tucker (153) found that the quantity of amylolytic enzymes from unmalted wheat, bran, or granular wheat flour which may be made available is markedly increased by washing the grain in a 0.05 per cent sodium sulfite solution. Extracts having a Lintner value equal to that of a medium-grade barley malt were obtained, and, by suitable mechanical treatment, the grain protein was obtained as a readily separable scum containing 86 per cent protein.

Activation of Malt Amylase by Hydrogen Sulfide. Chrzaszcz and Sawicki (154) have extensively studied the barley and malt amylases. They found that the amylase content of barley varies in the ratio of 36.8 to 434. The bound saccharifying amylase of barley may be increased from 18.6 to 734 per cent by treatment with hydrogen sulfide, depending on the nature of the barley. Well-sprouted (10-day-old) brewery malt contains 550 to 600 units of saccharifying amylase, varying only within this narrow limit. Hydrogen sulfide and papain have a negligible effect on this amylase.

Distillery malt (18 days old) has the same saccharifying amylase content as brewery malt. The dextrin-forming amylase and the liquefying function, however, increase continuously during malting and are considerably higher than those of the brewery malt. The dextrin-forming function is several hundred times higher than that of the barley itself. Peptone increases malt amylase activity to a considerable extent. The amylase content does not depend on the type or strain of the barley. Environmental and ripening conditions of the barley, however, have a direct effect.

Chrzaszcz and Sawicki prepared their amylase extracts by suspending 25 grams of well-ground barley or malt in 150 cc. of water, or in 150 cc. of water containing 3 grams of peptone or 3 grams of papain. Extraction was applied for 20 hours, with occasional shaking. When hydrogen sulfide was employed, the gas was introduced for 2 hours preceding the usual 20-hour extraction period. Malting was carried out at 10° to 13° C. for 10 days and 18 days, respectively. For drying at 30° to 35° C., the Faust-Heim apparatus was used (155, 156).

Holmbergh (157) found that malt amylase in a solution of 30 per cent alcohol, which was not well stoppered but was kept in a cool place, became inactive after 5 months. Eighty-six per cent of amylolytic activity was recovered by treatment of the inactive solution with hydrogen sulfide. The reactivation with sodium hyposulfite was some-

what less. Amylase that was inactivated by oxidation with iodine or alkaline hydrogen peroxide was reactivated by similar treatments.

These results strongly indicate the presence of an active sulfhydryl group in the enzyme molecule. Such active groupings had been found in urease, arginase, some fractions of crude papain, and many other enzymes. Highly purified pancreatic amylase, however, does not appear to require free sulfhydryl groups for its activity (108).

Soybean Amylases. Soybean seeds are a good source of β-amylase. They contain, however, no α-amylase or at best only a trace. Germination has no effect on the amylases. Unlike barley and wheat, soybeans do not contain bound β-amylase. The optimum *p*H of the soybean amylase is at 5.9 (158). Newton and Naylor (159) found that their purified soybean β-amylase had no α-amylase activity, as shown by the Wohlgemuth test, but had marked starch-liquefying power. The authors believe that the liquefaction was brought about by the β-amylase or that it may have been caused by an amylophosphatase (Waldschmidt-Leitz and Mayer).

Laufer, Tauber, and Davis (158) found the optimum *p*H for saccharogenic activity of soybean β-amylase to be between *p*H 5.18 and 6.38, with an apparent maximum value close to *p*H 5.90. The total saccharogenic activity of soybeans appears to be of the same order as that of barley or wheat; however, since all the soybean β-amylase is free, an aqueous extract shows much more saccharogenic activity.

Amylase Inhibitors

Amylase inhibitors have been described at various times. An amylase inhibitor has been shown to be present in potato plants (160), and ascorbic acid has been shown to be inhibitory to β-amylase (161). Bowman (162) found, in aqueous extracts of ground navy beans, a heat-labile fraction which retards the action of pancreatic amylase. Such extracts were progressively active with decreasing *p*H. The inhibitor was freed of most inert protein and was considerably active even in small concentrations. The partially purified preparations also showed a high antitryptic action. Kneen and Sandstedt (163) reported a study concerning an inhibitor in wheat endosperm, and in rye and sorghum, which inhibits the amylases of the saliva, pancreas, and those produced by certain bacterial isolates. The inhibitor was not destroyed by atmospheric boiling, but it became inactive on autoclaving. It is soluble in water, very soluble in dilute salt solutions and dilute ethyl alcohol, insoluble in concentrated ammonium sulfate solutions, in ether, and in 90 per cent ethyl alcohol. It does not diffuse

through cellophane membranes. The inhibitor is destroyed by pepsin (at *p*H 2.0) but not by pancreatic enzymes.

The amylase inhibitor from wheat flour was concentrated 750-fold by adsorption on, and elution from, aluminum hydroxide with phosphate solution (164). The purified inhibitor was digested by ficin, was precipitated by protein precipitants, and did not dialyze through viscous membranes. Thus, it is of protein nature. The purified inhibitor became inactive when dialyzed against distilled water. It remained active when dialyzation was carried out against tap water, sodium chloride, or phosphate. The inhibitor is sensitive to alkaline *p*H's but more resistant to an acid *p*H. Tryptophan appears to be essential for the activity of the inhibitor (164).

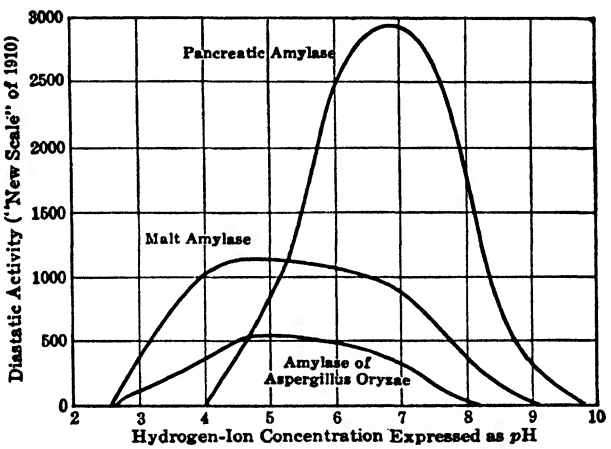


FIG. 9. Optimum *p*H of diastatic activity of amylases.

KINETICS OF STARCH HYDROLYSIS

Hydrolysis of starch by pancreatic amylase follows the course of a monomolecular reaction up to a saccharification of 40 per cent. Hanes (165) studied the effect of starch concentration upon the reaction velocity, using the amylase of germinated barley. He found that the relationship, as determined by the initial slope procedure, is in close agreement with that predicted by the Michaelis-Menten theory. The relationship between initial reaction velocity and enzyme concentration is linear over a wide range of enzyme concentration.

Optimum *p*H of Some Amylases. Sherman and associates (166) studied the influence of *p*H upon the activities of pancreatic amylase, malt amylase, and the amylase of *Aspergillus oryzae*. Their results are represented in Fig. 9. It may be seen that both *A. oryzae* amylase

and malt amylase were most active diastatically (maltose formation) at *p*H 4.5 to 5.0. Pancreatic amylase showed an optimum at about *p*H 7.0, however.

THE WOHLGEMUTH METHOD FOR THE DETERMINATION OF "α-AMYLASE" (TOTAL DEXTRINIZING) ACTIVITY

This is a modification of the older Wohlgemuth methods (167, 168). Although the results obtained by this method are not an exact measure of α-amylase activity, for practical purposes the procedure is very useful. A very small amount of α-amylase activity is produced by the β-amylase. True α-amylase activity may be determined by the method of Olson and coworkers (168), who have also published a modification for the determination of β-amylase in barley malt.

Reagents

Stock Iodine Solution. Eleven grams of iodine and 22 grams of potassium iodide are diluted to 500 cc. with water.

Dilute Iodine Solution (A) for Standard. Fifteen cubic centimeters of stock iodine solution and 8 grams of potassium iodide are diluted to 200 cc. with water.

Dilute Iodine Solution (B). Two cubic centimeters of stock iodine solution and 20 grams of potassium iodide are made up to 500 cc. with distilled water. (This solution must be made fresh daily.)

Standard Dextrin Solution. Six-tenths of a gram of Merck's "Reagent" dextrin (containing a trace of insoluble matter and 12 per cent water) is suspended in a small quantity of water, transferred to 900 cc. of boiling water, and, after cooling, made up to 1000 cc. The solution, preserved with a few cubic centimeters of toluene, keeps for several weeks in a refrigerator. One cubic centimeter of the dextrin solution is placed in 5 cc. of dilute iodine solution A in a ¼-inch test tube. This serves as a standard for color comparison.

Acetate Buffer. One hundred sixty-four grams of anhydrous sodium acetate is dissolved in 125 cc. of glacial acetic acid, and the solution is made up to 1 liter with water.

Starch Solution. Ten grams of Lintner soluble starch (dry basis) is mixed with 25 cc. of distilled water and stirred into 450 cc. of boiling distilled water. Boiling is continued for 2 minutes. The solution is cooled to 20° C., 25 cc. of acetate buffer is added, and the volume is made up to 500 cc. with water and mixed.

Procedure

Five grams of finely ground malt is extracted for 60 minutes at 30° C. with 100 cc. of water. Ten cubic centimeters of the filtrate is diluted to 100 cc. Five cubic centimeter portions of the dilute iodine solution is placed in each of 18 test tubes.

Ten cubic centimeters of the diluted malt extract at 30° C. is added to the 20 cc. of starch solution and mixed. The flask is placed in a constant-temperature water bath at 30° C. The time is recorded as soon as the malt extract comes into contact with the starch solution. After 10 minutes, 1 cc. of the mixture is added to the first iodine tube. This procedure is repeated at suitable intervals. The color of the tube that matches the standard is taken as the end point.

Calculation. In this method, 1 cc. of 2 per cent starch solution is hydrolyzed by 0.5 cc. of diluted malt extract or by 0.05 cc. of the original extract. Thus, 20 cc. of starch solution is hydrolyzed by 1 cc. of original extract. If hydrolysis of the starch to dextrin occurs in c minutes, 1 cc. of original malt extract will convert in 1 hour $(60/c) \times (1/0.05)$ cc. of starch solution. This is the formula for the α-amylase value.

DETERMINATION OF DIASTATIC POWER OF MALT

The American Society of Brewing Chemists recommends the following method for the determination of diastatic power of malt (169):

Preparation of Malt Infusion

Twenty-five grams of finely ground malt is placed in a container. To this is added 500 cc. of freshly distilled water, and the container is closed. The infusion is allowed to stand for 2½ hours at 20° C. (±0.2° C.) and is agitated at 20-minute intervals. At the end of 2½ hours the infusion is filtered. The first 50 cc. of filtrate is returned to the filter. The filtrate is collected until 3 hours have elapsed from the time that the water and ground malt were first mixed. Evaporation of the filtrate must be prevented as far as possible during the filtration period.

Actual Diastasis. Twenty cubic centimeters of the malt filtrate is diluted to 100 cc. at 20° C. Ten cubic centimeters of this diluted infusion is placed in a 250-cc. volumetric flask and brought to 20° C. Two hundred cubic centimeters of buffered starch solution is added from a fast-flowing pipet, all at 20° C. The " starch infusion " mixture

is maintained at 20° C. for 30 minutes. Twenty cubic centimeters of 0.5 *N* sodium hydroxide is added rapidly and mixed by inverting the flask. The solution is made up to the mark and mixed.

Solutions

(a) Buffer Solution. Sixty-eight grams of sodium acetate ($CH_3COONa \cdot 3H_2O$) is dissolved in 500 cc. of normal acetic acid and made up to 1 liter with water.

(b) Soluble Starch. Soluble starch of 10 to 12 per cent moisture and of low (0.75 per cent) reducing power should be used. Two grams of starch is mixed with not over 5 per cent of water to form a thin paste. This is poured, with stirring, into boiling water, representing not less than 75 per cent of the final starch solution, at such a rate that boiling does not cease. Boiling is continued for 2 minutes after the paste has been introduced. Ten per cent of the final volume of cold water is added, and the mixture is quantitatively transferred to a glass-stoppered 100-cc. volumetric flask. It is mixed, the sides are washed down, and the mixture is cooled to 20° C. Two cubic centimeters of butter is added for each 100 cc. of solution and made up to the mark. After mixing, it is kept at 20° C. until used.

(c) Alkaline Ferricyanide Solution. Sixteen and a half grams of pure potassium ferricyanide and 22 grams of anhydrous sodium carbonate are dissolved in water and diluted to 1 liter. The potassium ferricyanide normality is 0.05. This solution should be kept in a dark bottle away from the light.

(d) Sodium Thiosulfate Solution 0.05 *N*. Twelve and forty-one hundredth grams of pure dry $Na_2S_2O_3 \cdot 5H_2O$ crystals and 3.8 grams of borax are dissolved in water and diluted to 1 liter (borax prevents decomposition of $Na_2S_2O_3$ solution on long standing). Standardization is unnecessary if the solution is made up as directed.

(e) Acetic Acid Solution. Two hundred cubic centimeters of glacial acetic acid, 70 grams of potassium chloride, and 20 grams of $ZnSO_4 \cdot 7H_2O$ are dissolved and made up to 1 liter with water.

(f) Potassium Iodide Solution. To a 50 per cent aqueous solution of freshly prepared potassium iodide is added 1 or 2 drops of concentrated sodium hydroxide to prevent deterioration. The solution must be colorless.

(g) Half-Normal Solution of Sodium Hydroxide. This solution should be accurate to within 0.05 *N*.

Standardization of Ferricyanide Solution. To 10 cc. of the

$K_3Fe(CN)_6$ solution are added 25 cc. of acetic acid solution, 1 cc. of the potassium iodide solution, and 2 cc. of starch solution. This is titrated with the $Na_2S_2O_3$ solution, by means of a 10-cc. semi-micro buret, until blue starch iodine color is discharged.

Ferricyanide Titration

Five cubic centimeters of the digested starch solution is placed in a 150-cc. Erlenmeyer flask. Ten cubic centimeters of alkaline ferricyanide solution is added. The solution is shaken and immersed in a boiling water bath so that the liquid surface in the flask is slightly below the surface of the boiling water. After 20 minutes, the solution is removed and cooled under running water. Twenty-five cubic centimeters of acetic acid solution and 1 cc. of 50 per cent potassium iodide solution are added, and the iodine is titrated with 0.05 N sodium thiosulfate until the blue color is discharged. The starch blank is treated like the test solution except that the sodium hydroxide is added to the malt infusion before the starch is added. The procedure should be carried out continuously to avoid variation in results. The number of cubic centimeters of 0.05 N sodium thiosulfate solution required is designated as A.

Calculation of Diastic Power

A = cc. digested starch solution used in titration.
B = cc. digested starch solution used in blank correction.
M = per cent moisture in sample.

(a) Degrees Lintner:

$$°\text{L. (as is)} = (B - A) \times 23$$

$$°\text{L. (dry basis)} = \frac{°\text{L. (as is)} \times 100}{100 - M}$$

$$°\text{L.} \times 4 = \text{maltose equivalent}$$

The term "maltose equivalent" indicates the number of grams of reducing substances calculated as maltose that are produced by 100 grams of malt in a half-hour digestion of soluble starch at 20° C. under conditions as set forth in the method. Degrees Lintner times 4.0 gives maltose equivalent.

(*b*) Maltose equivalent:

$$\text{M.E. (as is)} = (B - A) \times 92$$

$$\text{M.E. (dry basis)} = \frac{\text{M.E. (as is)} \times 100}{100 - M}$$

$$\frac{\text{M.E.}}{4} = {}^{\circ}\text{L}.$$

A, *B*, and *M* are defined above.

POLYASES ACTING ON THE PLANT FRAMEWORK

Lichenase. This enzyme is present in *Aspergillus oryzae* (170), in the intestines of snails, in worms, in wood-eating insects, in corn, beans, hyacinths and other plants (171), and in wheat malt (172). The polysaccharide lichenin is closely related to cellulose. It occurs in Iceland moss. Lichenase hydrolyzes lichenin to glucose.

Cellulase. Cellulase is produced by various bacteria, molds, protozoans, and some invertebrates. Karrer, Schubert, and Wehrli (173) conducted interesting experiments with snail cellulase, using artificial silk, cellulose, and filter paper as a substrate. They found the filter paper most suitable. Pringsheim and Bauer (174) studied the effect of malt cellulase on chemically treated cellulose. Von Euler (175) noticed cellulase activity by the mushroom *Merculius lacrimans*. Schmitz (176) found cellulase in a great number of molds. The breakdown of cellulose in the intestinal tract of higher animals (e.g., of straw by cows) is due to certain intestinal bacteria and not to enzymes.

Grassmann, Stadler, and Bender (177) studied crude enzyme preparations from fungi. These preparations hydrolyzed cellulose, lichenin, and xylan readily, and also, to a certain extent, hydrated pectin, mannan, and inulin. After dialysis, they attacked only cellulose, lichenin, and xylan. Treatment with charcoal removed the xylanase. The filtrate readily split cellulose and lichenin, with an optimum *p*H of 4.5 for each substrate. These authors believed that mannanase, xylanase, and inulase are specific enzymes. Cellulase and lichenase are perhaps one enzyme.

Inulase. Inulase splits inulin into *d*-fructose. Inulase may best be obtained from molds such as *Aspergillus niger* and *Penicillium glaucum* (178). It is also present in bakers' yeast. Avery and Cullen (179) discovered inulase in pneumococci.

These polyases are usually found together and are difficult to separate. No extensive researches are available concerning this group of enzymes. The pectic enzymes are described in a special chapter.

MUCOLYTIC ENZYMES

This group of enzymes catalyzes the depolymerization of the highly polymerized mucopolysaccharides. These polysaccharides contain hexosamine. They are present in many internal and external structures of animals and microorganisms. The biological function of only a few of these polysaccharides is known. Like many other enzymes, mucolytic enzymes are of considerable aid in structure studies of little-known compounds.

Lysozyme. Lysozyme was discovered by Fleming (180) in 1922. He found this enzyme to be present in egg white, nasal mucus, tears, and leucocytes. Lysozyme is a bacteriolytic enzyme effecting the lysis of microorganisms, such as micrococci and sarcinae. Lysozyme is also present in certain molds (181) and in the latex of different plants (182). Feiner and associates (183) have published a more extensive description of the widely used test organism, *Micrococcus lysodeikticus* (Fleming), and extended our knowledge concerning the factors governing bacterial lysis by lysozyme of egg white and by latex lysozyme. The results indicate that the lysozymes of different sources differ in their specificities. Though egg white contains large quantities of this enzyme, crude ficin is also an excellent source. The proteolytic enzyme crystalline ficin, however, is very low in lysozyme activity (182). Papain, commercial and purified, is a poor source of lysozyme.

The role that these lysozymes play in the life process of these plants is not known. In the animal organism, lysozyme is said to act as a protective enzyme against bacterial invasion. But the most susceptible microbes are harmless saprophytes. Pathogenic organisms show little or no susceptibility. A few, however, have been found to be susceptible to egg-white lysozyme (184). Abraham and Robinson (185) prepared egg-white lysozyme in crystalline form. It appears to be a polypeptide.

Hyaluronidase. This enzyme is the best known of all mucolytic enzymes. It is present in type II pneumococcus, in group A hemolytic streptococcus, in certain strains of *C. welchii* and other microorganisms, in extracts of spleen or of the ciliary body, in testes, in heads of leeches, and in a number of snake venoms (184). Hyaluronidase depolymerizes and splits hyaluronic acid, a polymer of acetylglucosamine and

glucuronic acid. Hyaluronic acid is present in vitreous humor, in the umbilical cord, in synovial fluid, in exudates of pathological joints, etc. For the preparation of purified hyaluronidase, bull or ram testes are the best sources. This enzyme plays an important function in fertilization (186). The cumulus cells around the ovum are embedded in a jelly that contains hyaluronic acid. This jelly is liquefied by the hyaluronidase of the spermatozoa, thus permitting fertilization by a single spermatozoon.

Intraperitoneal injection of hyaluronidase was employed by Stewart and Meyer (187) in a case of mesenthelioma of the pleura and peritoneum, to aid in the removal of honeylike viscosity. Without hyaluronidase injection, paracentesis was incomplete and difficult. After injection of purified testicular hyaluronidase, the viscosity of the fluid was lowered and could be greatly reduced in a short time. The enzyme had no harmful effect, but continued growth of the malignant tumor finally caused the death of the patient.

A method for the preparation of highly active hyaluronidase from bull testes has been published by Hahn (188). He attained a 2000-fold concentration of the enzyme, but this product still did not behave like a homogeneous substance in the Tiselius electrophoretic apparatus. The isoelectric point of the purified enzyme was at *p*H 5.7. The enzyme was strongly mucolytic in concentrations as low as 0.1 gamma.

The Invasin Enzyme System of Haas. An enzyme that rapidly destroys hyaluronidase has been discovered by Haas (189) in normal blood plasma of mammals, birds, and fish. He named this new enzyme, which is an antiinvasion catalyst, " antinvasin I." For hyaluronidase, on account of its property of promoting spreading and invasion, he suggested the name " invasin." Haas extensively studied the distribution of antinvasin I. He found a pronounced decrease in the concentration of the enzyme in individuals with various infections. It appears that antinvasin I plays an important role in the defense mechanism of the body by preventing invasion of certain bacteria and venoms. Haas (190) observed that there is an interaction of various enzymes in the process of invasion and defense. He observed that pathogenic organisms produce simultaneously, with hyaluronidase, a second enzyme that acts by destroying antinvasin I, the defensive enzyme of plasma. Hyaluronidase, although normally destroyed by antinvasin I, remains intact, since it is protected by the second enzyme, which Haas named " proinvasin I." Haas (191) has also discovered a third new enzyme. This enzyme acts by destroying proinvasin I and has been named " antinvasin II " by Haas.

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CHAPTER IV

PHOSPHORYLASES AND RELATED ENZYMES

As early as 1899, Cremer (1) observed that yeast juice is capable of glycogen synthesis. Neuberg and Pollak (2) demonstrated in 1910 that sucrose may be changed by phosphorolysis using autolyzed yeast. Cori and Cori (3) were first to show that muscle tissue and muscle extracts form glucose-1-phosphate from glycogen. Because of this, the ester is called Cori ester. Later it was found that many animal and plant tissues contain this enzyme and that the reaction is reversible. In more recent years, other enzymes synthesizing various carbohydrates and their derivatives by phosphorylation have been described.

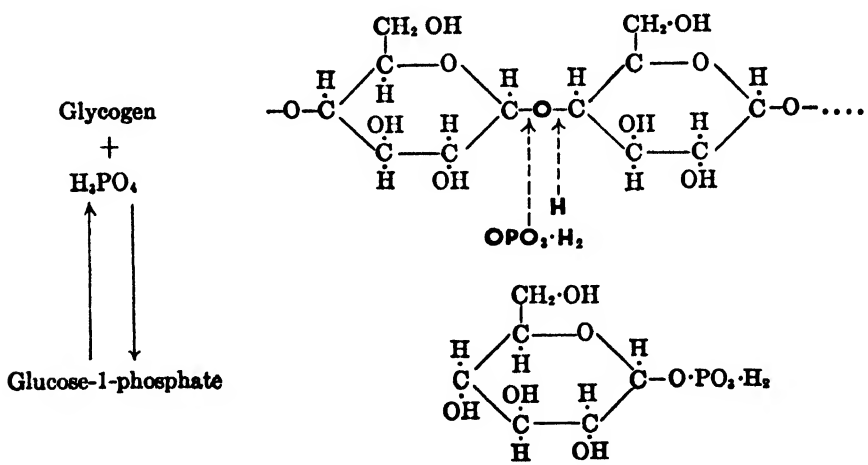


FIG. 10.

THE STARCH- AND GLYCOGEN-SYNTHESIZING ENZYMES

The reversible reaction: starch + phosphate $\rightleftarrows$ glucose-1-phosphate, as catalyzed by phosphorylase, was found to take place as a result of the phosphorolytic breakdown of successive terminal glucose molecules from the starch molecule, without the interaction of water. When the reaction is reversed and starch is formed from glucose-1-phosphate, a dephosphorylation with a simultaneous condensation takes place (see Fig. 10). Parnas called this type of starch breakdown " phosphor-

olysis " in order to differentiate it from hydrolysis, which indicates interaction with water.

Energy Requirements in Starch and Glycogen Synthesis (4)

Certain phosphorylated substances, such as adenosinetriphosphate, creatinephosphate, phosphopyruvate, and acetylphosphate, store energy liberated from oxidative steps, which is later used in certain metabolic reactions. These substances, although quite different in their chemical make-up, contain an equal quantity of energy, which is stored in the phosphate radical. On hydrolysis, these phosphoric acid esters, containing so-called " energy-rich phosphate bonds," furnish on the average 11,000 calories. Other organic esters, like hexosephosphate, glycerophosphate, and similar " ordinary " organic phosphates in which the phosphate radical is combined with an alcoholic hydroxyl, furnish only 3000 calories. Inorganic phosphate is the lowest energy level. Lipmann has coined the symbol ~ph for the energy-rich bond having an average energy of 11,000 calories and —ph for the ordinary ester bond furnishing an average of 3000 calories. The energy-rich phosphate bond ~ph is transferable from one energy-rich compound to another. This type of stored energy is distributed by the adenylic acid system. Metabolically available ~ph (acetyl phosphate, phosphopyruvate, etc.) interacting with the adenylic acid system is readily transferred. The transfer of ~ph to and from this system takes place by specific enzyme systems. Starch and glycogen synthesis from glucose occurs by the application of such phosphate bond energy. In the cell, glucose combines with phosphate by utilizing the energy drop from adenyl ~ph to glucose ester —ph. The ester bond of glucose-1-phosphate is then exchanged for a glucosidic radical on the same carbon, resulting in starch or glycogen synthesis.

When glucose is converted to glucose-6-phosphate, a loss of 8000 calories takes place. Energy-rich ~ph is converted into ordinary ester —ph. This step is practically irreversible. The other phases of the reaction are reversible, since only small quantities of energy are involved. The step that utilizes the most energy is the phosphorylation of glucose to glucose-1-phosphate. Glucose-1-phosphate is readily changed to starch or glycogen with little use of free energy (4–6).

Stepwise Polysaccharide Synthesis from Glucose

Colowick and Sutherland (7) have converted glucose, in the presence of adenosinetriphosphate, into polysaccharide by stepwise reactions.

First, glucose was changed to glucose-6-phosphate by hexokinase, obtained from bakers' yeast. Glucose-6-phosphate was converted to glucose-1-phosphate by rabbit muscle phosphoglucomutase, and the glucose-1-phosphate was finally changed to polysaccharide and inorganic phosphate by muscle phosphorylase. The work of Colowick and Sutherland is summarized in Fig. 11. In the living cell, adenosine-

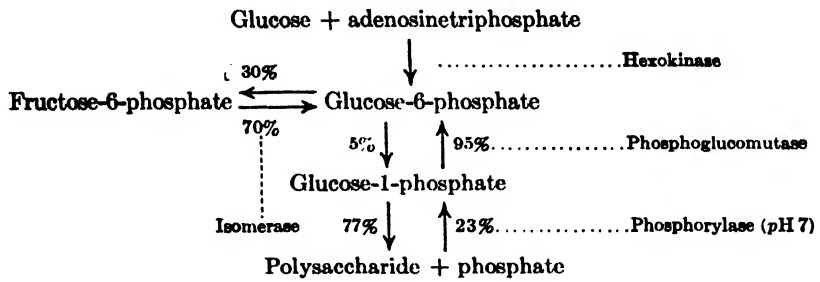


FIG. 11. Polysaccharide synthesis from glucose.

triphosphate loses its labile phosphates in this process and becomes adenylic acid. The living cell again rephosphorylates adenylic acid to adenosinetriphosphate. For the rephosphorylation reaction, oxidation of aldehyde to acid furnishes the high-energy phosphate (8).

Crystalline Muscle Phosphorylases and Their Relation to Other Phosphorylases

Cori and associates (9, 10) obtained crystalline phosphorylase from skeletal muscle. This enzyme is firmly bound to adenylic acid. It is an euglobulin with a molecular weight of 340,000 to 400,000. Cysteine increases the solubility and the activity of this enzyme, showing in its presence about 65 per cent of its maximum activity, without having adenylic acid in the digest (9). The muscle contains an enzyme which has the ability to remove the adenylic acid from the crystalline enzyme (phosphorylase *a*), changing it to a component (phosphorylase *b*) that is inactive without added adenylic acid (11). Trypsin acts similarly. Resting muscles of rabbits contain mainly phosphorylase *a*, as shown by the preparation of this enzyme in crystalline form in high yields. During strong muscular contractions produced by strychnine or by electrical stimulation, most of the phosphorylase *a* is converted to phosphorylase *b* by the adenylic acid removing enzyme *in vivo*. This explains why crystalline phosphorylase cannot be prepared from extracts of muscles stimulated to fatigue. Cori (12) believes that the

temporary inactivation of phosphorylase *a* by the enzymatic removal of its prosthetic group represents a regulatory function preventing the exhaustion of glycogen stores in fatigue.

Crystalline phosphorylase, supplemented by a second enzyme prepared by Cori and Cori (13) from heart or liver, produces a branched type of polysaccharide resembling glycogen. These investigators suggest that this enzyme is probably another kind of phosphorylase which is able to make 1–6 glucosidic linkages. Meyer (14) found that potato phosphorylase can open only 1–4 linkages, while yeast phosphorylase, which synthesizes a glycogenlike carbohydrate, contains enzymes that are capable of breaking both 1–4 and 1–6 linkages.

The Effect of Priming Agents on Polysaccharide Synthesis

The synthesis of starch or glycogen from glucose-1-phosphate, with the aid of purified muscle or potato phosphorylase, requires the addition of a small quantity of starch, glycogen, or dextrin. These polysaccharides function as priming agents. Without them, no phosphorylation takes place unless impure phosphorylases, containing small quantities of the priming agents, are used in the experiments. The activating power of the polysaccharides varies with the source of phosphorylase. The activating power of glycogen is much greater for muscle phosphorylase, and that of amylopectin is much more pronounced for potato phosphorylase (15, 16).

Some investigators believe that liver phosphorylase forms glycogen from glucose-1-phosphate, and that muscle phosphorylase and plant phosphorylases form compounds closely related to the two starch components (17, 19).

Sumner and associates (20) found that jack bean or potato phosphorylase may be primed with cornstarch amylose, cornstarch amylopectin, glycogen, erythrodextrin, achroodextrin, or either of the two water-soluble polysaccharides of sweet corn. Maltose has no priming property. These investigators observed that the nature of the end product of synthesis by plant phosphorylase depends on the kind and amount of the priming agent added. A small quantity of achroodextrin will influence the formation of a substance giving a blue color with iodine. A large amount of this dextrin will cause the formation of a compound that gives a red color with iodine, whereas a still larger quantity of the dextrin will produce a substance that gives no color with iodine. In each case, the amount of inorganic phosphate set free was the same.

Hidy and Day (21) published a highly interesting study con-

cerning the priming action of two sources of polysaccharides, using purified potato phosphorylase as the enzyme material. One source of activator was a polysaccharide synthesized by potato phosphorylase and hydrolyzed with potassium hydroxide. The other source was the non-dialyzable cornstarch dextrins which had been hydrolyzed to the achromic point with hydrochloric acid and purified by dialysis and methanol precipitation. The color given by iodine and the carbohydrate synthesized during the first few minutes was almost always the same as that given by the priming polysaccharide. The color of the activating polysaccharide varied from blue to colorless, depending on the degree of acid hydrolysis applied to the cornstarch. However, extension of the polysaccharide-synthesizing reaction resulted in products that stain blue with iodine irrespective of the nature of the activator. Hidy and Day conclude that activators of potato phosphorylase need not contain more than 6 or 7 glucose units per molecule.

Synthesis of the Two Starch Components by Potato Phosphorylase

The procedures that follow describe the synthesis from glucose-1-phosphate of a polyglucose with amyloselike properties. This synthesis is carried out by a phosphorylase first identified in potatoes by Hanes. Another enzyme, quite different from the phosphorylase just mentioned, has been isolated by Bourne and Peat (19), in a fraction of potato juice. This new enzyme, acting in conjunction with the purified phosphorylase, converts glucose-1-phosphate into amylopectin, which is the major component of whole starch. The synthetic amylopectin does not reduce, does not retrograde from solution, gives a red-purple color with iodine, and is changed by β-amylase to maltose until 46 per cent conversion is attained. This amylopectin is said to contain 20 glucose units.

Preparation of Potato Juice. One kilo potatoes is peeled, sliced, and soaked for 30 minutes in 1 liter of water containing 0.5 per cent hydrosulfite and 0.5 per cent toluene. The potatoes are drained, washed, minced, and pressed. The light yellow juice is clarified by centrifuging, and after addition of 0.5 per cent toluene it is stored at 0° C.

Qualitative Test for Synthetic Action. To 0.1 cc. of enzyme solution are added 0.2 cc. 0.1 *M* glucose-1-phosphate and 0.1 cc. citrate buffer of *p*H 6.0. The mixture is incubated for 15 minutes at 25° C. Three drops of an *N*/60 iodine-potassium iodide solution are added. The formation of a colored starch-iodine complex signifies phosphorylase action.

Purification of Phosphorylase. Fifty grams of ammonium sulfate is dissolved in 100 cc. of water, and the *p*H is adjusted to 6.0 with ammonium hydroxide. The solution is saturated with toluene. The potato juice is fractioned at 0° C. as follows: To each 100 cc. of juice 47 cc. of ammonium sulfate solution is added; this is to give a concentration of 16 grams per 100 cc. The precipitate is removed by centrifuging and is discarded. To the supernatant, 180 cc. of ammonium sulfate is added to make the concentration 35 grams per 100 cc. The mixture is allowed to stand for 4 hours. The phosphorylase which precipitates is collected by centrifuging. The supernatant is discarded. The precipitate is redissolved in the minimum amount of water and refractionated twice. The phosphorylase activity may be determined by the method of Green and Stumpf (22). The dry phosphorylase may be stored for at least 6 weeks at 0° C.

Synthesis with Purified Phosphorylase. The mixture contains 25.0 grams glucose-1-phosphate, 1.0 gram dry phosphorylase (containing ammonium sulfate), 10 cc. toluene. The final volume is 2 liters. *p*H is to be maintained at 6.0 with acetic acid. The temperature should be 20° C. The changes in inorganic phosphate are shown in Table XII.

TABLE XII

CHANGES IN INORGANIC PHOSPHATE DURING PHOSPHORYLATION

Incubation Time in Hours	Inorganic P in Grams	Conversion %	Incubation Time in Hours	Inorganic P in Grams	Conversion %
0	0.008	0	70	1.240	60
24	0.412	20	120	1.680	81
46	0.788	38	168	1.728	83

When equilibrium at 83 per cent conversion is attained, the " granular synthetic starch " may be separated by centrifuging, washed with water and ether, and dried. Yield: 5.75 grams.

Preparation of the Amylopectin-Synthesizing Enzyme. Seventy-six cubic centimeters of the clarified potato juice (described above under " Preparation of Potato Juice ") is shaken for 15 minutes with 20 cc. of *p*H 5.1 buffer and 5.4 grams of kaolin suspended in 32 cc. of water. The mixture is centrifuged, and the kaolin, which should contain any amylase present in the potato juice, is discarded. To the supernatant, 63 cc. of ammonium sulfate is added. The resulting precipitate is removed by centrifuging, redissolved in 40 cc. water, and reprecipitated by adding 20 cc. ammonium sulfate. This enzyme converts starch into a reddish purple color giving polysaccharide in 24 hours. This color remains for at least 9 days.

Amylopectin Synthesis. Phosphorylase prepared from 100 cc. of potato juice is redissolved in 60 cc. of water. The amylopectin-synthesizing enzyme prepared from 150 cc. potato juice is dissolved in 30 cc. of water. The two enzyme solutions may be used in the experiments to follow at 38° C. At intervals, 2 drops from each experiment may be removed, diluted with 0.25 cc. of water, and tested with 1 drop of $N/60$ iodine solution. These results are recorded in Table XIII.

TABLE XIII

AMYLOPECTIN SYNTHESIS

5 cc. 0.5 *M*-glucose-1-phosphate plus 2 cc. citrate of *p*H 6.0 plus	Iodine Color						
	0 min.	1 hr.	2 hr.	1 day	2 days	5 days	10 days
1 cc. phosphorylase	C	B(F)	B	B	B	B	B
1 cc. amylopectin syntase	C	C	RP	RP	RP	R	R
1 cc. phosphorylase plus 1 cc. amylopectin syntase	C	R(F)	RP	R	R	R	R

Key: B = blue; R = red; P = purple; (F) = faint; C = no color.

Synthesis of Polysaccharide with Untreated Potato Juice (19). To 5 grams of glucose-1-phosphate (K_2 salt), 20 cc. citrate buffer of *p*H 6.0, 20 cc. crude potato juice, and 60 cc. of water are added. Equilibrium is reached in 6 hours, 86 per cent of the ester-phosphorus being changed into inorganic phosphorus. Yield: 0.45 gram of polysaccharide.

Synthesis of Amylopectin by Kaolin-Treated Potato Juice. Potato juice is treated with kaolin, and then 35 grams of ammonium sulfate is added per 100 cc. of juice. The precipitate, containing both phosphorylases, is dissolved in the minimum amount of water and may be used for synthesis.

In this work, Bourne and Peat (19) did not employ polysaccharides for priming. This would indicate that their synthesizing enzymes must have contained traces of some polysaccharide.

PREPARATION OF GLUCOSE-1-PHOSPHATE

Sumner and Somers (23) published the following modification for the preparation of the Cori ester.

Preparation of Potato-Cyanide Extract. Three hundred twenty-five grams of potatoes is sliced, immediately placed into 100 cc. of 0.01 *N* hydrocyanic acid (KCN neutralized with hydrochloric acid), and disintegrated in a "Power-master" blender. Then the mixture

is pressed in cheesecloth, and the juice, amounting to about 125 cc., is centrifuged to free it from starch and cellular matter.

Indicators. Thymol blue paper and Congo red paper are made by dipping filter paper in solutions of these dyes and then drying the paper.

Preparation of the Ester. Eight grams of soluble starch is boiled in 100 cc. of water. After cooling, 12 grams of Na_2HPO_4 (anhydrous), 5 grams of KH_2PO_4 (anhydrous) dissolved in 300 cc. of water, and 100 cc. of potato-cyanide extract are added. The mixture is diluted to 1000 cc., some toluene is added, and, after mixing, the mixture is kept at 20° to 25° C. for 24 hours. The phosphorylase is destroyed by adding 0.1 *N* iodine solution until, upon mixing, the solution gives a permanent reddish brown color. The iodine is removed by adding 0.1 *N* sodium thiosulfate until the brown color is entirely gone. Ten to twenty cubic centimeters of an active 2 per cent pancreatin solution is added, and the solution is allowed to stand for 4 hours at room temperature or until a sample gives a negative test for dextrin when treated with 4 drops of 0.01 *N* iodine solution. Now 40 grams of barium acetate and about 8 cc. of 28 per cent ammonia (alkaline to phenol red) are added. The solution is mixed, then centrifuged to remove the barium phosphate, and the supernatant is filtered through cotton. To each volume of solution, 2 volumes of 95 per cent alcohol is added.

The precipitated barium salt of glucose-1-phosphate is collected by centrifuging. The supernatant is discarded. The precipitate is stirred with 2 *N* sulfuric acid and 30 to 60 cc. of water. Just enough sulfuric acid is added to produce a pink color with thymol blue paper. Now, saturated potassium hydroxide is added until the mixture just fails to give a blue or brown color with Congo red paper. Six grams of trichloroacetic acid is added, and, after mixing, 2 volumes of 95 per cent alcohol is added to every volume of the suspension. After mixing, the precipitate is centrifuged and the supernatant is decanted. Saturated potassium hydroxide is added to the supernatant until the solution is distinctly alkaline to phenol red. The dipotassium salt of glucose-1-phosphate usually separates in the form of an oil. When chilled overnight at 5° to 0° C., crystals of $C_6H_{11}O_9PK_2 \cdot 2H_2O$ separate. The crystals are washed several times with 95 per cent alcohol, then with acetone, and are dried at 40° to 50° C. The yield is about 3.5 grams.

The ester prepared by this method is about 85 per cent pure and contains potassium sulfate. It can be obtained practically pure by recrystallization, according to Hanes: The ester is dissolved in 20 times its weight of distilled water and is filtered. One volume of 95 per cent alcohol is added. After mixing and chilling, the solution is placed

overnight in a refrigerator having a temperature of 0° C. The crystals which separated may now be filtered and treated as described above. This product has a specific rotation with sodium light of about +79° and a phosphorus content of about 8.33 per cent.

SEPARATION OF AMYLOSE AND AMYLOPECTIN OF STARCH (24)

The principle of this method is the precipitation of amylose as a water-insoluble complex. The precipitant is thymol, and the operation is carried out at room temperature.

The starch (1 to 3 per cent) is dispersed in a small volume of water and then poured into boiling water with vigorous mechanical stirring. The stirring is continued at the boiling point for half an hour. Sodium chloride is added to make a 0.1 per cent solution. The mixture is cooled rapidly to room temperature. Powdered thymol is now stirred into the solution to saturation (0.13 per cent). In short time, the thymol-amylose complex begins to separate. Precipitation is complete in 48 hours. The precipitate is removed by centrifuging. It is washed rapidly with (*a*) water saturated with thymol, (*b*) absolute alcohol, and (*c*) ether. These steps must be carried out rapidly and the product dried in a vacuum; otherwise a water-insoluble amylose is obtained.

The amylopectin component is obtained by concentrating the mother liquid to one-fourth of its original volume at 60° C. and treating it with 1 volume of methylated spirits. The precipitate is washed and dried with alcohol and ether. The yield of amylose varies between 20 and 22 per cent of the weight of potato starch used in the separation.

Nonhydrolyzing Phosphorylation of Starch and Glycogen by Alkaline Phosphatase

Van Thoai and coworkers (25) obtained in crude state, from dog intestinal mucosa, an alkaline phosphomonoesterase which was capable of phosphorylating cornstarch and glycogen without hydrolysis. Thus, this enzyme differs from phosphorylase, which catalyzes a " lytic " type of phosphorylation. The enzyme was concentrated by dialyzing an extract of dog intestinal mucosa at 37° C. against redistilled water for 4 hours followed by precipitation with acetone.

Production of Extracellular Starch in Cultures of Capsulated Yeasts

Aschner, Mager, and Leibowitz (26) reported the interesting observation that *Torulopsia rotundata*, a capsulated yeast, when grown in

a simple inorganic salt medium with glucose as source of carbon, ammonium sulfate as the source of nitrogen, and a supplement of thiamin as a growth factor, produced a substance which gave a steel-blue color with iodine. The substance was found in the medium as well as in the large capsules by which cells of this species are usually surrounded, but it could not be detected within the cells. Several grams of the substance were prepared. It had all the properties of amylose. Twenty-five different yeast strains belonging to various taxonomic groups produced no amylose under similar conditions. Among the capsulated *Torulopsis* yeasts, the only species other than *T. rotundata* available to these investigators was the pathogenic *T. neoformans*, also known as *T. histolytica* or *Cryptococcus hominis*. This strain formed amylose in exactly the same manner as *T. rotundata*. There was no statement in this paper concerning the necessity of phosphate in this synthesis.

Bacterial Production of an Amylopectinlike Polysaccharide from Sucrose

Hehre and Hamilton (27) obtained a polysaccharide in quantities as large as 3 to 5 grams per liter from cultures grown in 5 per cent sucrose broth, using bacteria of the *Neisseria* genus. The polysaccharide was soluble in cold water but not in 50 per cent alcohol. It gave a purple-red color with iodine, which reaction was rapidly lost upon treatment with saliva. In other respects the polysaccharide resembled glycogen and amylopectin. Other sugars, such as glucose, glucose and fructose, maltose, lactose, trehalose, melibiose, raffinose, or melizitose, could not be converted in a similar fashion. The enzyme responsible for this reaction has not yet been isolated.

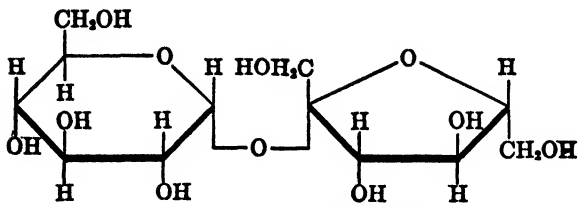
Bacterial Synthesis. Washed bacterial cells obtained from glucose broth cultures of *Neisseria sp.* are incubated with 0.05 *M* solution of sucrose in maleate buffer of *p*H 6.4.

SUCROSE PHOSPHORYLASE

The bacterium *Pseudomonas saccharophila* contains a sucrose phosphorylase, which catalyzes the reversible reaction (28):

$$\text{Glucose-1-phosphate} + \text{fructose} \rightleftarrows \text{sucrose} + \text{inorganic phosphate}$$

Hassid and collaborators (28) isolated sucrose from this reaction mixture in pure state. This experiment explains how sucrose is formed in nature and gives strong support for the accepted structure of the disaccharide:



STRUCTURAL FORMULA FOR SUCROSE (α-D-GLUCOPYRANOSIDO-β-D-FRUCTOFURANOSIDE.)

Preparation of Sucrose Phosphorylase. The enzyme is extracted from dried cells of *P. saccharophila* with *M*/30 phosphate buffer at *p*H 6.64. It is separated from invertase by repeated fractional precipitation with ammonium sulfate. When the solution is one-third saturated with ammonium sulfate, the invertase precipitates, but not the sucrose phosphorylase. The optimum *p*H of this enzyme is at 6.4 to 7.0. Glucose inhibits the enzyme but not 0.2 *M* sodium fluoride (29, 30).

Hassid and coworkers (28) recommend the following simple method for the enzymic synthesis of sucrose:

To 15 grams of K-glucose-1-phosphate and 15 grams of fructose is added 6 grams of phosphorylase made from dry *P. saccharophila*. The *p*H is adjusted to 6.8 with acetic acid. Barium acetate is added to make the final concentration 0.133 *M*, and the total volume is made up to 300 cc. The *p*H is maintained at 6.85 during incubation (12 hours at 37° and 12 hours at 29° C.). The yield is 3 grams of sucrose.

This phosphorylase can also combine glucose-1-phosphate with other ketose monosaccharides, such as *l*-sorbose and *d*-ketoxylose, to form the corresponding disaccharides. Thus, two new crystalline nonreducing disaccharides have been synthesized and their structure has been found to be analogous to that of sucrose (31).

Phosphorylation of Pyruvic Acid

Lardy and Ziegler (32), using a dialyzed extract of acetone-precipitated rat muscle extract, have shown that the reaction Pyruvate + ATP $\rightleftarrows$ phosphopyruvate + ADP is reversible. Pyruvate was enzymatically phosphorylated when high-energy phosphate was continually supplied by the oxidation of glyceraldehyde-3-phosphate. Potassium ions and magnesium or manganous ions are needed in much lower concentrations for the oxidation of glyceraldehyde-3-phosphate and the transfer of high-energy phosphate to the adenylic system than for the transfer from phosphoglycerate through phosphopyruvate to adenylate. Potassium ions have an important function in the synthesis of phos-

phopyruvate from pyruvate. Lardy and Ziegler have demonstrated by the distribution of P^{32}, and by direct synthesis, that pyruvate may be enzymatically phosphorylated by ATP in the presence of K^+ and Mg^{++} to produce ~pHPy (high-energy phosphopyruvate), and that it is not necessary to postulate 4-carbon intermediates in this synthesis. Yeast probably contains an enzyme of similar nature (33).

Phosphate Transfer via Aldehyde Oxidation

3-Phosphoglyceraldehyde is oxidized to 1,3-diphosphoglyceric acid (Warburg). The oxidation brings about the inclusion of the inorganic phosphate into carboxyl phosphate, which can then react with ADP (adenosinediphosphate) to form ATP (adenosinetriphosphate). The energy derived from this oxidation is stored in the ATP. Such an ATP synthesis occurs during the fermentation of glucose in intact cells (34).

Hexokinase

Hexokinase is present in all glucose-fermenting cells. It catalyzes the transfer of one phosphate radical from adenosinetriphosphate to hexoses (glucose, fructose, and mannose) with the liberation of 1 hydrogen equivalent of acid in the following manner (35, 36):

Hexose + adenosinetriphosphate =
hexose-6-phosphate + adenosinediphosphate

Preparation. Meyerhof (37) prepared hexokinase by plasmolyzing bakers' yeast with toluene, extracting the liquefied yeast with water at 35° C., and precipitating the active material in 50 per cent ethyl alcohol at 0° C. Berger and coworkers (38) improved this procedure by plasmolyzing in the presence of 1 per cent glucose and 0.05 *M* acetate at *p*H 5.2 to 5.4. The glucose protects the hexokinase from inactivation. Kunitz and McDonald (39), using a yeast extract prepared in this manner, and concentrating and fractionating the yeast proteins with ammonium sulfate and alcohol, prepared hexokinase in a pure crystalline state. The enzyme is an albumin. It has an isoelectric point at *p*H 4.8 and a molecular weight of 96,000. Hexokinase is most stable in dilute buffers at *p*H 5.0 and requires magnesium ions for its activity.

Phosphoglucomutase

This phosphomutase is generally found in all plant and animal cells (40). It had been prepared free of other enzymes (41). Phosphoglu-

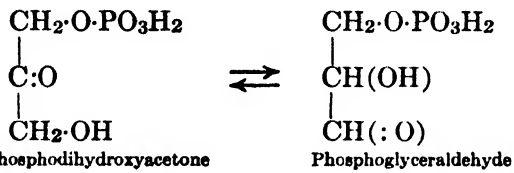
comutase changes glucose-1-phosphate (Cori ester) into glucose-6-phosphate. The conversion is very extensive, since 95.5 per cent of the Cori ester is changed to glucose-6-phosphate at 30° C. The reaction is reversible. The activity of the enzyme is increased by Mg^{++}, Mn^{++}, or Co^{++}. The optimum *p*H is at 7.5 to 9.2. Phosphoglucomutase does not act on mannose-1-phosphate or galactose-1-phosphate (42).

Phosphohexose Isomerase

This enzyme is present in muscle extracts and in yeast and other plants. It catalyzes the equilibrium between glucose-6-phosphate and fructose-6-phosphate. The equilibrium mixture contains 70 per cent glucose-6-phosphate (43, 18).

Triosephosphate Isomerase

Muscle tissue, yeast, and some bacteria contain an enzyme that forms an equilibrium between dihydroxyacetone phosphate and phosphoglyceraldehyde:



Phosphodihydroxyacetone — Phosphoglyceraldehyde

The equilibrium with either purified isomerase or crude aldolase-containing preparations is at 20 to 25 (44). A highly active triosephosphate isomerase preparation has been obtained from rabbit muscle tissue by Meyerhof and Beck (45). The major steps were fractional precipitation with ammonium sulfate, adsorption on cupric oxide, and elution with phosphate buffer at *p*H 7.2. This product was free of phosphohexose isomerase and of aldolase.

Phosphoglyceromutase

This enzyme is present in most cells. It converts 3-phosphoglyceric acid into 2-phosphoglyceric acid (46). The quantitative composition of the equilibrium mixture is not known. However, 3-phosphoglyceric acid is present in larger quantity. This enzyme interacts with enolase, which converts 2-phosphoglyceric acid into the enol form of phosphopyruvic acid.

The Role of Acetylphosphate in Metabolism

Work carried out in several laboratories indicates that acetyl phosphate plays a very important role in metabolism. Ingested acetate, when observed by the isotope procedure, appears in a great variety of substances. Fatty acids, cholesterol and other sterols, hemin, and glycogen have been found to be synthesized from some of the ingested acetate. These studies are being extensively expanded in order to establish the role of acetate in metabolism, chemo- and photosynthesis, etc. Lipmann (47) has reviewed the subject in great detail.

PREPARATION OF SOME COMPOUNDS BY PHOSPHORYLATION WITH YEAST

Preparation of *d*-Fructose-1,6-Diphosphate ($C_6H_{10}O_4(PO_4Ca)_2 \cdot H_2O$) with the Aid of Yeast (48, 49)

To a solution of 400 grams of sucrose, 83 grams of monosodium phosphate ($NaH_2PO_4 \cdot 2H_2O$), and 44 grams of sodium bicarbonate in 2000 cc. of tap water, contained in an 8-liter bottle, are added 600 grams of fresh bottom yeast (certain bakers' yeasts may also be used) and 100 cc. of toluol (ether, benzene, or carbon tetrachloride). The mixture is shaken until homogeneous and placed in an incubator at 37° C. until phosphorylation is complete (2 to 4 hours). Completion of this process may be determined by adding 3 cc. of 2.5 per cent ammonia and 1 cc. of 10 per cent ammonium chloride to 2 cc. of the filtered fermentation mixture, and then adding magnesia mixture. A precipitate does not form immediately when the reaction is complete.

When phosphorylation is complete, a few cubic centimeters of a 10 per cent solution of octyl alcohol in ethanol are added to the fermentation mixture. The container is immersed in a boiling water bath until the proteins are coagulated, and the mixture is centrifuged or filtered (in a refrigerator). The filtrate is neutralized to phenolphthalein by the addition of 4 *N* sodium hydroxide and immediately treated with a solution of 55 grams of calcium chloride in 100 cc. of water. The solution is immersed for a short time in boiling water to complete precipitation, and then it is filtered with suction while hot. The precipitated calcium-*d*-fructose-1,6-diphosphate is washed with warm water. The yield is 44 grams of crude salt.

The moist, crude calcium salt may be purified by dissolving it in 500 cc. of 2 *N* acetic acid and adding 250 cc. of water. To the filtered solution, 2 *N* sodium hydroxide is added, until neutral to phenolphthalein, and then it is heated in a boiling water bath for a short time. The

precipitate is filtered and washed with warm water. The yield is about 80 per cent of the crude salt.

The barium salt may be prepared in a similar manner, by using slightly more than 1 mole of barium chloride for each mole of monosodium phosphate employed in the salt-sugar mixture.

Calcium and barium fructosediphosphate dissolve readily in solutions of ammonium salts, such as the acetate, chloride, nitrate, and thiocyanate, whereas calcium and barium phosphate are insoluble. Thus, this property serves as a purity test.

Properties of *d*-Fructosediphosphoric Acid. Hexosediphosphoric acid was first described independently by Harden and Young and by Ivanoff in 1905. It is called the Harden-Young ester. The free ester may readily be obtained by grinding the barium salt with an equimolar quantity of ice-cooled sulfuric acid. Fructosediphosphoric acid is a tetra basic acid, stronger than orthophosphoric acid. It is slightly dextrorotatory ($[\alpha]_D = +3.55°$). The hydrolyzed product, however, is slightly less levorotatory than fructose. The free acid, as well as its salts, readily reduces boiling Fehling solution. Fructosediphosphoric acid and its salts do not form a precipitate with magnesia mixture in the presence of dilute ammonia. On boiling, the calcium and barium salts form precipitates which dissolve again on cooling.

Preparation of Fructose-6-Phosphate, $C_6H_{11}O_5PO_4H_2$ (50)

Thirteen and seven-tenths grams of calcium fructosediphosphate is placed in 150 cc. of *N* oxalic acid and boiled for 30 minutes. Thereafter, the calcium oxalate is filtered off. The solution is neutralized with barium carbonate and again filtered. From the filtrate, the barium salt of fructose-6-phosphate is precipitated by the addition of alcohol. The yield is 9 grams.

The barium salt is readily soluble in water; it reduces Fehling solution; and it gives a positive Selivanoff reaction. Fructose-6-phosphoric acid is called the Neuberg ester.

Preparation of Crystalline *d*(-)3-Phosphoglycerate, $C_3H_5O_7PBa \cdot 3H_2O$, with the Aid of Yeast (51)

One hundred twenty grams of sucrose, 22.2 grams of $NaH_2PO_4 \cdot H_2O$, and 6.6 grams of sodium bicarbonate are dissolved in 600 cc. of water. Three hundred grams of fresh National Grain (bakers') yeast and 90 cc. of carbon tetrachloride are added. After mixing, the sugar is allowed to phosphorylate for 24 hours at room temperature (the clear

filtrate should show a negative test with magnesia mixture). With fresh bottom yeast, however, phosphorylation is completed in 2 to 4 hours. At this stage, 840 cc. of 2 per cent acetaldehyde solution, 140 cc. of 0.2 *M* sodium fluoride solution, 1200 cc. of 10 per cent glucose solution, 380 grams of fresh yeast, and 20 cc. of carbon tetrachloride are added. After shaking, the mixture is allowed to stand for 24 hours at room temperature.

For deproteinization, 5 cc. of glacial acetic acid is added, and the mixture is heated in a boiling water bath for 30 minutes. Then it is centrifuged. Per 100 cc. of clear fluid, 5 cc. of glacial acetic acid and 7 cc. of 50 per cent barium acetate solution are added; the mixture is quickly filtered. The clear filtrate is placed in a refrigerator. Crystallization begins soon and is complete after 48 hours. The crystals are filtered under suction and washed with water. The yield of crude substance is 16.8 grams.

Purification. Four grams of the crude barium salt is dissolved in 280 cc. of 0.05 *N* hydrochloric acid; the mixture is then warmed and filtered. To the clear filtrate, 560 cc. of alcohol are added. The precipitate, which is at first milky, soon changes, on stirring, into lustrous crystals. The substance is allowed to crystallize in the refrigerator. It is filtered by suction and washed with alcohol until free from chlorine. The yield is 3.5 grams of pure acid barium salt of phosphoglyceric acid.

Properties of *d*(-)3-Phosphoglyceric Acid. This acid is readily changed to various compounds, biologically. It is, however, very resistant to acids and alkalis. The free ester is levorotatory: $[\alpha]_D^{20} = -14.5°$. It does not reduce. The crystalline salt is only slightly soluble in water. The properties and biological importance of the ester have been discussed by Neuberg and Lustig (51).

Analysis of Phosphoric Acid Esters (52)

The best method for the analysis of phosphoric acid esters is to follow the velocity of hydrolysis of the ester in *N* hydrochloric acid at 100° C.

Velocity of Hydrolysis by Hydrochloric Acid. The salts are dissolved in *N* hydrochloric acid to make a solution of *M*/100 — P. Barium may be removed by adding an equimolar quantity of potassium sulfate. The barium sulfate is centrifuged off, and total and inorganic phosphorus are determined. Then 1.2-cc. samples of the solution are placed in 3-cc. ampules, which, after sealing, are immersed in a covered, vigorously boiling water bath. Inorganic phosphate is deter-

mined in samples of 0.5 to 1 cc. of the solution. The method is applicable only if the components of the ester mixture show distinct hydrolysis constants

$$\left(k = \frac{l}{t} \log \frac{a}{a - x}\right)$$

Velocity of Hydrolysis by Sodium Hydroxide. In $N/5$ sodium hydroxide, fructosediphosphate is hydrolyzed 100 per cent in 3 minutes, whereas hexose-6-phosphate is split only 60 per cent under these conditions.

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CHAPTER V

NUCLEASES, AMIDASES, AND DEAMINASES

NUCLEASES

Ribonuclease

This enzyme is present in the pancreas, pancreatic juice, liver, lungs, spleen, and in blood. Ribonuclease depolymerizes yeast or ribonucleic acid (a tetranucleotide):

$$\left\{\begin{array}{l}\text{Phosphoric acid} - \text{Ribose} - \text{Guanine} \\ \text{Phosphoric acid} - \text{Ribose} - \text{Uracil} \\ \text{Phosphoric acid} - \text{Ribose} - \text{Cytosine} \\ \text{Phosphoric acid} - \text{Ribose} - \text{Adenine}\end{array}\right\}$$

In the living cell, the nucleic acids are further polymerized by unknown bonds. Nucleases hydrolyze nucleic acids into mononucleotides (phosphate-ribose-organic base). Ribonuclease acts only on yeast nucleic acid. Thymonucleic acid is hydrolyzed by desoxyribonuclease. In contrast to the nucleic acids, the mononucleotides are not precipitated by glacial acetic acid. Acidic groups are liberated, but not phosphoric acid. Kunitz (1) observed that there is a shift in the ultraviolet absorption spectrum of the substrate towards the shorter wavelengths. He applied this change in the absorption spectrum for the quantitative determination of ribonuclease activity. The manometric method may also be used, since acidic groups are liberated (2). The viscosimetric procedure is not accurate for measuring the enzymic decomposition of ribonucleic acid into smaller fragments, since salts alone are able to cause a degradation (3).

Preparation of Crystalline Ribonuclease (4). Fresh beef pancreas is chopped and extracted with 0.25 *N* sulfuric acid. Solid ammonium sulfate is added to make the filtrate 0.7 saturated. The mixture is filtered, and more saturated ammonium sulfate is added in order to make the solution 0.8 saturated. The mixture is filtered by suction. The precipitate containing the enzyme is dissolved in water (10 cc. per 10 grams filter cake) and filtered. To the filtrate, saturated ammonium sulfate is added until it becomes slightly turbid. The *p*H

is adjusted to 5 with *N* sodium hydroxide and readjusted to *p*H 4.2 with *N* sulfuric acid. The solution is allowed to remain at 20° C. for a day or two. The ribonuclease first precipitates in an amorphous state, which gradually turns into crystalline needles and later into plates. The enzyme may be recrystallized by dissolving in water and adding ammonium sulfate.

Properties of Crystalline Ribonuclease. Kunitz has shown that this enzyme is an albumin. Its molecular weight is 15,000. The enzyme is quite resistant to heat. There is no activity at 85° C., but all the enzyme activity returns on cooling. The ribonuclease may even be boiled at *p*H 2 without being destroyed. At *p*H's closer to neutrality, the enzyme is much more sensitive to heat, and at *p*H 5.0 it is rapidly denatured and destroyed. The enzyme is most active at *p*H 7.6.

Desoxyribonuclease or Thymonucleodepolymerase

The substrate of this enzyme, thymonucleic acid, consists of the four nucleotides guanylic acid, adenylic acid, cytidylic acid, and thymic acid; each of the four contains desoxyribose. Desoxyribonuclease is present in the pancreas, in the intestinal mucosa, in grains, and in seeds (5). Desoxyribonuclease hydrolyzes thymonucleic acid into mononucleotides. Acid-soluble phosphorus is also liberated. This may be used as a basis for determining the activity of the enzyme (6).

Preparation. McCarty (7) published a method for the purification of desoxyribonuclease from beef pancreas. The fresh glands are obtained at the slaughter house and at once immersed in cold 0.25 *N* sulfuric acid. Then the glands are ground and suspended in 2 volumes of cold 0.25 *N* sulfuric acid. Extraction is allowed to proceed in the refrigerator overnight. The mixture is filtered, and the filtrate is brought to 0.2 saturation with solid ammonium sulfate. The precipitate is discarded. After the filtrate is brought to 0.4 saturation with solid ammonium sulfate, the precipitate is allowed to separate for 1 or 2 days in the refrigerator. All the desoxyribonuclease is contained in the precipitate. The solution contains ribonuclease, chymotrypsinogen, and trypsinogen.

The precipitate at 0.4 saturation is redissolved in a small volume of water and brought to 0.17 saturation by the addition of saturated ammonium sulfate solution. A small amount of precipitate is formed; this is discarded. The filtrate is brought to 0.3 saturation with ammonium sulfate. The precipitate is dissolved in water and refractionated between 0.17 and 0.3 saturation with ammonium sulfate. The final 0.3 saturated precipitate is dissolved in water and dialyzed in the cold

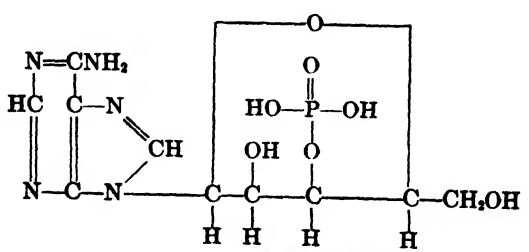
against 0.002 *N* sulfuric acid. The dialyzed solution is dried *in vacuo* from the frozen state. The yield of dried enzyme from 10 pounds of pancreas is 1 to 2 grams.

Properties. The dried enzyme is water soluble. At *p*H 4 to 5, the enzyme is fairly stable, but above *p*H 7 it is rapidly destroyed. This is the result of small amounts of proteolytic enzymes present in the preparation. Highly dilute solutions are unstable and must be used immediately. The enzyme is most active at *p*H 6.8 to 8.2. It has an isoelectric point at *p*H 5 to 5.2. This preparation is highly active. Its action can be measured in concentrations as low as 0.01 microgram per cc. It requires magnesium or manganese ions for its activity. Citrate is a powerful inhibitor of the magnesium-activated enzyme. Desoxyribonuclease does not attack ribonucleic acid. McCarty measured the activity of this enzyme by a viscosimetric procedure, using sodium desoxyribonucleate from calf thymus as substrate.

Complete Decomposition of Nucleic Acids by Tissue Extracts. Greenstein and collaborators (8) studied the time course of the appearance of ammonium and inorganic phosphate in digest of ribosenucleic and desoxyribosenucleic acids, of purines, pyrimidines, nucleosides, and nucleotides with fresh and with dialyzed extracts of various animal tissues. This paper contains many interesting facts concerning the catabolism of nucleic acids.

Nucleotidases

These enzymes hydrolyze purine and pyrimidine nucleotides into nucleosides and phosphoric acid. For instance, yeast adenylic acid is split into adenosine and phosphoric acid.



Yeast adenylic acid

Nucleotidases appear to be identical with "alkaline" and "acid phosphatase." Thus, the phosphatases having an alkaline optimum *p*H for phosphorolysis (liver, intestinal mucosa) act best also on nucleotides at that *p*H, whereas the "acid phosphatases" of plants

(rice, bran, molds, roots, leaves, seeds) act best at their respective optimum *p*H's on nucleotides. Neither the " acid phosphatase " nor the " acid nucleotidase " reaction can be activated by magnesium ions, but both are inhibited by sodium fluoride. However, the " alkaline phosphatase " and " alkaline nucleotidase " reactions require magnesium ions (9).

Nucleosidases or Purine Nucleoside Phosphorylases

Kalckar (10) has shown that the nucleosidases are actually phosphorylases. The action of the purine nucleoside phosphorylases is a phosphorolytic process resulting in the following type of reversible reaction:

$$\text{Ribose-1-purine} + \text{phosphate} \rightleftarrows \text{Ribose-1-phosphate} + \text{purine}$$

Kalckar prepared the purine nucleoside phosphorylase from rat liver. He found it to be a highly soluble protein which is inactivated by dialysis. The activity was restored by the addition of phosphate or arsenate. He named the enzyme purine nucleoside phosphorylase. When a purine nucleoside (inosine or guanosine) is hydrolyzed, one mole of phosphate is bound into an acid-labile organic linkage for each mole of purine liberated. When ribose-1-phosphate is incubated with hypoxanthine or guanine in the presence of liver nucleoside phosphorylase, a rapid synthesis of purine nucleoside occurs. The position of equilibrium favors the synthesis of purine riboside.

Nucleosidases have been frequently studied. They are present in liver, heart, lung, and small intestine (11). Small amounts of nucleosidases occur in leaves, fruits, seeds, and roots (9). However, emulsin of sweet almonds does not hydrolyze nucleosides. The intestinal nucleosidases readily split pyrimidine nucleosides. For methods for the preparation and estimation of these enzymes see reference 10.

AMIDASES

Amidases open carbon-nitrogen linkages. Most of these enzymes liberate ammonia from their substrates; some, however, act in a different manner.

Arginase

Arginase was discovered by Kossel and Dakin in 1904. It decomposes *l*(+)-arginine into ornithine and urea. This reaction is an

important phase of protein metabolism. It is responsible for the conversion of the catabolic amino acid nitrogen into urea in the mammalian liver. The best source of arginase is the male mammal's liver. Traces of arginase are present in other organs (12). It is also present in seeds, such as jack bean, and in molds, such as *Neurospora crassa* (13).

Preparation. Mohamed and Greenberg (14) have developed a method for the preparation of a highly active arginase, using the liver of various animals. The main steps were: the extraction of the enzyme from fresh minced liver with 5 per cent sodium acetate solution containing 5 milligrams Mn^{++} ions ($MnSO_4$) per cc. and the removal of inert proteins by lead acetate (calculated volumes of 0.024 *M* $Pb(C_2H_3O_2)_2 \cdot 3H_2O$) and then by acetone. The loss in activity in the final step amounted to only about 6 per cent of the enzyme content in the initial extract. The enrichment of arginase activity was more than 20-fold. Van Slyke and Archibald (15) have purified arginase by electrophoresis. Their preparation contained 35 per cent active enzyme protein. Arginase is a very stable enzyme. Its solutions may be kept in the refrigerator unchanged for months.

Activation of Liver Arginase. Mohamed and Greenberg have shown that activation of liver arginase depends upon a measurable reaction between enzyme and ion activator, and not between substrate and ion. Cobaltous and manganous ions are the best activators. The maximum activation was obtained after 3 hours' incubation at 40° C. This type of activation is reversible (removable by dialysis), governed by time, temperature, *p*H, and the type and the concentration of the activating ion. Arginase is stable in the presence of most metal ions; only Ag^{+} and Hg^{++} were found to be destructive. Weil (12) has shown that cysteine-Fe^{++} activates purified as well as crude liver arginase.

Rossi and Ruffo (16) are of the opinion that hog-liver arginase requires, in addition to Mn^{++} ions, a diffusible organic compound for full activity.

Activation of Jack-Bean Arginase. Anderson (17) found that the concentration of Co^{++} ions necessary for complete activation of jack-bean arginase is directly proportional to the amount of enzyme. The optimum *p*H also varies with Co^{++} ion concentration, increasing from 7.5 to 9.0 with rising concentration. The optimum *p*H with Mn^{++} ions is about 8.8 and is independent of the Mn^{++} concentration. Large amounts of Mn^{++} produce some inhibition. The Fe^{++} ion activates at *p*H 8.8, inhibits at *p*H 8.2, and is necessary in relatively high concentrations. Cysteine increases the Fe^{++} activation at *p*H 8.8 and

activates also at *p*H 8.2. The active substance is in the globulin fraction of the jack-bean extract.

Kinetics of Liver Arginase. The optimum *p*H of liver arginase is close to 10. With purified liver arginase and in the presence of manganous and cobaltous ions, hydrolysis of arginine closely follows the first-order reaction equation. The Michaelis-Menten (enzyme substrate dissociation) constant varies with the *p*H, yielding a U-shaped curve, with the minimum value at *p*H 8.0. On the assumption that the active enzyme intermediate is composed of the monovalent cation of arginine and the anionic portion of arginase, Greenberg and Mohamed (18) obtained the following equation for the true enzyme substrate dissociation constant:

$$Ks \text{ (true)} \left(\frac{K_1}{C_H} + 1\right)\left(\frac{C_H}{K_2} + 1\right) = Ks \text{ (experimental)}$$

Here, *Ks* (experimental) represents the enzyme substrate dissociation constant based on experiments at different *p*H values, K_1 is the dissociation constant of arginine (9×10^{-10}), and K_2 is the dissociation constant of the enzyme (approximately 1×10^{-7}). With the aid of this equation, *Ks* (true) was found to be 4.5×10^{-3}.

Bach, Crook, and Williamson (19) studied the participation of arginase in urea synthesis in the liver. They conclude that, in addition to the "ornithine cycle," there must exist another synthesizing mechanism in the mammalian liver. Van Slyke and Archibald (20) published a gasometric and photometric procedure for the determination of arginase activity.

Histidase

This enzyme occurs only in vertebrate liver, cat liver being the best source. Edlbacher and von Bidder (21) gave the following values of histidase activities, in terms of cat-liver histidase: cat, 100; guinea pig, 42; rabbit, 29; pigeon, 42; rat, 35 to 40. The enzyme may be prepared by grinding the fresh tissue with sand, shaking the mixture with water, and centrifuging. Though this solution is quite active, it is difficult to obtain precipitates by the usual procedures. Walker and Schmidt (21) obtained highly active histidase preparations by the following method:

Cat liver is frozen at —17° C. under toluene, thawed at room temperature, passed through a meat grinder, and extracted with 5 parts of distilled water by shaking for several hours. The mixture is filtered on a Büchner funnel with the aid of Supercel. To each 100 cc. of the

extract, 52 grams of phosphate buffer, prepared by dissolving 3.42 moles K_2HPO_4 and 0.38 mole of KH_2PO_4 in water and diluting to 1 kilogram, is added, and the mixture is stored in a refrigerator overnight. The precipitate is collected with the aid of Supercel. The cake is washed several times with a mixture of 100 cc. water and 60 grams phosphate buffer. This precipitate may be dialyzed against 0.05 *M* $KHCO_3$ without great loss of enzyme activity, indicating that histidase does not require a coenzyme. Dialysis against water, however, destroys the enzyme.

This purified histidase is free from arginase, liver esterase, and various dehydrogenases but contains some catalase. Histidase was believed to convert histidine to ammonia, glutamic acid, and formic acid. Walker and Schmidt, however, suggest that the end product is α-formamidino glutaric acid. The substance produced from histidine by the action of histidase could not be isolated.

Asparaginase

This enzyme is present in bacteria, yeast, higher plants (22), the small intestine, and the liver (23). Asparaginase removes the amide nitrogen from *l*-β-asparagine, converting it to *l*-aspartic acid:

$$CONH_2CH_2CHNH_2COOH + H_2O = COOHCH_2CHNH_2COOH + NH_3$$

Busch (24) has shown that the asparaginase content of bacteria can be considerably increased by the addition to the culture medium of alanine, glycine, *p*-aminobenzoic acid, and asparagine and aspartic acid. Samples of asparaginase of various sources appear to be identical, since they all show optimum action at *p*H 8 and sensitivity to acids and organic solvents. This enzyme is absolutely specific, and other enzymes do not attack asparagine.

Aspartase

This amidase may be prepared free of cells from *Bacillus fluorescens liquefaciens* (25). It is also present in resting *B. coli* and other organisms, as well as in some higher plants and in yeast (26). Aspartase deaminates aspartic acid, yielding fumaric acid:

$$COOHCH_2CHNH_2COOH \rightleftarrows COOHCH{:}CHCOOH + NH_3$$

Quastel and Woolf (27) have shown that this reaction is reversible. Aspartase has an optimum *p*H at 7 to 7.5. The enzyme is absolutely specific. Other amino acids are not attacked.

Lichstein and Umbreit (28) have found that bacterial aspartase and the enzymes that deaminate serine and threonine may be resolved and that the lost activity is restored specifically by biotin.

Hippuricase or Histozymase

The synthesis and hydrolysis of benzoylated amino acids, such as hippuric acid (benzoyl glycocoll), into benzoic acid and glycocoll was attributed by Schmiedeberg to a specific enzyme which he named "histozyme." This enzyme is present in muscle, liver, pancreas, and other organs of mammals. Hippuricase splits acid radicals from peptides and amino acids of the type RCONHCHR'COOH. Only derivatives of natural amino acids are hydrolyzed, and racemic mixtures may be separated by the use of the enzyme (29). Hippuricase also splits glycoholic and taurocholic acids (30).

Hippuric acid is not synthesized by tissue extracts containing hippuricase (31). However, synthesis can be performed using slices of rabbit liver and, with lower yield, using liver pulp. The concentration of the liver pulp is of more importance than the integrity of the cells. Dilution inactivates the enzyme and, therefore, lowers the yield in hippuric acid. In the dog, the same organs (liver, kidney) produce hydrolysis and synthesis.

In order to determine the steps by which nicotinic acid is metabolized, Lanfranchi (32) subjected nicotinuric acid to the action of hog intestine hippuricase. In 48 hours, 96 per cent of the compound was cleaved. Under the same conditions, hippuric acid was 99 per cent hydrolyzed.

Urease

The ammoniacal fermentation of urine, the change of urea to ammonium carbonate by *Micrococcus ureae,* has been known a long time. Later, urease was prepared from various bacteria, from molds, and from mushrooms. Urease is found in all leguminous plants (33) and in varying amounts in other plants. Jack bean (*Canavalia ensiformis*) is the best source. Weil (34) found that red blood cells of rat, rabbit, and man possess urease activity. Similarly, rat liver and rat spleen contained some urease, but none was found in kidney, gastric mucosa, pancreas, brain, thymus, or muscle. The function of urease in animals is not known. In plants, however, urease interacts in the nitrogen cycle.

Action and Specificity. In the presence of buffers, urease decomposes urea into ammonium salts and carbonic acid. However, when buffers are not present, ammonium carbamate is formed. The carbamate slowly decomposes into ammonium carbonate (35). Urease is absolutely specific. It acts only on urea. Methyl urea, thiourea, guanidine, and related substances are not attacked, nor do they show any affinity to urease (36).

Inhibition and Inactivation. Schmidt (37) found that heavy metals inactivate urease in the following order: silver, mercury, copper, zinc, cadmium, uranium, gold, lead, cobalt, nickel, cerium, manganese. Crystalline urease is extremely sensitive to traces of heavy metals. One gram-atom of silver inactivates more than 40,000 grams of urease (38). Proteins, amino acids, hydrophylic colloids, hydrogen sulfide, and some other substances have a protective action on this enzyme. Sumner and Poland (39) reported that the sulfhydryl group is a part of the catalytic function of the urease molecule. However, Hellerman (40), by studying the inactivating action of porphyrindin on urease, found that the more readily traceable sulfhydryl radicals can be oxidized without impairing the activity of the enzyme. Sizer and Tytell (41) found crystalline urease to be most active in a medium containing weak reducing agents. It is destroyed by strong reductants and by oxidants. Their findings appear to indicate that urease contains necessary sulfhydryl groupings.

Mapson (42) has investigated the mechanism of inhibition of urease by copper salts in the presence of ascorbic acid and related substances. He concludes that urease requires sulfhydryl groups for its activity. This enzyme does not require a coenzyme (43). Urease that had been inactivated by some heavy metals, such as silver, or by oxidizing agents, such as iodine, may be reactivated by treatment with hydrogen sulfide. Some inactivations, however, are irreversible. Quastel (44) found that all basic dyes inhibit urease action but that the acidic dyes do not.

Kinetics. Urease activity depends upon the type of buffer, the temperature, *p*H, urea concentration, and salt concentration. Thus, Howell and Sumner (45) have shown that crystalline urease is most active in the presence of 1 per cent urea and *M*/8 citrate at *p*H 6.5. In the presence of 2.5 per cent urea and phosphate, the optimum *p*H is at 6.9. The decomposition of urea by urease in the presence of buffer follows the course of a zero-order reaction.

Preparation of Crystalline Urease. By crystallizing urease in 1926, Sumner (46) was the first to obtain an enzyme in crystalline form. The following is a later modification of Sumner's method (47, 48):

One hundred grams of jack-bean meal (Arlco) is stirred 7 minutes, with 500 cc. of 32 per cent acetone at 25° C. The mixture is poured on the filter, and when about 100 cc. of filtrate comes through it is allowed to filter overnight in a refrigerator. Microscopic octahedric urease crystals separate. The urease crystals are centrifuged, preferably in a cold room. The supernatant is decanted. The crystals are dissolved and transferred to a 15-cc. centrifuge tube, using (2 cc. at a time) a total of 6 cc. of water. The mixture is centrifuged at high speed (1 to 2 hours) until the liquid is almost clear. The urease solution is pipetted into a Pyrex test tube. The enzyme may be recrystallized by adding, for every 20 cc. of supernatant, 1 cc. of 0.5 *M* citrate buffer of *p*H 6.0 (95 volumes of 0.5 *M* trisodium citrate and 5 volumes of 0.5 *M* citric acid) and 0.2 volume of pure acetone with stirring. The solution is placed in a refrigerator. Crystallization is almost complete after 30 minutes. Urease is a water-soluble protein. It has a molecular weight of 483,000 (49).

Other Amidases

Allantoinase, an enzyme present in amphibians and fish, hydrolyzes allantoin into simpler compounds. The exact course of this reaction is not yet known (50). Another enzyme that acts on allantoin, converting it to allantoic acid, has been described by Fosse and coworkers (51). This enzyme is present in amphibians, fish, and plants. It had been named *allantoicase.* Two different *glutaminases* have been described by Krebs (52). These enzymes are found in different animal tissues. They convert *l*-β-glutamine to *l*-glutamic acid. The two enzymes have different *p*H optima.

DEAMINASES OR NUCLEIN DEAMINASES

The deaminases add oxygen and remove ammonia from their substrates. Gaseous oxygen or hydrogen acceptors are not necessary for these oxidations.

Guanase and Adenase. Guanase is found in most animal tissues (53). Adenase, however, is present only in cows' milk and in cows' muscle (54). Adenase converts adenine to hypoxanthine and ammonia, whereas guanase changes guanine to xanthine. Schmidt (53) found that the action of guanase increases from 5 to 9.2, having no definite *p*H optimum. Kalckar (10) has published a method for the preparation of guanase which is free of nucleoside phosphorylase.

Adenosine Deaminase. This enzyme is present in heart, liver, spleen, muscle, pancreas, intestinal mucosa, nerve tissue, and erythro-

cytes (55). This enzyme is present in most mammalian tissues (55, 56). Conway and Cooke (55) found that adenosine deaminase and adenylic acid deaminase occur mostly together. However, liver, kidney, smooth muscle, and ventricle muscle of the heart contain only adenosine deaminase. This enzyme changes adenosine (adenine riboside) into hypoxanthosine (hypoxanthine riboside) and ammonia.

Kalckar (10) has improved the method of Schmidt and Thannhauser for the preparation of calf intestinal deaminase. He succeeded in developing a method by which other enzymes were removed and the preparations so obtained showed very high activity. These preparations were most active at *p*H 7 and were almost two-thirds as active at *p*H 9 and at *p*H 6 as at the neutral point. Kalckar described a spectrophotometric method for the quantitative determination of various purines. By this method spectral changes in the ultraviolet region are measured as induced by the addition of specific purine-splitting enzymes.

Zittle (56) found that purified calf intestinal phosphatase contains a very active deaminase which acts at *p*H 5.9 on adenosine and desoxyriboadenosine but not on adenylic acid, ribonucleic acid, or desoxyribonucleic acid. This enzyme had an optimum *p*H at 7.0. It is very sensitive to silver ions, being 50 per cent inhibited by 4.0×10^{-7} mole of silver per liter.

Mitchel and McElroy (57) studied the adenosine deaminase of *Aspergillus oryzae*. This enzyme has an optimum *p*H at 5 to 8. The deamination of adenosine appeared to be a pseudo unimolecular reaction up to 50 per cent conversion.

Guanosine Deaminase. This enzyme is present in rat spleen, kidney, node, brain, and liver (58). However, adenase is absent from all these tissues. The optimum *p*H of guanosine deaminase is at 7.0 (59).

Adenylic Acid Deaminase. This enzyme converts adenylic acid to inosine-5-phosphoric acid and ammonia. The enzyme is most active at *p*H 5.7 to 6.15 (60). It is present in most tissues of the rabbit, and the spleen and kidney of the cat (55, 58). Adenylic acid deaminase deaminates only adenylic acid (adenosine-5-phosphate). The end products are inosine-5-phosphate and ammonia. Kalckar (59) has been able to reverse this reaction. Adenylic acid deaminase is present in most tissues of the rabbit and the spleen and kidney of the cat (55, 58–60). This enzyme has so far not been prepared in true solution but only as finely dispersed suspensions. The addition of salts or alkalis prevents flocculation of the enzyme. Kalckar (59) found that dilute enzyme solutions as used in his optical test do not form precipitates even at an acid *p*H. The enzyme is not very stable even at a

low temperature. This enzyme has a very sharp optimum at *p*H 5.9 (10, 60). The activity at *p*H 7.0 is less than one-fifth of that at *p*H 5.9; thus it is important that studies with this enzyme be carried out with well buffered solutions. Kalckar (10) recommends the use of succinate owing to its high buffering capacity at *p*H 5.9 to 6 and its inertness in the ultraviolet region. He has also published a method for the preparation of adenylic acid deaminase using the skeletal muscle of the rabbit.

Guanylic Acid Deaminase. Schmidt (53) described a specific enzyme in rabbit liver that deaminates guanylic acid to xanthylic acid and ammonia. He found that the enzyme is most active at *p*H 5.3 to 6.5, and that it is considerably inhibited by small amounts of fluoride. However, Levine and Dmochowski (62) could not find this enzyme in pig liver. These investigators stated that guanylic acid is a very labile substance, being decomposed to an extent of 40 per cent in 48 hours under the conditions of the experiment.

Cytidine Deaminase. This deaminase changes cytidine (cytosine riboside) to uridine (uracil riboside) and ammonia. This enzyme is present in rabbit liver and intestinal mucosa (63).

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CHAPTER VI

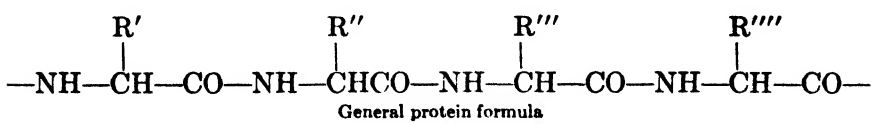
PROTEOLYTIC ENZYMES

All proteolytic enzymes attack peptide linkages. These enzymes are divided into proteinases and peptidases. The proteinases hydrolyze intact proteins to proteoses, peptones, peptides, and some amino acids. The products of proteinase action are further hydrolyzed by peptidases to amino acids, and in the case of leucine peptidase, ammonia is also formed. This classification, however, is only arbitrary, since proteinases also split peptides. For practical use, however, the old classification of proteolytic enzymes should be retained. Bergmann and associates have proposed a more exact classification of this group of enzymes, which uses synthetic compounds as the substrates for the proteolytic enzymes. The new classification will be presented in detail.

It is important to note that in nature proteolytic enzymes exist as mixtures of this and other types of enzymes. Cells (organisms) are often classified according to the types of enzymes they are able to produce.

The Protein Molecule

Chemically, proteins are built of hundreds of amino acid residues bound to one another through peptide linkages to form long chains. These long chains consist of 15 to 25 different amino acids combined in varying proportions:



General protein formula

The chain of the peptide linkages has been called the " backbone " of the protein molecule by Bergmann and associates. From the backbone, the R groups or side groups project. The character of the R groups depends upon the nature of the amino acids making up the backbone. Thus, at the point in the chain where an alanine portion appears, the R group is a methyl group; where a leucine portion appears, it is an isobutyl group; where a glycine portion appears, it is just a hydrogen atom, and so forth.

It seems obvious from this that the nature of the protein molecule is determined by the type, relative number, and sequence of the R groups in its molecule. The nature of the R groups is well known, but not their relative number.

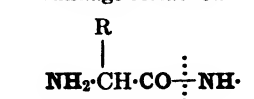
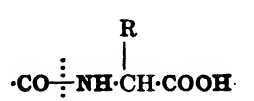
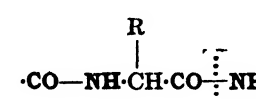
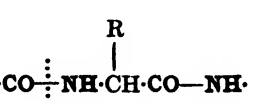
Specificity and Classification

In recent years Bergmann and associates, using a large number of synthetic substrates, have made important contributions concerning the specificity of proteolytic enzymes. On the basis of these researches, and by extending the findings of earlier workers, they have proposed the following classification for the proteolytic enzymes (1–3):

The substrate for a proteolytic enzyme contains two necessary points of attack: one is the CO·NH group or some portion of it; the other is in the "backbone" of the substrate molecule and differs with the type of the enzyme. The nature of the R group and its relation in the backbone to the sensitive peptide linkage offers the basis for the classification of proteases into four major groups, as shown in Table XIV (3).

TABLE XIV

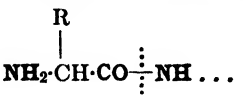
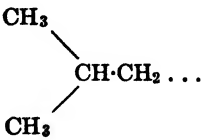
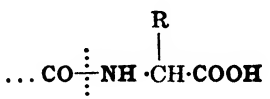
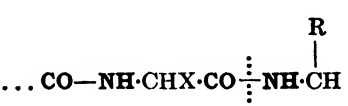
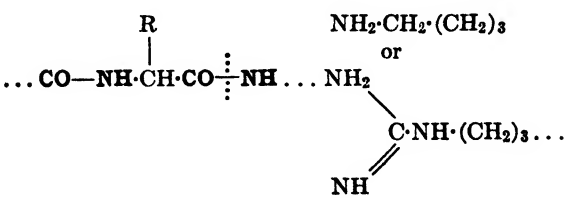
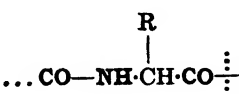
Classification of Proteolytic Enzymes (Bergmann-Fruton)

Group No.	Linkage Attacked	Classification	
I		Aminopeptidases	Exopeptidases
II		Carboxypeptidases	
III		Proteinases	Endopeptidases
IV		Proteinases	

Groups I and II (Table XIV) contain enzymes that can attack substrates only at the end of the peptide chain. The peptidases, such as aminopeptidase, belonging to group I, act on the chain at the peptide linkage adjacent to the amino end of the chain. The pepti-

TABLE XV

SPECIFICITY OF PROTEOLYTIC ENZYMES (BERGMANN-FRUTON)

Enzyme	Requisite Groups in Substrate Backbone	Requisite Groups in Substrate Side Chain
	Peptidases (Exopeptidases)	
Leucine aminopeptidase from intestinal mucosa, beef spleen, beef kidney, and swine kidney		
Chymotrypsin aminopeptidase	Same	$HO{\cdot}C_6H_4{\cdot}CH_2\ldots$ or $C_6H_5{\cdot}CH_2\ldots$
Other aminopeptidases	Same	
Carboxypeptidase from pancreas, beef spleen, beef kidney, and swine kidney		$HO{\cdot}C_6H_4{\cdot}CH_2\ldots$ or $C_6H_5{\cdot}CH_2\ldots$
Other carboxypeptidases	Same	
	Proteinases (Endopeptidases)	
Pepsin		
Pepsinases from beef spleen, beef kidney, and swine kidney		$HO{\cdot}C_6H_4{\cdot}CH_2\ldots$ or $C_6H_5{\cdot}CH_2\ldots$
Trypsin		
Tripsinases from beef spleen, beef kidney, swine kidney, and papain		$NH_2{\cdot}CH_2{\cdot}(CH_2)_3$ or
Chymotrypsin		$HO{\cdot}C_6H_4{\cdot}CH_2\ldots$ or $C_6H_5{\cdot}CH_2\ldots$

dases of group II, such as carboxypeptidase, act on the chain at the peptide linkage adjacent to the carboxyl end of the chain. These enzymes cannot hydrolyze bonds that are centrally placed in the peptide chain, and they are called exopeptidases. The necessary

groups are set in bold-face type, and the sensitive peptide linkage is shown by a dotted line.

All protein-hydrolyzing enzymes whose backbone necessities have been established belong to group III. These enzymes, owing to their action on central peptide linkages, are called endopeptidases. They require a peptide bond in the substrate close to the carbonyl group of the peptide linkage, which is split by the enzyme. No proteolytic enzyme belonging to group IV has yet been identified. The suggestion has been made, however, that an enzyme of the intestinal mucosa which hydrolyzes leucylglycylglycine may belong to this group (4). As stated above, it is not sufficient for a substrate containing indispensable groups in the backbone to be attacked by an enzyme. Each proteolytic enzyme tested also requires the presence in the substrate of a certain type of side group (R) in an exactly defined position. In the second column of Table XV (3), the required location of side group R is shown for each of the enzymes, and the nature of the R groups is listed in the third column.

The enzymes (listed) in order to react with a given substrate must contain, for instance, one of the following side groups: isobutyl, as in leucine; benzyl or *p*-hydroxybenzyl, as in phenylalanine or tyrosine; aminobutyl or guanidopropyl, as in lysine or arginine. In Table II, enzymes of varied origin are placed in the same group; this has been done because they split the same synthetic substrates. Such groups of enzymes (pepsinases, trypsinases, leucine aminopeptidases), with identical backbone and side-chain requirements, are called homospecific enzymes.

Criticism Concerning Bergmann's Classification of Proteolytic Enzymes

Harington and Rivers (5) have published results which seem to be contradictory to Bergmann's view concerning the requirement of more than one free carboxyl group in the vicinity of the labile peptide bond, and the inhibitory effect of free amino groups in substrates for pepsin. They found that tyrosylcysteine, tyrosylcystine, cysteinyltyrosine, and cystinyltyrosine were significantly split by crystalline pepsin. The hydrolysis of these free peptides occurred at *p*H 1.8 and 4.0, ranging from 0 to 31 per cent. These peptides, however, were hydrolyzed somewhat more slowly than the corresponding N-carbobenzoxy derivatives. Harington and Rivers found that peptides with free thiol radicals were more readily attacked by pepsin than other peptides. This fact should be compared with the increased

readiness to peptolysis, which results in some proteins upon denaturation, and the appearance of sulfhydryl groups. Bergmann and Fruton (1), however, have stated that pepsin substrates must contain at least two free carboxyl groups for optimal splitting, though they have demonstrated that this requirement is not absolute. Thus, carbobenzoxy-*l*-glutamyl-*l*-tyrosineamide is hydrolyzed 15 per cent in 48 hours, as compared to a 53 per cent hydrolysis of carbobenzoxy-*l*-glutamyl-*l*-tyrosine in 24 hours. A specificity that is less than 100 per cent is not rare in enzymology.

Roche and Mourgue (6) reported that papain liberates most of the total leucine from casein with a small amount of valine and short-chain polypeptides. Pepsin frees a small quantity of leucine and large amounts of valine and short-chain substances. These investigators believe that leucine is located at the end of the protein chains, whereas valine is not, and that this property of leucine explains the facility of transpeptidation by labeled *l*-leucine after its ingestion. They conclude that pepsin has chiefly endopeptidase activity, while papain has both endo- and exo-peptidase action. Bergmann classified papain as an endopeptidase. It is quite difficult, however, to distinguish between qualitative and quantitative differences in this respect when proteins are used as the substrates. Bergmann and coworkers employed synthetic peptides.

Studies with purified proteinases have shown that they do not liberate terminal peptide bonds of the polypeptide chain in proteins to any considerable degree. Only a small quantity of amino acids is formed (7, 8). The peptidases, on the other hand, are most active in hydrolyzing peptide linkages (2, 9). It should be noted that Bergmann (2) and more recently Synge (10) have called attention to complications that may occur in the application of this classification, owing to simultaneous synthesis during partial hydrolysis of proteins by the various proteolytic enzymes.

PEPSIN

The protein-digesting power of gastric juice was first observed in 1836 by Schwann, who called the enzyme responsible for this effect pepsin. This enzyme is secreted in great amounts by the chief cells of the gastric mucosa. It had been shown that the gastric mucosa also contains small quantities of other proteolytic enzymes, such as gelatinase and cathepsin.

The Action of Pepsin. Pepsin hydrolyzes native proteins, but not keratin, silk fibroin, ovomucoid, mucins, conchiolin, and spongin.

Egg albumin is attacked only slowly by pepsin. The end products of peptolysis are mostly proteoses and peptones and small quantities of amino acids. The optimum *p*H of peptic action is between 1.8 and 2.2, depending on the substrate (11). Fruton and Bergmann (12) reported the hydrolysis of a synthetic peptide, carbobenzoxy-*l*-glutamyl-*l*-tyrosine, to carbobenzoxyglutamic acid and tyrosine, by crystalline swine pepsin. The *p*H optimum of this action is 4.0; at *p*H 1.8 the reaction is hardly noticeable. It has been stated that certain diketopiperazines are split by pepsin. However, diketopiperazines with acidic or basic side chains do not function as proteinase substrates.

Pepsinogen

Pepsinogen, the precursor of pepsin or pepsinase, as formed by the gastric cells, is completely inactive. If the ground gastric mucosa of a pig is extracted with a suspension of calcium carbonate in water and some of the filtrate is added to milk, no clot will form. Adjustment of a sample of the extract to about *p*H 2.0 converts all the pepsinogen instantaneously to pepsin, which digests protein and also has a high milk-clotting power. A similarly prepared extract of the calf's fourth stomach behaves the same way, with the exception that its protein-digesting power is very low and is probably due to the presence of small amounts of pepsin.

Pepsinogen and pepsin appear to be different proteins. Tauber and Kleiner (13) found that, when propepsin is exposed to 50 per cent ethyl alcohol for 24 hours, it is completely destroyed. It cannot be activated with hydrochloric acid. Pepsin, however, is quite resistant under similar conditions.

Preparation of Crystalline Pepsinogen. Herriott and Northrop (14) have isolated pepsinogen in crystalline form from alkaline, 0.45 saturated ammonium sulfate extracts of the swine fundus mucosae. The crystals were of protein nature. Pepsin prepared by the acidification of this pepsinogen can be crystallized, and its crystalline form is identical with pepsin crystallized from commercial pepsin.

Crystalline Pepsin

Among the early papers concerning the purification of pepsin, the article by Pekelharing (15) published in 1902 is the most important one. He purified gastric juice and acid extracts of the gastric mucosa of the pig by dialysis and found that the pepsin separated in refractive

globules. This preparation was very active. Northrop (16) repeated the work of Pekelharing and found that the precipitate which formed in the dialyzing sac appeared in more or less granular form and filtered very readily, as though it were on the verge of crystallization. Northrop found that the pepsin precipitate dissolved on warming to 45° C. and crystallized on slow cooling of the filtrate. He has developed a method for the large-scale crystallization of pepsin.

Preparation of Crystalline Pepsin (16)

Five hundred grams of Parke, Davis and Co. pepsin U.S.P. 1: 10,000 is dissolved in 500 cc. water, and 500 cc. of *N* sulfuric acid is added. To this, 1000 cc. saturated magnesium sulfate is added with stirring. The solution is filtered through fluted paper and then filtered with suction. The filtrate is discarded. The remaining precipitate is stirred with water to a thick paste, and *M*/2 sodium hydroxide is added to form a complete solution. Local excess of sodium hydroxide must be avoided, and the *p*H should never be more than 5.0. *M*/2 sulfuric acid is next added with stirring until a heavy precipitate forms (*p*H about 3). The solution is allowed to stand from 3 to 6 hours at 8, then filtered with suction. The filtrate is discarded. The precipitate is stirred to a thick paste at 45° C., then *M*/2 sodium hydroxide is added carefully until the precipitate dissolves. The solution is filtered, if necessary, and the precipitate is discarded. The beaker is placed in a vessel containing 4 liters of water at 45° C. and allowed to cool slowly. This should require from 3 to 4 hours. A heavy crystalline precipitate forms at about 30° to 35°. The solution is kept at 20° for 24 hours to complete crystallization. It is filtered with suction and washed with small amounts of cold water and then with magnesium sulfate at 5°.

Recrystallization. The crystalline paste is filtered with suction and washed three times with cold *M*/500 hydrochloric acid. The filter cake is stirred to a paste with half its weight of water at 45°, and *M*/2 sodium hydroxide is added with constant stirring until the solution is faintly turbid. A few crystals are added, and the solution is allowed to cool slowly, as before. In 24 hours, a heavy crop of crystals separates. The suspension is then warmed to 45°, and sulfuric acid is added until *p*H 3.0. The solution is cooled slowly and after 24 hours is filtered. The crystals may be washed with *M*/500 hydrochloric acid until free of sulfate ions.

Chemical Nature of Crystalline Pepsin. Crystalline pepsin appears to be an albuminlike substance. Its molecular weight, as determined

by the ultracentrifuge, is 35,500 (17). Northrop reported 2.7 for the isoelectric point of pepsin. Tiselius and coworkers (18), however, stated that the pepsin is negatively charged between *p*H 4.4 and 1.0. These investigators found that the activity of crystalline pepsin could be increased up to 69 per cent by electrophoresis. (See also reference 19.) Herriott and coworkers (20) prepared from pepsinogen a pepsin concentrate which had twice the activity of the most active crystalline pepsin previously isolated. This "highly active" pepsin was much less stable than ordinary crystalline pepsin. About 50 per cent of its activity was destroyed in 15 hours at 25° C., as compared to only 5 to 10 per cent of the ordinary crystalline pepsin. Thus, it appears that it is quite difficult to obtain crystalline pepsin free of impurities and of highest activity. Owing to this fact, the following methods for the preparation of amorphous pepsins will also be given.

Preparation of Pepsin of Higher Activity than Crystalline Pepsin

McMeekin (21) and Freeland (22) prepared pepsin 2 to 4 times as active as crystalline pepsin by adsorption on specially prepared egg white at *p*H 3.0 and elution at *p*H 6.1 with disodium phosphate. The following simple adsorption and elution method, based on McMeekin's procedure, was described by Borgstrom and Koch (23).

Preparation of Highly Active Amorphous Pepsin

A solution of 10 grams of Cudahy 1 : 10,000 U.S.P. pepsin (or varying amounts of crystalline pepsin) in 100 cc. of 0.01 *N* hydrochloric acid is cooled in the icebox for 30 minutes, then adsorbed on 4 grams of egg white, treated according to McMeekin, by shaking in a 100-cc. Erlenmeyer flask for 2 hours at 7° C. in a rotary shaker. The pepsin-egg white is filtered off and the pepsin eluted by the addition of 100 cc. of 0.4 per cent disodium phosphate, shaken in the cold as before, and filtered. The eluate is adjusted from *p*H 6.1 to *p*H 3.0. Ammonium sulfate is added slowly, with stirring, to half saturation. The precipitate is allowed to settle at 8° C. The following morning, 5 grams of Hyflo is added to the precipitate, with stirring, and the mixture is filtered. The filter cake is transferred to a 250-cc. centrifuge cup and stirred for 15 minutes, after the addition of 100 cc. of 0.3 per cent hydrochloric acid. This is centrifuged and the supernatant poured off. Ninety cubic centimeters thereof is placed in a ¼-inch

cellophane dialysis bag and dialyzed against 0.3 per cent hydrochloric acid for an hour in the icebox and then against several changes of distilled water until the dialysate shows a negative Nessler's test. All solutions should be kept in an icebox until ready for use. The final pepsin samples are frozen and evaporated in the frozen state.

"Protein-Free" Pepsin

Kraut and Eusebio (24) have re-examined the nature of Brücke's "protein-free" pepsin and compared it with Northrop's crystalline pepsin. Both enzymes were prepared from commercial pepsin. Only 1 per cent of the original peptic activity could be recovered by Brücke's method, and this enzyme was considerably different from crystalline pepsin. It had a lower nitrogen content and gave fainter protein tests, and its chemical composition is unknown. It hydrolyzed casein which had been first digested by crystalline pepsin. It is well known that commercial pepsins are prepared by the autolysis of gastric mucosa and, because of this, contain admixtures of small amounts of cell enzymes, such as cathepsin and gelatinase, and probably other proteolytic enzymes. Crystalline pepsin, as prepared by Northrop's procedure from commercial pepsin, contains almost all the proteolytic activity present in the commercial product.

Preparation of Alcohol-Soluble Pepsin

Albers and coworkers (25) have obtained from pig gastric mucosa, by extensive autolysis, a peptonelike pepsin that was soluble in 65 per cent alcohol and could not be precipitated by basic lead acetate or by sulfosalicylic acid. This pepsin diffused through collodion membranes and contained more tryptophane than crystalline pepsin. The optimum *p*H with casein as the substrate was at 1.50. It was inactive in alkaline medium.

Preparation. The fundus portion of 20 pig stomachs is finely ground, suspended in 8 liters of 0.5 per cent hydrochloric acid, and allowed to autolyze at 37° C. for 30 hours. Then the mixture is centrifuged, filtered, and concentrated in vacuum at 25 to 30° C. to one-third of its original volume. The filtrate is placed in a refrigerator and kept there for several months. A 1200-cc. portion of the filtrate is adjusted to *p*H 3.2 with strong ammonium hydroxide. While the solution is extensively stirred, 800 cc. of saturated magnesium sulfate solution is slowly added. The mixture is placed in a refrigerator at 0° C. for 24 hours. The precipitate (30 grams net weight) is collected

suspended in 200 cc. of 70 per cent alcohol, and extracted for 3 hours at room temperature by shaking frequently. The insoluble residue (fraction II) is washed with 96 per cent alcohol and with ether, and is dried in vacuum. The filtrate is placed in a refrigerator at —18° C. for 24 hours. The precipitate which forms is collected and washed with ether (fraction III).

Albers and associates claim that, owing to autolysis, the pepsin changes into low-molecular-weight pepsins. Thus, fraction III is soluble in 65 per cent alcohol at room temperature, whereas fraction II is insoluble. The vacuum-dried preparations are very stable. Both fractions II and III are water soluble and as active as Northrop's crystalline pepsin. More recently, Northrop (26) repeated the work of Albers and collaborators. He found that the pepsin soluble in 65 per cent alcohol is still precipitated by hot trichloroacetic acid, and sulfosalicylic acid does not precipitate crystalline pepsin; the two pepsins are identical in chemical nature and specific activity.

Preparation of Commercial Pepsin

Most commercial pepsin products are prepared from the pig's gastric mucosa. Often, however, beef-stomach mucosae are used. Pig stomachs weigh 600 grams and yield 200 grams of mucosa. Beef stomachs weigh 2.5 kilograms and yield 1.2 kilograms of mucosa. The fundus or center portion of the stomach contains much more pepsin than either the cardiac end or the pylorus. The stomachs are cut through the length and are washed and brushed; the mucosa is separated and minced. The muscular wall is worked up to peptone. It is best to extract the mucosa with 3 volumes of 0.5 per cent hydrochloric acid or 1 per cent phosphoric acid (or a suitable concentration of citric acid). The mixture is allowed to autolyze for 24 to 48 hours at 40° C., with continuous stirring. The fatty substances may be removed by extraction with ether or other fat solvents. The clear filtrate is dried in vacuum at 45° C. The yield is 12 per cent of the weight of mucosa. This crude pepsin may be further purified by redissolving it right after precipitation and then precipitating it with alcohol or acetone. This procedure yields 200 to 300 grams of pepsin per 20 kilograms of mucosa.

A series of purification methods and an extensive bibliography may be found in reference 27.

Uses of Pepsin. Pepsin is used medicinally for gastric disturbances, in the manufacture of peptones, and in the digestion of the gelatin in the recovery of silver from photographic films in the moving-picture industry.

RENNIN OR RENNET

Rennin is the milk-clotting enzyme secreted by the fourth stomach (*abomasum*) of young calves and lambs. It appears that the mucosa of these animals also contains a small amount of pepsinlike proteinase. The property of milk-clotting apparently is displayed by most proteolytic enzymes of the animal and plant world. Although rennin is the most extensively used enzyme in the manufacture of cheese (see chapter entitled "Enzymes in Dairy Products"), in some countries certain plants are employed by the cheese industry. To what extent pure peptidases are able to coagulate milk has not been investigated.

The Chemistry of Milk-Clotting. When rennin acts on casein, it changes it to paracasein, and, in the presence of a necessary quantity of soluble calcium ions (as in milk), the insoluble gel calcium-paracaseinate precipitates. Paracasein, itself, is soluble. Lundsteen (28), working with calcium-free casein, found the optimum for rennet action at *p*H 5.35, for pepsin at 5.25, and for chymotrypsin at 7.00. Tauber and Kleiner (29) have shown that crude trypsin solutions clot milk only within a certain range. Concentrated crude trypsin solutions, like those used in protein-digestion experiments, change the casein molecule so rapidly beyond the paracasein stage that the milk will not clot, even after the subsequent addition of a very active rennin solution, unless the *p*H is unfavorable to proteolysis. Similar results were obtained by Clifford (30). Crystalline trypsin, however, does not clot milk.

Rennin and Pepsin are Different Enzymes

A comparison of the ratio of proteolytic power and milk-clotting activity of the young mammal's gastric juice as compared to that of the adult's has been the object of several investigations. Holter and Andersen (31) verified and extended the earlier findings of Hammarsten, i.e., that the proteases of the gastric juice of various animals differ, and that the difference is most pronounced between the calf's protease and that of the other mammals.

The peptic activity of the pepsinases parallels the milk-clotting power in the adult mammal as well as in the young. An exception is the calf's stomach. Here separation of the two activities is possible. The ratio of pepsin-rennin activity as expressed in units, however, differs in various mammals' gastric secretions. In calves, the quotient was 0.13–0.26, in cows 1.6; in children 2.7, and in adults 2.5; in young dogs 11.5, and in grown dogs 12.5; in grown pigs 0.50. These

results mean that the gastric enzymes do not vary in the young and adult human or dog as they do during various ages in the calf (31).

The present author (32) has shown that the gastric protease differs with each species. From the gastric mucosa of the pig, rabbit, and calf's fourth stomach may be prepared selective chymoinhibitors which point to the " individuality " of the rabbit's and the pig's pepsinase and of rennin, and also show that there is no rennin in the pig's and adult rabbit's gastric mucosa. The early conception of Hedin (33) that gastric proteases are kind-specific is now corroborated by the researches of Tauber, and of Holter and Andersen. Rennin is the only typical milk-clotting enzyme, and it exists only in the fourth stomach of the calf, associated with a small amount of pepsin, from which it is separable.

Estimation of Rennet Activity. A milk of *p*H 5.0 is most practical. Since the *p*H of cows' milk varies, the addition of an equal volume of *M* acetate buffer of *p*H 5 (containing 42 grams of sodium hydroxide and 115 cc. of 80 per cent acetic acid) furnishes an excellent substrate for the measurement of rennet activity. In each of a series of test tubes, 10 cc. of this milk is placed and is allowed to adjust to 20° C. in a water bath with a shaking device. Varying amounts of the enzyme solution to be tested, having a temperature of 20°, are added. The test tubes are then corked, placed in the water bath, and shaken. The amount of enzyme which clots 10 cc. of the buffered milk in 10 minutes at 20° is determined. A greater delay of the clotting time will yield results not proportional to amount of the enzyme. The use of a strongly buffered milk was first suggested by Ege and Menck-Tygesen.

Preparation of Prorennin

Prorennin, as contained in the fourth stomach of the calf, is inactive. In this respect it resembles propepsin. An extract of prorennin may be readily prepared according to the method of Kleiner and Tauber (34): To 75 grams of mashed and minced mucosa of the fourth stomach of the calf, 150 cc. of a 2 per cent suspension of calcium carbonate in water is added; the mixture is stirred for 8 minutes and filtered. This filtrate contains a trace of active rennin and pepsin which can be destroyed by adjusting the *p*H of the solution to 9.0 at 22° C. and by keeping it at this *p*H for 40 minutes. The prorennin may be precipitated with solid magnesium sulfate and dried in vacuum, or it may be converted into active rennin by adjusting the *p*H to about 4.5.

Preparation of Purified Amorphous Rennin according to Tauber and Kleiner (35)

Twelve fourth stomachs of calves are washed in cold water. The mucosa is dissected from the muscular wall and from fat. The pyloric end is discarded. The mucosa is minced as finely as possible. About 1 kilogram of minced mucosa is obtained from twelve stomachs. One liter of 0.04 *N* hydrochloric acid is added, and the mixture is stirred for 8 minutes. The mixture is squeezed through toweling and is filtered through filter paper. The clear filtrate should have a *p*H of about 5.2. It is dialyzed against distilled water, which is frequently changed until the *p*H is 5.4. This requires about 4 hours. Then the solution is transferred to a large beaker and stirred, and 95 per cent ethyl alcohol is added to make the extract 50 per cent alcoholic. After a few minutes, two-thirds of the solution may be decanted, and the remainder is centrifuged. The precipitate is extracted with 150 cc. of distilled water and centrifuged. The insoluble material, consisting mainly of mucin, is discarded. To the opalescent solution, alcohol is added to make it 50 per cent alcoholic. The precipitate, when dissolved in 0.04 *N* hydrochloric acid, shows very high rennin activity, and it increases in potency when the above procedure is repeated. The precipitated rennin may be dried at room temperature *in vacuo* over sulfuric acid, although drying inactivates about half of the enzyme. The yield is about 1 gram of dry substance per kilogram of mucosa.

Saturated salt solutions, such as sodium chloride, magnesium sulfate, and ammonium sulfate, precipitated this rennin. It contained neither phosphorus nor chlorine, but 14.4 per cent nitrogen and 1.19 per cent sulfur. Because of these and the other properties mentioned, Tauber and Kleiner suggested that their rennin is probably a thioproteose.

Crystallization of Rennin according to Hankinson

Hankinson (36) prepared crystalline rennin from commercial rennin as follows: The *p*H of the commercial rennet extract is adjusted to approximately 5.0 with concentrated hydrochloric acid. Then the extract is saturated with sodium chloride. The salted-out rennin is centrifuged off. These steps are based on the patent of Keil and Stout (37). The precipitate is dispersed in one-half the original volume of water and dissolved by adjustment to *p*H 5.7–6.0. This procedure is repeated four times, the precipitate being dissolved each time in one-half the preceding volume of water at *p*H 5.7 to 5.0. The fourth precipitate is dispersed in water and dialyzed 24 hours against running distilled water until free from sodium chloride. The dialyzed suspen-

sion is diluted to 0.05 per cent solids concentration, adjusted to *p*H 5.7 to 6.0, and filtered. Toluol is added to prevent bacterial growth.

The *p*H is adjusted with *N*/10 hydrochloric acid until definite turbidity is shown. After 10 minutes' standing, more acid is added to decrease the *p*H 0.1 unit. More acid may be added at 10-minute intervals until a heavy white turbidity appears. At this stage of the method, many white needle-shaped crystals are to be obtained. On standing for several hours, the crystalline suspension may be collected by centrifuging. According to Hankinson, "Recrystallization may be effected by repeating the crystallization procedure, but this has been found to be unnecessary, because of losses of active material with no further increase in activity per unit weight." The crystals may be dissolved in a small volume of 20 per cent sodium chloride at *p*H 5.7 to 6.0 and stored in the refrigerator.

Hankinson states: "The crystals were discovered to be dissolving slowly when placed on a glass slide under the microscope, some fields of crystals completely disappearing in 5 minutes." No explanation could be given for the phenomenon. This, however, is a minor matter. Hankinson described a very simple procedure for purifying and crystallizing rennin. It should be very suitable for modern specificity studies.

This crystalline rennin showed an activity 18 to 21 times the commercial rennet extract on a total-nitrogen basis. The preparation was isoelectric at *p*H 4.45 to 4.65. It contained 1.46 per cent sulfur. That purified rennin contains large amounts of sulfur had been shown in 1932 by Tauber and Kleiner (35), whose vacuum-dried preparation contained 1.19 per cent sulfur but was free of phosphorus and chlorine.

Two procedures for the crystallization of rennin, which are quite different from that of Hankinson's have been published by Berridge (38). This investigator, too, makes use of commercial products as the starting materials.

Crystallization of Rennin according to Berridge (38)

In this method, a crude commercial rennin solution is obtained by extracting dried salted calf abomasa with 10 per cent sodium chloride, adsorption on alumina, and precipitation with potash alum and sodium hydroxide at *p*H 6. From the resulting precipitate, the rennin is eluted with 0.2 *M* sodium phosphate of *p*H 6.6. The eluate is precipitated by saturation with sodium chloride; the precipitate is redissolved in water and again salted-out. Alkali is added until the precipitate dissolves (at *p*H 7.0), followed by the addition of acid and warming to

produce faint turbidity. The slightly active precipitate is removed. This procedure is repeated. Then the solution is purified by salting out, a rotating semipermeable membrane being used. "A highly active precipitate consisting almost entirely of spheroids" is obtained.

In a second procedure, Berridge employs a commercial rennet solution for the crystallization of rennin. Sodium chloride, ammonium sulfate, and magnesium sulfate are used at various stages of the procedure as the precipitants. According to the author, crystallization is difficult and one filtration requires 4 days. The method is quite lengthy and will not be given here in detail.

Berridge states that the pepsin which was present in the commercial rennet solution was completely removed by his method. He found that, with hemoglobin as the substrate, rennin has an optimum *p*H at 3.7 and pepsin at 1.8, and he believes that both enzymes are proteases. The rennin crystals have a very low solubility in water and can be dried without decomposition.

"Low-Nitrogen Rennin" of Lüers and Bader (39)

In view of the recent findings concerning the chemical nature and specificity of rennin just reviewed, the early work of Lüers and Bader may be noted. These investigators attempted the purification of rennin by adsorption on aluminum hydroxide. They described a rennin preparation that gave a negative ninhydrin and Millon test. The salt-free dry substance contained only 0.678 per cent nitrogen. According to these workers, their preparation was extremely active. The rennet activity increased 39 times and the peptic activity 21 times when compared with the liquid commercial starting product. Lüers and Bader conclude that, owing to experimental difficulties and complications in the procedure of purification, the difference in activity is too small to allow a decision of two distinct enzymes, rennin and pepsin.

Note on the Activity of Various Rennins

The milk substrates, in regard to *p*H, added salts, and milk concentration, differ considerably in each rennin investigation. In some cases, fresh cows' milk was used; in others, some sort of dried milk was employed. Thus, it is exceedingly difficult to draw conclusions concerning the potency of the various rennins described. It should be highly desirable to compare the several pure rennins by the same assay procedure. Numerous properties of the two enzymes, rennin and pepsin, prove that they are distinct enzymes. Rennin is the milk-coag-

ulating enzyme of calves and lambs. It has very little proteolytic power. Pepsin is the gastric proteinase of mammals, having both high milk-clotting and high proteolytic power. Both enzymes are proteins.

Preparation of Commercial Rennin

Keil (40) employs the following procedure for the preparation of commercial rennin:

Eight hundred and ninety parts by weight of fresh calf stomachs is hashed in the usual manner; 19.6 parts by volume of 24 per cent hydrochloric acid is added. This results in a *p*H of about 2 to 3 and effects a liberation and activation of the rennin. Heating the mixture to 42–46° C. hastens activation. To this mixture is added 25 parts by weight of trisodium phosphate. The *p*H is now changed to about 5.5. Sodium citrate may also be used for *p*H adjustment. At this *p*H, the muscle tissue becomes colloidally dispersable in water. Then the mixture is evaporated in vacuum or in a current of air while spread out on pans. The drying temperature should not exceed that at which rennin is destroyed. If it is desired to remove the fat, the dried product is extracted with a suitable fat solvent, such as gasoline or hexane, and the solid residue is again dried in a current of air. The dry residue is ground to a fine powder.

For a very interesting and extensive discussion of the chemistry of rennin, the book by Associates of L. A. Rogers (41) should be consulted.

TRYPSINS

Extracts of fresh pancreas or freshly secreted pancreatic juice have no proteolytic activity. The preparations become active when mixed with the enterokinase of the small intestine or when the pancreas is kept in a medium of slight acidity. Several crystalline trypsins are now known.

Specificity of Trypsin. Trypsin splits peptide linkages, and basic groups are necessary for its action. Whereas pepsin acts readily on native proteins, trypsin decomposes only slowly such proteins as ovalbumin, serum globulin, hemoglobin, and collagen. However, after some type of denaturation, such as cooking, tryptic action proceeds readily. The end products are polypeptides, amino acid, and some ammonia. Polypeptides may form, owing to the synthetic action of trypsin and other enzymes that may be present in crude or impure trypsin preparations.

Enterokinase; Activation of Trypsin

In the pancreatic juice, trypsinogen is accompanied by prokinase, which does not become active within the gland. It is activated in the cells of the intestinal mucosa. The intestinal juice does not contain any enterokinase, and its appearance here depends upon the pancreatic juice. After pancreatectomy, no appreciable amount of enterokinase is found in the intestinal mucosa (42).

Waldschmidt-Leitz (43) succeeded in concentrating enterokinase from the intestinal mucosa 100 times. He found that the kinase is destroyed at 50°. Pace (44) has described a method for the isolation of the precursor of enterokinase. This precursor, however, could not be separated from dipeptidase. The prokinase becomes active on standing. Pace believes that dipeptidase activates the prokinase. Bates and Koch (45) showed that an aqueous extract of fresh hog pancreas becomes completely active if kept at room temperature for 24 hours at *p*H 4 to 5. These authors have obtained trypsin-free trypsinogen by keeping the finely ground hog pancreas suspended in water at *p*H 1.8 for 3 hours at 37° and adjusting the filtrate with sodium hydroxide to *p*H 6.0. A slight precipitate which formed was discarded, as was the one which formed on 45 per cent acetone concentration. The precipitate on 75 per cent acetone concentration contained the final trypsinogen. Bates and Koch also developed a method for preparing enterokinase and demonstrated that enterokinase acts as an enzyme in the activation of trypsinogen.

According to Willstätter and Rohdewald (46), dried pancreas, extracted several times with water-free glycerol, leaves about 30 per cent trypsin bound to the tissue. They call this " desmotrypsin," and the small amount of peptidase accompanying it " desmopeptidase." Desmotrypsin is inactive and may be activated by enterokinase. The kinase is also present in the form of an inactive precursor. If the pancreas is extracted slowly with aqueous glycerol, a spontaneous activation takes place which is complete in several weeks. Desmotrypsin may be fractionated into an α fraction, which is soluble in the presence of electrolytes, and a β fraction, soluble only in diluted sodium carbonate or hydrochloric acid. The soluble, or " lyotrypsin," carries much protein ballast; likewise, the desmotrypsin. The latter, however, if subjected to " autotryptic " activation, yields a purer lyotrypsin which gives only slight protein tests. These tryptases are not claimed to be pure.

Specificity of Crystalline Trypsin. Hofmann and Bergmann (47) observed that crystalline trypsin is a very powerful catalyst for the

hydrolysis of hippuryllysineamide. The split products are ammonia and hippuryllysine. Crude trypsin preparations often contain carboxypeptidase, which then hydrolyze hippuryllysine to hippuric acid and lysine. This and other experiments of Bergmann and associates have shown that basic groups are necessary for the action of trypsin.

Crystalline Trypsin

Northrop and Kunitz (48) described the preparation of a crystalline protein from frozen beef pancreas which had been allowed to thaw overnight (spontaneous activation). It had a high tryptic activity, which remained constant, as did the optical activity, under various conditions. The loss in activity corresponded to the decrease in native protein when the protein was denatured by heat, digested by pepsin, or hydrolyzed in diluted alkali. This enzyme, which Northrop and Kunitz named " trypsin," does digest casein, gelatin, edestin, peptone, and denatured hemoglobin but is inactive to native hemoglobin. Peptides which were readily hydrolyzed by the original extract were not changed by the crystalline enzyme. Apparently the peptidases are separated or rendered inactive by the procedure. This trypsin does not clot milk. Experiments of Tauber and Kleiner have shown that crude trypsin can clot milk only under certain limited conditions (29). Enterokinase does not increase the activity of crystalline trypsin. It has an isoelectric point between *p*H 7 and 8, and optimum *p*H of casein digestion from 8 to 9. The stability is optimum at 1.8; that of crude trypsin is *p*H 6.5 (48).

Since crystalline trypsin follows a different hydrolysis course from crude material, Northrop and Kunitz advise against the method used by Willstätter and his associates for the determination of tryptic activity. There are enzymes in crude preparations which carry the hydrolysis further.

The preparation of crystalline trypsin by the direct method (48) is extremely difficult. It is much easier to prepare crystalline trypsinogen first. From this, crystalline trypsin may be obtained more readily (see below) than by the direct method.

The Effect of the Substrate Concentration on Tryptic Hydrolysis (49)

The digestion of gelatin and casein (Fig. 12) with crude and crystalline trypsin has been followed with varying amounts of the substrates

at 35° C. For about 35 per cent of the reaction, the amount of digestion with crude trypsin is the same for 2.5 and 5 per cent protein concentration. The amount of digestion, instead of being proportional to the substrate concentration, becomes independent of it. This, of

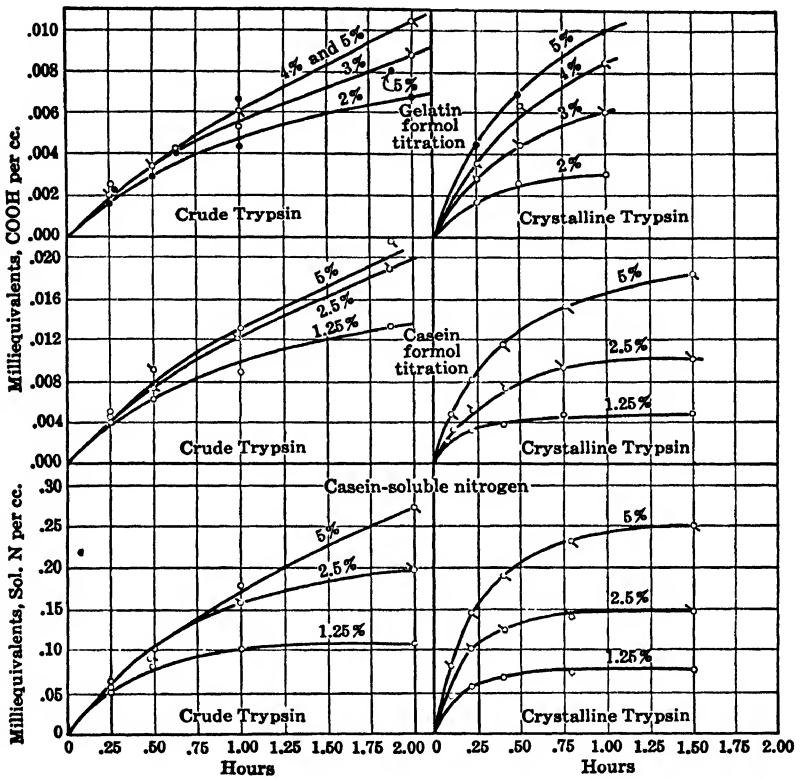


FIG. 12. Digestion of various concentrations of gelatin and casein with crude and crystalline trypsin.

course, occurs often with enzymes, and may be due to the formation of an intermediate compound. With crystalline trypsin, however, this anomaly is much less marked and the result is about that expected from the substrate concentration.

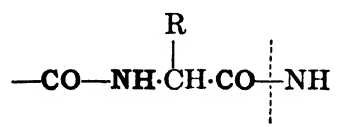
CRYSTALLINE CHYMOTRYPSINOGEN AND CRYSTALLINE CHYMOTRYPSIN

Specificity of Chymotrypsin

Fruton and Bergmann (50) have shown that crystalline chymotrypsin hydrolyzes simple substrates at peptide linkages that concern

the carbonyl group of an aromatic amino acid. Thus, benzoyl-*l*-tyrosylglycineamide is split to benzoyl-*l*-tyrosine and glycineamide. Because the point of attack is at an interior peptide linkage, they classified it as an endopeptidase or proteinase. This group of enzymes split only substrates that contain at least two peptide bonds in close proximity to each other.

In subsequent work, Fruton and Bergmann (51) have found that this enzyme splits glycyl-*l*-tyrosinamide and glycyl-*l*-phenylalanineamide at the peptide linkages involving the carbonyl group of the aromatic amino acid residue. The site of splitting of these two substrates indicates that chymotrypsin requires the following arrangements of groups in the " backbone " of its substrate:



where R is the side chain of tyrosine or phenylalanine. Thus, chymotrypsin is a carbonylproteinase. Chymotrypsin also slowly splits *l*-tyrosinamide and *l*-phenylalaninamide. This is a typical aminopeptidase action. Therefore, chymotrypsin may function as an endopeptidase, a carbonyl proteinase, and an aminopeptidase.

Kunitz and Northrop (52) attempted to isolate the inactive precursor of crystalline trypsin from fresh inactive (cattle) pancreatic extracts. They obtained a crystalline inactive protein and named it " chymotrypsinogen." Enterokinase does not activate it, but crude or crystalline trypsin changes it into an active enzyme which they called " chymotrypsin." This enzyme was also crystallized. It differs from chymotrypsinogen in the form of its crystals, in optical activity, and in number of amino groups. It is less stable and more soluble. The molecular weight is the same as that of chymotrypsinogen. This new enzyme does not clot blood like crystalline trypsin and has a weaker effect on protamins. Like crystalline trypsin, it attacks proteins in weak alkaline solution. It clots milk well, which makes it differ from trypsin.

It has been demonstrated by Waldschmidt-Leitz and Akabori (53) that pancreatic proteinase (the trypsin of Waldschmidt-Leitz and associates) is probably a mixture of trypsin and chymotrypsin. These findings seem to confirm Vernon's experiments, which showed that in activated pancreatic extract there are at least two proteinases. The experiments of Kunitz and Northrop indicate that there are at least

two inactive zymogens, chymotrypsinogen and trypsinogen, in fresh pancreatic extracts. Enterokinase changes trypsinogen to trypsin, and the trypsin transforms chymotrypsinogen into chymotrypsin. The formation of chymotrypsin from chymotrypsinogen is followed by a change in optical activity and an increase in amino nitrogen. There is no change in the non-protein nitrogen fraction formed and in the molecular weight. Kunitz and Northrop believe that the activation may be caused by an internal molecular rearrangement.

According to these investigators, neither of these two crystalline substances changes its properties after repeated recrystallization. Tests such as denaturation and hydrolysis indicate that the enzyme and its precursor are pure proteins.

Preparation of Crystalline Chymotrypsinogen. Ten cattle pancreases are immersed in cold 0.25 *N* sulfuric acid immediately after removal. The pancreas is freed from fat and connective tissue, and minced in a meat grinder. Two volumes of ice-cold 0.25 *N* sulfuric acid is added, and the suspension is allowed to stand at 5° C. overnight. It is then strained through gauze on a Büchner funnel, and the precipitate is suspended again in 0.25 *N* sulfuric acid and refiltered. The combined filtrates are brought to 0.4 saturation with solid ammonium sulfate and filtered through a soft fluted filter at a low temperature. To the filtrate, ammonium sulfate is added to 0.7 saturation, and the suspension is allowed to settle in the cold for 48 hours. The supernatant fluid is decanted, and the suspension is filtered with suction. The filter cake is dissolved in 3 volumes of water and 2 volumes of saturated ammonium sulfate is added. The volume of the semi-dry cake is determined by weight, and the specific volume is assumed to be equal to 1. The suspension is filtered and the precipitate discarded. The filtrate is brought to 0.7 saturation with solid ammonium sulfate. The suspension is filtered with suction. The filter cake is dissolved in 1.5 volumes of water and brought to ¼ saturated ammonium sulfate by the addition of saturated ammonium sulfate solution. The solution is adjusted to *p*H 5.0 (brick-red color with methyl red on test plate) with 5 *N* sodium hydroxide. About 1.5 cc. per 100 cc. is necessary. The solution is kept at 20° for 2 days. A heavy crop of crystals gradually forms; they are filtered with suction.

Recrystallization. The crystalline filter cake is suspended in 3 volumes of water, and 5 *N* sulfuric acid is added, with stirring, until the precipitate is dissolved. The solution is brought to ¼ saturated ammonium sulfate by the addition of 1 volume of saturated ammonium sulfate. Five *N* sodium hydroxide is added, with stirring, until the

solution reaches *p*H 5; the solution is kept at 20°. In an hour, crystallization should be completed. Yield: 15 grams of crystallized filter cake.

For the preparation of active chymotrypsin, the crystallization should be repeated eight times. Otherwise, it would be difficult to obtain the active enzyme in crystalline form.

It has been shown by Kunitz and Northrop that the properties of the crystalline chymotrypsinogen are constant through ten fractional recrystallizations.

Activation of Chymotrypsinogen. The activity of recrystallized chymotrypsinogen is extremely slight — only about 1/10,000 of chymotrypsin. It cannot be activated by enterokinase, calcium chloride, pepsin, inactivated trypsin, of chymotrypsin. It can be activated by commercial trypsin preparations and by crude active pancreatic extracts. The spontaneous activation is only 1 per cent in 1 month at 5°. The active trypsin-nitrogen used for the activation should be about 1/200 of the chymotrypsinogen-nitrogen, and the *p*H should be between 8 and 9.

Isolation and Crystallization of Chymotrypsin. Ten grams of eight-times-recrystallized chymotrypsinogen filter cake is suspended in 30 cc. of water and dissolved by the addition of a few drops of 5 *N* sulfuric acid; 10 cc. *M*/2, *p*H 7.6, phosphate buffer and a quantity of *M* sodium hydroxide equivalent to the acid are added. About 0.5 milligram of crystalline trypsin is added, and the solution is left at about 5° for 48 hours. The equivalent of any active trypsin may be used instead of the crystalline trypsin. After 48 hours, the solution is brought to *p*H 4.0 by the addition of about 5 cc. *N* sulfuric acid. Twenty-five grams of solid ammonium sulfate is added, and the precipitate is filtered with suction. The filter cake is dissolved in 0.75 volume of *N*/100 sulfuric acid and filtered if necessary. The clear filtrate is inoculated and allowed to stand at 20° for 24 hours. About 5 grams of crystalline filter cake should be obtained.

Recrystallization. The crystalline filter cake is dissolved in 1.5 volumes *N*/100 sulfuric acid; 1 volume of saturated ammonium sulfate is then added carefully until crystallization commences. If the solution is allowed to stand at room temperature, complete crystallization should take place. The properties of the enzyme remain constant through three fractional crystallizations.

Crystalline Trypsinogen

Kunitz and Northrop (54) developed a method for the preparation of crystalline trypsinogen from the mother liquor of chymotrypsinogen.

Magnesium sulfate at *p*H 7 to 8 activates this zymogen. The active trypsin can also be crystallized. The following is the procedure for the preparation of crystalline trypsinogen: All the solutions must be cooled to about 5°, and all operations must be carried out in the icebox. The mother liquid from the chymotrypsinogen crystallization is adjusted to *p*H 4.0 with 2.5 *M* sulfuric acid, brought to 0.7 saturated ammonium sulfate, and filtered. One hundred grams of the precipitate is dissolved in 300 cc. of water, brought to 0.4 saturated ammonium sulfate, and filtered. The filtrate is brought to 0.6 saturated ammonium sulfate by slow addition of saturated ammonium sulfate and is filtered with suction. The precipitate is washed twice with saturated magnesium sulfate. Ten grams of filter cake is dissolved in 10 cc. 0.4 *M* borate buffer of *p*H 9.0; 17 cc. saturated magnesium sulfate is added, and the solution is allowed to stand at 6°. Short triangular pyramids form in the course of 2 to 3 days. Inoculation of the solution hastens crystallization. Sometimes the solutions become active, and crystallization stops or crystals of the active trypsin may appear.

Conversion of Trypsinogen to Trypsin in the Presence of Calcium Chloride and Crystallization of Trypsin

McDonald and Kunitz (55) improved the method for the crystallization of trypsin by permitting the autocatalytic conversion of trypsinogen into trypsin to proceed in the presence of calcium ions. The new conditions result in a quantitative conversion of trypsinogen to trypsin, and crystallization is easy. The quality and yield of crystalline trypsin are greatly improved by the new method.

1. Conversion of Trypsinogen into Trypsin. Thirty grams of dried crystalline trypsinogen, or 50 grams of semi-dry filter cake, is dissolved in 200 cc. 0.005 *M* hydrochloric acid. A previously prepared ice-cold mixture of 100 cc. 1 *M* calcium chloride, 250 cc. 0.4 *M* borate buffer of *p*H 8.0, and 400 cc. distilled water is added. The mixture is made up to 1000 cc. in volume and left in a cold room at about 5° C. for 18 to 24 hours.

Stock Borate Solution. This solution contains 49.6 grams boric acid and 80 cc. 5 *N* sodium hydroxide per 1000 cc. 0.4 *M* borate buffers, *p*H 8.0 and 9.0, are made up by mixing 100 parts stock borate and 78.6 and 17.6 parts 0.4 *M* hydrochloric acid respectively.

2. Removal of Calcium Ions. Two grams of standard Supercel (Johns-Manville Corporation) are added, and the mixture is filtered in a cold room, using suction and soft paper, such as Eaton-Dikeman No. 303. The filter cake is rejected. The filtrate is adjusted to *p*H 3.0 with 5 *N* sulfuric acid (about 4 cc. of acid is required). Solid

ammonium sulfate is added to make 0.4 saturation; 242 grams per 1000 cc. is required. After 2 days in the cold room, the calcium sulfate precipitate is filtered off, with suction, using Whatman No. 3 paper. The precipitate is rejected.

3. Precipitation of Amorphous Trypsin. Solid ammonium sulfate is added to the filtrate until it is 0.7 saturated, using 202 grams per 1000 cc. The mixture is filtered, with suction, using hardened paper, such as Schleicher and Schull No. 575. The filtrate is rejected. The filter cake (about 50 grams) is dissolved in 150 cc. distilled water, and the trypsin is reprecipitated by the slow addition, while stirring, of 350 cc. saturated ammonium sulfate, by means of a dropping funnel. The precipitate is filtered, with suction, through hardened paper, such as S. and S. No. 575, size 18.5 cm. or larger, and washed with saturated magnesium sulfate in 0.02 *N* sulfuric acid to remove excess ammonium sulfate. Yield: 50 grams.

4. Crystallization of Trypsin. The semi-dry filter cake is dissolved in ice-cold 0.4 *M* borate buffer, *p*H 9.0 (10 cc. per 10 gram filter cake), at 2° to 5° C. (in an ice water bath). Crystals of fine needles form almost at once. Crystallization is allowed in cold room for 24 hours. The crystals are filtered, with suction, using S. and S. No. 575. They are washed several times with cold 0.5 saturated magnesium sulfate in 0.1 *M* borate buffer, *p*H 8.0, and then, at room temperature, with saturated magnesium sulfate in 0.1 *N* sulfuric acid. Yield: 15 to 20 grams.

5. Recrystallization of Trypsin. The semi-dry filter cake is dissolved in 0.02 *N* sulfuric acid, 10 cc. per 10 grams of filter cake being used. To avoid foaming, the filter cake must be gradually mixed into the acid. The mixture is filtered clear through small fluted No. 3 Whatman paper, and the precipitate is washed with several cubic centimeters of 0.02 *N* sulfuric acid. The filtrate is cooled to 5° C. and adjusted to *p*H 8.0 with cold 0.4 *M* borate buffer, *p*H 9.0 (about 8 cc. per 15 cc. solution). Crystallization takes place almost at once. The solution is kept cold for 24 hours, then filtered and washed as described for the first crystallization. Yield: 10 to 15 grams. The filter cake is dried in a dry refrigerator for several days, ground to a fine powder, and stored in a cold room.

β- AND γ-CHYMOTRYPSIN

Kunitz (56) found that chymotrypsin in solution changes irreversibly into new enzymes. He isolated two of the enzymes in pure crystalline state and called them β- and γ-chymotrypsins. They are proteins and enzymically indistinguishable from the original chymotrypsin,

but they differ from it in molecular weight, crystalline form, stability in acid or alkali, solubility, etc. Kunitz believes that the new enzymes form from chymotrypsin by slight hydrolysis, as evidenced by the loss of protein during the process of formation of the new enzymes and by the fact that their molecular weights are lower than that of the original chymotrypsin.

Reznitschenko and coworkers (57) isolated from pancreatin a crystalline proteinase that transforms gelatin into compounds soluble in trichloroacetic acid without splitting any peptide bonds.

Crystalline Trypsin Inhibitor and Crystalline Inhibitor-Trypsin Compound. The activity of trypsin is influenced markedly by the presence, in pancreatic extracts, of a substance that inhibits trypsin digestion. This substance is present in the mother liquor from the trypsinogen crystallization. It has been isolated in a pure and crystalline state, and as a compound with trypsin by Northrop and Kunitz (58, 59). The inhibitor trypsin compound has also been crystallized. The trypsin inhibitor compound contains approximately 80 per cent trypsin and 20 per cent inhibitor. In acid solution the trypsin is liberated, and, if added to protein solutions at *p*H 8.0, the protein is digested. No digestion of the protein will take place if the compound is neutralized before it is added to the protein solution. The inhibitor has a molecular weight of about 5000 and appears to be a polypeptide.

Crystalline Soybean Trypsin Inhibitor. Kunitz (60) obtained a protein trypsin inhibitor in crystalline form from commercial soybean meal that had previously been defatted by solvent extraction. The meal is washed with 80 per cent alcohol, and the inhibitor is extracted from the meal with 0.25 *N* sulfuric acid. The acid extract is treated with a small amount of bentonite, which removes some of the inert protein; this bentonite fraction is rejected. A second adsorption with a large amount of bentonite removes all the inhibitor protein. The inhibitor is eluted from the bentonite with the aid of a dilute aqueous pyridine solution, and the pyridine is removed by dialysis. The dialyzed solution is adjusted to *p*H 5.3, and the precipitated inert material is removed by filtration. The inhibitor is precipitated from the filtrate by adjusting to *p*H 4.65 at 5–10° C.

Crystals, in the form of fine needles, and thin plates form by adjusting a concentrated solution of the amorphous substance with acid to *p*H 5.1 and leaving at 36° C. The protein may be recrystallized from dilute alcohol. The dried protein retains its crystalline structure and inhibitory power. One gram of crystals is obtained from 1000 grams of meal.

Autolytic Trypsin. Kleiner and Tauber (61) autolyzed fresh ground pancreatic tissue in 30 per cent ethyl alcohol for 18 months. After dialysis and acetone precipitation, the water-soluble fraction was dried in vacuum. A solution of this dry preparation gave a slightly positive xanthoproteic test, a positive Folin-Denis test, and a positive ninhydrin test. The biuret test, the Millon test, and the Hopkins-Cole test were negative. The heat-coagulation test, on prolonged boiling, was slightly positive. Saturated solutions of neutral salts gave a precipitate. The isoelectric point of this trypsin was 6.2. It dialyzed readily through cellophane.

These properties are those of a polypeptide. The proteolytic activity of this trypsin is about that of crystalline trypsin. Its rennet activity is 1 : 420, as compared to 1 : 4,550,000, the potency of the highly active rennin of Tauber and Kleiner. It has been pointed out that proteolytic activity of trypsin must be depressed when the rennet activity is determined; otherwise, casein is digested without calcium paracaseinate formation. This preparation showed no amylolytic or lipolytic activity (62). A trypsin of similar nature has been obtained by Willstätter and Rohdewald.

Preparation of Pancreatin

Pancreatin preparations contain the enzymes of the pancreas gland, such as trypsin, amylase, and lipase. The best source for the preparation of pancreatin is the pancreas gland of the pig; the beef pancreas, though sometimes used, is a much poorer source. The pig pancreas weighs about 100 grams; the beef pancreas, 250 grams.

The glands are minced and mixed with water containing a small quantity of chloroform. To effect complete activation of the trypsinogen, pancreatic extracts should be kept at room temperature for 24 hours at *p*H 4 to 5 (45). Then the mixture is pressed, and from the press juice the enzymes are precipitated with alcohol or acetone. The precipitate is dried at 40° C. *in vacuo* or in a current of air. This product is only partly soluble. A soluble product may be obtained by extracting the alcohol or acetone precipitate with water. Gum arabic is added to the centrifuged clear solution. The solution is then evaporated *in vacuo*. For some purposes, the dried whole gland, powdered and sieved, may also be used.

Preparation of Technical Pancreatin. The salted or fresh glands are minced, mixed with water, pressed, and filtered. The filtrate is evaporated to dryness in vacuum or in an air current at 40° C. For use as bating in the leather industry, the press juice is mixed with sawdust before it is evaporated.

Uses of Pancreatin. In addition to its applications in medicine and in the tanning industry, pancreatin is extensively used in the manufacture of gelatin and glue. Some is employed in the textile industry and in laundries. This and other proteolytic enzyme products are frequently used in the manufacture of peptones. For a review of the literature, see reference 27.

The Influence of *p*H on Initial Velocity of Hydrolysis of Crude Pancreatic Proteinase. Pancreatic proteinase includes a number of proteinases and peptidases. Typical data for the crude proteolytic mixture are not necessarily identical with those characteristics for the crystalline components.

Farber and Wynne (63) studied the crude pancreatic proteinase. They prepared dry fat-free pancreas powder, using acetone and ether as the solvent (64, 65). Eight grams of the pancreas powder was extracted with a phosphate-borax buffer of *p*H 8.7. The extract was then centrifuged and passed through a Seitz filter. This solution showed constant activity for 2 weeks when kept at 8° C.

The substrates were casein (0.80 per cent solution), hemoglobin (0.79 per cent solution), and fibrin (0.55 per cent solution). All three proteins displayed the same optimum, *p*H 8 to 9.

The initial rate of proteolysis varied directly with the concentration of the enzyme over a wide range. The reaction was of zero order during the proteolysis of most of the protein.

CATHEPSIN

Cathepsin is present in most animal tissues, the best source being those cells concerned with the synthesis of cellular proteins. Beef spleen and beef kidney are very convenient sources. The optimum *p*H of catheptic protein digestion is close to 4.00, whereas the *p*H of living tissues is much closer to neutrality. Digestion of proteins by crude tissue extracts is very extensive.

Waldschmidt-Leitz and associates (66) and Grassmann and coworkers (67) found that cathepsin can be activated by sulfhydryl compounds. Anson (68) believes that cathepsin is the only proteinase present in animal tissues, and that it is not activated by cysteine. Fruton and collaborators (69), however, have shown that aqueous extracts of beef spleen contain four cathepsins of widely different specificity: cathepsin I, splitting carbobenzoxy-*l*-glutamyl-*l*-tyrosine, and requiring no cysteine; cathepsin II, splitting benzoyl-*l*-argininamide, requiring cysteine; cathepsin III, hydrolyzing *l*-leucinamide, and requiring cysteine or ascorbic acid; cathepsin IV, hydrolyzing carbobenzoxyglutamyltyrosine on addition of cysteine. The distribution of

the cathepsins, however, is not the same in all tissues. For instance, swine kidney extracts contain much more cathepsin IV than beef spleen extracts.

Cathepsin III and IV are proteinases, and cathepsin I and II are peptidases.

Zamecnik and Stephenson (70) found that the activity of catheptic enzymes was much higher in the hog kidney cortex than in the medullary region. An enzyme solution of good activity was prepared by lyophilization of fresh tissue and extraction of the dried tissue powder with 50 per cent glycerol containing 0.1 *M* citrate buffer at *p*H 5. These investigators were able to confirm the work of Fruton and coworkers (69) concerning the presence in hog kidney of at least four distinct catheptic enzymes. The same types of key substrates were used in the present work, e.g., carbobenzoxy-*l*-glutamyl-tyrosine for pepsinase, carbobenzoxyglycyl-*l*-phenylalanine for carboxypeptidase, benzoyl-*l*-argininamide for trypsinase, and *dl*-leucylglycine for aminopeptidase. See also reference 71.

PEPTIDASES

According to the early literature (72–74), "intestinal erepsin" can hydrolyze polypeptides, peptones of peptic and tryptic digestion, protamins, histones, and casein. Waldschmidt-Leitz and Schäffner (75), however, believed that these statements were incorrect, since only peptides are attacked by the "intestinal erepsin." In 1925, Waldschmidt-Leitz and associates separated "erepsin," the peptide-hydrolyzing fraction, from "pancreatic trypsin." In 1926, Willstätter and Grassmann (76) separated the proteolytic enzymes of yeast from the peptidase fraction, using an adsorption method similar to that of Waldschmidt-Leitz and Harteneck for the fractionation of pancreatic proteases (proteinase + carboxypolypeptidase). Now it is known that "erepsin" consists of a number of specific polypeptidases (70–80).

Aminopolypeptidase

Specificity. The relative activity of intestinal aminopolypeptidase, prepared by acetone precipitation of a glycerol extract followed by adsorption on ferric oxide, has been studied by Johnson (81). This investigator found that, while polypeptides were very rapidly split, the dipeptides alanylglycine, diglycine, prolylglycine, and leucylglycine were fairly rapidly hydrolyzed, by this enzyme. Alanylglycylamine (decarboxylated alanyldiglycine) was also split, which indicated that a carboxyl group is not required. Monomethyl substitution of the

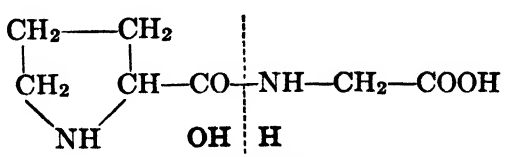
amino group does not hinder splitting, but N-dimethylalanyldiglycine is not hydrolyzed. Most of the leucyldiglycine-splitting activity of " erepsin " is not due to aminopolypeptidase but to leucylpeptidase. Bergmann and Fruton (82) stated that a peptide, to be split by aminopeptidase, must contain a free hydrogen atom on the peptide nitrogen atom, but that a free carboxyl group is not essential. It hydrolyzes the peptide linkage adjacent to the amino end of the peptide molecule. The optimum *p*H of the enzyme is at 8.0 (83).

Agner (84) has obtained a highly active aminopolypeptidase from hog's pyloric mucosa. This preparation was free of dipeptidase, was of protein nature, and had an isoelectric point at *p*H 4.6. Agner and Waldenström (85) believe that this enzyme is identical with the intrinsic (antipernicious anemia) factor of Castle. This is in harmony with the prevailing view that the intrinsic factor is of enzymic nature. The Swedish authors support their hypothesis with clinical observations. It had been previously demonstrated that the enzyme was not identical with pepsin, rennin, cathepsin, or lipase. Agner's highly purified hog-pyloric mucosa aminopolypeptidase does not digest preparations containing the antipernicious liver principle. Agner offers this as additional evidence that the two factors are identical (85). However, the results of Andersen and Faber (86) do not support this view.

Prolinase or Prolylpeptidase

According to Grassmann and collaborators (87), if the amino group of the α-amino acid of the peptide chain is replaced by the imino group of prolin, a specific polypeptidase, a so-called prolinase, acts upon it. Abderhalden and Zumstein (88) found that not all prolylpeptides can be hydrolyzed by this intestinal enzyme. Grassmann and coworkers (88–90) reported that any prolylpeptide is split by the prolinase. They stated that the polypeptides of Abderhalden and Zumstein were not free of protein, were not pure crystalline compounds, and were probably contaminated with silver salts, which were used for the preparation of the peptides and had powerful inhibitory properties.

An example is

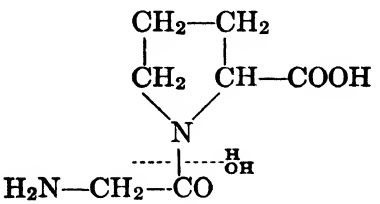


Prolylglycine

Prolinase may be obtained free from aminopolypeptidase but not from dipeptidase. The optimum *p*H is 7.8 when prolylglycine is used as a substrate.

Prolidase (4, 82, 91–94)

Bergmann and associates have shown that in compounds in which the cyclic nitrogen of the proline combines with the carboxyl group of a peptide, as, for example, in glycylproline, enzymic hydrolysis may take place. This is in support of the theory that proteins may be built of



Glycylproline

cyclic compounds instead of peptides. In this glycylproline hydrolysis, the imino group of proline is set free. This group cannot be estimated by the Van Slyke method. Prolidase attacks synthetic polypeptides having a —CO—N=R— group instead a —CO·NH— radical (82). Such linkages are present in collagen and gelatin. This enzyme hydrolyzes glycylproline to glycine and proline. Prolidase is not precipitated when *l*-leucine aminoexopeptidase (leucyl peptidase) is prepared from crude aqueous extracts of intestinal mucosa on the addition of acetone, according to the method of Smith and Bergmann (4). This enzyme is activated by Mn^{++} but not by Mg^{++}. Smith and Bergmann described a method for the concentration of prolidase. This preparation also contains enzymes that rapidly split *l*-leucyldiglycine, *l*-alanylglycylglycine, diglycylglycine, diglycyl-*l*-proline, and diglycyl-*l*-hydroxyproline. Here no activation occurred by Mn^{++} ions, and sometimes a definite inhibition was noticed. The enzyme that hydrolyzes *l*-leucylglycylglycine acts at the peptide bond adjacent to the free amino group, forming leucine and glycylglycine. Thus, this enzyme is not identical with *l*-leucine aminoexopeptidase. It splits *l*-leucylglycylglycine 80 times faster than *l*-leucylglycine. Smith and Bergmann named this enzyme *imidopeptidase*. These investigators stated that there are no exclusive dipeptidases in nature.

Carboxypeptidase

Waldschmidt-Leitz and Purr (95) showed that carboxypeptidase hydrolyzes only a few dipeptides. It readily splits polypeptides and peptic digests as well as certain synthetic peptides. Chloracetyl-*l*-tyrosine has been generally regarded as the most suitable substrate for carboxypeptidase (96). However, the use of this substrate offers certain difficulties. During enzyme action free tyrosine crystallizes out, thus causing interference with the quantitative determination of hydrolysis. Of greater importance is the inhibitory action of the liberated chloroacetic acid. Owing to this fact the hydrolysis of chloroacetyl-*l*-tyrosine by carboxypeptidase does not follow the course of a first-order reaction (97). Hofmann and Bergmann (97) found that carbobenzoxyglycyl-*l*-phenylalanine represents an ideal substrate for the determination of carboxypeptidase activity. This enzyme is most active at *p*H 7.4.

Crystalline Carboxypeptidase

Carboxypeptidase has been obtained in crystalline form from bovine pancreas by Anson (98). Putnam and associates (99) have also prepared crystalline carboxypeptidase by Anson's method. They found that electrophoretic homogeneity was achieved only by seven recrystallizations. The isoelectric point of the pure enzyme was at *p*H 6. The molecular weight, calculated from viscosity and diffusion data, was 32,000.

Method of Crystallization (98)

To the spontaneously activated fluid which exudes from frozen pancreas at 5° C., 5 *N* acetic acid is added until yellow cresol green turns green. The acid fluid is kept at 37° for 2 hours and filtered. The filtrate is diluted with ten times its volume of water. The precipitate which settles is filtered and is suspended in water so as to give an activity twice that of the original fluid. Then barium hydroxide is added until the suspension is pink to phenolphthalein. The barium hydroxide dissolves only a part of the protein but all the carboxypeptidase. Sodium hydroxide dissolves all protein matter. The undissolved protein is removed by centrifuging, and to the supernatant fluid 1 *N* acetic acid is added until orange to phenol red. Globulin crystals appear on short standing. The enzyme can be dissolved in sodium hydroxide and recrystallized by neutralization. Destruction due to

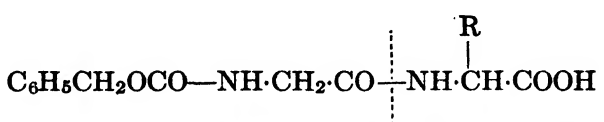
coagulation by heating is proportional to the protein coagulated. Contrary to the claims of Anson (98), this enzyme has been found to be destroyed by formaldehyde at 40° and also at 25° (97).

Anson (100) has also published a method for the preparation of crystalline carboxypeptidase from ground frozen beef pancreas, using 2 per cent sodium chloride for extraction, salting out with solid ammonium sulfate, and dialyzing. From the dialyzed solution, the precipitated enzyme is collected and treated in a manner similar to the precipitate which is obtained by dilution of the frozen autolytic pancreas exudate.

Specificity of Crystalline Carboxypeptidase

Bergmann and Fruton (82) showed that the crystalline enzyme did not attack leucylglycine or leucyldiglycine. Carbobenzoxyglycyl-*l*-alanine, however, was hydrolyzed very fast, and the *d*-form was attacked extremely slowly. Hofmann and Bergmann (97) reported that the thrice-crystallized carboxypeptidase very rapidly hydrolyzed acylated peptides at the linkage adjacent to the carboxyl group provided that the amino acid in carboxyl position was tyrosine or phenylalanine. The acyl compound carbobenzoxyglycyltyrosine was hydrolyzed 20,000 times faster than the same compound without the acyl group (glycyltyrosine). The importance of the terminal carboxyl radical was evident by the rapid splitting of carbobenzoxyglutamyl-*l*-phenylalanine and the inertness to hydrolysis of the amide of this substance.

Stahmann and associates (101) have reinvestigated the specificity of crystalline pancreatic carboxypeptidase. The rate of reaction of the enzyme, using various carbobenzoxyglycylamino acids, was found to be dependent upon the terminal amino acid of the substrate (R in general formula):



General formula of carbobenzoxyglycylamino acids

Thus, to obtain comparable rates of hydrolysis, 340 times as much enzyme was necessary for the hydrolysis of carbobenzoxyglycyl-*l*-alanine as was required for carbobenzoxyglycyl-*l*-phenylalanine. Neither carbobenzoxyglycylsarcosine nor carbobenzoxyglycyl-*l*-proline was split by carboxypeptidase, even at very high concentrations. This shows that the presence of the " peptide hydrogen " in the substrate is

necessary for the action of this enzyme. Carboxypeptidase exhibits antipodal specificity. Only such amino compounds are attacked as contain a terminal amino acid of the levo configuration. Partial inactivation of the enzyme by heat or alkali resulted in a parallel decrease in the enzymic activity toward several substrates, indicating that the different activities were not due to the presence of several enzymes in the preparation. The presence of substances structurally related to carbobenzoxyglycyl-*l*-phenylalanine or carbobenzoxy-*d*-phenylalanine inhibited the enzyme.

More recently, Putnam and collaborators (99) published kinetic data for the hydrolysis of (figures in parentheses are corrected unimolecular constants divided by enzyme concentration): carbobenzoxyglycyl-*l*-phenylalanine (9.6), chloracetyl-*l*-tyrosine (3.8), chloracetyl-*l*-phenylalanine (4.2), carbobenzoxyglycylglycine (0.00035). Benzoyl-*l*-arginine amide, acetylglycine, and *dl*-leucylglycine were not attacked.

Leucyl Peptidase or *l*-Leucine Aminoexopeptidase

This enzyme is present in many plants, bacteria, and animal tissues. It is not produced by molds and yeast (102). The enzyme of the intestinal mucosa acts rapidly on *l*-leucylglycine and *l*-leucylglycylglycine. It was discovered by Linderstroem-Lang (103), who named it leucylpeptidase. The enzyme was extensively investigated by Smith and Bergmann (4), who called it *l*-leucine aminoexopeptidase. They believed that this enzyme requires a free amino group but not a free carboxyl group. The enzyme of the intestinal mucosa is dependent on manganous and magnesium ions (104).

Preparation (4). Three hundred and forty-five grams of mucosa, from twenty-five 2- to 3-foot lengths of the upper portion of hog duodenum, is ground with 35 grams of sand and 350 cc. of water. The extract is centrifuged. The mucosa is re-extracted with 350 cc. of water; 650 cc. of combined extract is obtained (preparation A).

The precipitate obtained by the addition of an equal volume of cold acetone to chilled preparation A is collected and washed with acetone. This crude dry material may be kept in the desiccator for months without loss of activity. The dry powder (16.8 grams) is twice extracted with water at 40° C., and the solution is clarified by filtration with Celite. The volume of this solution is 360 cc. (preparation B).

To 350 cc. of preparation B is added 84.7 grams of ammonium sulfate to make a 40 per cent saturation. The precipitate is discarded. To the clear solution (Celite filtrate) is added 48 grams of ammonium

sulfate to make a 60 per cent saturation. The precipitate is collected and dissolved in water. After dialysis in the cold against distilled water, the inactive precipitate is filtered off and discarded. Sixty-six cubic centimeters of a clear, faintly yellowish solution is obtained (preparation C), representing a 34-fold purification and a yield of 31 per cent of the original activity of preparation A.

TABLE XVI

ACTIVITIES OF CRUDE AND OF PURIFIED *l*-LEUCINE AMINOEXOPEPTIDASE PREPARATIONS TOWARDS VARIOUS SUBSTRATES

For the crude extract (preparation A), no activator was added for the tests, since no activation was detectable. For the other tests, the enzyme was incubated with 0.02 *M* $MnSO_4$ buffered at *p*H 8.0 at 40° for 3 to 4 hours. A test aliquot was then added to the buffered peptide solution.

	Proteolytic Coefficient, *C*			
Substrate	Preparation A	Preparation B	Preparation C	Yield,* per cent
l-Leucylglycine	0.062	0.35	2.1	31
d-Leucylglycine	0.00076	0.0010	<0.0001	
l-Leucylglycylglycine	0.11†	0.55†	2.2	18
l-Leucinamide	0.022		1.0	42
Glycylglycine	0.025		0.0065	0.24
Benzoyl-*l*-argininamide	0.0005		<0.0001	
Glycyl-*l*-leucine	0.034		0.012	0.32
l-Alanylglycine	0.26	0.042	0.17	0.60
Glycyl-*l*-proline	0.015		0.035	2.1
l-Prolylglycine	0.035		0.027	0.71
Glycyl-*l*-alanine			0.021	
l-Leucyl-*l*-leucylglycine			1.6	
Benzoyl-*l*-leucylglycine			<0.0001	

* Yields are given as $\frac{100 \times \text{total activity of preparation C}}{\text{total activity of preparation A}}$.

† Initial *C* values.

Table XVI shows the effect of the purification procedure on the original extract. For instance, 99 per cent of the activity towards *l*-alanylglycine and glycylglycine had been removed. Thus, this activity must be due to action or actions distinct from leucine aminoexopeptidase. The action towards *d*-leucylglycine had been reduced very considerably, showing that two stereoisomeric peptides are split by two distinct enzymes.

The kinetics of the purified enzyme is complicated by the enzyme's responsiveness to activation by Mn^{++} or Mg^{++}. For other important facts concerning this enzyme, see reference 94.

DIPEPTIDASES

1. Yeast Dipeptidase. Grassmann and associates (105) described the isolation from yeast autolysate of a fraction which splits dipeptides but not polypeptides. This fraction they called yeast peptidase. Johnson (106) found that leucylglycine hydrolysis by this enzyme is activated by halide ions. Berger and Johnson (107) reported that prolylglycine, diglycine, and leucylglycine hydrolysis is strongly activated by manganese ions and by cysteine. Alanylglycine hydrolysis, however, is inhibited by these activators. These data indicate that there are several dipeptidases in yeast, and that yeast "prolinase" may be identical with one of them.

2. Intestinal Dipeptidase. The literature seems to indicate that intestinal dipeptidase is not an entity. It is also stated that much dipeptidase action is not due to intestinal aminopolypeptidase and leucylpeptidase. In 1931, Balls and Köhler (108) obtained fractions that hydrolyzed dipeptides much more rapidly than polypeptides. Gailey and Johnson (94) have reinvestigated the specificity of intestinal dipeptidases. These investigators came to the conclusion that three of the dipeptidases could be identified as (*a*) an enzyme hydrolyzing alanylglycine rapidly, for which action no activator was found; (*b*) an enzyme hydrolyzing diglycine, considerably activated by cobalt; and (*c*) a manganese-activated enzyme (not identical with leucylpeptidase) capable of attacking substrates lacking a free amino group of *l* configuration, such as prolylglycine, *N*-methylleucylglycine, and *d*-leucylglycine. Since separation experiments were not very successful, these conclusions are only provisional.

Dehydropeptidases (109). There are two kinds of dehydropeptidases. Dehydropeptidase I converts glycyldehydroalanine to glycine, ammonia, and pyruvic acid:

$$NH_2CH_2CONHC(=CH_2)COOH \rightarrow NH_2CH_2COOH + NH_3 + CH_3COCOOH$$

This enzyme has been found in many normal and neoplastic mammalian tissues and in a large number of plants. The second enzyme, dehydropeptidase II, hydrolyzes chloroacetyldehydroalanine. It is found mainly in kidney and liver and in small amounts in various plants. Mushrooms contain both dipeptidases in large quantities. Dehydropeptidase action may be observed by measuring the evolution of ammonia in the digest. The universal distribution of these enzymes suggests that peptides of α, β-unsaturated amino acids may play an important role in intermediary protein metabolism.

PROTEOLYTIC ENZYMES OF PLANTS

The proteolytic enzymes of plants vary according to their origin. Some depend on reducing agents, such as sulfites, sulfides, cysteine, and cyanides; others do not require activators. Some of proteinase-containing plants have milk-clotting enzymes; others do not. However, the proteinase itself may coagulate milk. In some cases, the milk-clotting enzyme was separated from the proteinase. The *p*H optima of the plant proteinases may differ considerably, varying from a distinctly acid to a distinctly alkaline *p*H. Some of these products are used in medicine and in industry.

FIG. 13. Collecting latex from green papaya.

PAPAIN

Papain is the name of the powdered latex of the green fruit of *Carica papaya*. The fresh latex contains a very powerful proteolytic enzyme system. Owing to the sensitivity of this proteinase to oxidation, half of the proteolytic power is soon lost. It is believed that the crude enzyme mixture contains some substance that brings about the rapid loss of potency. This inactivation may at first be reversed by treatment with reducing agents such as hydrogen sulfide and other sulfides, hydrogen cyanide, and sulfites (110, 111). Prolonged oxidation, however, renders the proteinase system permanently inert. The papaya proteinases contain readily oxidizable groups. The chemical nature of the groupings has not yet definitely been established. Some fractions contain SH groups; thus, commercial papain preparations cannot be kept too long. This defect is compensated for, however, by the fact that, in normal times, papain is the cheapest source of commercial proteolytic enzyme. Another desirable property of papain is its relative resistance to heat.

Preparation of Papaya Latex. Latex is prepared by making three or four longitudinal scratches in the skin of the green fruit while it is on the tree. See Fig. 13 (112). The latex either drips down into a container held under the fruit or clots on the fruit (112). The liquid

latex rapidly coagulates and is dried in the air. Vacuum drying yields a superior product, however. The scratching process may be repeated several times, as long as the fruit is green. The yield of dry latex is about 0.1 per cent of the weight of the green fruit.

About one-half of the total solids of latex consists of enzyme protein (110). Balls and associates (111) found that the addition of sodium chloride to the moist latex, followed by partial removal of the water, yielded a better and more stable product than the sulfide-treated latex — about as good, weight for weight, including the salt and water of the paste. These experiments are summarized in Table XVII (112).

Precipitation Procedures. Balls and coworkers reported that the proteases may also be prepared from the press juices of the stalks, flowers, and stems of the papaya plant. The proteases may be precipitated from the acidified (*p*H 4 with sulfuric, hydrochloric, or acetic acid) or filtered juice on the addition of 5 volumes of 92 per cent alcohol. Acidification of the press juice inhibits the action of natural oxidizing agents. The precipitate must be rapidly dried *in vacuo* because of the destructive action of the alcohol.

The enzymes may also be precipitated by the addition of 2 volumes of saturated ammonium sulfate. The precipitate is allowed to settle overnight in the cold and is filtered the next morning. The pressed and vacuum-dried product contains 50 per cent ammonium sulfate. Not all the enzymes are removed by this methed, however. The precipitation methods are probably too expensive to be of commercial use.

For measuring proteolytic activity, any of the usual methods may be employed. The milk-clotting test is also applicable and most rapid (113). Unless the papain concentrations are very small, the clotting time is inversely proportional to the amount of enzyme employed in the test.

Like proteinase activity, the milk-clotting power is activated by hydrogen sulfide, cysteine, and cyanide.

Crystalline Papain

Papain has been crystallized by Balls and Lineweaver (114) and chymopapain by Jansen and Balls (115). In both cases, the crystals consisted of a mixture of active and inactive enzyme. Papain has a molecular weight of 27,000 to 30,000; chymopapain, of about 45,000. Chymopapain has half the proteolytic power of papain but the same milk-coagulating activity. There are, however, other proteolytic enzymes in the papaya latex.

Crystalline papain is a prolaminelike protein, being soluble in 70 per cent ethyl alcohol. It is activated by sulfhydryl compounds and

TABLE XVII

PERCENTAGE OF THE ORIGINAL ACTIVITY* OF THE UNDRIED LATEX REMAINING AFTER THE STORAGE OF VACUUM-DRIED AND PARTLY DRIED PREPARATIONS OF LATEX AT ROOM TEMPERATURE

Preparation	Activated Enzyme after Storage of:					Naturally Active Enzyme after Storage of:				
	0 days	33–36 days	45–50 days	130–135 days	190 days	0 days	33–36 days	45–50 days	130–135 days	190 days
	Per cent	Per cent	Per cent	Per cent	Per cent	Per cent	Per cent	Per cent	Per cent	Per cent
Dried latex	92	..	41	..	..	91	..	17	..	..
Dried latex containing 10 per cent of Na_2S	85	..	49	..	..	37	..	34	..	..
Dried latex containing 10 per cent of NaCl	78	..	59	..	..	76	..	55	..	..
Partly dried latex: Papaya solids 35 per cent, NaCl 20 per cent, Water 45 per cent	82	80	80	74	75	73	74	74	67	65
Partly dried latex: Papaya solids 35 per cent, Na_2S 10 per cent, NaCl 10 per cent, Water 45 per cent	100	58	..	..	..	30	19	..	..	..

* The activity of the original latex was 1.1–1.3 milk-clotting units per milligram of dry weight without activation and 1.2–1.5 units after activation with sodium cyanide. The percentage of activity is calculated on the weight of papaya solids only, thus excluding such additions as salt and sulfide.

by cyanide. Like other proteinases, it hydrolyzes certain peptides and splits hyppurylamide. It acts much faster, however, on native proteins. This enzyme is very stable at *p*H 10.5 but is inactivated below *p*H 4.5.

α- and β-Papain

Irving and associates (116) found that papain contains a cysteine-activable proteinase that splits benzoyl-*l*-argininamide. Because this kind of specificity is also shown by crystalline pancreatic trypsin, this enzyme has been named papain trypsinase. It contains two inactive forms, which have been called papain-α-trypsinase and papain-β-trypsinase. Only the β form may be activated by hydrogen cyanide. The α form may be changed into the β form by traces of sulfhydryl compounds, such as hydrogen sulfide or cysteine. The activation of the β-form by an excess of hydrogen cyanide or hydrogen sulfide is reversed when the activator is removed in vacuum. The activator papain trypsinase compounds formed with various activators represent different enzymes, depending on the activator used. Winnick and associates (117), however, observed that no inactivation of papainlike enzymes takes place if the activator is removed in the absence of air. These investigators are of the opinion that the role of activators (cyanide, cysteine, etc.) is to protect the proteinase from atmospheric oxidation by effecting combination with heavy metals. Anaerobic dialysis removes cysteine but does not inactivate the proteinase. When the dialysis is carried out in the presence of air, the enzyme becomes reversibly inactive. These discrepancies will no doubt be clarified by new evidence concerning the active groupings in enzymes of this type.

Papain Aminopeptidase. Agner (118) reported that papain preparations contain an aminopolypeptidase with a substrate specificity similar to that of the corresponding enzymes present in hog's pyloric and duodenal mucosa.

Bromelin

Bromelin is the papainase of pineapple juice. This enzyme is similar to papain; it digests proteins and clots milk. Bromelin is completely precipitated from pineapple juice by alcohol and by ammonium sulfate. Although proteinase activity decreases with the ripening of the papaya fruit, the same is not true of the bromelin of pineapple.

The Preparation of Bromelin. Pineapple products are too expensive as a commercial source of proteinase. Balls, Thompson, and Kies

(119), on the basis of their laboratory experiments, recommended the preparation of bromelin from filtered third-press juice (a factory mid-product). In their method, bromelin is precipitated with alcohol, the alcohol is recovered, and the residual juice, owing to its sugar content, is utilized in the usual alcoholic fermentation. No large-scale experiments, however, have been carried out, and the authors state that they are unable to say whether such a process would be profitable.

Preparation of Bromelin from Fresh Pineapple Fruit. Three liters of fresh juice is filtered, with the aid of supercell, and adjusted to *p*H 6 with ammonia. Solid ammonium sulfate is added to saturation. The precipitated crude enzyme is centrifuged, placed on a Büchner filter, and washed with 0.6 saturated ammonium sulfate. The

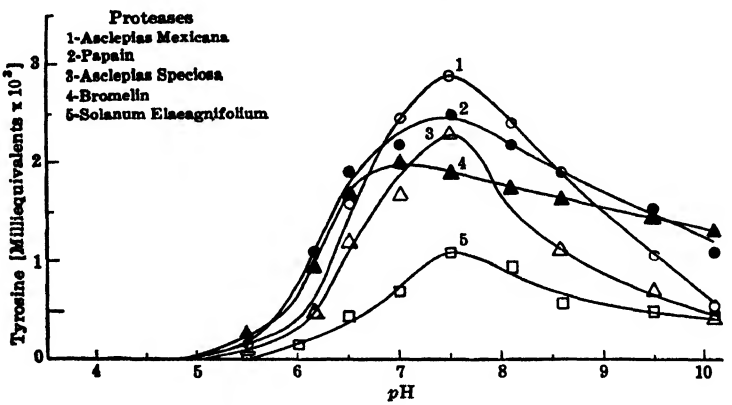


FIG. 14. *p*H activity curves of plant proteases on denatured ovalbumin. The quantity of enzyme in 6 cc. of digestion mixture was 0.5 milligram in asclepain *m* (curve 1), papain (curve 2), and asclepain *s* (curve 3), and 1.0 milligram in digestions with bromelin (curve 4) and solanain (curve 5).

precipitate is dissolved in a liter of 0.02 *M* sodium cyanide (*p*H 6), and the solution is again made 0.6 saturated with ammonium sulfate. The drained precipitate is redissolved in 600 cc. of 0.02 *M* sodium cyanide. Then the enzyme is precipitated with 3 volumes of acetone. It is collected and dried *in vacuo*. The yield is 5 grams of almost colorless enzyme (120).

Bromelin and papain are similarly affected by a series of reagents (120). All the SH compounds do not produce the same degree of activation. Cysteine, cyanide, and hydrogen sulfide probably activate by the same mechanism, however. CN- is oxidized to CNO-, and H_2S to S. Na_2S and H_2S are less powerful activators than cysteine and cyanide. They are more destructive. Oxidized sulf-

hydryl groups appear to be less readily reduced by sulfides. It should be noted, however, that some proteinases of papain may not contain sulfhydryl groups but may have other oxidizable radicals.

The *p*H Optima. Greenberg and Winnick (121) found the optimum *p*H of papain and the optimum of proteinases of the weeds *Asclepias mexicana, Asclepias speciosa,* and *Solanum elaeagnifolium* to be at 7.5 when ovalbumin was the substrate. Bromelin showed an optimum at *p*H 7.0 (Fig. 14). With different substrates, different optima and *p*H activity curves may be expected. For instance, Willstätter and Grassman (122) found the optimum *p*H for gelatin hydrolysis by papain at 5.0.

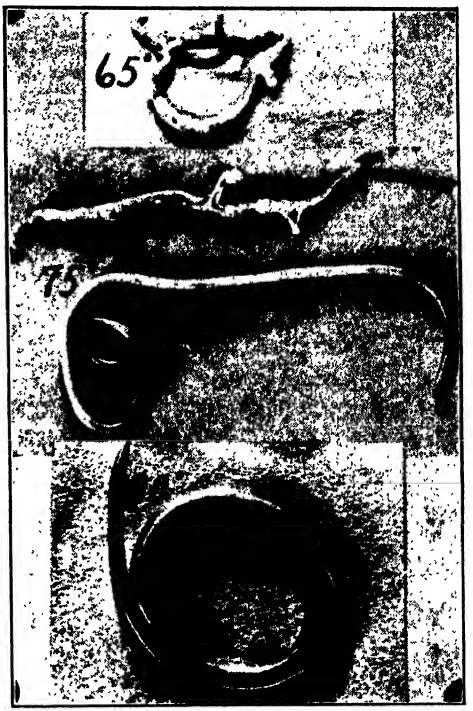


FIG. 15. A comparison of *Ascaris*, after 2 hours' incubation in 0.2 per cent solutions of the active ficin, which had been heated to temperatures of 65°, 70°, 75°, and 80° C for 5 minutes. A temperature of 75° C. for 5 minutes destroys the digestive action of the material on *Ascaris*.

FICIN

This papainase is present in various concentrations in the latex of certain fig trees. The latex is widely used as a general anthelmintic by natives of Central and South America. It is especially effective against *Trichuris trichiura.*

Robbins and Lamson (123) examined the latex of sixteen species of the genus *Ficus.* Only in two was the enzyme concentration high enough to digest *Ascaris* in a 2.2 per cent solution of the latex. The action was tested by incubating fresh worms (from the pig) at 35° C. in 100 cc. of Ringer's solution containing 2 cc. of the sap.

Latex from four genera of the family *Moraceae* showed only one-fifth of the proteolytic activity displayed by *Ficus carica* L. Sap from six South American trees had high proteolytic activity.

Robbins and Lamson's results are summarized in Table XVIII. Figure 15 shows the action of ficin on *Ascaris* (124).

TABLE XVIII

ACTION OF SAP FROM CUBA, ALABAMA, AND SOUTH AMERICA UPON GELATIN AND *Ascaris*

	Family	Genus and Species	Increase in Amino N by Action of 1 cc. of Sap on 100 cc. of 2 Per Cent Gelatin Solution for 24 Hours at 35° C.	Action on *Ascaris*
			mg. N_2	
1. From Cuba (classification by Mr. Robert M. Grey, botanist at Harvard Botanical Gardens, Soledad, Cuba)	Moraceae	*Artocarpus foret*	0.0	
		Brosimum alicastrum	7.5	
		Broussonetia papyrifera	10.0	None in 24 hr.
		Ficus aurea	0.0	
		" *benghalensis*	0.0	" " 24 "
		" *benjamini*	9.0	" " 24 "
		" *brevifolia*	2.5	
		" *carpensis*	0.0	
		" *crassinervia*	1.2	
		" *elastica*	0.0	
		" *glabrella*	1.2	
		" *glamarata*	0.0	
		" *nitida*	10.0	" " 24 "
		" *nota*	2.5	
		" *religioso*	0.0	
		" *spragueana*	1.2	
		" *vogelii*	0.0	
		" unknown	24.0	Digested in 24 hr.
		Morus nigra	2.5	
2. From Alabama		*Ficus carica*, L.	65	" " 1½ "
3. From South America (local names)		Higueron hembra	60	" " 1½ "
		Ficus glabrata	52	" " 2 "
		Higueron liso	50	" " 2½ "
		" rojo	45	" " 2½ "
		" " hembra	42	" " 6 "
		" milagroso	0	No digestion in 12 hr.

Purification of Sap. The active principle of the latex is precipitated by the addition of 3 volumes of acetone or 5 volumes of alcohol. The precipitate may be dissolved in water, reprecipitated with acetone, washed with acetone, and dried *in vacuo*. One hundred cubic centimeters of latex yields 11 to 12 grams of a light yellowish powder.

Robbins showed that the enzyme precipitate ficin digests *Ascaris* in a dilution of 0.1 per cent in 1½ to 2 hours, and that enzyme activity and the *Ascaris-liquefying* principle are the same substance.

Berger and Asenjo (125) have found that fresh pineapple juice digests *Ascaris lumbricoides* and *Macracanthorynchus hirundinaceus*, obtained from hogs' intestines, at the end of 24 hours. This indicates that there is some scientific basis for the use in some countries of pineapple juice as an anthelmintic.

Crystalline Ficin. Walti (126) described a very convenient method for the crystallization of ficin. The clarified latex is adjusted to *p*H 5 with normal sodium hydroxide and placed in a refrigerator at about 5° C. for several weeks. The crystals which precipitate are dissolved in approximately 0.02 *N* hydrochloric acid and filtered through a filter cell if necessary. On neutralization to *p*H 5 crystals appear. The crystalline enzyme was found to be a protein. Activity to hydrolyze gelatin or benzoylglycylamide disappeared upon treatment with phenylhydrazine, and reappeared with the addition of cysteine. The ficin was also inactivated by hydrogen peroxide and by iodine. Thus, this enzyme is a papainase.

Walti confirmed the findings of Robbins, and of Robbins and Lamson, concerning the identity of the proteolytic power with the anthelmintic principle.

PROTEOLYTIC ENZYMES OF MILKWEEDS AND THEIR RELATION TO OTHER PROTEASES

Asclepain. Greenberg and Winnick (120, 121, 127) have published a series of interesting papers concerning the properties of two milkweed proteinases. One of the proteinases of the milkweed, *Asclepias mexicana*, they named " asclepain *m*." The second milkweed enzyme, that of *Asclepias speciosa*, they called " asclepain *s*. " These papers also contain important information on the plant proteinases bromelin, papain, and solanain, the protease of the horsenettle, *Solanum eleagnifolium*.

Bromelin, asclepain *m*, and asclepain *s* resemble papain in their dependence on sulfhydryl groups. Solanain, however, is not affected by oxidizing or reducing agents. This enzyme is not a papainlike proteinase. Papain probably contains essential phenolic and sulf-

hydryl groups. *p*H activity curves, as obtained by Greenberg and Winnick, are given in Fig. 14 for five proteinases.

These investigators also determined the Michaelis constants for papain, bromelin, asclepain *m*, asclepain *s*, and solanain. In every case, the enzyme-substrate intermediary compound contained 1 molecule each of enzyme and protein. The heat inactivation of asclepain *m* and solanain followed a first-order reaction course. These enzymes had high critical thermal increments, similar to papain and bromelin.

The Proteolic Enzymes of the Leaves of the Milkweed *Asclepias syriaca*

Tauber and Laufer (128) investigated the proteolytic enzymes of the leaves of a species of milkweed, *Asclepias syriaca*, which grows in abundance in the State of New York, sometimes reaching a height of 5 to 6 feet. They found that this plant contains at least one specific proteinase, which after dialysis becomes inactive and is greatly activated by sulfite. It also contains at least one polypeptidase and a milk-clotting enzyme (chymase). The proteinase is not identical with the other two active principles. The milk-clotting enzyme resembles chymotrypsin and the chymase of papain, which also have only weak proteolytic activity.

Preparation of Extract from Leaves and Its Properties. Four hundred forty grams of milkweed leaves are cut into small pieces and mixed with 220 cc. of an activator solution containing 1 gram of sodium citrate, 0.4 gram of citric acid, and 2 grams of sodium bisulfite per liter. The mixture is twice passed through a meat chopper. Thereafter, the liquid is removed by straining through a cloth, and the solid particles are separated by centrifuging one hundred seventy cubic centimeters of clear green extract, of *p*H 5.90, is obtained. Preliminary experiments have shown that extraction with the activator solution yields an extract of higher proteolytic activity. An aliquot of the extract is kept for 24 hours at 5° C. In the cold, the clear green extract separates into a green precipitate and a clear, light-colored solution. An aliquot of the mixture is tested. The precipitate is centrifuged off, and both supernatant and precipitate are tested. The greenish precipitate appears to be a chlorophyll-protein complex. The mixture and precipitate showed high activity on gelatin, but the supernatant was almost completely inactive, indicating that all the proteinase activity is present in the precipitate. Milk-clotting tests show that 75 per cent of the milk-clotting enzyme is in the supernatant. This indicates that a separate enzyme is involved.

The Optimum *p*H. The optimum *p*H of the proteinase, using gelatin as the substrate in citrate buffer, is at 5.45. The enzyme, however, is quite active between 5.45 and 8.45. With casein as the substrate, the proteinase shows a flat optimum between *p*H 7 and 9.

Separation of Papainaselike Proteinase (Asclepain *sy*) from Polypeptidase. Forty cubic centimeters of the original extract of milkweed leaves was dialyzed in a cellophane tubing against a solution containing 100 grams of dextrin in 700 cc. of water (129) at 5° C. for 24 hours. After dialysis, the volume of the milkweed extract decreased to 8 cc. On dialysis, a green precipitate formed. This was mixed with the supernatant, and the mixture was tested, using gelatin as a substrate. It was almost inactive. An aliquot of the dialyzed mixture was centrifuged. Both precipitate and supernatant were only very slightly active. To an aliquot of the dialyzed mixture, a small amount of sodium bisulfite was added. This sample became highly active. The dialyzed inactive precipitate also became active on the addition of bisulfite, but the supernatant of the dialyzed mixture did not (Table XIX). When the same type of tests were applied using

TABLE XIX

FRACTIONATION AND REVERSIBLE INACTIVATION OF THE *A. syriaca* PROTEINASE MIXTURE BY DIALYSIS (128)

Experiment No.		Volume of Enzyme Solution, cc.	Cubic Centimeters of *N*/20 KOH per 2 cc. Aliquot of Digest
1	Original (whole) extract	1	1.08
2	Dialyzed extract, mixed	0.4	0.28
3	Green precipitate	0.4	0.09
4	Supernatant fluid	0.4	0.06
5	Dialyzed (whole) extract, plus 5 milligrams sodium bisulfite	0.4	1.11
6	Green precipitate, plus 5 milligrams sodium bisulfite	0.5	0.75
7	Supernatant fluid, plus 5 milligrams sodium bisulfite	0.4	0.10

The digest contained 6 per cent gelatin in *M*/15 citrate buffer of *p*H 5.45 in total volume of 20 cc. Hydrolysis was tested after 24 hours at 37° C. Toluene was used as the antiseptic.

dl-leucylglycylglycine as the substrate, almost all the activity was found to be in the supernatant (Table XX). Thus, it is obvious that a separation of the two activities, proteinase and polypeptidase, took place. The polypeptidase action did not require bisulfite activa-

tion. The dialyzing procedure resulted in a considerable concentration of the enzymes. It is suggested that the name asclepain *sy* be given to the proteinase, that the milk-clotting enzyme be called chymoasclepain, and that the peptidase be called " asclepain peptidase."

TABLE XX

A. syriaca POLYPEPTIDASE ACTIVITY IN DIALYZED FRACTIONS (128)

Experiment No.		Cubic Centimeters of *N*/20 KOH per 5 cc. Digest
1	Dialyzed extract	2.34
2	Green precipitate	0.38
3	Supernatant fluid	2.30

The digests contained a *M*/15 concentration of *dl*-leucylglycylglycine adjusted to *p*H 7.0 with sodium hydroxide, 2 cc. *M*/15 phosphate buffer, and 0.5 cc. of dialyzed enzyme solution, its precipitate or supernatant. The total volume was 5 cc. The temperature was 37° C. The incubation time was 24 hours. Toluene was the antiseptic. Here, no sulfite was added to any of the samples.

Crystalline Asclepain. Carpenter and Lovelace (130) published a very simple method for the crystallization of asclepain from the press juice of the roots of the milkweed *Asclepias syriaca.* Their method consists in salting out the enzyme with half-saturated ammonium sulfate at 5° C. The proteinase crystallizes in yellow, microscopic plates, when its solution in phosphate buffer is placed in a dialyzing tube attached to a stirrer and suspended in a saturated solution of ammonium sulfate. The enzyme is activated by the usual reducing agents, and it has an isoelectric point at *p*H 3.11. Whether this preparation has milk-clotting power or peptidase activity is not mentioned. Both the genus and species of the milkweed employed by Carpenter and Lovelace are misspelled. The correct spelling is *Asclepias syriaca.* The error has been repeated in a recent review (131).

MEXICAIN

Castañeda and coworkers (132) have identified a new papainase in *Pileus mexicanus* and named it mexicain. This enzyme is present in the latex of the fruit and leaves of the plant. *Pileus mexicanus* is an arboreous plant about 8 meters high, a member of the family of Caricaceae. It grows wild and abundantly in the tropical regions of Mexico (states of Morelos, Guerrero, Colima, Campeche, and Yucatan) and is known as " cuaguayote. " The latex of the fruit is collected like that of the papaya fruit — longitudinal incisions are made in the

fruit. It clots rapidly and becomes yellow. The vacuum-dried (45° C.) latex may be readily pulverized to a white powder.

In vitro, mexicain shows high anthelmintic power. It clots milk and digests proteins slightly faster than papain. The fresh latex and dry-weight ratio is 30 per cent for *Pileus mexicanus* and only 20 per cent for papain.

Crystalline Mexicain. More recently, Castañeda-Agulló, Hernández, Loaeza, and Salazar (133) obtained mexicain in crystalline form by the following procedure:

Two volumes of water is added to 1 volume of fresh latex. The *p*H is adjusted to 7.5 with 0.5 *N* sodium hydroxide, and the solution is chilled at 5° C. for 24 hours. The solution is filtered through Hyflo Super-Cel, the *p*H is adjusted to 5.5 with 0.05 *N* hydrochloric acid, and the opalescent solution is chilled to 5°. After 24 hours crystals form. They are protein in nature and homogeneous, and they have a proteolytic activity and milk-clotting power 4 to 5 times that of vacuum-dried latex. The crystalline enzyme is very soluble. A solution at *p*H 5.8 may be kept at room temperature without losing its activity. Sodium cyanide and cysteine are not required for activation. A latex sample that was a year old yielded identical crystals with those obtained from fresh latex, but their activity was lower.

The Proteinase of the Osage Orange

Large crops of osage oranges are produced annually in the United States on female osage orange (*Maclura pomifera* Raf.) trees. The tree is naturally distributed in southeastern Oklahoma and eastern Texas and is widely planted in other states for hedges and for ornament. Tauber and Laufer (134) found that the osage orange contains a powerful proteolytic enzyme system that has not yet been described in the literature.

Preparation of the Proteinase. One green osage orange weighing 670 grams is cut into several parts. The exuding latex is collected in a small beaker, and the cut-up fruit is finely ground in a meat chopper and pressed with the aid of a hydraulic press; 180 cc. of press juice is obtained, or 26 cc. per 100 grams of fruit. To 90 cc. of centrifuged juice, 180 cc. of 95 per cent ethyl alcohol is added. The precipitate is centrifuged off and dried in vacuum at room temperature; 1.3 grams of yellowish-green powder is obtained, or 0.19 per cent on basis of the green fruit.

The latex is very similar in appearance to the latex of the papaya

fruit but contains a large amount of gummy substance. It is dried in vacuum and then twice extracted with 15-cc. portions of chloroform. The degummed latex is dried in vacuum. One-half gram of an almost white powder is obtained, or 0.08 per cent on the basis of green fruit. The quantity of press juice obtained varied with the stage of development of the osage orange. Green fruits contain more press juice than those nearing the stage of ripeness.

General Properties of the Osage Proteinase. The osage proteinase is not activated by sodium sulfite, indicating that it is not a papainase. This proteinase is not quite as active as fresh papaya latex, and the osage latex clots milk less rapidly than papain. The optimum *p*H of the osage proteinase, with gelatin as the substrate, is at 6.45. Osage proteinase is still considerably active at *p*H 8.45 but much less so at *p*H 4.32. Thus, its *p*H activity curve is also somewhat different from that of papain. Tauber and Laufer propose the name " pomiferain " for this proteinase.

Papainase of Soybeans

Tauber and Laufer (135) prepared a fairly active proteinase by extraction of defatted soybean with 4 volumes of 30 to 50 per cent glycerol for 24 hours at 30° C. This enzyme was activated by sodium sulfite, indicating that it is a papainaselike enzyme. The optimum *p*H of this proteinase is at 6.78 with casein and at 7.20 with gelatin as the substrate using sodium citrate as the buffer. However, the decrease is very slight in the alkaline range up to *p*H 9.00. The proteinase increases considerably during germination. In Table XXI

TABLE XXI

Effect of Germination on Soybean Protease
(*p*H 7.0 — Citrate buffer)

Experiment No.	Type of Material	Proteolytic Activity Expressed in Cubic Centimeters of *N*/20 KOH per 10 Grams — Soybean Variety Y	I	C
1	Soybean, original	1.63	1.37	1.21
2	Soybean, germinated 6 days	4.03	2.95	2.43
3	Soybean, germinated 12 days	6.86	5.85	5.39

the values for three varieties of soybeans are given as obtained by using 50 per cent glycerol extraction (136) and gelatin as the substrate. The proteinase of soybean was named "soyin. "

Tabernamontanain

Jaffe (137) has shown that a papainlike proteinase is present in the juice, bark, and green fruit of the Venezuelan shrub *Tabernamontana grandiflora*. He named the enzyme " tabernamontanain. " The proteinase was obtained by acetone precipitation, and in this crude form it was many times more active than crude papain as calculated by its milk-clotting power and its activity on gelatin or peptone. Plant material collected in April was activated by cyanide and cysteine; that collected in July did not become more active by the reducing agents. It was concluded that the first-obtained material contained no natural activators. Like most papainases, tabernamontanain digests living intestinal parasites.

Arachain

Irwing and Fontaine (138) prepared, in a purified state, the proteinase arachain from peanut meal. Papain activators had no effect on the activity of the enzyme when benzoyl-*l*-argininamide was the substrate, and the activity was greatest at *p*H 6 to 7.5.

Euphorbain

This name was suggested by Castañeda and collaborators (139) for the proteolytic principle of the latex of *Euphorbia cerifera*. This proteinase was activated by cysteine and cyanide and inactivated by hydrogen peroxide. A similar proteinase has been prepared from the latex of the weed, *Euphorbia lathyris* by Ellis and Lennox (140). The enzyme was prepared by extraction of the acetone-precipitated latex. This enzyme was as active as commercial trypsin or papain. It was also named " euphorbain."

Many of the so-called " proteinases " of plants require further purification and testing of their specificity. In Table XXII, some properties of a series of plant proteinases are shown.

PROTEOLYTIC ENZYMES OF YEAST

Dernby (143) reported that yeast contains a proteolytic enzyme having an optimum at *p*H 4.5, a trypsinlike enzyme with an optimum at *p*H 7.0, and a peptidase. Contrary to these findings, Willstätter and his associates (144) and Grassmann and his associates (145) reported that yeast contains only one proteinase with an optimum *p*H at 5.0, a polypeptidase, and a dipeptidase acting at *p*H 7 to 7.8. The

TABLE

PROPERTIES OF SOME

Name	Botanical Origin		
	Common Name of Plant	Genus and Species	Source of Material
Papain	Papaya	*Carica papaya*	Latex of green fruit
Ficin	Fig	*Ficus carica, glabrata, doliaria*	Latex
Bromelin	Pineapple	*Anana sativa*	Fruit, leaves
Pinguinain	Maya	*Bromelia pinguin*	Fruit
Asclepain	Milkweed	*Asclepias speciosa, mexicana, syriaca*	Latex, roots, leaves
Mexicain	Cuaguayote	*Pileus mexicanus*	Leaves, fruit
Tabernamontanian		*Tabernamontana grandiflora*	Sap, fruit
Euphorbain	Caper Spurge	*Euphorbia cerifera, lathyris*	Latex
Solanain	Horsenettle	*Solanum eleagnifolium*	Fruit
Hurain	Jabillo	*Hura crepitans*	Sap
Soyin	Soya beans	*Soja hispidus*	Germinated beans
Pomiferain	Osage orange	*Maclura pomifera* Raf.	Fruit
Arachain	Peanut	*Arachis hypogea*	Seed

proteinase requires the presence of hydrocyanic acid or hydrogen sulfide for full activity. The yeast polypeptidase, however, is strongly inhibited by these reagents (146).

Hecht and Civin (147), using ether-plasmolyzed yeast autolyzate, have reported that yeast contains a pepsinlike enzyme acting at *p*H 1.8, and a trypsinlike enzyme acting at *p*H 7.8. The trypsin concentration was found to be much less than that of pepsin. The substrate was hemoglobin, and the tyrosine liberated was used as a measure of proteolytic activity. These investigators found that, of a series of organic solvents used for autolysis of yeast, ether gave the best results. Other reagents such as are usually employed to destroy the yeast cells (ethyl

XXII

PLANT PROTEINASES

Stability in Acid or Alkali	Response to Cysteine and HCN	*p*H of Optimum Protein Digestion*	References
Unstable below *p*H 2.5 and above 12.0	+	7–7.5	120
Stable at *p*H 2	+	7	117
Stable at *p*H 2–3	+	6–7	119
	+	3 (milk)	141
Unstable in acid or alkali	+	7–7.5 5.45 (gelatin)	120 128
Stable at *p*H 5.8	–		132
	Depends on season	5.6 (gelatin)	137
	+	6	139
Stable in dilute alkali	–	8.5	141
Stable in dilute alkali	–	8 (gelatin)	149
	+	6.78 (gelatin)	135
	–	6.45 (gelatin)	134
	–	6†–7.5	138

* Casein, ovalbumin, and denaturated hemoglobin.
† Benzoyl-*l*-argininamide.

acetate, toluene, and chloroform) produced values of pepsin from zero to two-thirds of those obtained with ether.

Hecht and Civin employed the following method for the autolysis of yeast. To 250 grams of yeast (Fleischmann), 50 cc. of ether was added, and the mixture was allowed to stand at room temperature for 18 hours. The mixture was centrifuged in a Sharples centrifuge, and the heavier liquid phase was used. Ninety cubic centimeters of liquid was obtained. This solution contained the total peptic activity that was demonstrable in the yeast. The yeast solution retained

full activity for 2 hours when kept in ice water. This same yeast extract contained all the tryptic activity that could be demonstrated in the yeast.

The results of Hecht and Civin are extremely interesting, since the presence of a pepsinlike enzyme with optimum *p*H at 1.8 had not previously been described in yeast.

Macrae (148) studied the liberation of the proteolytic enzyme of English top yeast and Dutch bakers' yeast. He used ethyl acetate as the plasmolyzing agent, added water, and adjusted the *p*H close to 7.0 with dilute ammonium hydroxide during autolysis. These yeasts showed differences in the liberation of the proteolytic enzymes when compared with results obtained with German beer yeasts. Macrae found that the proteolytic enzymes could be precipitated from the clear autolyzate with acetone. Seventy-five cubic centimeters of autolyzate treated with 150 cc. of acetone yielded 25 mg. of dry enzymes which had good proteolytic activity when gelatin of *p*H 5.0 was used in the substrate. In many respects the yeast proteinases resemble the papainlike enzymes.

PROTEOLYTIC ENZYMES OF MOLDS

Berger, Johnson, and Peterson (149) examined the proteolytic enzyme systems of 30 common molds. These molds contain at least one proteinase and at least five peptidases in widely varying amounts, great variation being found between individual members of the same genus. The proteolytic activity varies with the incubation period. When the molds were grown on an organic medium, their proteolytic activity was found to be higher than when a synthetic medium was employed. The optimum *p*H for the proteinase of the molds *Aspergillus oryzae, A. alliaceus,* and *A. wentii,* with gelatin as substrate, was about 7.0.

PROTEOLYTIC ENZYMES OF BACTERIA

Maschmann (150, 151), by using young (3- to 12-hour) cultures of aerobic and of anaerobic bacteria, obtained four different proteinases. All four have an optimum *p*H at 7. Three do not require cysteine, being fully active under aerobic conditions; the fourth is an anaerobic proteinase and requires cysteine *in vitro.* The purification procedure consists in the precipitation of the proteinases from the bacterial filtrates by ammonium sulfate, methyl alcohol, or acetone. The precipitates are further purified by dialysis against mild alkaline buffer. By this method, the proteinases are concentrated 1000 to 2000 times.

PROTEOLYSIS UNDER VARIOUS CONDITIONS

Partial Hydrolysis Products of Proteolysis. Winnick (117), by using casein as the substrate and by digesting it separately with pepsin, trypsin, chymotrypsin, ficin, papain, and carboxypeptidase, made a study of the hydrolytic products. After digestion, the average non-protein molecules contained from five to seven amino acid residues, with 1.5 to 4.5 per cent of the total nitrogen in the form of free amino acids. Inorganic electrolytes were removed from the digests by electrodialysis, and nitrogen, specific rotation, and average molecular weight were determined. Carboxypeptidase reacted further with the protease digests. This was interpreted as a splitting of free amino acids from the ends of polypeptide chains. The initial products from short periods of protein digestion were compared with those from prolonged protease action, and the specific rotations and ratios of amino to total nitrogen were found not to differ significantly. These findings are in agreement with the view that relatively few protein molecules are split rapidly in each time interval in enzymic hydrolysis.

Hydrolysis of Soybean Oil Meal by Proteolytic Enzymes. Evans (152) found that maximum yield of amino nitrogen (37 per cent of total nitrogen present) in soybean oil meal was obtained from autoclaved meal by successive digestions with commercial pepsin, trypsin, and "erepsin." Optimum results were obtained on mild autoclaving (120° C. for 30 minutes). The article contains a detailed description of the composition of the various digests and of the other experimental conditions.

Proteolysis in the Presence of Ethyl Alcohol. Risley and associates (153) published an interesting study concerning the tryptic digestion of bovine serum and other proteins in the presence of ethyl alcohol. They found that 20 per cent solutions of dried bovine serum were resistant to the action of trypsin and that this resistance could be overcome by the use of digestion mixtures containing 20 per cent ethyl alcohol. It is suggested that the ethyl alcohol may inhibit the action of antiproteases present in the serum. Tryptic digestion of serum proceeded smoothly in concentrations of 10 to 30 per cent ethyl alcohol for as long as 2 weeks, at which time approximately 80 per cent of the potential amino groups were liberated. Concentrations of ethyl alcohol higher than 30 per cent inhibited tryptic digestion of serum, but appreciable hydrolysis occurred even in 60 per cent ethyl alcohol. Efficient digestion of other proteins (casein, lactalbumin, soybean protein, egg albumin) was obtained in 20 per cent ethyl alcohol. Digestion at 60°, instead of the more usual 37°, is less rapid and is complete

when 20 per cent ethyl alcohol is present in the digestion mixture. Ethyl alcohol was found to be a useful reagent for preventing putrefaction during tryptic digestion.

The Effect of Papain on Proteins. Papain digests most proteins to proteoses, peptones, and peptides. Hoover and Kokes (154) studied the extent of digestion of casein by activated papain at *p*H 7.00, *p*H 5.00, and *p*H 2.50. At *p*H 7.00 and *p*H 2.5, about 25 per cent of the peptide bonds were hydrolyzed, resulting in the liberation of 14 per cent of the amino acids. At *p*H 5.00 (the optimum for the initial rate of hydrolysis), about 50 per cent of the peptide bonds were split and 30 per cent of the amino acids were set free. At first, peptides averaging 4 to 6 units were formed. This was followed by the release of amino acids without much change in the average length of the peptides present.

It is very important from a practical standpoint that sulfhydryl groups present in the protein substrates are liberated during hydrolysis, as during the digestion of meat. These groups will change an almost inactive papain to a highly active product (155).

TABLE XXIII

AMINO ACIDS ISOLATED FROM FIBRIN-PAPAIN-HCN DIGEST

Amino Acid	Amount Isolated per cent	Amount In Fibrin per cent
Tyrosine	3.2	3.5
Tryptophane	1.6	5.0
"Leucines"	6.7	15.0
Leucine	3.1	
Isoleucine	0.7	
Phenylalanine	0.3	2.5

Extensive Digestion of Protein by Papain. It has frequently been reported that amino acids and synthetic peptides are liberated from proteins by the action of proteinases. On this basis, it may be expected that an exhaustive digestion of proteins by papain should yield large quantities of amino acids. Bergmann and Niemann (156), using the butyl alcohol method of Dakin, isolated from a cattle fibrin papain digest all the tyrosine, large quantities of the phenylalanine, tryptophane, leucine, and isoleucine present in the fibrin. In this digest, 64 per cent of the peptide linkages were split. The following conditions were employed by Bergmann and Niemann.

Preparation of Digest: 1172 grams of cattle fibrin, 1635 cc. of an aqueous solution containing 27 grams of papain and 6.5 grams of HCN,

1635 cc. of 0.2 *M* disodium phosphate, 6500 cc. of water, and 20 cc. of toluene were incubated at 37° C. for 20 days. The digest, which had a *p*H of 6.4, was brought to 80° and filtered, and the residue was several times extracted with hot water. The concentrated washings were combined with the original filtrate and the solution was made up to 14 liters. The amino acid yields are shown in Table XXIII.

METHODS FOR THE DETERMINATION OF PROTEOLYTIC ACTIVITY

The Estimation of Proteolytic Activity of Papain (157)

Preparation of Sample. (*a*) *Unactivated.* If the enzyme preparation is a solid, it is finely divided by grinding to smooth paste in small mortar with a little freshly boiled cold water. Then the enzyme is suspended in cold boiled water in the proportion of 10 milligrams of original preparation per cubic centimeter. After 5 to 10 minutes, the suspension is centrifuged and the sediment discarded.

(*b*) *Activated.* The procedure is carried out as directed in (*a*), but half-saturated hydrogen sulfide water is used instead of boiled water. After centrifuging, the enzyme solution is incubated at 40° C. for 1 hour to complete activation.

Reagents. (*a*) *Casein Solutions.* A 6 per cent solution of Hammarsten's casein is made by rubbing 60 grams with a little water in a mortar and gradually adding 60 cc. of 1 *N* sodium hydroxide and water until the volume totals 1 liter. The viscous solution is heated for 30 minutes in a bath of boiling water, cooled, and filtered (with glass wool), if necessary.

(*b*) *Buffer Solution.* A 0.2 *M* monosodium citrate solution is prepared by partial neutralization of citric acid with sodium hydroxide.

(*c*) *Titrating Solution.* One-tenth *N* alcoholic potassium hydroxide.

(*d*) *Indicator.* One per cent alcoholic solution of thymolphthalein.

Determination. Ten cubic centimeters of the casein solution and a small charge of 4-millimeter-diameter glass beads are placed in each of several 125-cc. glass-stoppered bottles, and bottles and contents are brought to 40° C. The desired volume of the prepared enzyme solution is added, but not more than 4 cc. If this quantity is insufficient (see later), a more concentrated solution of the enzyme is prepared. Immediately, exactly 3 cc. of the buffer solution (*p*H of system should then be 5.0, plus or minus 0.1) is added. The bottle is vigorously shaken for a few seconds and placed in a constant-temperature water bath at 40° C.

The mixture is incubated for 20 minutes at 40° C., counting time from addition of buffer; 1 cc. of the indicator is added, and titration is

begun with the titrating solution. As soon as a deep blue color appears, the bottle is shaken until the color is discharged or the precipitate is completely dissolved. (It is usually best to add the alkali in portions of about 0.5 cc.). When all precipitated casein has been brought into solution, the contents of the bottle are transferred to a 400- to 500-cc. flask and the bottle is rinsed 2 or 3 times with alcohol, a total of 25 cc. being used for this purpose. Enough alcoholic potassium hydroxide is added to restore blue color in the titration, then 175 cc. of boiling alcohol is added. More alcoholic potassium hydroxide is carefully added until a pale but distinct blue color persists in the solution.

A control titration is made exactly as described, immediately after the addition of the buffer and, therefore, without any incubation time. The difference between the titration of the undigested sample and that of the digested sample is a measure of the proteolytic activity of the enzyme.

Calculation of Proteinase Unit. For smaller quantities of enzyme, the extent of hydrolysis determined by the titration described is a straight-line function of the amounts of papain used. For accurate work, this straight line is determined by making several titrations with different quantities of enzyme. If the quantities of papain used are too large, the straight-line relationship will no longer hold; if they are too small, the determination will be inaccurate. Quantities of enzyme giving titration differences of 0.6 to 1.2 cc. of 0.1 *N* potassium hydroxide are recommended.

The unit of papain may be considered the quantity of enzyme that produces, under the conditions outlined, a titration difference of 1 cc. of 0.1 *N* potassium hydroxide determined either graphically or arithmetically. The value of the original preparation is then expressed in units per milligram, or as milligrams of the papain preparation necessary to make 1 unit (157).

OTHER METHODS FOR THE ESTIMATION OF PROTEOLYTIC ACTIVITY

The Formal Titration Method (158)

One cubic centimeter of enzyme solution of varying activity is added to a series of test tubes containing 5.0 cc. of standard protein solution [6 per cent gelatin solution (159), or 5 per cent casein solution of desired *p*H]. At various intervals during 1 hour an aliquot is removed and strong sodium hydroxide is added to the mixture. The concentration of the sodium hydroxide is prepared so as to require 5.0 cc. to

adjust the sample to faint pink to phenolphthalein. The indicator is a 0.5 per cent alcoholic solution. One drop of it is sufficient. Then 1.0 cc. of 40 per cent formaldehyde solution is added and the titration is concluded with $N/50$ sodium hydroxide. If the whole titration were carried out with $N/50$ sodium hydroxide, the volume required would be too large and the end point would be difficult to determine.

A blank titration is carried out with boiled enzyme solution added to the protein solution. The activity of the enzyme is determined from the time curves (65).

The Gross-Fuld Method (160)

This is a very simple and rapid procedure often used, in conjunction with other methods, for testing commercial enzyme products.

Reagents

1. *Casein Solution.* To 0.1 gram of casein, 5 cc. of $N/10$ NaOH and 25 cc. of distilled water are added. The mixture is brought to boiling, cooled, and adjusted to the desired *p*H with $N/10$ HCl. Then the solution is diluted to 100 cc.

2. *Alcoholic Acetic Acid Solution.* This is a mixture of 1 part acetic acid, 49 parts water, and 50 parts 96 per cent alcohol.

Procedure. Increasing amounts of the enzyme solution are placed in ten test tubes. Then the volume is made up to 4 cc. with distilled water. To each sample of enzyme solution, 2 cc. of the casein solution is added. All solutions should be at 38° C. After mixing, the test tubes are incubated for 1 hour at 38° C. Then the digests are cooled, and 6 drops of the alcoholic acetic acid solution is added to each sample. The tube in which clarification begins is the basis for calculating proteolytic activity.

Grob (161) modified Wunderly's method for the estimation of proteolytic activity. Here the undigested casein is treated with 20 per cent sulfosalicylic acid and the turbidity of the resulting suspension of undigested casein is determined with the aid of a photoelectric nephelometer. The value for the digested casein is subtracted from the undigested concentration. By employing the law of Schütz, the proteolytic activity is calculated. This paper also contains a comparison of the activities of crude trypsin, crystalline trypsin, crude papain, and leucoprotease and the effect of a series of activators and inhibitors on these enzymes.

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CHAPTER VII

INTRODUCTION TO OXIDIZING ENZYMES AND THEIR CLASSIFICATION

All functions of the living cell require energy. The cell obtains the energy from foods by two different methods. One is burning or oxidation; the other, fragmentation or fermentation. Oxidation requires atmospheric oxygen in the more complex organisms; fermentation does not. Fermentation is a simpler process than oxidation, but about ten times as much energy is liberated by oxidation. Fermentation can maintain only the simplest kind of life. A human being cannot live without oxygen for more than about three minutes. Molecular oxygen cannot oxidize foods to any degree. The oxidations in nature are carried out by oxidation-reduction systems, so called because there is no oxidation without simultaneous reduction. However, in the energy-producing mechanisms of higher forms of life, fermentation processes (glycolysis) do play an important role (skeletal muscle). The relationship, as will be seen, appears to be quite close. Pasteur discovered that, if oxidation is suppressed by lack of oxygen, fermentation commences. If oxidation is permitted to take place again, fermentation discontinues. This phenomenon is known as the "Pasteur reaction."

Our knowledge of the mechanism of biological oxidation is still incomplete. However, we know today that biological oxidation, or respiration, as it is called, represents a definite chain of reactions. Enzyme systems have been isolated from various tissues which activate the stable atmospheric (molecular) oxygen, and other enzyme systems have been found which activate the hydrogen of foods. Flavoproteins, several cytochromes and cytochrome oxidase, the pyridine nucleotides, the diphosphothiamin proteids and other enzymes, coenzymes and intermediates (hydrogen carriers) take part in these "oxidations." These enzyme systems, called oxidases for convenience, are the energy producers. They burn foods to carbon dioxide and water, involving a series of reactions, transferring electrons from the substrate to molecular oxygen by a stepwise process. It may be readily seen that such processes require a variety of complex oxidases. There are some oxidases that oxidize their substrates, and they, in turn, are oxidized

by molecular oxygen. Most oxidases require several oxidation-reduction systems in order to transfer electrons from their substrate to molecular oxygen.

Oxidases act quite rapidly on their substrates at body temperature. Some of these substances are among the most stable compounds known to the organic chemist. For instance, the purine derivatives are not readily oxidized by even concentrated nitric acid and potassium permanganate. The recrystallization of uric acid from concentrated sulfuric acid is a well-known laboratory experiment. Many amino acids are quite stable in strongly acid or alkaline medium.

CLASSIFICATION

The following classification is based on the functions and the chemical composition of the oxidizing enzymes: A, oxidases, B, dehydrogenases, C, other oxidases. These groups may be subdivided:

A. Oxidases

Group I. These are the iron-porphyrin enzymes. They contain hematin, or a substance closely related to hematin, as their prosthetic group. Examples: catalase, peroxidase, verdo peroxidase, cytochrome oxidase, and cytochrome peroxidase.

Group II. These are the copper-containing enzymes: ascorbic acid oxidase, tyrosinase, and laccase.

B. Dehydrogenases

These oxidases have been divided into three distinct groups, depending on the type of hydrogen acceptor through which they react with molecular oxygen (1). The dehydrogenases remove two hydrogen atoms (or two protons and two electrons) from their substrates.

Group I. Dehydrogenases requiring codehydrogenase I and II belong here. Through these coenzymes, which act as hydrogen transmitters or carriers, they react with certain flavoproteins, which in turn react with cytochrome c and molecular oxygen. These dehydrogenases do not function directly with flavoproteins, cytochrome c or molecular oxygen, or with artificial carriers such as methylene blue. Examples: alcohol, lactic, glycerophosphoric, β-hydroxybutyric, and many other dehydrogenases.

Group II. This group contains flavoproteins, which do not require the addition of coenzymes, the prosthetic flavin group functioning as

the acceptor. Some of these enzymes (aerobic dehydrogenases) react directly with molecular oxygen; for others (diaphorases), the mediation of the cytochrome system is necessary. Examples: Old yellow enzyme, liver aldehyde oxidase, *l*-amino acid oxidase, and other oxidases.

Group III. These dehydrogenases are cytochrome-linked or the cytochrome-reducing dehydrogenases; so named because they readily reduce cytochrome c. However, this may not be a direct reaction, since these enzymes are impure. They are difficult to purify, since they are firmly bound to the insoluble portion of the cell. They do not require coenzymes, and they readily reduce methylene blue in their absence. Examples: succinic, glycerophosphoric, formic, and other dehydrogenases.

C. Other Oxidases

Other oxidases not belonging to the above groups: Luciferase, uricase, tyramine oxidase, etc.

For the study of oxidases, Warburg's manometric procedure and the Thunberg technique may be used. These methods have been ably described in an appropriate manual by Umbreit, Burris, Stauffer, and their collaborators (2).

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CHAPTER VIII

THE IRON-PORPHYRIN ENZYMES

These enzyme systems contain hematin or a substance closely related to hematin. The hematin is bound to various proteins to form the specific, active enzymes. This group of enzymes includes catalase, peroxidase, verdoperoxidase, cytochrome oxidase, and cytochrome peroxidase. Many other hematin proteins, such as hemoglobin and myoglobin, exist in nature. These substances have only very slight catalytic effects. Hemin has also a slight catalase activity.

CATALASE

Catalase is found in most cells. Horse liver and erythrocytes are the best sources. Catalase is not an oxidizing enzyme. It is now being classified with oxidases because it is chemically related to several oxidases and also because it takes part in respiration. It decomposes the cell poison, hydrogen peroxide, into water and oxygen.

Chemical Nature, Inhibitors, and Action

Catalase is an iron porphyrin compound. Sumner and associates (1) found that crystalline beef-liver catalase has a molecular weight of 225,000. The enzyme has an isoelectric point at *p*H 5.7 (2). The visible absorption bands are at 627, 536, and 502 μ. The band at 502 μ is difficult to see. Unlike other porphyrins, catalase is not reduced by dithionite. This enzyme may be kept for several weeks in an icechest provided that the solution is not too dilute. Concentrated solutions are almost black, whereas dilute solutions are yellow. Catalase is inhibited by hydrogen cyanide and by sulfhydryl compounds, such as Na_2S, NaSH, and *l*-cysteine, and by reduced glutathione (3). It is quite unstable in an acid medium (*p*H 3.0). Trypsin digests catalase rapidly.

Agner and Theorell (4) made the important observation that the hemin iron in horse liver and blood catalase is dissociably combined with a hydroxyl group which is necessary for catalase activity. The hydroxyl group can be displaced by anions with a resultant reduction in the activity of the catalase and a corresponding change in the

absorption spectrum of the enzyme. The hydroxyl group is apparently particularly susceptible of replacement by formate ions. The inhibitory effect of formations is 800 times stronger than that of acetate. The difference in the heat of ionization of the iron hydroxyl and iron formate compounds in catalase is about 12,400 calories.

Catalase resembles acid methemoglobin in its color, its absorption spectrum, and its property of combining reversibly into well-defined compounds with HCN, H_2S, HN_3, HF, NH_2OH, and NO. However, it differs from methemoglobin in three important ways: (*a*) the color and absorption spectrum of its derivative with azide (NaN_3); (*b*) the reaction with hydrogen peroxide is a violent one, during which no spectroscopic changes can be observed; (*c*) the stability of its trivalent iron, which is not reduced even by $Na_2S_2O_4$. In this last property, catalase is quite different from other hematin derivatives (5). Catalase combines reversibly with azide. Like that of free catalase, the hematin iron of azide catalase is trivalent. One molecule of azide combines with one atom of the catalase hematin iron. The azide catalase compound resembles free catalase in color, absorption spectrum, magnetic susceptibility, and resistance to reducing agents ($Na_2S_2O_4$). On addition of hydrogen peroxide to azide catalase, the color of the derivative turns from greenish brown to red, and its absorption band in the red is replaced by two bands at 587 and 559 μ. Keilin and Hartree (5) give a series of experimental facts showing that the peroxide-treated azide catalase contains iron in the divalent state. They conclude that the close relationship between the properties of azide catalase and the free catalase indicates that the catalytic decomposition of hydrogen peroxide by catalase involves a cyclical reduction and oxidation of its iron. In harmony with their work, Keilin and Hartree proposed the two following tentative equations for the decomposition of hydrogen peroxide by catalase:

$$4Fe^{+++} + 2H_2O_2 = 4Fe^{++} + 4H^+ + 2O_2 \qquad (1)$$

$$\frac{4Fe^{++} + O_2 + 4H^+ = 4Fe^{+++} + 2H_2O}{2H_2O_2 = 2H_2O_2 + O_2} \qquad (2)$$

Kinetics. Catalase is one of the most powerful enzymes. One molecule decomposes 2.6×10^6 molecules of hydrogen peroxide per minute at 0° C. The optimum *p*H of liver and erythrocyte catalase is very close to 7.0. Euler and Josephson (6) express the activity of a catalase preparation as follows:

$$\text{Cat. f.} \quad \frac{\text{reaction constant } k}{\text{grams of enzyme in 50 cc.}}$$

" Cat. f." indicates " Katalase Fähigkeit " or catalase activity (0.005 to 0.015 M H_2O_2, 0° $M/150$ phosphate buffer of 6.8); k is the constant of the monomolecular reaction and a measure of the relative concentration of the enzyme in the reaction mixture. Catalase action follows nearly a monomolecular reaction course. This is true, however, only for short reaction times, because some of the enzyme is gradually destroyed by the hydrogen peroxide. Sizer (7) studied the effect of temperature on crystalline catalase over the range from 2° to 68° C. Below 53° the catalase action increased with temperature, in accordance with the Arrhenius equation, the activation energy being 4200 calories per mole; the entropy of activation was —23 calories per degree per mole. Above the optimum temperature (53° C.), catalase action decreased in rate with the temperature, owing to increase in enzyme destruction with temperature. Similarities were found between the kinetics of catalase, peroxidase, and hemoglobin.

Function. Keilin and Hartree (8) have published a highly interesting study concerning the interaction of catalase with oxidases, which catalyze oxidation by molecular oxygen by reducing oxygen to hydrogen peroxide. Such enzymes are xanthine oxidase, uricase, tyraminase, *d*-amino acid oxidase, glucose oxidase (notatin of *Penicillium notatum*), and others. The total oxygen uptake of such systems should be double the quantity calculated for the substrate, but, in the presence of a small quantity of catalase, the uptake is only the theoretical amount, because the hydrogen peroxide is decomposed and oxygen is formed. When ethyl alcohol is added to such catalase-containing oxidizing systems, the oxygen uptake is doubled because the hydrogen peroxide that is formed in the primary reaction is used in the secondary reaction for the coupled oxidation of ethyl alcohol. The ethyl alcohol is oxidized by the catalase. Purified catalase also catalyzes the oxidation of methyl alcohol, ethylene glycol, and β-aminoethyl alcohol, and it slowly catalyzes propyl alcohol and isobutyl alcohol. With xanthine oxidase and aldehyde, purified catalase catalyzes the cyclic oxidation of ethyl alcohol and acetic acid, which progresses until all the alcohol is oxidized. In the oxidation of ethyl alcohol by catalase, the hydrogen peroxide of the primary reaction can be replaced by barium peroxide or cerium peroxide with proper care. The hydrogen peroxide of the primary reaction, however, is the best mechanism.

The authors conclude that the biological function of catalase is the catalysis of coupled oxidation by means of hydrogen peroxide produced in primary oxidations. The concentration of catalase in liver tissue is about 1000 times higher than that necessary for the decomposition

of hydrogen peroxide and of about the same order as is necessary for the catalysis of coupled oxidation. This shows why crude enzyme preparations containing traces of catalase give theoretical oxygen uptakes with their substrate and yet cannot catalyze the coupled oxidation of ethyl alcohol. Only a few compounds have been found to take part in this type of coupled oxidation. It strongly suggests, however, that other biologically important substances may also undergo *in vivo* oxidations of a similar nature.

Preparation of Crystalline Beef Liver Catalase (4, 9)

Beef liver is ground 5 to 10 times, and 300-gram portions are mixed with 400-cc. portions of 35 per cent dioxane. After being extracted for 1 hour at room temperature, the mixture is filtered through fluted filters (Schleicher and Schüll, No. 595) in an icechest. The next day, 20 cc. of dioxane is added per 100 cc. of filtrate. The preparation is placed in the icechest for 12 hours. The mixture is filtered as before, and 10.2 cc. of dioxane is added per 100 cc. of filtrate. The solution is stirred and placed in the icechest for another 12 hours. The precipitated catalase is filtered. The moist precipitate is collected with a spatula and mixed with enough water to form a cream. A little saliva is added to digest the glycogen present. Saturated ammonium sulfate is added until the catalase solution is faintly hazy. The solution is placed in the icechest. The catalase will separate in the form of silky crystals. These may be obtained by removing first amorphous impurities on short centrifugation, and the crystals on centrifuging from 30 to 60 minutes.

Recrystallization. The crystals are dissolved in small amounts of water and a little 9.6 per cent neutral phosphate (3 cc. for crystals from 5 pounds of liver). The solution is centrifuged until clear. To the supernatant fluid, about 6 cc. of saturated KH_2PO_4, or twice the volume of neutral phosphate, is added. Now saturated ammonium sulfate is added, drop by drop, until the solution is faintly hazy. On cooling, crystals of catalase form. Crystalline catalase may also be prepared from beef erythrocytes (10).

The Alleged Reversible Hydrolysis of Liver Catalase. Agner (11) purified catalase from the horse liver by extraction of the tissue with water, precipitation with ethyl alcohol-chloroform, adsorption on tricalcium phosphate, elution with secondary sodium phosphate, and dialysis. This final catalase preparation was hydrolyzed into two components by allowing it to dialyze against hydrochloric acid. One

component dialyzes through cellophane and is colored (hemin), whereas the other one, within the dialyzing bag, is colorless and is a protein. By themselves, the two components are inactive. If brought together, however, they very rapidly decompose hydrogen peroxide. The hydrolysis of the catalase was carried out similarly to the hydrolysis of the oxidation ferment by Theorell. Tauber and Kleiner (12) were not able to confirm the results of Agner, using the catalase of beef, rabbit, and rat liver, respectively.

Estimation of Activity. Catalase activity may be determined by measuring the formation of oxygen gas by volume, by titrating the undecomposed hydrogen peroxide with potassium permanganate, or iodometrically, or by using the polarograph (13).

PEROXIDASES

These hematin-containing enzymes occur mostly in the roots and sprouts of higher plants. The best source is horseradish and the sap of the fig tree (14). It is not certain whether animal tissues contain peroxidase. Tests for this enzyme are also given by cytochromes, hemoglobin, and other tissue constituents. Bancroft and Elliott (15), however, reported that the spleen and lung are good sources of peroxidase. The peroxidase of milk has been extensively investigated. This enzyme oxidizes persulfate, but plant peroxidase does not (16). Peroxidase is seldom found in lower forms of life. However, *Acetobacter peroxidans* contains peroxidase but does not contain catalase. It never forms free hydrogen peroxide (17).

Peroxidase, in the presence of hydrogen peroxide, oxidizes many aromatic amines and phenols. It sets free iodine from iodides, and it oxidizes such thiols as 2-thiouracil, 2,6-dithiouracil, glutathione, and cysteine (18). Sumner and Nymon (19) found that peroxidase oxidizes bilirubin to biliverdin if the *p*H is within a narrow zone around *p*H 7.4.

Substrates and Estimation. Pyrogallol is oxidized to purpurogallin, benzidine to *p*-quinone di-imide, *o*-phenylene diamine to phenazine, leucomalachite green to malachite green, tyrosine to a yellow solution, adrenaline to a red solution, *o*-cresol to a green solution, *p*-cresol to a milky suspension and *m*-cresol to a pink solution, and catechol to *o*-quinone (20). Some of these substrates are being used for the quantitative determination of peroxidase. Willstätter and associates (21) measured the color produced from pyrogallol colorimetrically or weighed the ether-soluble purpurogallin. The colorimetric method, by which the oxidation of leucomalachite green is followed, is very

convenient for plant peroxidase estimation (21); the microgasometric procedure may also be used (22). Sumner and Gjessing (23) have published a modification of Willstätter's method using a small volume of reaction mixture containing phosphate buffer and a relatively high concentration of hydrogen peroxide. After the addition of sulfuric acid to stop enzyme action, the purpurogallin formed is extracted with ether, the solution is filtered, and the ethereal solution is read in an electrophotometer. Peroxidase action is proportional to the amount of enzyme used provided that an excess of substrate is present and that the hydrogen peroxide concentration is low.

Crystalline Horseradish Peroxidase

Willstätter and Stoll (24) prepared peroxidase from horseradish and other plants that had considerable activity. Elliott (22) obtained a very active peroxidase free from catalase by fractional precipitation with ammonium sulfate from milk. Theorell (25) has succeeded in isolating horseradish peroxidase in pure and crystalline state. In his method, 200-kilogram portions of horseradish were ground, extracted with 200 liters of water, and strained through cloth. The residue was further extracted with water and with the aid of an hydraulic press. The clear extract was concentrated to a syrup in vacuum at 20° C. The syrup was subjected to fractional precipitation with ammonium sulfate, dialysis, fractional precipitation with alcohol, and a second dialysis, followed by electrophoresis for 9 days in the Tiselius apparatus. The peroxidase moved only slowly towards the anode; impurities moved faster. A second type peroxidase was discovered which moved slowly towards the cathode. This cathode type, which was called " type I " or " paraperoxidase," separated gradually from the anode type or " type II " peroxidase and impurities. The fact that the paraperoxidase could be precipitated by 55 per cent saturation with ammonium sulfate, but not the anode type or type II, afforded a method of purification. By adding (drop by drop) saturated ammonium sulfate solution to the paraperoxidase-free enzyme solution, the peroxidase separated in a dark brown crystalline state. The crystals (transparent microscopic needles) were recrystallized by careful addition of water until nearly all of them dissolved, and by slowly increasing the ammonium sulfate concentration. Theorell states that paraperoxidase did not occur in all horseradish batches that he examined. He remarks, however, that this failure might have been due to the extreme lability of the paraperoxidase. In one experiment, the yield of crystalline peroxidase per 200 kilograms of horseradish was 2.8

grams; in a second experiment, 130 kilograms yielded 0.5 gram of peroxidase. The hemin content was 1.48 per cent. Sedimentation tests in the ultracentrifuge and diffusion measurements showed perfect homogeneity. The molecular weight of this peroxidase was found to be 44,100. The two peroxidases have different absorption bands.

Hydrolysis of Horseradish Peroxidase into Protein and Hemin. Theorell (26) was able to separate pure horseradish peroxidase (type II) reversibly into hematin and a colorless protein component by adding acetone and hydrochloric acid (1 volume of concentrated hydrochloric acid and 1000 volumes of acetone) at —15° C. to a cooled solution of peroxidase. The hematin dissolved in acetone as hemin, whereas the protein precipitated. After 10 minutes at —15° C., the hydrolysis of peroxidase was complete. The colorless precipitate was washed at —15° C. with acidified acetone and then dissolved in 1 per cent sodium bicarbonate solution. In order to restore the activity, blood hematin in phosphate buffer of *p*H 7.0 was added to a portion of the enzyme protein solution. After 20 to 40 minutes, all the peroxidase activity and the typical absorption bands were regained. Theorell and associates (27) tested a series of iron porphyrins for their ability to replace hematin. Only deuterohemin and mesohemin showed some activity.

Gjessing and Sumner (28) cleaved impure peroxidase preparations according to the method of Theorell and associates and tested the reactivation resulting from the addition to the protein component of Cu-, Co-, Mn-, and Ni-protoporphyrin, and also Fe-meso- and Fe-hematoporphyrin. The copper, cobalt, and nickel derivatives gave no activity. Manganese protoporphyrin showed 28.4 per cent activity. Some activity was shown by the ferrimesoporphyrin and by the ferriprotoporphyrin "catalase." Theorell later (29) repeated the manganese hemin experiment of Gjessing and Sumner and concluded that the manganese compound is entirely inactive with the peroxidase protein, and that only iron hemins formed active enzymes, as previously shown by him.

Kinetics of Horseradish Peroxidase. Horseradish peroxidase has an optimum *p*H at 4.5 to 6.5 when guaiacol is the substrate. With *o*-cresol it is at 3.5 to 5.0, and with pyrogallol the optimum cannot be determined since the polyphenol formed is autoxidizable (30). Elliott (22) showed that milk peroxidase is active over a wide range in the *p*H scale. In an alkaline medium, from *p*H 8 to nearly 10, the activity decreases, but it recovers immediately on neutralization. At *p*H 10, practically all the enzyme is destroyed. In acid medium, at *p*H 4.2 to 3.8, a precipitate is formed and a decrease in activity takes

place; between *p*H 3.6 and 3.2, the precipitate redissolves and the enzyme is completely inactivated. On adjustment to about *p*H 7.0 and standing overnight, the peroxidase is wholly active again.

Schwimmer (31) observed that when a peroxidase solution was heated a precipitate formed. When it was separated, the precipitate and the supernatant fluid were inactive. Regeneration occurred when the two were combined. The regeneration reaction was complete in 4 hours and was primarily a function of the heating rate. For further data concerning the kinetics of peroxidase action, see the study by Chance (32).

Interaction of Ascorbic Acid and Peroxidase (33). Undialyzed peroxidase preparations and peroxide oxidize ascorbic acid with great rapidity; dialyzed solutions, however, do not act on the vitamin. In the undialyzed peroxidase solution, certain substances are present which form quinones with peroxide peroxidase. The quinones oxidize the ascorbic acid, and they are, in turn, reoxidized by peroxide peroxidase. The oxidation of ascorbic acid and reduction of quinones proceed until all the peroxide is reduced, resulting in the decomposition of the physiologically toxic peroxide. The oxidized ascorbic is readily reduced again by glutathione (34).

Milk Peroxidase

Theorell and Akeson (35) prepared a very active peroxidase from milk. The milk was fractionated with ammonium sulfate in order to remove casein (Elliott). The filtrate was heated to 70° C. and dialyzed. Inert proteins were removed by lead precipitation. Then the solution was dialyzed and concentrated in vacuum, and ½ volume of acetone was added to remove impurities. The acetone was removed, and further impurities were separated by electrophoresis at *p*H 5.9. The peroxidase moved cathodically. The yield was 0.2 gram per 100 liters of milk, or approximately 2 per cent of the total activity present in the milk. This enzyme, too, is a hemin proteid, as was suggested by Elliott. The enzyme solutions are brownish green in the ferri and emerald green in the ferro state, resembling in this respect the verdoperoxidase isolated by Agner (36). However, the milk peroxidase has different absorption bands.

Verdoperoxidase

This enzyme is present in leucocytes. Agner (36) has prepared it in pure and highly active form. The enzyme could be found only

in mycloic cells, amounting to about 1 per cent of the dry weight of the cells. The iron content of this peroxidase is 0.1 per cent. The enzyme is an iron porphyrin compound. It is assumed that the iron enters into the prosthetic group of the enzyme. Unlike the plant peroxidases, this enzyme cannot be separated into protein and hematin by acetone-hydrochloric acid. Its activity is only about 1/10 to 1/20 that of the plant peroxidases. Verdoperoxidase is resistant to 0.1 *N* alkali, to 1 *N* acetic acid, and to alcohol and formaldehyde. It is inactivated by 1 *N* alkali and by 0.1 *N* hydrochloric acid. Like plant peroxidases, verdoperoxidase exhibits dihydroxymaleic acid oxidase activity under aerobic conditions.

Dihydroxymaleic Acid Oxidase

In 1938, Banga and associates (37, 38) found, in plants, a new enzyme, "dihydroxymaleic acid oxidase," which oxidizes dihydroxymaleic acid to diketosuccinic acid. Theorell and Swedin (39) have shown that this enzyme is identical with peroxidase. In solution, dihydroxymaleic acid oxidizes spontaneously in the presence of air, producing hydrogen peroxide. On the addition of peroxidase, further quantities of the substrate are oxidized. If catalase is added, peroxidase action does not occur, owing to the destruction of the hydrogen peroxide. When crystalline peroxidase is split into protein component and hemin, the dihydroxymaleic acid oxidase activity is destroyed. When the peroxidase is resynthesized, both activities reappear (26).

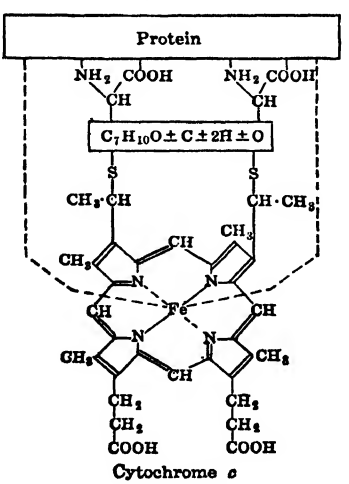
Cytochromes

In 1886, and the following years, MacMunn described several iron porphyrin pigments which he observed spectroscopically in various animal tissues. He named them "myohematins" and "histohematins," and stated that they have respiratory functions since the pigments in the reduced state showed typical absorption spectra that disappeared when the pigments became oxidized. MacMunn believed that his pigments had some interrelationship with hemoglobin. Hoppe-Seyler cast serious doubt on the value of MacMunn's observations. Forty years later, Keilin (40) called attention to the importance of MacMunn's findings and showed that these pigments are much more important than was anticipated by MacMunn. He has shown that these pigments are present in every order of the animal kingdom, in the tissues of plants, and in the cells of lower organisms such as

bacteria and yeast. Keilin named these pigments cytochromes. There are three different cytochromes — " a," " b," and "c." These cytochromes are found together in the cells. By using a spectroscope, the three typical bands given by these cytochromes may be readily observed in a suspension of yeast cells. These are the bands of reduced cytochromes: " a " at 6000 to 6050 and 5200 Å, " b " at 5640 and 5300 Å, and " c " at 5500 Å. On passing air through the yeast suspension, the absorption bands disappear.

Theorell and Akeson (41) have extensively studied the chemical properties of cytochrome c. They found that it is an iron porphyrin protein, having a molecular weight of 13,000 and an iron content of 0.43 per cent. The absorption spectrum of the ferri form shows five different absorption lines. Ferricytochrome c does not combine with hydrogen cyanide unless its solution is more alkaline than *p*H 13. Nor does cytochrome c combine with carbon monoxide at *p*H 3 to 10. Cytochrome c is a very stable compound; it is resistant to boiling, to 0.1 *M* hydrochloric acid, and to 0.1 *M* sodium hydroxide. Between *p*H 4.0 and 10, cytochrome is not oxidized by molecular oxygen. It is readily reduced by reducing agents, such as hydrosulfite, cysteine, ascorbic acid, and adrenaline, and by a yellow enzyme, cytochrome reductase (42). Reduced cytochrome c is oxidized by cytochrome oxidase, by cytochrome peroxidase and hydrogen peroxide, or by leucoflavin. The oxidized form contains ferric iron, whereas the reduced form contains ferrous iron.

Theorell proposed the following structure for cytochrome c:



Cytochrome *c*

In contrast to cytochrome c, the other two cytochromes, a and b, are labile compounds. Yakushiji and Okunuki (43) claim to have isolated

cytochrome a from heart muscle, and Yakushiji and Mori (44) reported the isolation of cytochrome b from bakers' yeast.

Preparation of Cytochrome c

The following is a modification for the preparation of this protein from horse heart muscle (45). By this procedure, a solution containing 0.34 per cent iron may be obtained. When this preparation was compared with one containing 0.43 per cent iron, it showed the same catalytic activity per gram of iron. Both can be used equally well in enzyme experiments.

A horse heart is cut open as soon as possible after collection from the slaughterhouse, and the blood is allowed to drain out. The muscle is freed from fat and ligaments and finely minced. To the minced tissue is added 0.145 N trichloroacetic acid at the rate of 1 liter per kilogram of tissue. The trichloroacetic acid is a 20 per cent solution and should be made up by titration with normal sodium hydroxide, phenolphthalein being used as the indicator. The acid mixture is allowed to stand for 4 hours. Then it is squeezed out in a press, and the cloudy extract is adjusted to pH 7.3 with 10 per cent sodium hydroxide. Ammonium sulfate is added at the rate of 500 grams per liter. When it is dissolved, the mixture is filtered overnight through paper. To the filtrate, 50 grams of ammonium sulfate is added per liter and the solution is left overnight at 0° to 5° C. If any precipitate forms, it is filtered off. The cytochrome in the cold filtrate is precipitated by the addition of a 20 per cent trichloroacetic acid solution, using 20 cc. per liter of filtrate. Within a few minutes, the protein cytochrome precipitates and is collected by centrifugation. The red precipitate is suspended in about 300 cc. of saturated ammonium sulfate and centrifuged again. It is now suspended in the minimum quantity of distilled water and transferred to a cellophane bag, in which it is dialyzed against 0.5 per cent sodium chloride until free from ammonium sulfate. The suspension is shaken with a few drops of chloroform, and the precipitate is filtered off; 30 to 40 cc. of a dark red solution of cytochrome-c is obtained, containing about 1 per cent protein. When saturated with chloroform and stored in a well-stoppered container at 5° C. this cytochrome c solution keeps indefinitely. See reference 45 for a simple method for the preparation of cytochrome c with an iron content of 0.43 per cent. However, the catalytic activity of the two proteins is exactly the same.

Keilin and Hartree use the manometric method for the measurement of the catalytic potency of the two cytochromes. The tests are carried out at 39° C. and pH 7.3 on two enzyme systems obtained from a

kidney preparation containing cytochrome oxidase and succinic dehydrogenase:

1. Cytochrome oxidase + cytochrome c + ascorbic acid.
2. Cytochrome oxidase + cytochrome c + succinic dehydrogenase + succinic acid.

When the enzymes and the substrates are in excess, the oxygen uptakes of the systems serve as measures of the catalytic power of cytochrome c.

Functions of the Cytochromes. Most of these iron porphyrin proteins do not autoxidize. They are oxidized by cytochrome oxidase. The reduced oxidase is then oxidized by molecular oxygen. Cytochrome oxidase performs the last function in the gradual oxidation (removal of hydrogen) of most foods. For instance, in the final reactions ferricytochrome c is reduced by the reduced form of the flavin enzyme, cytochrome reductase. Ferrocytochrome c is oxidized by the ferri form of cytochrome oxidase. The resultant ferrocytochrome oxidase is oxidized by molecular oxygen. A large number of oxidases require the cytochrome systems for their action. Some of these are: pyruvate oxidase, succinic acid oxidase, choline oxidase, α-hydroxy acid oxidase, and glycerol oxidase. Bach, Dixon, and Zerfas (46) have shown that a new hemochromogen protein, cytochrome b_2, is an essential part of the yeast lactic dehydrogenase system. However, in certain cells that do not contain cytochrome, oxidases function without cytochromes (47).

Cytochrome Oxidase (Indophenol Oxidase)

This enzyme (48, 49) is present in all kinds of cells. It acts only on phenols and amines indirectly, by first oxidizing cytochrome c. The cytochrome c oxidizes the compounds just mentioned. Cytochrome oxidase oxidizes only cytochromes, and only in the presence of gaseous oxygen. Haas (50) has been able to obtain cell-free solutions of cytochrome oxidase. His method involved prolonged grinding, autolysis, and ultrasonic radiation. The cytochrome oxidase of Haas consists of two enzymes. Component I loses 80 per cent of potency in 15 minutes at 50° C.; component II may be kept for 15 minutes in boiling water without loss of potency (51). Component II in ammonium buffer of *p*H 9.0 loses 40 per cent of its activity on standing overnight at 0° C.; the unseparated oxidase, under similar conditions, remains unchanged for weeks. Both enzymes are proteins concerned with the oxidation of cytochrome c. On the basis of these results, Haas concludes that cytochrome oxidase and Warburg's oxygen-trans-

ferring enzyme are separate enzyme systems. Cytochromes have an important function in biological oxidations. Haas (52) has shown that cytochrome c in yeast could account for all the oxygen consumption of the yeast cell. It is not known, however, how the other cytochromes interact in the various phases of biological oxidation-reduction reactions.

Cytochrome c Peroxidase

This is also a hemin-protein enzyme. It is a constituent of brewers' and bakers' yeast. Cytochrome c peroxidase oxidizes reduced cytochrome c to oxidized cytochrome c in the presence of hydrogen peroxide:

$$2\ \text{Cytochrome c}-\text{Fe}^{++} + \text{H}_2\text{O}_2 + 2\ \text{H}^+ = 2\ \text{cytochrome c}-\text{Fe}^{+++} + \text{H}_2\text{O}$$

In the presence of catalase, this enzyme is inactive. The absorption spectrum of cytochrome peroxidase consists of an intense band at 4100 Å and two shallow bands at 5000 and 6200 Å. The enzyme has a molecular weight of 60,000 (53).

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CHAPTER IX

OXIDASES CONTAINING COPPER

ASCORBIC ACID OXIDASE

An enzyme free of phenolase and peroxidase action, and specific for the direct oxidation of ascorbic acid, was first described by Tauber and Kleiner (1, 2) in 1935. They found this enzyme in squash and named it ascorbic acid oxidase. The findings of Tauber and Kleiner have been verified by numerous investigators (3–10). Although ascorbic acid oxidase has been reported to be present in many plants (4), squash became the favorite starting material for many important investigations concerning this enzyme.

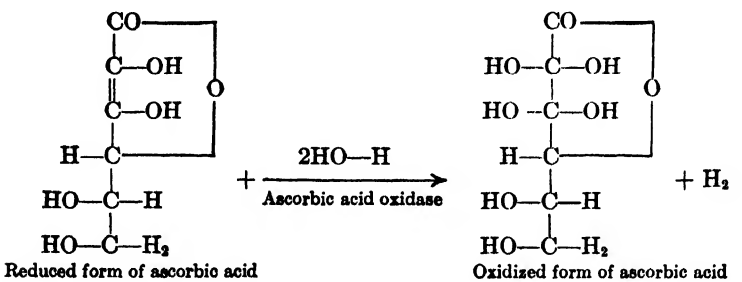
Ascorbic acid may be oxidized by three different enzymic reactions. An example for the first group is the specific enzyme ascorbic acid oxidase of squash, cucumber, drumstick tree, etc. In the second group belong phenolases, which oxidize phenols (present in the plant juices). The oxidized phenols, in turn, then oxidize ascorbic acid (11, 12). The third group is represented by peroxidases. These enzymes oxidize ascorbic acid only in the presence of quinone-forming compounds (mostly present in plants) and hydrogen peroxide (13). The quinones are reduced by the ascorbic acid, and they are reoxidized by peroxide peroxidase. A very small quantity of quinone suffices for this catalysis. The oxidation of ascorbic acid and reduction of quinones continue until all the ascorbic acid is oxidized or all the hydrogen peroxide is reduced, resulting in the destruction of the physiologically toxic peroxide.

Ascorbic acid oxidase is not present in various animal tissues, in cows' milk, or in yeast (14, 15).

Function and Properties

Ascorbic acid oxidase (1, 2) of squash (any type) and certain other plants oxidizes ascorbic acid very rapidly and completely, without the intermediary action of another substance. Oxygen is required for the reaction. After ascorbic acid has been oxidized by the oxidase, it may be reduced by hydrogen sulfide. When the hydrogen sulfide is removed by nitrogen, its original reducing power is

regained. This enzyme is quite sensitive to potassium cyanide. In contrast to a dialyzed cabbage leaf extract (16), which also oxidizes ascorbic acid, squash preparations do not give color reactions with tyrosine, benzidine, guaicol, pyrogallol, catechol, phloroglucinol, resorcinol, and vanillin (2). Nor does the ascorbic acid oxidase affect glutathione, cysteine, or adrenaline (2). Ascorbic acid oxidase oxidizes ascorbic acid by introducing two hydroxyl groups at the double bond. The liberated hydrogen combines with atmospheric oxygen:



Lampitt and Clayson (17) reported that a solution containing calcium bicarbonate, an alkali phosphate, and a trace of ionized copper behaves as a synthetic ascorbic acid oxidase, and the catalytic action disappears on heating to 55° C. $Ca_3(PO_4)_2$ gel containing Cu^{++} can be brought into solution by passing in carbon dioxide and can be reprecipitated by removing the carbon dioxide with heat or by the addition of solvents, such as acetone, that reduce the carbon dioxide solubility in water. Reprecipitation is followed by loss of activity. However, cabbage juice did not behave in this manner, and it is postulated that the citric acid present in the cabbage juice must have a "regulatory effect on ascorbic acid oxidation." These authors have not studied the real sources of ascorbic acid oxidase (1, 2), and they give no data concerning the *p*H range of activity of the synthetic oxidase. They conclude that the evidence in support of the enzymic nature of the ascorbic acid oxidase is insufficient.

Steinman and Dawson (18), however, found three years earlier that, in contrast to the aerobic oxidation of ascorbic acid when catalyzed by cupric ions, hydrogen peroxide is not a reaction product of the aerobic oxidation by squash ascorbic acid oxidase. Stotz (19) showed, as early as 1940, that glycine at *p*H 6.0 completely inhibits the action of non-enzymic copper but not that of the copper protein enzyme.

Chemical Nature of the Oxidase and the Function of Copper. In 1940, Ramasarma and associates (20) reported an approximate paral-

lelism between ascorbic acid oxidase activity and copper content, in the course of a series of purifications of the oxidase, using extracts of drumstick and cucumber as their source material. Their preparations contained less than 0.03 per cent copper and were not very active. In the same year, Stotz (7) and Lovett-Janison and Nelson (8) obtained preparations containing 0.25 to 0.15 per cent copper from cucumber and squash respectively. The preparation of Lovett-Janison and Nelson was four times more active than that of Stotz. The high copper content in the preparation obtained by Stotz would indicate that the cucumber enzyme either contained much non-enzyme copper or inactivated copper, or that the cucumber enzyme is different from the squash enzyme. The preparation which Lovett-Janison and Nelson obtained after extensive purification showed that its enzyme activity became proportional to the copper content. Further purification steps did not change this ratio. Their most active preparation contained 0.15 per cent copper. It was free of peroxidase and phenolase activity. These findings have definitely proved that ascorbic acid oxidase is a specific copper protein enzyme. Lovett-Janison and Nelson's preparation oxidized ascorbic acid over 1000 times faster than an equivalent quantity of ionic copper.

Powers, Lewis, and Dawson (10), using a modification of the method of Lovett-Janison and Nelson (8), obtained from squash an ascorbic acid oxidase preparation containing 0.24 per cent copper. It had an activity one and a half times greater than that of the preparation of Lovett-Janison and Nelson, but the activity per microgram of copper was the same. The preparation of Powers and associates rapidly lost its activity when diluted. However, added inert proteins stabilized the enzyme considerably. In concentrated solution, this highly purified enzyme is blue. From concentrated solutions, when dialyzed until free of salts, the enzyme precipitates in the form of an amorphous green-blue protein. This protein rapidly dissolves in 0.1 *M* secondary sodium phosphate, and the solution is stable for many months provided that it is kept in a refrigerator and that the protein concentration is more than 0.1 per cent.

Kinetics. The optimum *p*H of the squash enzyme is at 5.56 to 5.93 with 0.15 *M* phosphate-citrate buffer, and at 5.38 to 5.57 with sodium acetate buffer (2). The drumstick oxidase has an optimum *p*H at 4.6 to 5.6 in phosphate-citrate buffer, and 5.3 to 5.6 in phosphate buffer (3). Auto-oxidation of ascorbic acid in acetate buffer is considerable; in citrate buffer it is negligible (17).

Constant quantity of squash enzyme produces a constant amount of

oxidation within the range of 1 to 5 milligrams of ascorbic acid (2). The rate of change is proportional to the amount of enzyme used in experiments with phosphate-citrate buffer (duration of experiments 5 minutes). The kinetics of squash ascorbic acid oxidase shows that a single enzyme is involved (2, 10).

Purification. The method of Powers and associates (10) appears to yield the most active and purest preparation of summer-squash enzyme. The procedure is too lengthy to be described here in detail. The main stages of preparation are: removal of the juice from the pericarp (edible portion) of the squash, with the aid of a hydraulic press; adjustment of the *p*H to 7.6 with sodium borate; clarification with barium acetate to remove extraneous proteins; fractionation by ammonium sulfate and by magnesium sulfate; adsorption of impurities on alumina; fractionation by lead subacetate and cold acetone; finally, adsorption on alumina, and dialysis.

Tadokoro and Takasugi (21) claim that they have been able to obtain ascorbic acid oxidase in crystalline state from pumpkin juice. They state that the crystals were highly active and had properties of an albumin. However, Dawson and Mallette (22) contest this claim by stating that the method of preparation employed by Tadokoro and Takasugi was such that only a product of low activity could have been obtained, and that the identity of the crystals with the enzyme was not definitely proved.

Enzymic Estimation of Ascorbic Acid. Tauber and Kleiner (23) described an enzymic method for the estimation of ascorbic acid, utilizing squash ascorbic acid oxidase for the oxidation of the vitamin. In this procedure, the total reduction is determined volumetrically with sodium 2,6-dichlorobenzenone indophenol. A second aliquot is titrated with the indicator after enzyme action is stopped. The true ascorbic acid is computed by substracting the second titration figure from the first. Rönnerstrand (24) has used this method extensively in his studies of the ascorbic acid content of algae. It had been stated that isomers of ascorbic acid are also oxidized by ascorbic acid oxidase. However, those compounds do not occur in nature. Details for the removal of reducing substances that act on 2,6-dichlorobenzenone indophenol have been given by Tauber and Kleiner. Rönnerstrand found that the interference by most reducing substances may be eliminated by using metaphosphoric acid for the extraction of the vitamin, by allowing the oxidase to act for short periods, and by conducting rapid titrations. A respirometer may also be employed for measuring the action of ascorbic acid oxidase.

TYROSINASE (MONOPHENOL OXIDASE, POLYPHENOL OXIDASE, CATECHOLASE, CRESOLASE)

Tyrosinase was discovered by Bourquelot and Bertrand in the fungus *Russula nigricans*. Bertrand (25) found that this enzyme oxidizes tyrosine to a black pigment (melanin), and that peroxidase and laccase are not able to do this. The enzyme is very abundant in plants and vegetables, occurring together with tyrosine. This is the reason for the darkening of the cut surface of many plants. Tyrosinase may be prepared from potato peelings, wheat bran, the mealworm (*Tenebrio molitor*), and various mushrooms. It is also present in tissues of invertebrates.

Specificity. The earlier view was that tyrosinase deaminizes tyrosine. Raper and Wormal (26), however, have shown that tyrosinase does not liberate ammonia from tyrosine and that the black pigment melanin contains more nitrogen than tyrosine. Happold and Raper (26) and Szent-Györgyi (26) found independently that *o*-quinones are produced by the action of tyrosinase upon phenol, *m*-cresol, *p*-cresol, catechol, and homocatechol. *o*-Quinones, however, are not the final products. Tyrosinase is not a very specific enzyme. It also acts on adrenaline, pyrogallol, and dopa (3,4-dihydroxyphenylalanine), but not on guaiacol, *o*-cresol, *p*-phenylenediamine, hydroquinone, or on ascorbic acid. Dalton and Nelson (27) reported that purified as well as crystallized tyrosinase obtained from the edible mushroom *Psalliota campestris* oxidizes *p*-cresol 10 times faster than catechol. Tyrosinase requires gaseous oxygen. Hydrogen peroxide is not produced.

Preparation. Mallette (29) has carried out important investigations on the purification and the unimolecular nature of *P. campestris* tyrosinase. His work is too extensive to be given here in detail. His conclusion is that, after correlating the activity, copper, and homogeneity data, it was possible to explain all the known properties of tyrosinase in terms of a single copper-protein entity. His work showed that different types of tyrosinase arise as the result of fragmentation of the protein molecule during the preparative procedures. He gives detailed methods for the preparation of purified, highly active catecholase and high-cresolase-type tyrosinase preparations. In view of this, the Columbia University workers (30) name tyrosine preparations obtained from *Psalliota* and having much more activity towards catechol than towards cresol " catecholase," and those with a higher activity towards cresol " cresolase." Since the rate of the initial reaction is linear, these investigators use the initial reaction ve-

locity as the basis for the estimation of enzyme activity. Their paper contains a discussion of methods in use for the estimation of tyrosinase.

Chemical Nature. Kubowitz (31) prepared tyrosinase from potato peelings by extraction with water, fractionation with acetone and ammonium sulfate, purification with silver acetate and aluminum hydroxide, and dialysis. The final purified tyrosinase contained 0.19 to 0.20 per cent copper and 14.4 per cent nitrogen. The enzyme solutions were yellow but showed no specific absorption bands. The copper of the enzyme was removed by dialysis of the solution containing some added potassium cyanide. Upon addition of copper salts, the enzyme became active again.

Dalton and Nelson (32) obtained the tyrosinase of *Lactarius piperatus* in crystalline form. Their method consists of fractionation with ammonium sulfate and acetone, purification with aluminum oxide and charcoal, and a final fractionation with ammonium sulfate. The precipitate was dissolved in water and adjusted to *p*H 5 with acetic acid. On standing in the cold, the tyrosinase crystallized in the form of hexahydric plates. These crystals contained 0.25 per cent copper and 13.6 per cent nitrogen. The activity of these crystals was much less than that of some non-crystalline material.

LACCASE

This oxidase is present in the latex of such trees as the Japanese lacquer tree (*Rhus vernicifera*) and an Indo-Chinese lacquer tree (*Rhus succedanea*), and in some fungi (33). Laccase is responsible for the darkening of the latex. The phenols present in the latex and giving the reaction are the pyrocatehol derivatives lacol and urushiol (34). Laccase oxidizes a great variety of ortho- and paraphenols, diamines, and amino phenols in the presence of oxygen. It does not oxidize monophenols, such as *p*-cresol, nor does it act on tyrosine. Keilin and Mann (35) differentiate in the following manner between laccase of *Rhus succedanea* and the polyphenol oxidase of cultivated mushrooms (*Psaliota campestris*):

1. Laccase is blue: polyphenol oxidase is yellow.
2. Laccase oxidizes *p*-phenylenediamine better than catechol; polyphenol oxidase acts on it only slightly.
3. Laccase, when pure, does not act on *p*-cresol; polyphenol oxidase acts on it after a lag period.
4. Laccase, in contrast to polyphenol oxidase, is not poisoned by carbon monoxide.

Like polyphenol oxidase, laccase is also a copper-protein enzyme. Laccase is strongly inhibited by potassium cyanide, hydrogen sulfide, sodium azide, and diethyl dithiocarbamate. Laccase preparations contain a substance which inhibits the oxidation of *p*-cresol by crude extracts of potatoes and mushrooms. Boiled laccase, however, does not show this inhibition (36).

The most active laccase preparations, which Keilin and Mann (36) obtained from the latex of *Rhus succedanea,* contained 0.24 per cent copper and no iron and manganese. Bertrand (37), however, claims that he has now obtained preparations from the same source having higher activity. Examination of the absorption spectrum showed that the enzyme is not necessarily a protein. This, of course, is not in harmony with our present conception concerning the chemical nature of enzymes. Spectroscopic studies showed that manganese is present, whereas only 0.04 per cent copper could be detected, and this amount might have been introduced by the reagents used. Bertrand's preparations were free of blue color.

Laccase, like tyrosinase and peroxidase, forms purpurogallin from pyrogallol. This reaction is also employed for the estimation of laccase.

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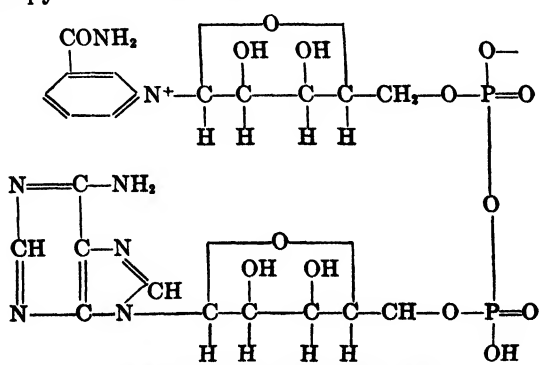
CHAPTER X

DEHYDROGENASES CONTAINING CODEHYDROGENASE I AND II

It has been stated in the above classification of the oxidizing enzymes that the dehydrogenases remove two hydrogens from their substrates, and that they have been divided into groups, depending on the type of hydrogen acceptor through which they react with molecular oxygen. In the following, the dehydrogenases will be reviewed in such separate groups. The function of the dehydrogenases containing codehydrogenase I and II is the catalysis of the transfer of protons and electrons to the pyridine nucleotides, which thereby become reduced. Normally, the reduced nucleotides are reoxidized by molecular oxygen through the mediation of flavoproteins and cytochromes. The reduced nucleotides may also be reoxidized by dyes having a higher oxidation-reduction potential: methylene blue, pyocyanine, dichlorophenolindophenol, ferricyanide, and others. Before reviewing the dehydrogenases, a few words concerning the codehydrogenases should be of interest.

Codehydrogenase I and II

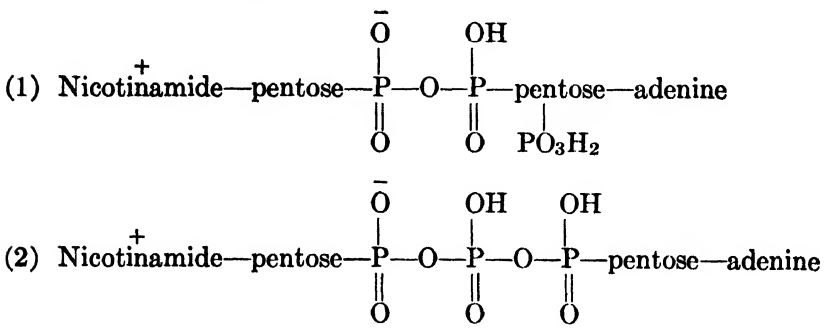
Codehydrogenase I (Coenzyme I, Cozymase, Diphosphopyridine Nucleotide). Cozymase, the "coenzyme of alcoholic fermentation," was discovered by Harden and Young in 1905. Shortly after Warburg and associates had shown that their second coenzyme, now called codehydrogenase II, is a triphosphopyridine nucleotide, it was found in collaborative investigations by Warburg and Christian, and by Euler, Albers, and Schlenk, that nicotinic acid amide is also a part of cozymase. Euler and Schlenk (1) proposed that cozymase is identical with diphosphopyridine nucleotide:



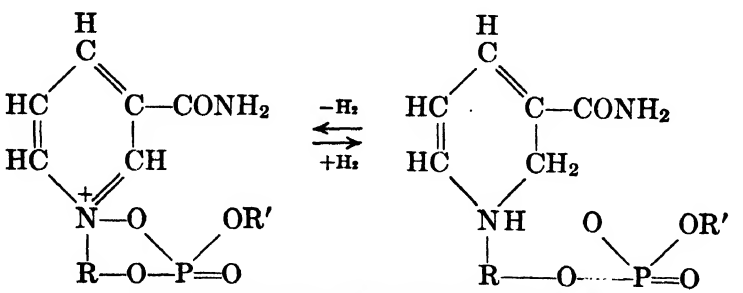
Diphosphopyridine nucleotide (cozymase)

Both coenzymes contain one molecule of adenine, one molecule of nicotinic acid amide, and two molecules of *d*-ribose. In addition, codehydrogenase I contains two molecules of orthophosphoric acid, whereas codehydrogenase II contains three molecules of orthophosphoric acid.

Codehydrogenase II. This coenzyme was discovered by Warburg and Christian in 1932 (2). It is a triphosphopyridine nucleotide. Schlenk (3) suggested that, in codehydrogenase II, the third orthophosphoric acid molecule is attached to one of the two pentose molecules (formula 1), this being the only difference between the two coenzymes. Another alternative would be the pyrophosphoric acid linkage (formula 2) so often encountered in nature. However, this could not be proved by experimental means.



In both codehydrogenase I and codehydrogenase II, it is the pyridine ring that is concerned with hydrogen transfer, undergoing reversible oxidation-reduction involving two electrons and one proton (one proton is transferred to the medium):



Reversible reduction of the nicotinamide nucleotides

Properties of Codehydrogenase I and Codehydrogenase II. Codehydrogenase I is found in small amounts in almost all cells, yeast being the best source. Codehydrogenase II is also widely found in

nature, but in much smaller concentrations. Warburg and Christian (4) used horse erythrocytes for the preparation of codehydrogenase II. A method for the preparation of pure codehydrogenase I (cozymase) from yeast has been published by Jandorf (5). (See chapter on yeast.)

Yeast phosphatases rapidly destroy this coenzyme. It is for this reason that during the first phase of cozymase preparation the temperature is maintained at 80° to 85° C. The same type of codehydrogenase destruction has been noticed by several authors, using minced tissues.

Codehydrogenase I has a molecular weight of 663 and an absorption band at 260 μ. Codehydrogenase II has a molecular weight of 743 and the same absorption band as codehydrogenase I. Very dilute solutions of the coenzymes do not keep well. However, solutions containing 2 to 20 milligrams per cubic centimeter will remain unchanged for some time if they are kept in a refrigerator (6).

Alcohol Dehydrogenase of Plants

This enzyme is found in yeast, molds, bacteria, and in some higher plants. It oxidizes ethyl alcohol reversibly to acetaldehyde. The two hydrogens of the alcohol are taken up by codehydrogenase I as follows:

$$CH_3CH_2OH + \text{codehydrogenase I} \rightleftarrows CH_3CHO + \text{reduced codehydrogenase I}$$

Various types of alcohols are oxidized by this enzyme (7, 8). This dehydrogenase is very considerably inhibited by $M/3000$ iodoacetic acid, indicating that it contains a necessary sufhydryl group (9). Negelein and Wulff (10) obtained this alcohol dehydrogenase in crystalline form using Lebedew juice of brewers' yeast as the starting material.

Alcohol Dehydrogenase of Animals

Batteli and Stern (11) were the first to study the oxidation of alcohol by animal tissues. They found that liver and kidneys were the only sources of this enzyme. This dehydrogenase converts ethyl alcohol into acetaldehyde, and isopropyl alcohol into acetone. All primary alcohols are converted to aldehydes, and secondary alcohols to the corresponding ketones (12, 13):

$$R{\cdot}CH_2OH \rightleftarrows R{\cdot}CHO; \qquad \begin{matrix} R_1 \\ \diagdown \\ \quad CHOH \\ \diagup \\ R_2 \end{matrix} \rightleftarrows \begin{matrix} R_1 \\ \diagdown \\ \quad CO \\ \diagup \\ R_2 \end{matrix}$$

Lactic Dehydrogenases of Animals

A specific lactic enzyme dehydrogenase is present in a great many tissues. It is not identical with the lactic dehydrogenase of plants (bacteria and yeast). Lactic dehydrogenase oxidizes lactic acid to pyruvic acid:

$$CH_3CHOHCOOH + \text{codehydrogenase I} \rightleftarrows CH_3COCOOH + \text{reduced codehydrogenase I}$$

The reaction is reversed on the addition of pyruvic acid. Natural $l(+)$lactic acid is more rapidly dehydrogenated than the $d(-)$lactic acid (14). Straub (15) obtained beef heart lactic apodehydrogenase in crystalline form. He extracted the ground tissue with water and adsorbed the enzyme on tricalcium phosphate. Then, the enzyme was eluted with buffer of *p*H 7.2 and fractionated with acetone and ammonium sulfate. The crystals separated when the ammonium sulfate concentration reached 0.5 saturation. Straub's apodehydrogenase required codehydrogenase I and flavoprotein for the dehydrogenation of lactic acid by the methylene blue method. He added cyanide in order to bind the end product of oxidation, pyruvic acid. The reduced coenzyme is oxidized by flavoprotein, and the reduced flavoprotein is oxidized by methylene blue. Leucomethylene blue, in turn, is oxidized by atmospheric oxygen. Mehler and associates (16) have studied the specificity of several dehydrogenases toward pyridine nucleotides by measuring the changes spectrophotometrically at 340 μ. They found that crystalline lactic dehydrogenase reacts one hundred times faster with codehydrogenase I than with codehydrogenase II (16).

The optimum *p*H of heart lactic dehydrogenase is at 9.0, whereas the yeast dehydrogenase is most active at *p*H 5.2 (17). Yeast lactic dehydrogenase does not require codehydrogenase I. It interacts with methylene blue and with cytochrome. Unlike the animal lactic dehydrogenase, it does not interact with flavoprotein (17, 18). The lactic acid dehydrogenase of *B. coli* requires codehydrogenase I and acts in the absence of flavoprotein (19). For a more detailed discussion of yeast lactic dehydrogenase, see Chapter XI.

l-α-Hydroxy Acid Dehydrogenase (Animal)

Iselin and Zeller (20), using crude extracts of vacuum-dried frozen rat kidney tissues, found that such preparations attacked a series of higher hydroxy acids according to the following equation:

$$RCH(OH)COOH + O_2 = RCOCOOH + H_2O_2$$

Higher members of the group, like *l*-phenyl lactic acid, were much faster oxidized than *l*-lactic acid. This enzyme is claimed to be not identical with lactic dehydrogenase of heart muscle, since the kidney enzyme does not require codehydrogenase I and the heart dehydrogenase does not attack the higher hydroxy acids. This enzyme has been placed in this group on account of its similarity to the lactic dehydrogenase of animal tissues.

Soluble α-Glycerophosphoric Dehydrogenase

Animal tissues are claimed to contain two different α-glycerophosphoric acid dehydrogenases (21, 22). The present enzyme, which is soluble, transfers hydrogen to codehydrogenase I. The other animal tissue dehydrogenase is insoluble and requires cytochrome c as the hydrogen carrier. The insoluble enzyme is described in the section dealing with cytochrome-linked dehydrogenases. Both dehydrogenases act on α-glycerophosphoric acid changing it to 3-phosphoglyceric aldehyde. The soluble enzyme predominates in animal tissues. This enzyme is not inhibited by cyanide or by iodoacetic acid.

The soluble dehydrogenase may readily be prepared from any animal tissue except brain. The tissue is ground in a Latapie mill and extracted by further grinding with sand and 0.1 *M* Na_2HPO_4. The filtrate of the mixture, containing some cell suspensions, is clarified by adjusting the *p*H to 5.5 with 0.5 *M* KH_2PO_4 and by centrifuging. The supernatant fluid contains, in addition to the soluble apo α-glycerophosphoric dehydrogenase, apo lactic dehydrogenase and apo maleic dehydrogenase. Yeast and seeds of plants also contain an α-glycerophosphoric dehydrogenase, but this enzyme has not yet been purified to any great extent.

Isocitric Dehydrogenase and Carbon Dioxide Fixation

Citric acid is oxidized by extracts of many plant and animal tissues (Thunberg, Battelli and Stern), provided that methylene blue is present. However, this oxidation is not catalyzed by one single enzyme, citric dehydrogenase. Martius (23, 24) has shown that citric acid is first dehydrated by a liver preparation to *cis*-aconitic acid, which is hydrated to *l*-isocitric acid. Here, the equilibrium is far to the side of citric acid. Both reactions are catalyzed by aconitase, a specific enzyme. The *l*-isocitric acid is then dehydrogenated to α-keto-β-carboxyglutaric (oxalosuccinic) acid. This reaction requires codehydrogenase II and manganese or magnesium ions (24). The α-keto-β-car-

boxyglutaric acid was supposed to change spontaneously at a rapid rate into α-ketoglutaric acid and carbon dioxide. Ochoa (25) has shown that a dialyzed extract of washed, acetone-dried pig heart was free of aconitase but contained two specific enzymes. One enzyme catalyzed the reaction 1:

(1) $\cdot COOH\cdot CH_2\cdot \underset{\underset{\text{Isocitric acid}}{|\ COOH}}{CH}\cdot CHOHCOOH$ + codehydrogenase II ⇄

$COOH\cdot CH_2\cdot \underset{\underset{\text{Oxalosuccinic acid}}{|\ COOH}}{CH}\cdot COCOOH$ + reduced codehydrogenase II

The second enzyme catalyzed reaction 2:

(2) $COOHCH_2\underset{\underset{\text{Oxalosuccinic acid}}{|\ COOH}}{CH}COCOOH \rightleftarrows COOHCH_2CH_2COCOOH + CO_2$

α-Ketoglutaric acid

The first reaction is catalyzed by isocitric dehydrogenase and takes place in the absence of Mn^{++}. The second reaction is catalyzed by *oxalosuccinic carboxylase,* a new enzyme (27). Summation of the two activities result in reaction 3:

(3) $COOHCH_2\underset{|\ COOH}{CH}CHOHCOOH$ + codehydrogenase II ⇄

$COOHCH_2CH_2COCOOH + CO_2$ + reduced codehydrogenase II

Reaction 1 is reversed on the addition of an excess of oxalosuccinic acid provided that Mn^{++} ions are absent. In the presence of Mn^{++}, reoxidation can be induced by α-ketoglutaric acid and carbon dioxide (reaction 3). If codehydrogenase II is reduced with glucose-6-phosphate and glucose-6-phosphate dehydrogenase, reoxidation is brought about by α-ketoglutaric acid and carbon dioxide only after the addition of the heart enzyme and in the presence of Mn^{++} ions. Thus, the experiments of Ochoa indicate that the equilibrium of reaction 3 can be shifted to the left, toward carbon dioxide fixation, by combination with glucose-6-phosphate dehydrogenase. Codehydrogenase II is reduced according to reaction:

Glucose-6-phosphate + codehydrogenase II →

6-phosphogluconate + reduced codehydrogenase

This results in the dismutation: α-ketoglutarate + CO_2 + glucose-6-

phosphate = *l*-isocitrate + 6-phosphogluconate. Further shifting of equilibrium, favoring carboxylation of α-ketoglutaric acid, takes place in the presence of aconitase.

Ochoa (28) has demonstrated that the isocitric dehydrogenase system can be linked with the cytochrome system by cytochrome reductase. In this reaction, cytochrome c is reduced in the presence of purified isocitric dehydrogenase and oxalosuccinic carboxylase when isocitrate, manganese ions, codehydrogenase II, and cytochrome reductase are added.

Reversible Oxidative Decarboxylation of Malic Acid

Ochoa and associates (29) have isolated a new enzyme from pigeon liver. Its isolation helps to explain carbon dioxide fixation in this organ. This enzyme, in the presence of Mn^{++}, catalyzes the reversible reaction:

$$\underset{\text{Malic acid}}{COOHCH_2CHOHCOOH} + \text{codehydrogenase II} \rightleftarrows \underset{\text{Pyruvic acid}}{CH_3COCOOH} + CO_2 + \text{reduced codehydrogenase II}$$

The enzyme has been obtained by fractionation of a pigeon-liver extract with alcohol at low temperature and fractional adsorption on alumina gel. It is specific for codehydrogenase II. There is no reaction with codehydrogenase I, fumarate, or phosphopyruvate. Orthophosphate and adenosine triphosphate do not take part in this reaction (see also chapter on decarboxylating enzymes).

Diphosphoglyceric Aldehyde Dehydrogenase or Triosephosphate Dehydrogenase

Warburg and Christian (30) obtained this enzyme in crystalline form from a specially prepared yeast juice. Cori and associates (31) isolated the enzyme of rabbit muscle in crystalline form. The latter investigators obtained a yield of 7 per cent of the extracted rabbit muscle protein. The activity of the enzyme was of the same order as that of the yeast enzyme of Warburg and Christian. The muscle enzyme, however, requires a reducing agent for full activity. Caputto and Dixon (32) have also obtained, independently and about the same time as Cori and coworkers, the crystalline triosephosphate dehydrogenase from rabbit muscle.

In the presence of inorganic phosphate, diphosphoglyceric aldehyde dehydrogenase oxidizes its substrate to 1,3-diphosphoglyceric acid.

This enzyme requires codehydrogenase I, adenosine diphosphate, and inorganic phosphate for both reactions. Glyceric aldehyde is also oxidized. The oxidation of triosephosphate is considerably faster than the oxidation of glyceric aldehyde.

The following reactions take place:

Phosphoglyceric aldehyde + inorganic phosphate + codehydrogenase I

$\rightleftarrows$ 1,3-diphosphoglyceric acid + reduced codehydrogenase I

The energy-rich carboxylphosphate of 1,3-diphosphoglyceric acid is labile; it shifts its phosphate to adenosinediphosphate to reform adenosinetriphosphate:

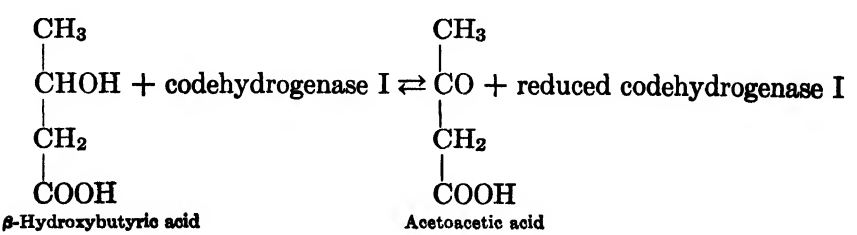
1,3-diphosphoglyceric acid + adenosinediphosphate $\rightarrow$

3-phosphoglyceric acid + adenosinetriphosphate

The postulated substrate for this enzyme, 1,3-diphosphoglyceric aldehyde, has never been prepared. The work of Meyerhof (33) appears to indicate that 1,3-diphosphoglyceric aldehyde is not formed in this reaction.

β-Hydroxybutyric Dehydrogenase

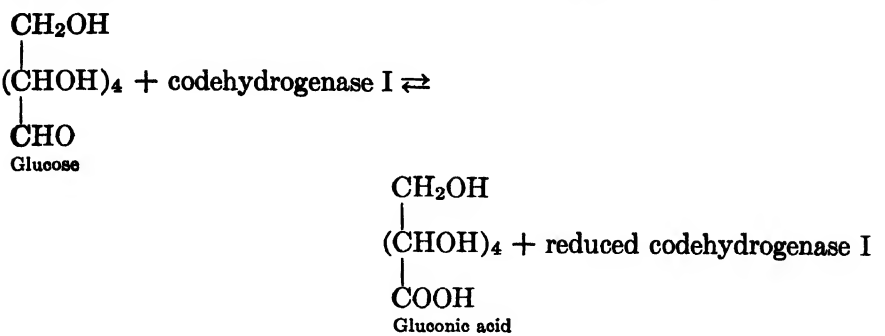
This enzyme is present in liver, heart, and kidneys. Wishart (34) has shown that the dehydrogenase may be extracted from the tissues by a disodium phosphate solution, and that it reduces methylene blue in the presence of β-hydroxybutyric acid. The two hydrogen atoms removed are taken up by codehydrogenase I (35). The end product is acetoacetic acid. It is interesting to note that the *d*-β-hydroxybutyric acid is dehydrogenated 3 to 5 times faster than the natural *l*-β-hydroxybutyric acid (36). This reaction is reversible:



Glucose Dehydrogenase of Animals

This dehydrogenase is present in animal tissues. It was first obtained by Harrison (37) from liver extracts. It is not identical with the glucose dehydrogenase of molds and bacteria. This oxidase is

highly specific for glucose, which it oxidizes to gluconic acid (38, 39):



Hexose-6-Monophosphate Dehydrogenase

Barron (40) found that erythrocytes and methylene blue oxidize glucose. Warburg and Christian (41) prepared an enzyme from horse erythrocytes. They called it "Zwischenferment." This enzyme, in the presence of a coenzyme, now known to be codehydrogenase II, and methylene blue, dehydrogenated hexose-6-phosphate (Robinson ester). The end product of this reaction is phosphogluconic acid. Negelein and Gerischer (42) prepared this dehydrogenase in pure state from bottom yeast. These workers found that the apodehydrogenase varies with the source. The yeast enzyme had an isoelectric point at *p*H 4.82, whereas the corresponding apoenzyme from erythrocytes was isoelectric at *p*H 5.85 (43).

Glutamic Acid Dehydrogenases

This enzyme supposedly oxidizes *l*(+)glutamic acid to α-ketoglutaric acid and ammonia. However, Euler and associates (44) claim that this dehydrogenase converts *l*(+)glutamic acid to iminoglutamic acid, which changes spontaneously to α-ketoglutaric acid and ammonia. This enzyme is present in yeast and requires codehydrogenase II. Damodaran and Nair (45) discovered a different glutamic dehydrogenase in germinating beans and peas. Their enzyme oxidizes *l*(+) glutamic acid to α-ketoglutaric acid and ammonia, in the presence of methylene blue or atmospheric oxygen. The enzyme of Damodaran and Nair requires codehydrogenase I. A third kind of dehydrogenase is present in various animal tissues and may be prepared in a soluble state (46, 47). This dehydrogenase acts like Euler's enzyme, supposedly converting *l*(+)glutamic acid to iminoglutaric acid. The latter acid undergoes spontaneous decomposition to α-ketoglutaric acid

and ammonia. This dehydrogenase requires either codehydrogenase I or II.

Formic Dehydrogenase of Plants

Dried peas contain a soluble dehydrogenase that decomposes formic acid into carbon dioxide and hydrogen:

$$HCOOH + \text{codehydrogenase I} \rightleftarrows CO_2 + \text{reduced codehydrogenase I}$$

Adler and Sreenivasaya (48) prepared the soluble formic apodehydrogenase from dried peas by soaking in phosphate solution, filtration, precipitation of the enzyme from the filtrate with ammonium sulfate, and dialysis. This enzyme solution was very active. It could be kept unchanged for 10 days in the refrigerator. Formic dehydrogenase transfers hydrogen to codehydrogenase I. Thus, it is different from the formic dehydrogenase of *Bacterium coli*, which does not require coenzymes, but transfers hydrogen to cytochrome c. (See Chapter XI, Dehydrogenases Transferring Hydrogen to Cytochromes.)

Malic Dehydrogenase

This dehydrogenase is present in muscle, heart, liver, kidney, brain, certain bacteria, yeast, and higher plants. Malic dehydrogenase dehydrogenates *l*(—)malic acid to oxaloacetic acid:

$$\begin{array}{l} CH_2COOH \\ | \\ CH_2OHCOOH + \text{codehydrogenase I} \rightleftarrows \\ \qquad\qquad\qquad\qquad CH_2COOH \\ \qquad\qquad\qquad\qquad | \\ \qquad\qquad\qquad\qquad COCOOH + \text{reduced codehydrogenase I} \end{array}$$

The reaction is reversed on the addition of oxaloacetic acid. Codehydrogenase I acts as a hydrogen acceptor. The reduced coenzyme may be oxidized by diaphorase. This enzyme is specific for malic acid. Natural or *l*(—)malic acid is oxidized much faster than the unnatural form (49). Traces of oxaloacetic acid inhibit the oxidation of malic acid by malic dehydrogenase, owing to reversibility of the reaction. The equilibrium is far to the side of malic acid. Mehler and coworkers (16) found, by spectrophotometric measurement of pyridine nucleotides at 340 μ, that malic dehydrogenase from pig heart reacted 15 times more rapidly with codehydrogenase I than with codehydrogenase II. Potter (50) has published a method for the determination of malic dehydrogenase in animal tissues.

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CHAPTER XI

DEHYDROGENASES TRANSFERRING HYDROGEN TO CYTOCHROMES

Dehydrogenases belonging to this group transfer two electrons to cytochrome c and two H^+ to the medium (one H^+ at a time). The system requires cytochrome oxidase, which reoxidizes the reduced cytochrome. Cyanide inhibits the cytochrome oxidase. However, this oxidase may be replaced by methylene blue, and then cyanide does not inhibit. In a large number of aerobic bacteria and in yeast, these dehydrogenase systems have an important function. Warburg and Keilin have demonstrated that practically all the respiration of these microorganisms is catalyzed by these systems, forming an important link between the various metabolic processes. Only a few enzymes belong to this group.

Succinic Dehydrogenase

This enzyme is found in most plants and animals. The liver, heart, and kidney contain large amounts. Succinic dehydrogenase oxidizes succinic acid to fumaric acid, two electrons being transferred to cytochrome c and two H^+ to the medium. The cytochrome c is reoxidized by cytochrome oxidase by means of atmospheric oxygen. Such dyes as methylene blue, toluene blue, indophenol blue, and others may function as hydrogen acceptors.

Keilin and Hartree (1) have shown that all succinic dehydrogenase systems can be stimulated by methylene blue, but stimulation by the dye is considerably less effective than by cytochrome c. Potter (2) found that a complete succinic dehydrogenase system is inhibited by methylene blue, and that the dye was about 1900 times less effective than cytochrome c as a hydrogen carrier. He found that the usual method for preparing the succinic dehydrogenase system, precipitating tissue extracts at *p*H 4.6, results in the loss of most of the enzyme on the basis of comparison with the original tissue.

Keilin and Hartree reported that codehydrogenase I in a very low concentration strongly inhibits the oxidation of succinic acid. This inhibition is brought about by the formation, in the presence of the

coenzyme, of a small amount of oxalacetic acid, which functions as a very strong competitive inhibitor of succinic dehydrogenase. Pyocyanine has also a strong inhibitory action on this enzyme system. Axelrod, Swingle, and Elvehjem (3) observed that calcium ions strongly activate the succinic oxidase system in rat-liver homogenates. Potter, however, noticed that, while the calcium ions did activate at 38° C., they had no effect or only a negligible one at 24° C. Confirming the earlier work of Adler and coworkers (4), Zittle (5) found that ribonucleic acid, adenylic, and guanylic acids inhibited succinic acid dehydrogenase. Inhibition was not immediate and appeared to be a slow chemical reaction, non-enzymic in nature. This author concludes that ribonucleic acid and its hydrolytic products may govern enzymic oxidation-reduction systems within the living cell.

α-Ketoglutaric Dehydrogenase

This enzyme was first described by Ochoa (6). It is present in extracts of washed heart tissue. In the presence of 0.025 *M* malonate, which inhibits the succinic dehydrogenase, the following reaction takes place:

$$\alpha\text{-Ketoglutaric acid} + 0.5O_2 = \text{succinate} + CO_2$$

The optimum *p*H of this reaction is at 7.5. However, after 4 to 5 hours of dialysis, very little α-ketoglutaric acid is utilized. Catalytic action is restored to values approaching those displayed before dialysis by the addition of (*a*) inorganic phosphate (but not arsenate) and (*b*) muscle adenylic acid or adenosine triphosphate (but not yeast adenylic acid or adenosine). Addition of fumarate has no influence on the rate of α-ketoglutarate utilization. This indicates that C_4 dicarboxylic acids are not concerned in the transport of hydrogen from α-ketoglutarate to oxygen. This enzyme reacts with cytochrome c.

Lactic Dehydrogenase of Yeast and Bacteria

These dehydrogenases catalyze the oxidation of lactic acid and certain other α-hydroxy acids to the corresponding keto acids:

$$CH_3CHOHCOOH + \text{acceptor} \rightarrow CH_3COCOOH + \text{reduced acceptor}$$

Yeast Lactic Dehydrogenase

This enzyme was first obtained in soluble state by Bernheim (7), by treating Delft bakers' yeast with acetone and extracting the dry residue

with alkaline phosphate solutions. Sometimes the acetone yeast is so acid that an alkaline *p*H cannot prevail, and the presence of the enzyme in the extract cannot be demonstrated. If the solution is kept alkaline, however, the enzyme can be extracted. According to Bach, Dixon, and Zerfas (8), even under the best conditions, only small quantities of the enzyme are extracted by this procedure. Bach and associates have disclosed a method for obtaining solutions several thousand times as active as those described previously. Although their final solution was 100 times more active than the original Lebedew juice used as the starting material, they state that their enzyme is not to be considered pure.

The following are the main steps employed by Bach and coworkers in the purification of lactic dehydrogenase from Delft yeast: The yeast was air-dried at room temperature, dispersed in warm tap water, and kept at 37° for 4½ hours. The mixture was centrifuged. To the supernatant Lebenew juice, alumina C_γ was added, and the *p*H was kept at 5 to 6. The alumina removed some impurities and cytochrome c. Sodium lactate was added, and the mixture was heated to 53° C. This step removes a large amount of protein. Without lactate, the enzyme was destroyed by heating to 40° C., and only a small amount of protein was precipitated. The dehydrogenase was removed from the clear solution by adsorption on calcium phosphate gel suspension. The enzyme was then eluted from the calcium phosphate by extraction with 7.2 phosphate containing 10 per cent ammonium sulfate. The enzyme solutions were concentrated on Bechhold ultrafilters. Instability of the enzyme began to be noticeable at this point and increased with further purification. The enzyme solution was now fractionated with ammonium sulfate in neutral solution and in acid solution, in order to remove traces of cytochrome c. This procedure is applicable only to Delft yeast. A different method is given for using Manchester yeast.

In the paper just cited, the Cambridge investigators also describe the new hemochromogen protein which is closely related to cytochrome b, and which they named cytochrome b_2. It accompanies their yeast lactic dehydrogenase. The authors conclude, from spectroscopic and other evidence, that cytochrome b_2 forms an essential part of the enzyme system, either as the dehydrogenase itself or as an essential carrier between lactate and methylene blue. An essential factor is required to enable this system to react with cytochrome c. The highly purified lactic dehydrogenase does not depend on soluble coenzymes or on flavin groups.

This purified enzyme has an optimum at *p*H 5.2, and it is most stable

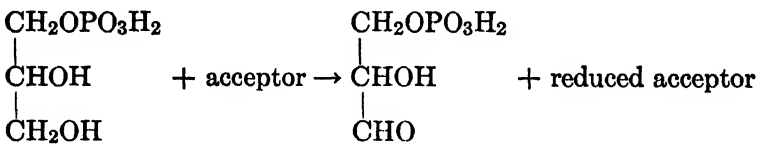
at this *p*H. The enzyme is rapidly destroyed below *p*H 4.5. The Thunberg method offers a rapid and accurate quantitative test for measuring the activity of this dehydrogenase.

Bacterial Lactic Dehydrogenase

This dehydrogenase may be prepared by autolyzing washed suspensions of *E. coli* in the presence of 1 per cent fluoride for a few days. The insoluble cellular material is removed by filtration through kieselguhr, and the enzyme is precipitated by saturation with ammonium sulfate. In this procedure, a soluble product is obtained, but there is considerable loss in enzyme activity (9).

Insoluble α-Glycerophosphoric Dehydrogenase

This enzyme is present in various animal tissues (10, 11), rabbit brain being a very good source. It converts α-glycerophosphoric acid into 3 phosphoglyceric aldehyde:



Cytochrome c serves as the hydrogen carrier. It oxidizes only the natural isomer of α-glycerophosphoric acid. β-Glycerophosphoric acid and glycerol are not affected. The reduction of methylene blue is not inhibited by cyanide, iodoacetic acid, azide, or fluoride.

Green (11) prepared this enzyme by passing rabbit skeletal muscles through a coarse meat mincer twice, and washing exhaustively with tap water. The washed mince was mixed with sand and water, and ground to a paste in a mortar. The sand and debris were filtered off through muslin. *M*/10 acetate buffer of *p*H 4.6 was added to the filtrate, and the precipitate was centrifuged. The supernatant fluid was discarded, and the precipitate was resuspended in 100 cc. *M*/5 phosphate of *p*H 7.2. This suspension retains most of its activity for 10 days if kept at 0° C. The vacuum-dried preparation is quite stable.

Glutathione Dehydrogenase

Ames and Elvehjem (12) prepared a cell-free enzyme solution from mouse-kidney homogenates, which, in the presence of cytochrome c oxidizes reduced glutathione. This system is not catalyzed by cyto-

chrome oxidase but is catalyzed by a new unknown copper enzyme. Codehydrogenase I increases the oxidation rate in the absence of cytochrome c, and it decreases the induction period either in the presence or absence of cytochrome c. Ascorbic acid also has a stimulating action on this enzyme. In contrast to cytochrome oxidase, this new enzyme is not inhibited by azide. However, it is strongly inhibited by cyanide, diethyl dithiocarbamate, and iodoacetate. Ames and Elvehjem postulate that glutathione acts as a coenzyme in certain systems involving the oxidation by molecular oxygen of fixed sulfhydryl radicals occurring in tissue preparations.

Sarcosine Oxidase

Bernheim and Bernheim (13) have shown that broken cell preparations (tissue ground with an equal volume of 0.05 *M* phosphate of *p*H 7.8 filtered through muslin) of rat, rabbit, and guinea-pig liver, but not kidney or muscle, contain an enzyme that oxidatively deaminates sarcosine (N-methylglycine) to glycine and formaldehyde:

$$CH_3NHCH_2COOH + \tfrac{1}{2}O_2 \rightarrow CH_2OHNHCH_2COOH \rightarrow NH_2CH_2COOH + HCHO$$

The optimum *p*H of this enzyme is at 7.8. The enzymic oxidation of sarcosine goes through the cytochrome-cytochrome oxidase system. Sarcosine oxidase does not require sulfhydryl groups for its action. The enzyme is not identical with succinic dehydrogenase. This oxidase, with succinic dehydrogenase, choline oxidase, *l*-proline oxidase, and other enzymes, is bound to the insoluble protein of liver. Thus, sarcosine oxidase has not yet been obtained in pure state, whereas succinic dehydrogenase is present in other tissues from which it can be prepared in a purified state for comparison.

Formic Dehydrogenase of Bacteria

This enzyme decomposes formic acid into carbon dioxide and hydrogen:

$$HCOOH + \text{methylene blue} = CO_2 + \text{leucomethylene blue}$$

Gale (14), who has extensively studied the formic dehydrogenase of *Bacterium coli*, found that the enzyme cannot be liberated from the bacterial cell. Cyanide inhibits, and formate inactivates, the enzyme system. He believes that this enzyme interacts with cytochrome c and cytochrome oxidase, which act as the hydrogen carriers. This

dehydrogenase differs from the soluble formic dehydrogenase of plants since that enzyme requires codehydrogenase I (15).

Fatty Acid Oxidase

Liver contains a labile enzyme system which oxidizes lower fatty acids, such as butyric acid. The oxidizing system is destroyed more rapidly in the absence of oxygen, and the addition of fumarate increases the rate of oxidation. A method for the preparation of this oxidase system has been described by Muñoz and Leloir (16), who used guinea-pig liver as the source material. Inorganic phosphate, fumarate, cytochrome c, adenylic acid, and magnesium or manganese ions were found to be required components. Fluoride, iodoacetate, arsenate, and malonate inhibit the oxidation. These authors claim that their preparation does not attack higher fatty acids as does the enzyme described by earlier investigators. Thus, Lang and Mayer (17) described an enzyme that dehydrogenates stearic acid to oleic acid.

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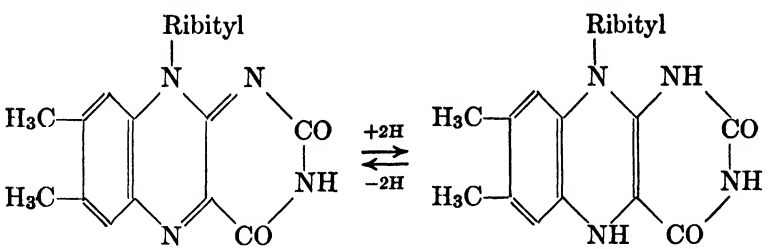
CHAPTER XII

FLAVOPROTEINS

The flavoproteins or yellow enzymes are a series of respiratory catalysts widely found in nature. They contain as their prosthetic groups either isoalloxazine mononucleotide (riboflavin phosphate) or isoalloxazine adenine dinucleotide (composed of riboflavin phosphate and adenylic acid). Riboflavin (vitamin B_2) was obtained from heart muscle by Szent-Györgyi and associates in 1932. It was synthesized, simultaneously, in the laboratories of Karrer and of Kuhn two years later. Riboflavin orthophosphate may be prepared by treating riboflavin with phosphoryl oxychloride (1) or enzymatically by phosphorylation with intestinal epithelial powder (2). Riboflavin in aqueous solution is yellow and becomes colorless on reduction. Riboflavin acts as a reversible oxidation-reduction system:



Isoalloxazine mono- and dinucleotide combine with specific enzyme proteins to form the flavoprotein enzymes, which are important components of several oxidation systems. The two formulas for the nucleotides may be written as follows:

For flavin mononucleotide: Isoalloxazine-*d*-ribose-phosphate.

For flavin dinucleotide: Isoalloxazine-*d*-ribose-phosphate-phosphate-*d*-ribose-adenine.

The Old Yellow Enzyme

In 1932, Warburg and Christian (3, 4) isolated this enzyme from bottom yeast. It has not yet been obtained from any other source. The aqueous solutions of this and the other flavoproteins are yellow and become colorless on reduction. The yellow color returns if the

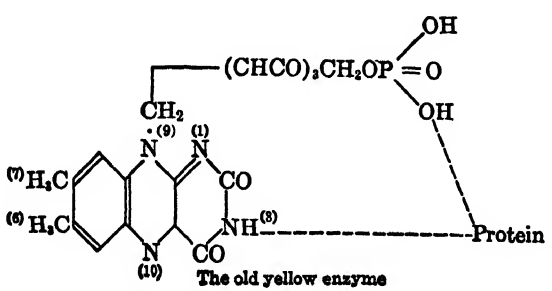
solution is shaken with oxygen or with hemin systems in the ferric state. This enzyme has been named the "old" yellow enzyme because there are now many yellow enzymes.

Preparation of the Old Yellow Enzyme. The following are the main steps used by Warburg and Christian for the preparation of their yellow ferment: The fresh juice of bottom yeast (Lebedew) was treated with lead subacetate, and the excess lead was removed by the addition of phosphate. An equal volume of acetone was added and the mixture filtered. The filtrate contained the enzyme. The filtrate was then saturated with carbon dioxide at 0° C. The pigment enzyme precipitated in the form of a yellow oil. By repeating the precipitation from an aqueous solution with acetone and carbon dioxide, followed by precipitation from aqueous solution by methyl alcohol, the substance was obtained in the form of a yellow powder.

Crystallization of the Old Yellow Enzyme. Theorell (5) obtained, by the method of Warburg and Christian, about 30 grams of the crude enzyme, which he further purified by cataphoresis at *p*H 4.2 to 4.5. This procedure caused a loss of only 10 per cent. By fractionation with ammonium sulfate at *p*H 5.2 and dialysis against 2 volumes of saturated ammonium sulfate plus 1 volume acetate buffer of *p*H 5.2, a crystalline product was obtained, the yield being 60 per cent. The constancy in pigment content indicated that the substance is pure. It contained 15.5 per cent nitrogen.

Theorell (5, 6) was able to hydrolyze the old yellow enzyme reversibly. He removed the coenzyme riboflavin phosphate from the protein, or the so-called apoenzyme, by dialyzing the enzyme solution against dilute hydrochloric acid. The protein component precipitated, and the coenzyme remained in solution. The protein precipitate was dissolved on neutralization. When the riboflavin solution was added to the apoenzyme, the active enzyme was re-formed.

The Structure of the Old Yellow Enzyme. Theorell has shown that the yellow enzyme is riboflavin monophosphoric acid combined with a specific protein:



The old yellow enzyme

The nitrogen atoms in the 1- and 10-positions are hydrogen transporters. The 6- and 7-position methyl radicals diminish the toxicity of the flavin; without them the compound is inactive. The nitrogen atom in the 9-position serves to combine the isoalloxazine nucleus with the pentose molecule, which is bound to phosphoric acid. The phosphoric acid and the nitrogen atom in the 3-position bind the prosthetic group to the protein component of the enzyme. The nitrogen atom in the 3-position is responsible for extinguishing the fluorescence of the flavinphosphate when combined with the specific protein (7).

Mechanism of the Old Yellow Enzyme. This enzyme system transfers hydrogen from codehydrogenase II to molecular oxygen as follows:

(*a*) Hexosemonophosphate + codehydrogenase II = phosphohexonic acid + dihydrocodehydrogenase II

(*b*) Dihydrocodehydrogenase II + old yellow enzyme = codehydrogenase II + reduced old yellow enzyme

(*c*) Reduced old yellow enzyme + molecular oxygen = old yellow enzyme + H_2O_2

In reaction (*a*), codehydrogenase II is bound to hexosemonophosphate dehydrogenase, called " Zwischenferment " by Warburg.

Xanthine Dehydrogenase (Xanthine Oxidase, Schardinger Enzyme, Aldehydrase)

This oxidase does not always occur where purine metabolism is involved. Morgan (8) has extensively studied the distribution of the enzyme in the tissues of a large number of animals. Thus, in the horse, the spleen is the only organ that contains it. In the hen, it is present in the liver and kidneys. The reduction of methylene blue by aldehydes, in the presence of fresh milk and the absence of oxygen, was described by Schardinger in 1902. The general contention is that xanthine oxidase and the aldehyde-oxidizing enzyme are identical (9). Milk xanthine oxidase had been prepared in a highly purified state by Ball (10). He found that the golden-brown enzyme contained isoalloxazine adenine dinucleotide, and that it could be reversibly split. The oxidase had an isoelectric point at *p*H 6.2 and a molecular weight of 74,000. It oxidizes xanthine to uric acid, and aldehydes to the corresponding acids, removing two hydrogen atoms in the dehydrogenation. Xanthine oxidase also oxidizes reduced codehydrogenase I. The purified enzyme uses oxygen, as well as methylene blue and other

dyes, as the hydrogen acceptor. Cyanide acts as an irreversible inhibitor. Plant aldehyde dehydrogenase is a different enzyme, because it does not act on purines.

Liver Aldehyde Oxidase

This enzyme occurs in mammalian liver. It oxidizes aliphatic and aromatic aldehydes to acids. Unlike xanthine oxidase, this enzyme does not attack purines. Gordon and coworkers (11) found that liver contains aldehyde mutase, xanthine oxidase, and aldehyde oxidase. These investigators separated liver aldehyde oxidase from the oxidases just mentioned. The purified enzyme had a yellowish brown color and contained adenine dinucleotide as the prosthetic group.

Fumaric Hydrogenase

This flavoprotein catalyzes the reduction of fumaric acid to succinic acid in the presence of a leuco dye. The leuco dye is oxidized to the colored dye:

$$\begin{array}{l} \text{HCCOOH} \\ \| \\ \text{HOOCCH} \end{array} + \text{leuco dye} = \begin{array}{l} \text{COOH} \\ | \\ \text{CH}_2 \\ | \\ \text{CH}_2 \\ | \\ \text{COOH} \end{array} + \text{colored dye}$$

The activity of the enzyme is measured by determining the rate of color appearance. Fumaric dehydrogenase can be reversibly split into apoenzyme and isoalloxazine dinucleotide. The combination of protein and coenzyme is not as firm as in the other yellow enzymes (12). This enzyme occurs in crude fractions of the old yellow enzyme of Warburg and Christian.

l-Amino Acid Oxidase (*l*-α-Hydroxy Acid Oxidase)

This enzyme catalyzes the oxidation of the natural or *l*-forms of leucine, phenylalanine, norleucine, norvaline, isoleucine, valine, cystine, histidine, tyrosine, methionine, alanine, and tryptophane. N-methylleucine is oxidized to the corresponding keto acid and methyl amine. The pyrrolidine ring of proline is split open by oxidation, with the formation of α-amino-δ-ketovaleric acid. In this reaction, one molecule of oxygen is taken up for each molecule of amino acid, resulting

in one molecule of each keto acid, ammonia, and hydrogen peroxide. When catalase is also present, however, 1 atom of oxygen is taken up per molecule of amino acid oxidized. Methylene blue functions as a hydrogen acceptor in this oxidation. The enzyme is inhibited by ammonium ions, benzoate, and iodoacetate (13, 14). The best source for the preparation of this enzyme is rat kidney, liver tissue, certain bacteria, and snake venom.

Preparation of Rat-Kidney *l*-Amino Acid Oxidase. Blanchard and coworkers (15) obtained *l*-amino acid oxidase in a pure state. Rat-kidney tissue was minced in a Waring blender, poured into 4 volumes of acetone, and cooled to —5° C. with Dry Ice. The tissue was filtered with suction, washed with cold acetone, dried, and pulverized. The powder was extracted with 10 volumes of water for 30 minutes. The filtrate was purified by an extensive series of salt fractionations and dialysis. This brought the enzyme to electrophoretic homogeneity. The oxidase was found to be a flavoprotein with riboflavinphosphate as the prosthetic group. By ultracentrifuging, two components were obtained possessing the same catalytic function, the heavy component being an aggregate of 4 molecules of the light one. The light component contains 2 molecules of flavin, and the heavy component 8. This enzyme also oxidizes *l*-α-hydroxy acids, such as lactic acid.

Zeller and Maritz (16) have previously observed that rat kidney and liver contain an enzyme that oxidizes *l*-phenyllactic acid.

Bacterial *l*-Amino Acid Oxidase

Bernheim and associates (17) were the first to report that *Proteus vulgaris* was able to oxidize all the known natural amino acids. Stumpf and Green (18) verified these observations, provided that the freshly harvested bacterial cells were used in the test. When a suspension was kept for 2 weeks at 0° C., only 11 of 22 amino acids were oxidized. The gradual disappearance of activity is explained by assuming that there are several amino acid oxidases in the bacterium. Green and Stumpf obtained this enzyme in cell-free form by disintegrating the bacterial cell with supersonic vibrations. A similar enzyme was found in *Aerobacter aerogenes* and *Pseudomonas pyocyaneus.*

Snake Venom *l*-Amino Oxidase

Zeller and Maritz (19, 20) found a powerful and very soluble *l*-amino acid oxidase in various snake poisons. This enzyme, however,

is different in many respects from mammalian *l*-amino acid oxidase. A variety of amino acids are oxidized. *l*-Proline, *l*-hydroxyproline, N-methyl-*l*-leucine, and N-methyl-*l*-phenylalanine, however, were not oxidized. Natural aspartic and glutamic acids were deaminated by oxidation. Dimedon and carbonyl reagents, such as semicarbazide-HCl, hydroxylamine-HCl, and 2,4-dinitrophenylhydrazine, inhibit in low concentrations. Zeller and coworkers (21) found a similar enzyme in various organs of snakes. The best source was lung tissue.

d-Amino Acid Oxidase of Animal Tissues

The oxidase is present in most animal tissues, kidney and liver being the best sources. *d*-Amino acid oxidase removes the α-amino group from *d*-amino acids, replacing it by oxygen. This results in the formation of the corresponding α-keto acid and ammonia ($RCHNH_2COOH + O_2 = RCOCOOH + NH_3$). Glycine, *dl*-lysine, and *dl*-cystine, however, are not attacked by the oxidase (22). *d*-Proline, having no amino radical, is oxidized to α-keto-δ-aminovaleric acid (23). The function of this enzyme is unknown. *d*-Amino acids do not, as a rule, occur in nature.

Properties of Enzyme. This enzyme contains a protein and a flavin coenzyme (Straub). It was shown by Warburg and Christian (24) that the pure coenzyme is an isoalloxazine adenine dinucleotide. For the preparation of the pure *d*-amino acid oxidase, the paper by Negelein and Brömel (25) may be consulted. Edlbacher and associates (26) found that the purified kidney oxidase becomes 100 times more active in the presence of 0.001 *M* hydrogen cyanide when *d*-alanine is the substrate. Similar functions were shown by pyrophosphate. Semicarbazide acted as a powerful inhibitor. Under certain conditions, both *l*-amino acid oxidase and *d*-amino acid oxidase are powerfully inhibited by octylalcohol.

Hellerman and coworkers (27) prepared *d*-amino acid oxidase from lamb kidneys and separated the prosthetic group according to the method of Warburg and Christian. Quinine, atabrine, and related substances inhibited the *d*-amino acid oxidase strongly at low concentrations of the prosthetic group flavin-adenine-dinucleotide, but only slightly at high concentrations. This suggests inhibition by competition with the dinucleotide for the enzyme protein (apoenzyme).

Activation of the Oxidation of *d*-Amino Acids by *l*-Amino Acids. Edlbacher and Wiss (28), using a purified, dry swine-kidney *d*-amino acid oxidase preparation, have made some very interesting observations concerning the activation of oxidation of *d*-alanine, *d*-valine, *d*-leucine,

d-isoleucine, and *d*-phenylalanine with a series of *l*-amino acids (see Fig. 16). The degree of activation differs for each amino acid, depending on the purity and concentration of the enzyme and the quantity of *l*-amino acid used. Large quantities of *l*-amino acids produce an inhibition. Inactive proteins isolated during the purification of the oxidase are very powerful activators of the purified enzyme.

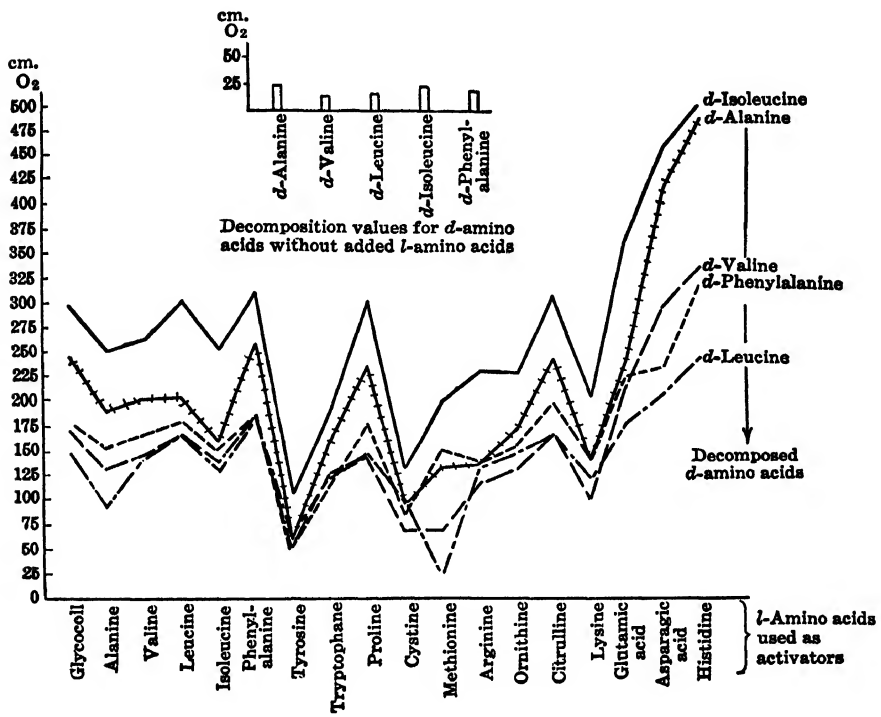


FIG. 16. Activation of *d*-amino acids by *l*-amino acids. The ordinate shows oxygen consumption in 1 hour. The abscissa indicates the added *l*-amino acids. Their concentration and those of the *d*-amino acids was *M*/50. The figure also shows the oxidation of the *d*-amino acids without added *l*-amino acids. The *p*H was 8.

Glycine Oxidase of Kidney

This oxidase may be prepared by neutral aqueous extraction of pig-kidney acetone powder. This enzyme oxidizes glycine to glycolic acid and ammonia, and sarcosine to glycolic acid and methyl amine. Dimethylglycine, phenylglycine, and acylglycines are not oxidized. The presence of flavin adenine dinucleotide is required for activity. This enzyme is most active at *p*H 8.3. Glycine oxidase prepared from kidneys of other animals, or from pig kidney by extraction at *p*H 4.8

of the acetone powder, does not require the addition of dinucleotide. Glycine oxidase is a flavoprotein (29).

Glucose Oxidase (Notatin) of Molds

Müller (30) has shown that the glucose-oxidizing enzyme of *Aspergillus niger* and certain *Penicillia* is a specific enzyme which oxidizes glucose to gluconic acid. Galactose and mannose were oxidized only slowly. Xylose, arabinose, fructose, and lactose were not oxidized. Sucrose, however, was attacked, since it was first hydrolyzed by the invertase, which was present in the crude enzyme preparations. Nord and Engel (31) found this enzyme in *Fusarium lini.* It had been reported that this mold also contains an enzyme that oxidizes xylose to xylonic acid, and arabinose to arabonic acid (32).

Franke and Deffner (33), working with a purer glucose oxidase preparation than earlier investigators, destroyed the catalase of their preparation with hydrogen sulfide. A quantitative amount of hydrogen peroxide was formed during the oxidation of glucose in the presence of molecular oxygen to gluconic acid:

$$C_5H_{11}O_5CHO + H_2O + O_2 = C_5H_{11}O_5COOH + H_2O_2$$

Also, they observed a proportionality between the activities of various preparations and their flavin content. They concluded that glucose oxidase must be a flavoprotein. Coulthard and associates (34) have published a very extensive study concerning the purification and antibacterial properties of glucose oxidase from *Penicillium notatum* Westling. They named the purified preparation " notatin." Franke's suggestions concerning the flavoprotein nature of the oxidase were confirmed. The antibacterial action is said to be due to the enzyme's ability to produce hydrogen peroxide from glucose and oxygen. This activity *in vitro* is very high, completely inhibiting the growth of *Staphylococcus aureus* in a dilution of even 1:1,000,000,000. *d*-Xylose and *d*-mannose also can be oxidized by the purified enzyme, but much less effectively than glucose. An identical enzyme has been obtained from *P. reticulosum.*

Green and Pauli (35) have demonstrated that the flavoprotein from milk, which catalyzes the oxidation of hypoxanthine to uric acid (xanthine oxidase), and which produces hydrogen peroxide, also has antibacterial action. This property is lost when minute amounts of catalase are added or as soon as the activity of the enzyme ceases. They conclude that there is little to choose between the flavoproteins from cows' milk and *P. notatum,* respectively, as far as antibacterial

action is concerned. The only difference is that notatin is a more stable enzyme. This would suggest that any flavoprotein enzyme system that produces hydrogen peroxide should have the same antibacterial action as notatin.

More recently, Keilin and Hartree (36), using a purified preparation of Coulthard and associates, have furnished new evidence showing that glucose oxidase is a yellow enzyme. They have combined the prosthetic group of glucose oxidase (isoalloxazine adenine dinucleotide) and the inactive protein fraction of amino acid oxidase. This brought the inactive protein of the amino acid oxidase back to its original activity. On addition of glucose to the enzyme, its characteristic yellow color is rapidly discharged, owing to the reduction of the isoalloxazine ring. When the mixture is shaken with air, the yellow color of the enzyme returns, as a result of the reoxidation of the isoalloxazine ring. Intact glucose oxidase, unlike diaphorase, shows no fluorescence in ultraviolet light, but the green fluorescence of the free dinucleotide appears on denaturation of the enzyme protein. The notatin preparation studied was about 90 per cent pure.

Preparation of Pure Glucose Oxidase. Coulthard and associates prepared their glucose oxidase (notatin) from a culture of *Penicillium notatum* Westling, using a modified Czapex-Dox medium. The culture filtrate was concentrated to one-fifth of its original volume. To the reddish brown concentrate, 2 volumes of acetone was added at 0° C. The precipitate was dissolved in water and again precipitated with acetone. This procedure was repeated once more. The concentrate may be purified by precipitation with tannic acid. The enzyme-tannate was decomposed by triturating with acetone. From the solution, the enzyme was precipitated with an excess of acetone. The solid was dried and powdered. Several procedures are given for the further purification of the concentrated enzyme.

Histaminase or Diamine Oxidase

This enzyme was first described in 1929 by Best (37), who believed that it is specific for histamine. However, Zeller (38, 39) has shown that the enzyme attacks almost all diamines by oxidative deamination and has suggested the following equation for the reaction:

$$RCH_2NH_2 + O_2 + H_2O = RCHO + NH_3 + H_2O_2$$

Zeller named this enzyme diamine oxidase. It is present in many tissues; the kidneys and the intestinal mucosa are the best sources. Usually, the defatted tissues are extracted with water, and the filtered

extract is concentrated *in vacuo*. Also the enzyme may be precipitated from aqueous extracts by the addition of acetone or alcohol; then the precipitate is dried *in vacuo*. These products are very soluble and highly active, but impure.

Diamine oxidase destroys toxic diamines, such as histamine, cadeverine, and putrescine. Histamine and other diamines form in the large intestine as a result of bacterial decarboxylation of amino acids, such as histidine, lysine, and ornithine. Histamine is a very toxic substance, having a pronounced pharmacological action.

Purification. Swedin (40) prepared histaminase by extracting hog kidney with $\frac{1}{15}$ *M* phosphate buffer of *p*H 7.7. The filtrate was fractionated with ammonium sulfate. The precipitate was dissolved in water which had been brought to *p*H 7.5 with ammonium hydroxide. The filtrate was dialyzed against distilled water. The residual mixture was heated for 10 minutes at 50° and then centrifuged. The filtrate was again fractionated with ammonium sulfate. The purified enzyme had all the properties of a flavoprotein. When the purified enzyme was subjected to dialysis it lost 80 per cent of its activity, but 32 per cent was restored on the addition of flavin. Treatment with methyl alcohol also caused destruction. If the alcoholic solution was made alkaline, irradiated with electric light, and neutralized, all activity was lost. When the enzyme acts on histamine, one atom of oxygen is used and no hydrogen peroxide is formed (41).

Codehydrogenase Oxidases (Diaphorases)

Two codehydrogenase oxidases are known at present. They are widely distributed enzymes. Both are isoalloxazine dinucleotides (42, 43). One oxidizes codehydrogenase I and the other codehydrogenase II. Both require methylene blue for the oxidation.

Cytochrome Reductase

This new flavoprotein enzyme was isolated from yeast by Haas and associates (44). Physiologically, it appears to be much more important than the diaphorases and the old yellow enzyme. The old yellow enzyme is probably a decomposition product of the reductase. This new enzyme can be rapidly reduced by dihydrocodehydrogenase II and oxidized by ferricytochrome c. Thus, the reductase establishes an important link in the chain of respiratory enzymes which transfer hydrogen to codehydrogenase II. Cytochrome reductase has the same prosthetic group as the old yellow enzyme, e.g., alloxazine mononucle-

otide; but the proteins differ. Like some of the other yellow enzymes, reductase may be reversibly split into enzyme protein (apoenzyme) and coenzyme. The reductase has a molecular weight of 75,000.

Haas and coworkers use the following system in their reductase studies: hexosemonophosphate, hexosemonophosphate dehydrogenase, codehydrogenase II, cytochrome-reductase, and cytochrome c. In this reaction, the codehydrogenase II is rapidly reduced, whereas the ferricytochrome c does not react until the reductase is added. Enzyme action may be demonstrated by spectroscopic means within a few minutes. Instead of ferricytochrome c, molecular oxygen may also be used for the reoxidation of reduced cytochrome reductase:

$$\text{Reductase}\cdot H_2 + O_2 \rightarrow \text{reductase} + H_2O_2$$

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CHAPTER XIII

OTHER OXIDASES

Luciferase

The enzyme luciferase was discovered in 1885 by Dubois in the elaterid beetle *Pyrophorus noctilucus,* and the mollusc *Pholas dactylus* Harvey (1) showed that luciferase is present in fireflies, in ostracod crustaceans, and in the worm *Odontosyllis.* According to Harvey, it occurs in forty different orders of animals, in certain classes of plants, and in bacteria and fungi. The firefly, however, is the best-known luminous animal. Some familiar examples of bioluminescence are the glowing of wood, the phosphorescence of the sea, and the shining of fish. They are all due to microscopic organisms. In some luminous animals, however, the presence of the luciferin-luciferase system could not be demonstrated (2). Bioluminescence is a distinct form of chemiluminescence, resulting from the energy change in a chemical reaction.

Luciferin, the substrate of luciferase, also was discovered by Dubois. Luciferin is a rare example of a substrate with an unknown chemical nature. The oxidation product of luciferin (after luciferase has been acted upon) has been named oxiluciferin by Harvey. The luciferin differs slightly with the species, and the luciferase acts only with luciferin of closely related animals.

Preparation. The best source for the preparation of luciferin and luciferase is an animal such as the ostracod crustacean *Cypridina hilgendorfii,* which has a large gland in the head region. This gland manufacturers and stores the oxidase as well as luciferin. By drying the animals quickly, the gland can be preserved for many years. This material shows strong luminescence on moistening. Anderson (3) purified *luciferin* considerably by extracting dry cypridinas with methyl alcohol. Then he added 10 per cent of butyl alcohol and removed the methyl alcohol *in vacuo.* The supernatant butyl alcohol extract was treated with benzoyl chloride. After 15 minutes, this solution was diluted with 10 volumes of water, and the inactive benzoyl-luciferin derivative was extracted with ether. After removal of the ether, the residue was hydrolyzed with hydrochloric acid. The free active luciferin was extracted with butyl alcohol. By repeating the benzoylation and hydrolysis, the luciferin was concentrated 2000-fold.

The *luciferase*, as used by the Princeton workers, was prepared from rapidly dried powdered cypridinas by extracting with water, and by dialyzing the extract against cold running water for 24 hours (2). A few drops of toluol added to the enzyme solution will preserve it for months in a refrigerator.

Mechanism of Luciferase Action (2). In the presence of luciferase and oxygen, luciferin produces light. The following four steps are involved:

(1) LH_2 (luciferin) + A (luciferase) → A·LH_2 (luciferin-enzyme compound)

(2) A·LH_2 + $\frac{1}{2}O_2$ → A·LH_2·O

(3) A·LH_2·O → A′ (excited luciferase) + L (oxidized luciferin + H_2O)

(4) A′ → A + $h\nu$ (a quantum of light)

When the exact chemical nature of this enzyme system is elucidated, this scheme will undoubtedly be modified.

The Chemistry of the Luciferin-Luciferase System (1, 2). Luciferin of *Cypridina* dialyzes through a membrane. It is not destroyed by trypsin, and it is soluble in water, in many organic solvents, in dilute salts, in acids, and in alkali. In alkaline medium it oxidizes readily, but it keeps for years in aqueous solution. Saturated ammonium sulfate precipitates the luciferin, but not magnesium sulfate or sodium chloride.

The oxidase *Cypridina* luciferase does not dialyze through membranes; it is destroyed by trypsin; it is insoluble in practically all organic solvents, and soluble in water, dilute salt solutions, dilute acid, and alkali. It is precipitated by the usual protein precipitants.

Giese and Chase (4) reported that 0.000033 *M* cyanide affects partially purified *Cypridina* luciferin, so that no light is produced when luciferase is added. Much higher concentrations of cyanide, however, do not affect luciferase. Crude luciferase is not inhibited by even 0.033 *M* potassium cyanide. Calculations based on the combining weight of luciferin with cyanide indicate that luciferin has a low molecular weight. Johnson and Chase (5) found that the luciferin-luciferase system is inhibited by small amounts of urethane, sulfanilamide, sulfathiazol, sulfapyridine, and *p*-aminobenzoic acid. The urethane inhibition is reversible except at high concentrations (approaching 1 *M*). Sodium azide progressively inhibits this enzyme system in concentration from 0.001 *M* to 0.1 *M*. The action of azide is almost entirely upon the luciferin and is a reversible inhibition (6). Chase (7) found that *Cypridina* luciferase, partially purified by dialy-

sis, is reversibly inactivated by short exposure to a temperature of 38° C. in the same manner that light intensity of luminous bacteria is reversibly quenched.

Kluyver and coworkers (8) have studied the inhibition of luminescence in *Photobacterium phosphoreum* by irradiation with blue light. They believe that the inhibition is due to the photochemical inactivation of dehydroluciferin. An examination of the inactivation spectrum indicates that this coenzyme may be a vitamin K derivative. More recently, Johnson and Eyring (9), however, have published results that suggest that the luminescent system of *Cypridina* consists of a pyridine nucleotide and flavoprotein. In view of these results, it may be said that the chemical nature of the luciferin-luciferase system deserves further study.

Uricase

This oxidase may be prepared from the liver, kidney, and brains of most mammals. It is not present in human organs. Keilin and Hartree (10) found that the respiratory quotient of this enzyme was consistent with the following reaction:

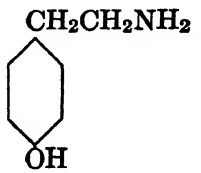
$$\text{Uric acid} + O_2 + H_2O \rightarrow \text{allantoin} + CO_2 + H_2O_2$$

Since this finding was not consistent with earlier views, Klemperer (11) has re-examined the enzymic oxidation of uric acid. He observed that the amount of carbon dioxide formed during this oxidation was only a fraction of one equivalent, indicating that compounds other than allantoin are formed in the reaction. From the quantity of nitrogen which these substances lost on treatment with nitrous acid, he concluded that these compounds are uroxanic acid and hydroxyacetylene-diurcine-carboxylic acid. These substances are not attacked by uricase. The primary product of uricase action is an unstable substance that decomposes spontaneously into the three reaction products. The relative amount of these products formed depends on the *p*H and the nature of the buffer used. It is not known whether this reaction occurs *in vivo*. The decomposition products uroxanic acid and hydroxyacetylene-diurcine-carboxylic acid have not been found so far in urine.

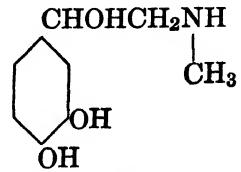
In this work, Klemperer employed pig-liver or beef-kidney uricase prepared according to the method of Keilin and Hartree. Dry tissue powder was extracted at 38° C. with Sørensen's borate buffer of *p*H 9.2, and the clear extract adjusted to a *p*H of 4.5 with 0.5 *N* acetic acid. The precipitate that formed contained the entire uricase activity. This enzyme has optimum *p*H at 9.2.

Tyramine Oxidase

Tyramine is one of the toxic amines. It raises blood pressure. It is structurally related to the blood-pressure-raising hormone adrenaline:



Tyramine



Adrenaline

Bernheim and Bernheim (12) have found that rat, rabbit, guinea-pig heart slices and intestinal muscle strips can both deaminate the side chain of tyramine and cause the disappearance of the hydroxy group on the ring. Kidney and liver slices, however, bring about only deamination. Dog and cat ventricles are much less active. None of these reactions take place anaerobically. Cocaine, ephedrine, caprylic alcohol, and high concentrations of cyanide inhibit the deamination but have little effect on the oxidation of the hydroxyl group (Table XXIV). Methylene blue inhibits both, whereas thiourea has

TABLE XXIV

Effect of Various Drugs on Disappearance of Amine and Hydroxy Groups of 2.0 Mg. of Tyramine Hydrochloride Incubated for 2 Hours with Rat Heart Slices

Drug	NH_2-N Disappeared from 0.164 Mg. Added as Tyramine HCl		OH Groups Disappeared from 0.2 Mg. Added as Tyramine HCl	
	mg.	per cent	mg.	per cent
	0.139	85	0.140	70
7.3×10^{-3} *M* cocaine HCl	0.072	44	0.134	67
5.9×10^{-3} *M* ephedrine SO_4	0.061	37	0.140	70
2.0×10^{-2} *M* caprylic alcohol	0.082	50	0.115	57
1.3×10^{-3} *M* methylene blue	0.044	27	0.014	7
0.85×10^{-2} *M* indole	0.057	35		
0.57×10^{-2} *M* indole acetic acid	0.082	50		
0.5×10^{-2} *M* sodium cyanide	0.070	43	0.130	65

no action on either. This indicates that a copper-containing enzyme is not involved. Indole and indole acetic acid inhibit deamination, but their effect on the oxidation of the hydroxyl radical could not be determined. The deamination of tyramine is more rapid than the removal of the hydroxyl groups. This is also true when the tyramine concentration is altered. All drugs, with the exception of methylene

blue, inhibit the deamination much more than the removal of the hydroxyl groups. However, Bernheim and Bernheim conclude that their findings do not necessarily indicate that two enzymes are concerned with the oxidative decomposition of tyramine. Animal tissues contain enzyme systems which are able to oxidize aliphatic as well as aromatic monoamines (13).

Fatty Acid Dehydrogenase

Various animal tissues contain an enzyme which dehydrogenates higher fatty acids. Liver appears to be the best source (14). According to Champougny and Le Breton (15) this enzyme requires adenosinetriphosphoric acid as a coenzyme. The hydrogenase has its optimum at *p*H 8.0. These workers suggest the following procedure for the quantitative estimation of liver fatty acid dehydrogenase:

Six grams of fresh liver tissue is ground in a mortar at 0° C. The homogenate is placed in a centrifuge tube without rinsing the mortar. The tube is dipped in hot water in order to raise the temperature to 37° within 3 minutes. As soon as 37° is reached, the tube is cooled quickly to 0° and centrifuged 25 minutes at 4000 r.p.m. in a centrifuge cooled below 5°. The supernatant is transferred to another centrifuge tube; 4.9 grams of ammonium sulfate is added, and the tube is centrifuged for 25 minutes at low temperature. The precipitate is dissolved in 10 cc. of Sørensen phosphate of *p*H 6.0. The temperature is kept low to avoid loss of activity. The enzyme activity is determined by Thunberg's method by mixing 1 cc. of phosphate of *p*H 8.0, 1 cc. of 0.01 *M* sodium palmitate, 0.8 cc. of a solution containing 0.08 milligrams of adenosinetriphosphoric acid in phosphate of *p*H 8.0, and 0.2 cc. of 0.0005 *M* methylene blue. One cubic centimeter of the liver enzyme solution is added, and the time required for the decolorization of the dye is noted. Samples from different lobes of the liver of the same rabbit differ considerably in their dehydrogenase activity. Without the coenzyme, no activity is shown.

Glucose Dehydrogenase of Germinated Seeds

Basu and Karkun (16) described an oxidizing enzyme of the germinated seeds of green and black grams (*Phaseolus radiatus* and *P. mungo* L.). This enzyme acts both aerobically and anaerobically on glucose, with gluconic acid as the probable end product. Galactose and mannose also are said to be oxidized by this enzyme, but not fructose, xylose, or arabinose. Methylene blue inhibits the enzyme

and cannot be employed as a hydrogen acceptor. 2,6-Dichlorophenol-indophenol, however, acts as an acceptor. Narcotics inhibit the dehydrogenase function, while potassium cyanide and hydrogen sulfide inhibit to oxidase action. Flavin, adrenaline, and ascorbic acid do not act as hydrogen carriers, but glutathione does to some extent. This enzyme appears to be different from Harrison's liver glucose oxidase and the glucose oxidase of certain molds.

Sulfide Oxidase. From tissues of the higher vertebrates, such as cat, dog, cow, and sheep, an enzyme may be extracted which oxidizes $S^=$ into $S_2O_3^=$. Der Garabédian (17) found that optimum extraction of sulfide oxidase may be obtained with phosphate buffer of *p*H 7.2. The enzyme may be precipitated from aqueous solutions by ethyl alcohol. The oxidase does not dialyze through cellophane. It has optimum *p*H at 7.2.

Cysteine Desulfurase. This enzyme is present in certain bacteria and in animal tissues. It catalyzes the liberation of hydrogen sulfide from cysteine. According to Fromageot and Grand (18), the action of cysteine desulfurase is completely suppressed by 10^{-3} *M* hydrocyanic acid and by reagents that combine with a carbonyl group, a carboxyl group, and a basic nitrogen group.

Dopa Oxidase. Dopa oxidase (19) is the enzyme that oxidizes 3,4-dihydroxyphenylalanine ("dopa") to melanin. It is found in the melanoblast of the epidermis. It may be detected by fixing skin sections in formaldehyde and treating them, for a time varying from a few hours up to a few days, with a 0.1 per cent solution of pure 3,4-dihydroxyphenylalanine buffered to *p*H 7.3 to 7.4. After the tissue has been kept in the reagent for 24 hours or so, the cells containing the oxidase will be deeply stained with melanin. Only the pigment-forming cells (melanoblasts) give this color reaction. Dopa oxidase, according to Bloch and Schaaf (20), does not give color reactions with monophenolic substances, with catechol derivatives, or with polyphenolic compounds. According to some authors, the dopa oxidase is stereo specific. It reacts only with the natural *l*-substrate (21). However, the specificity of dopa oxidase has been seriously questioned (22).

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CHAPTER XIV

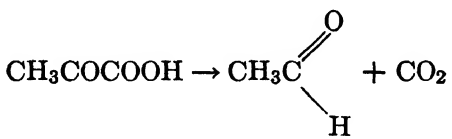
DECARBOXYLATING AND PHOSPHOPYRIDOXAL ENZYMES

DIPHOSPHOTHIAMIN ENZYMES

All animal tissues and many plants and bacteria contain diphosphothiamin enzyme systems that act on pyruvate in various ways. The best known, and the first to be recognized, is yeast carboxylase.

α-Keto Carboxylases of Plants

Yeast and other plants contain an enzyme that decarboxylates α-keto acids, such a pyruvic acid, forming the corresponding aldehyde; e.g., acetaldehyde (1, 2):



The function of α-keto carboxylase in yeast is important, since alcohol is formed from acetaldehyde by the action of alcohol dehydrogenase and reduced cozymase:

$$CH_3CHO + H_2 = CH_3CH_2OH$$

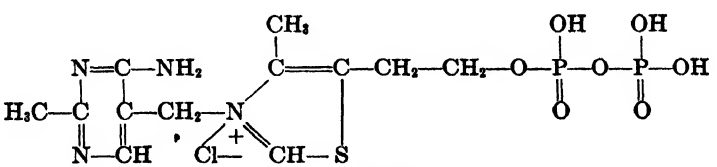
The general opinion has been that the decarboxylation of pyruvic acid in the yeast carboxylase system takes place in the manner just mentioned. However, Euler, Ahlström, and Högberg (3) reported that from pyruvic acid, dry yeast carboxylase produces 20 per cent lactic acid, 15 per cent carbon dioxide, and 50 per cent acetaldehyde. These authors state that pyruvic acid is first rearranged before it is acted upon by carboxylase, and that it will require further investigation to find out whether or not the carboxylase system itself or the interaction of a flavin enzyme is required for the formation of lactic acid from pyruvic acid. Kubowitz and Luttgens (4) reported that purified yeast carboxylase contains 1 gram atom of magnesium and 1 mole of diphosphothiamin to 75,000 grams of protein (apoenzyme). This enzyme is most active at *p*H 6.2.

Cocarboxylase (Diphosphothiamin)

In 1932, Auhagen (5) showed that yeast carboylase can be separated into two components, one a protein and the other a thermostable fraction. He found that, when brewers' yeast was washed with an alkaline phosphate solution, it lost its power to act on pyruvic acid, and in 1937 Lohmann and Schuster (7) isolated from yeast cocarboxylase, the carboxylase again became active. Auhagen obtained cocarboxylase in a semi-pure state. He suggested that the substance was probably a new phosphoric ester of an organic substance.

Simola (6) obtained some of Auhagen's cocarboxylase, with which he carried out experiments on rats. He found that the substance possessed vitamin B_1 potency and that tissues of rats kept on a vitamin B_1-free diet contained less cocarboxylase than those of an animal which received cocarboxylase.

In 1928, Kinnersley and Peters showed that yeast contained an unknown derivative of thiamin that was precipitable by lead acetate; and in 1937 Lohmann and Schuster (7) isolated from yeast cocarboxylase in pure crystalline state and identified it as the pyrophosphoric acid ester of thiamin. This work was supported by the findings of Stern and Hofer (8), who, by treating thiamin with $POCl_3$, obtained a demonstrable quantity of cocarboxylase which, however, was insufficient for isolation. A practical method for the synthesis of cocarboxylase was published by Tauber (9). By this method, which uses a new procedure for the synthesis of primary phosphoric acid esters, cocarboxylase may be prepared in large quantities. In this synthesis, thiamin and anhydrous sodium pyrophosphate are heated in a medium of dehydrated orthophosphoric acid. A study of the pure crystalline product showed that it was in every respect identical with the natural coenzyme (10). Thus, the important work of Lohmann and Schuster was fully verified by synthesis and analysis of the synthetic product.



Cocarboxylase

Weil-Malherbe (11) also has developed a method for the synthesis of cocarboxylase. It is based on the replacement of the alcoholic hydrogen group of thiamin with bromine by heating with alcoholic hydrogen bromide and allowing the bromothiamin to react with silver

pyrophosphate. He found, however, that the original method of Tauber (9) is more practical.

Karrer and Viscontini (12) have found that, from the reaction mixture of Tauber's (9) synthesis, the yield of cocarboxylase may be increased up to 55 per cent. The Swiss authors isolated the carboxylase as the phosphate, rather than the chloride, which was the procedure followed by Weijlard and Tauber (10), who, by dissolving the reaction mixture in hydrochloric acid, split much of the pyrophosphate into the monophosphate.

Thiamin or thiamin monophosphate cannot replace cocarboxylase. The function of this coenzyme is not well understood. Cocarboxylase is a very stable compound and is very soluble. It is formed from thiamin and phosphate by many cells.

Activators of the Carboxylase Enzyme System. Alkali-washed dry yeast is only slightly active when cocarboxylase is added to it. For full activity, magnesium ions or manganese ions (7) must be added. The latter, however, activate in much greater dilutions. Tauber has found that, in addition to the salts mentioned, other salts have some activating property. Sodium cyanide is a good activator, probably because it combines with the inhibitory end product, acetaldehyde, forming a more reactive cyanohydrin (enol) with the pyruvic acid. Greenberg and Rinehard (13) showed that cysteine, reduced glutathione, sodium bisulfite, and phenylhydrazine increase carboxylase activity. These compounds also combine with aldehyde and ketone groups.

Inhibitors of Carboxylase. This enzyme is inactivated by copper, silver, mercuric, and antimony ions, by iodine, and by bromine (14, 15). Sevag and collaborators (16) found that cocarboxylase antagonizes the inhibitory action displayed by sulfathiazole on the carboxylases of yeast and of *Staphylococcus aureus*. On molecule of cocarboxylase counteracted the inhibitory effect of 322 to 53,400 molecules of sulfathiazole on yeast carboxylase and the inhibitory effect of 215 molecules of sulfathiazole on *S. aureus*. Sulfathiazole and pyruvate compete for the active site of carboxylase. Presence or absence of inhibition depends on which of the two substances get to the active site of the carboxylase first. Para-aminobenzoic acid was antagonistic to sulfathiazole by maintaining a certain degree of inhibition of its own on the activity of carboxylase. However, certain concentrations of para-aminobenzoic acid display anti-inhibitory properties.

Synthesis of Cocarboxylase (9, 17). Two hundred milligrams of sodium pyrophosphate is placed in a Pyrex test tube and heated in order to remove water of crystallization. One-half cubic centimeter

of orthophosphoric acid (C.P. 85 per cent) is placed in another Pyrex test tube and heated until a small amount of solid forms on the side of the tube. Then the pyrophosphate is added and the mixture is gently heated until solution takes place. After a few minutes of cooling, 200 milligrams of thiamin hydrochloride is added, and the contents of the tube are well mixed. The tube is placed in an oil bath at 155° C. and kept at this temperature for 15 minutes, its contents being mixed occasionally. Then the tube is removed and allowed to cool. The solid mass is dissolved in 10 cc. of ice water and adjusted to *p*H 6.2 with cold *N* sodium hydroxide. The mixture is then diluted to 20 cc. One cubic centimeter of this solution is further diluted 100 times with a phosphate buffer of *p*H 6.2 (Sørensen). Both solutions are very stable. One cubic centimeter of the final solution is adequate in the cocarboxylase test. For a method of isolation of pure cocarboxylase from the reaction mixture, see references 10, 18, and 12.

Preparation of Yeast for the Cocarboxylase Test. Brewers' bottom yeast is extensively washed by suspension in water and centrifugation. Then the washed yeast is dried at room temperature with the aid of a fan. Washing with water alone, however, does not remove the cocarboxylase; alkaline washing must also be applied. To 2 grams of dry yeast in a 250-cc. centrifuge flask, 100 cc. of 0.1 *M* Na_2HPO_4 at 30° C. is added, and the flask is shaken in a shaking machine for 12 minutes (7). Then the mixture is centrifuged, and the supernatant fluid is discarded. This procedure is repeated, and the yeast is washed for 3 minutes with 100 cc. of water at 30° C. The cocarboxylase-free yeast is suspended in 20 cc. of phosphate of *p*H 6.2 and is now ready for use. For the cocarboxylase test, the manometric method of Warburg is used (7). One cubic centimeter of the yeast suspension and 1 cc. of cocarboxylase in phosphate of *p*H 6.2 are placed in the main compartment of a 17-cc. Warburg vessel. In the side arm is placed 0.5 cc. of sodium pyruvate (5 milligrams of pyruvic acid containing 0.1 milligram of magnesium as $MgCl_2$). The vessel is connected with a Warburg respirometer, and, after equilibrium has set in, the pyruvate is washed in from the side arm. Carbon dioxide formation may be measured in an atmosphere of air. Nitrogen may also be used as the gas. Decarboxylation is exceedingly rapid under both conditions. It differs somewhat, however, with the nature of the yeast and other conditions.

Pure, synthetic cocarboxylase is now commercially available (Merck and Co., Rahway, N. J.).

OTHER DIPHOSPHOTHIAMIN ENZYMES

Barron (19) lists a large number of reactions as being catalyzed by different diphosphothiamin enzymes, all acting on pyruvic acid, resulting in the following end products: acetaldehyde plus carbon dioxide; acetate plus carbon dioxide; lactate plus acetate plus carbon dioxide; acetate plus formate; acetylmethylcarbinol plus carbon dioxide; carbohydrate; citrate; acetoacetate; succinate; and α-ketoglutarate. In addition to these reactions, the conversion of α-ketoglutarate to succinate is said to be catalyzed by a specific diphosphothiamin-containing enzyme system.

THIAMIN METABOLISM OF ANIMAL TISSUES *IN VITRO*

In 1938, Tauber (20) showed that cocarboxylase is very rapidly hydrolyzed *in vitro* by beef-kidney tissue, and that, when large quantities of thiamin are ingested by humans, most of it is soon excreted in the unphosphorylated state. He has found also that acetone-treated pig duodenal mucosa has the ability to attach the pyrophosphate group to thiamin. Cedrangolo and Villano (21) reported that an extract of rat kidney in phosphate buffer of *p*H 8.4 containing a trace of arsenite converts thiamin readily to cocarboxylase.

Westenbrink and coworkers (22) have reported that the optimum *p*H for the hydrolysis of thiamin orthophosphate by crude autolyzates of ox kidney and by purified ox-kidney phosphatase is at 7.3 and at 6.5, respectively. The optimum *p*H for the removal of the first phosphate radical from thiamin pyrophosphate by purified kidney phosphatase was 5.8, whereas, when β-glycerophosphate was employed as the substrate, the optimum *p*H was at 9.0. This indicates that the enzyme preparation contained only "alkaline phosphatase" or the so-called "kidney phosphatase." The substrate appears to have a very pronounced effect on the optimum *p*H of the phosphatase. This enzyme removes the two phosphate groups of cocarboxylase one by one, thiamin orthophosphate being an intermediate in the reaction. Top-yeast phosphatase acts similarly. The decomposition of cocarboxylase is strongly inhibited by phosphate ions but not by thiamin. With top-yeast phosphatase, however, thiamin inhibits strongly, whereas phosphate ions have no effect on the enzyme. No organ was found that did not decompose cocarboxylase. The cocarboxylase in the tissues is firmly bound to protein, which is much less readily affected by the tissue phosphatases than free cocarboxylase. The optimum *p*H for the synthesis of cocarboxylase from thiamin by minced liver and kidney of rats and pigeons is at 6.5.

HYPOGLUCEMIC ACTION OF COCARBOXYLASE

Roux and Teysseire (23) found that in guinea pigs, rabbits, and dogs the intravenous or subcutaneous injection of 3 to 8 milligrams of cocarboxylase per kilogram caused a 20 to 40 per cent decrease in blood sugar after a delay of about 7 hours. Roux and associates (24) reported that the intramuscular injection of a single dose of 100 to 400 milligrams of thiamin pyrophosphate produced an important drop in blood sugar and glucosuria of the human diabetic. The effect was maximum between the eighth and twelfth hour after the injection. This would, in part, explain the favorable results reported in diabetes with bakers' yeast (see Chapter XVI).

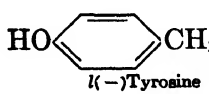
PHOSPHOPYRIDOXAL ENZYMES

Amino Acid Decarboxylases of Bacteria

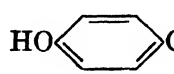
It has been known for many years that bacteria can decarboxylate amino acids. This type of decarboxylation results in the formation of the corresponding amine and carbon dioxide. The following are two examples:

(1) $\underset{l(+)\text{Lysine}}{NH_2CH_2CH_2CH_2CH_2CHNH_2COOH} \rightarrow NH_2CH_2CH_2CH_2CH_2CH_2NH_2 + CO_2$

Cadaverine

(2) 

$l(-)$Tyrosine



Tyramine

Other substrates that are rapidly decarboxylated by washed suspensions of bacteria are $l(+)$ornithine to putrescine, $l(+)$arginine to agmatine, $l(-)$histidine to histamine, $l(+)$glutamic acid to γ-aminobutyric acid, etc. If the reaction medium is kept at an acid *p*H, the carbon dioxide that is liberated may be conveniently measured manometrically. All bacterial decarboxylases are most active at *p*H 2.5 to 6.0.

Bacterial amino acid decarboxylases are present in various bacteria. Gale (25) lists the following conditions for the formation of these enzyme systems. The organism must possess such enzymes in its potential enzymic constitution; growth must take place in the presence of the specific substrate (adaptation). The organism must be able

to synthesize codecarboxylase or, if unable to do this, must contain certain factors necessary for the synthesis of codecarboxylase. The growth medium must be acid. Some organisms require a temperature lower than 30° C. in order to form the enzymes.

Gale (26) has described methods for obtaining cell-free bacterial preparations of *l*(+)arginine, *l*(+)ornithine, and *l*(+)glutamic acid decarboxylase. Arginine decarboxylase has its optimum *p*H at 5.25, ornithine decarboxylase at *p*H 5.25, and glutamic decarboxylase at *p*H 4.25. Arginine decarboxylase may be resolved into apoenzyme and codecarboxylase by precipitation with ammoniacal ammonium sulfate solution. Ornithine decarboxylase resolves spontaneously on standing into apoenzyme and codecarboxylase.

Tryptophanase

This enzyme may be prepared from *Bacterium coli* or from *Escherichia coli*. Wood and associates (27) prepared tryptophanase free of cells and semi-pure from *B. coli*. The enzyme has been resolved and shown to require pyridoxal phosphate as the coenzyme. Tryptophanase catalyzes the following reaction:

$$\text{Tryptophane} \rightarrow \text{indole} + \text{pyruvic acid} + \text{ammonia}$$

No oxidation takes place in this reaction. Alanine or serine do not occur as intermediates.

Codecarboxylase-Pyridoxal Phosphate

Members of the Department of Bacteriology at the College of Agriculture, Cornell University, and members of the School of Biochemistry, Cambridge, England, have found that derivatives of the vitamin B_6 group function as a coenzyme of a series of bacterial amino acid (lysine, tyrosine, arginine, glutamic acid, 3,4-dihydroxyphenylalanine or dopa, and ornithine) decarboxylases (25–29). Gunsalus and collaborators (28, 29) have shown that the coenzyme of tyrosine decarboxylase is pyridoxal orthophosphate.

Glutamic Acid Decarboxylase of Plants

Okunuki (30) discovered, in various higher plants, an enzyme which specifically decarboxylated glutamic acid and pyrolidone carbonic acid. Only in a few cases was pyruvic acid attacked and then only slightly. Schales and coworkers (31) have extensively studied this

enzyme. The distribution of glutamic acid decarboxylase in 34 different plants was determined. Squash, avocado, and green pepper were found to be the best sources for this enzyme. However, clear solutions of the decarboxylase could be obtained only from carrots. The enzyme was separated into apoenzyme and coenzyme by dialysis at *p*H 6.4. Pyridoxal and minute quantities of pyridoxal phosphate restored the activity of the apoenzyme. The glutamic acid decarboxylase from carrots is most active at *p*H 5.3 to 5.9. It is inactive beyond *p*H 4.0 and 7.5 (32). The reaction kinetics of this enzyme system has also been investigated.

Schales and Schales (33) have published a procedure which utilizes glutamic acid carboxylase of squash for the quantitative determination of *l*(+)glutamic acid. The unnatural isomers of glutamic acid and other amino acids are not attacked and do not interfere with the rapid liberation of 1 mole of carbon dioxide from 1 mole of glutamic acid. The method is useful in the determination of glutamic acid in protein hydrolyzates. This paper contains a simple procedure for the preparation of the enzyme in dry form from squash. Decarboxylation of glutamic acid is carried out in the Warburg apparatus at 37° C. and *p*H 6 to 7. Carbon dioxide liberation starts immediately and is completed after 20 minutes. The results obtained with this method check well with data obtained by chemical and microbiological procedures.

Glutamic acid appears to be an important metabolic link. The amino acid is present in most animal and plant proteins. About 4,000,000 pounds of monosodium glutamate is used annually, chiefly to impart a meat flavor to foods. Owing to these facts, the method just mentioned should find wide application.

Mammalian Decarboxylases

Histidine Decarboxylase. This enzyme is present in high concentrations in the kidney of guinea pig, rabbit, hamster, and mouse. However, the enzyme could not be found in the kidney of sheep, horse, cow, dog, or cat (34). Histidine decarboxylase changes *l*(+)histidine into histamine, a toxic substance. The optimum *p*H for this decarboxylation is at 8.6 to 9.0. The enzyme is reversibly inhibited by cyanide but not by carbon monoxide. Ketone reagents strongly inhibit histidine decarboxylase. It is believed that the enzyme contains an active carbonyl group (35).

Phenylalanine Decarboxylase. Schapira (36) found that guinea-pig liver, kidney, adrenals, heart, and muscle, and beef adrenal medulla

contain enzyme systems which at *p*H 7.7 simultaneously decarboxylate and deaminate *l*(—)phenylalanine and form phenylacetaldehyde, recognizable by its hyacinthlike odor. *d*(+)Phenylalanine is attacked only slightly or not at all, depending on the kind of tissue extract employed.

Other Mammalian Amino Acid Decarboxylases. Enzymes, which may or may not be specific decarboxylases, have been described to be present in mammalian tissues, acting on tryptophan, cysteic acid, dopa, and tyrosine (37–40).

TRANSAMINASES OR AMINOPHERASES

These enzymes are of major biological importance. The process of transamination in animal and plant tissues was first observed by Braunstein and Kritsman. These investigators (41) showed that this is a reaction between an α-amino acid and an α-keto acid, resulting in the transfer of the amino group of the amino acid to the α-keto acid (24):

$$R_1CH(NH_2)COOH + R_2COCOOH \rightleftarrows R_1COCOOH + R_2CH(NH_2)COOH$$

Their experiments suggested that the transamination of glutamic acid and aspartic acid requires two specific enzymes. Green and coworkers (42) have obtained two specific enzymes, in fairly pure state, from pig heart. One enzyme catalyzed the reaction:

$$\text{Glutamic acid} + \text{oxalacetic acid} \rightleftarrows \alpha\text{-ketoglutaric acid} + \text{aspartic acid}$$

This enzyme was named *aspartic-glutamic transaminase.* The other enzyme, *alanine-glutamic transaminase,* catalyzed the reaction:

$$\text{Glutamic acid} + \text{pyruvic acid} \rightleftarrows \alpha\text{-ketoglutaric acid} + \text{alanine}$$

The specificities of these enzymes are different from those described by other investigators. Neither of the two enzymes can catalyze transamination between the pair aspartic acid-pyruvic acid. Both enzymes differ only in their specificity for the second member of the pair. Green and associates (42) showed that both pig-heart transaminating enzymes require pyridoxal phosphate as a coenzyme.

Cohen and Hekhuis (43) studied the rate of the following reactions, using homogenized tissues of rat brain, liver, kidney, skeletal muscle, and heart muscle.

(1) $l(+)$Glutamic acid + oxalacetic acid $\overset{a}{\underset{b}{\rightleftarrows}}$ α-ketoglutaric acid + $l(-)$aspartic acid

(2) $l(+)$Glutamic acid + pyruvic acid $\overset{a}{\underset{b}{\rightleftarrows}}$ α-ketoglutaric acid + $l(+)$alanine

(3) $l(-)$Aspartic acid + pyruvic acid $\overset{a}{\underset{b}{\rightleftarrows}}$ oxalacetic acid + $l(+)$alanine

Reaction 1*a* proceeded at the fastest rate in all tissues studied, heart muscle being most active. Reaction 1*b* proceeded at a rate one-half to one-third that of reaction 1*a* in the different tissues. Reactions 2 and 3 were very slow as compared with reaction 1, values being considerable

TABLE XXV

Q_{TN} VALUES OF *Bacillus coli*, ANIMAL, AND PLANT TISSUES

Tissue	Q_{TN}
Bacillus coli	3,900
Oat seedlings (96 hr.)	5,650
Brain (rat)	2,800
Liver (rat)	2,200
Kidney (rat)	1,750
Heart muscle (rat)	3,330
Purified transaminase (beef heart muscle)	10,300

only with liver tissue. Lichstein and Cohen (44) reported experiments showing that a potent transaminase system, which catalyzes reaction 1, is also present in various bacteria and in higher plants. Some of these results are summarized in Table XXV and are expressed as Q_{TN} values.

$$Q_{TN} = \frac{\text{microliters aspartic acid formed}}{\text{milligrams N} \times \text{hours}}$$

Aspartic-alanine transaminase, as described by earlier workers, does not exist. The reaction attributed to this enzyme occurs through the combined action of aspartic-glutamic transaminase and alanine-glutamic transaminase with glutamic acid functioning as a link (45).

Oxalacetic Carboxylase or β-Carboxylase

This enzyme is present in most living things. The experiments of Werkman and of Evans and their coworkers have shown that oxalacetic

carboxylase is one of the enzymes responsible for the fixation of carbon dioxide:

$$CO_2 + CH_3COCOOH \rightleftarrows COOHCH_2COCOOH$$

Pyruvic acid — Oxalacetic acid

Here carbon dioxide is fixed to pyruvic acid, producing oxalacetic acid. However, carbon dioxide fixation is much more complex than this, since other substrates also interact. For an excellent discussion, a review by Wood (46) should be consulted. Vennesland and Felsher (47) found that the crystalline globulins of squash and pumpkin seeds showed oxalacetate carboxylase activity. Manganous chloride is necessary for the activity of the dialyzed enzyme. This enzyme does not appear to require cocarboxylase for its action.

Recent findings show that adenosinetriphosphate accelerates the rate of $C^{14}O_2$ fixation in oxaloacetate (48). There is evidence at hand indicating that a biotin-containing enzyme is involved in carbon dioxide fixation (49).

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CHAPTER XV

HYDRASES, MUTASES, AND OTHER ENZYMES

Hydrases and Mutases

These enzymes, unlike the dehydrogenases, do not remove hydrogen from their substrates, but add water to them. When the action is reversed, they remove water. In this manner, these enzymes can either oxidize or reduce. When water is transferred to the substrates, they become available for attack by other enzymes.

Aconitase. This hydrase is present in various animal and plant tissues. Human and dog prostate are very good sources (1, 2). Martius and Knoop (2) demonstrated that the aconitase enzyme system changes citric acid to cisaconitic and *l*-isocitric acid. The reaction is a reversible one. Barron and Huggins (1) prepared aconitase by grinding prostate glands with sand and 5 volumes of 0.1 *M* phosphate of *p*H 7.4. The mixture was centrifuged, and the clear supernatant fluid was diluted so that it contained about 5 milligrams of tissue per cubic centimeter. To 4 cc. of this solution were added 4 cc. of 0.92 *M* cisaconitic acid, neutralized in ice-cold water with solid $NaHCO_3$, 4 cc. of 0.1 *M* phosphate buffer of *p*H 7.4, and 4 cc. of water. The samples were incubated at 37.5° C., and the citric acid formed was determined at different intervals for 60 minutes. Under these conditions, citric acid formation was linear. In 60 minutes, 9.5 micromoles of citric acid was produced by the aconitase from human hypertrophic prostate. The aconitase from dog's prostate formed 4.7 micromoles. Johnson (3) reported the following aconitase values of tissue extracts: pigeon breast muscle, 120; rat liver, 61.5; rat kidney, 80; rat testis, 7.6; rat lung, 14.5; rat brain, 10; human hypertrophic prostate, 74; and dog's prostate, 43. Barron and Huggins found that there is a lack of citric acid oxidation in human prostatic tissue, and, owing to the high aconitase content, there is an accumulation of citric acid in this gland. These investigators found that the citric acid content of human prostatic adenoma was about 645 milligrams per 100 grams of tissue; that of human prostatic cancer, 74 milligrams; and that of dog's prostate, 8 milligrams.

Glyoxalase. This ketoaldehyde mutase is widely distributed in nature. Liver and yeast are good sources. Glyoxalase converts

methyl glyoxal into lactic acid:

$$CH_3COCHO + H_2O = CH_3CHOHCOOH$$

Lohmann (4) discovered that glutathione functions as a coenzyme of glyoxalase. He prepared glutathione-free glyoxalase by extracting liver tissue with sodium acetate buffer, adjusting the extract to neutrality with sodium bicarbonate, followed by dialysis. By adding 5 volumes of alcohol to the dialyzed extract and drying in vacuum, an enzyme preparation of only fair stability was obtained. Glyoxalase solutions are not very stable, either. They may not be kept for more than a few days in the refrigerator without losing most of their activity.

Fumarase. This hydrase is present in most living things. Bodur (5) found it in many fruits and seeds. The largest amounts of the enzyme were found in plum and apple fruits. Leaves and roots, however, were lacking in fumarase. Liver and muscle are very good sources (6). Fumarase acts as an oxidase when it adds water to fumaric acid, converting it to *l*-malic acid, and as a reductase when the reaction is reversed:

$$\begin{matrix} CHCOOH \\ \| \\ CHCOOH \end{matrix} + H_2O \rightleftarrows \begin{matrix} COOH \\ | \\ CHOH \\ | \\ CH_2 \\ | \\ COOH \end{matrix}$$

Clutterbuck (6) prepared muscle fumarase by extraction of the washed tissue with $M/15$ disodium phosphate, adjustment of the *p*H to 6.5 with acid in order to remove the succinic dehydrogenase, filtration, precipitation with ammonium sulfate, and dialysis. Fumarase is a quite stable enzyme. It does not require a coenzyme. The kinetics of the reversible reaction was extensively studied by Jacobsohn (7) and by Davydova (8). The latter investigator found that the formation of *l*-malic acid from fumaric acid is accelerated by phosphate, arsenate, and magnesium ions. The position of equilibrium is not altered. The ions Cl, NO_2, I, and CNS have an inhibitory action.

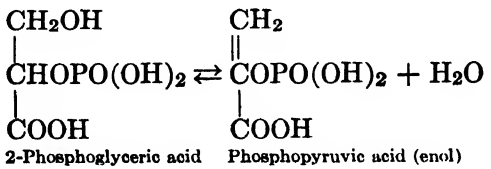
Aldehyde Mutase. This enzyme is present in yeast and in liver. It changes two molecules of any aliphatic aldehyde by simultaneous oxidation and reduction (Cannizzaro reaction) into one molecule of acid and one molecule of alcohol:

$$2CH_3CHO + H_2O = CH_3COOH + CH_3CH_2OH$$

Aldehyde mutase, unlike xanthine oxidase, does not attack aromatic

aldehydes. Iodoacetate destroys the mutase, but not the oxidase, whereas cyanide is toxic to xanthine oxidase but not to the mutase. Aldehyde mutase requires codehydrogenase I, but not the oxidase (9). Higher plants, such as peas, contain an aldehyde mutase that does not require codehydrogenase I. It acts on both aliphatic and aromatic aldehydes, and is inactivated by iodoacetate (10).

Enolase. Dialyzed muscle extract and yeast contain an enzyme which converts 2-phosphoglyceric acid into the enol form of phosphopyruvic acid (11):



2-Phosphoglyceric acid Phosphopyruvic acid (enol)

Warburg and Christian (12) obtained the mercury salt of enolase of yeast in crystalline form. The yield was 1 gram of crystalline enzyme per kilogram of dried brewers' yeast. This enzyme was inactive. However, when freed of mercury and combined with magnesium, manganese, or zinc ions, the enzyme became active. At equilibrium, the ratio of phosphopyruvic acid to 2-phosphoglyceric acid is 1.43. Fluorides inhibit the action of enolase provided that phosphate or arsenate is present. Pyrophosphate inhibits enolase considerably. This enzyme interacts with phosphoglyceromutase, which changes 3-phosphoglyceric acid into 2-phosphoglyceric acid by mutation.

Carbonic Anhydrase

This enzyme is found in traces in most tissues of all vertebrates and in the tissues of some invertebrates. The enzyme is present in very high concentrations in erythrocytes. Purified preparations of carbonic anhydrase have only 150 times as much activity as the erythrocytes when the two are compared on dry-weight basis (13). Large quantities of the enzyme are also present in the gastric mucosa, the pancreas, and the kidney cortex of vertebrates. Carbonic anhydrase plays an important role in the carbon dioxide transport of the blood, involving the acid-base equilibrium in the body. The enzyme appears to have an important function also in the shell-forming organs of birds. It has been reported that the enzyme also is present in some plants (14). Carbonic anhydrase was discovered by Meldrum and Roughton (15). It catalyzes the reversible reaction $H_2CO_3 \rightleftarrows CO_2 + H_2O$.

Preparation. Keilin and Mann (13) purified the carbonic anhydrase of erythrocytes. They washed red blood cells with 0.9 per cent sodium chloride and laked them with distilled water. The hemoglobin was precipitated with a mixture of alcohol and chloroform. The carbonic anhydrase remained in solution. This solution was further purified with lead acetate, calcium phosphate, and alumina gels. In this paper, a second procedure is given, which uses fractional precipitation with ammonium sulfate and purification with alumina gel.

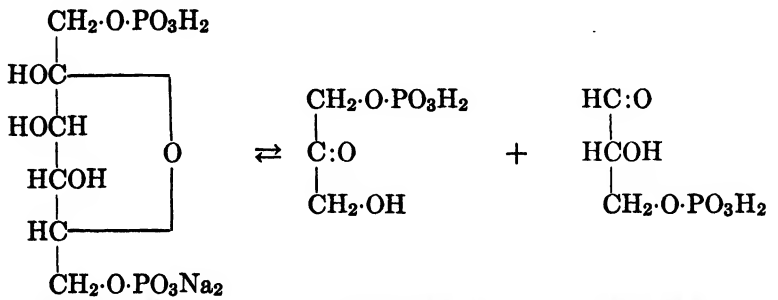
Properties. The purified enzyme is a zinc-protein compound containing 14.8 per cent nitrogen and 0.3–0.33 per cent zinc. Keilin and Mann consider this preparation as either pure or almost pure enzyme. Scott and Mendive (16, 17), however, claim that carbonic anhydrase contains only 0.2 to 0.23 per cent zinc. Their enzyme preparation appears to be 4 to 4.5 times more active than the purest preparation of Keilin and Mann. The latter authors (18) point out that Scott and Mendive estimated the anhydrase activity in the presence of peptone, which stabilizes and also doubles the activity of the freshly diluted purified enzyme. Keilin and Mann observed that crystalline carbonic anhydrase of Scott and Fisher (19) were merely compounds of the enzyme with the bases piperidine, isoamylamine, and *n*-amylamine, and that the crystals were completely inactive. The same type of crystals can be obtained even with gelatin and the bases, instead of the carbonic anhydrase.

Kinetics. Roughton and Booth (20) improved the manometric technique for measuring carbonic anhydrase activity, and they described applications of the procedure to the study of the kinetics of the enzyme. The Michaelis constant was found to be independent of *p*H, its value being at 0° C. 0.009 *M* $CO_2 \pm 0.001$. The *p*H activity curve, using the buffers cacodylate, phosphate, veronal, and dimethylglyoxaline, showed a minimum between 6.0 and 7.0 and was still rising at *p*H 10.0. The anions of a series of neutral salts, such as sulfate, chloride, bromide, nitrate, and iodide, were inhibitory at *p*H 7.4 and of 0.02 to 0.1 M concentrations. The anions of most carboxylic acids were inhibitory also. Potassium cyanide, hydrogen sulfide, and sodium azide inhibit in small concentrations. These compounds are known to react in this manner with metals (20). Shaking inactivates carbonic anhydrase solutions, both in air or in inert gas. Proteins and peptones exert more or less protective action, but not starch and glycogen (21). Cysteine, glutathione, histidine, and histamine increase the activity of undenatured carbonic anhydrase.

Davenport (22) has contributed a very extensive review concerning the distribution and role of carbonic anhydrase in various tissues.

Aldolase

The enzyme aldolase is widely found in nature. It splits fructose-1,6-diphosphate into the two triosephosphates, dihydroxyacetone phosphate and phosphoglyceric aldehyde:



This reaction is reversible. For the reaction to the left dihydroxyacetone phosphate is necessary, whereas for the reaction to the right fructose-1,6-phosphate is required. Warburg and Christian (23) have been able to obtain adolase in pure crystalline state from an aqueous extract of rat muscle by fractionation with acetone and ammonium sulfate. The pure enzyme is not affected by complex formers such as pyrophosphate, and cysteine. The partly purified enzyme of yeast, however, is inhibited by them.

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PART II

THE TECHNOLOGY OF ENZYMES

CHAPTER XVI

YEAST: PRODUCTION AND UTILIZATION

Yeasts are classified as budding fungi and are divided into spore-forming or true yeasts, such as the family of Saccharomycetaceae, and the non-spore-forming or false yeasts, such as the Torulopsidaceae, Rhodotorulaceae, and Nectaromycetaceae. There are many genera in the group of true yeasts, and a great number of species and strains. Commercially, only one genus is of interest, *Saccharomyces*. These are divided into top and bottom yeasts. The top yeasts, which are used in distilleries, and in the manufacture of compressed yeast and some beers, are so called because, at the end of fermentation, they collect at the top of the fermented mash. The bottom yeasts, which are used in the making of wine and certain beers, separate at the end of fermentation on the bottom of the fermenting vessel. Both types of yeast, however, contain many related strains of varying grades. The general opinion is that top yeasts are much more resistant than bottom yeasts.

THE YEAST CELL

The individual yeast cell supplies itself with food (from a suitable medium), respires, grows, and reproduces. We are interested mainly in the industrially important *Saccharomyces cerevisiae*. The form of the yeast cell depends upon the environment and age of the culture, as does the shape, which may be elliptical, spherical, ovoid, or elongated. The size of the yeast cell also varies, and it may be from 2 to 25 microns. The cell wall is non-living, permeable matter, containing hemicellulose and yeast gum. Next to the cell wall is the plasma membrane, a living, proteinous liquid structure. This membrane, the plasma membrane is permeable to certain solutions only. Protoplasm inside the cell contains protein, glycogen, lipids, amino acids, vitamins (growth factors), a very large number of enzymes, numerous other organic substances, and inorganic salts. Figure 17 shows the appearance of the yeast cell under various conditions (1).

THE REPRODUCTION OF YEAST

Sporulation Method for *S. Cerevisiae*. Lindegren and Lindegren (2) suggest the following presporulation medium:

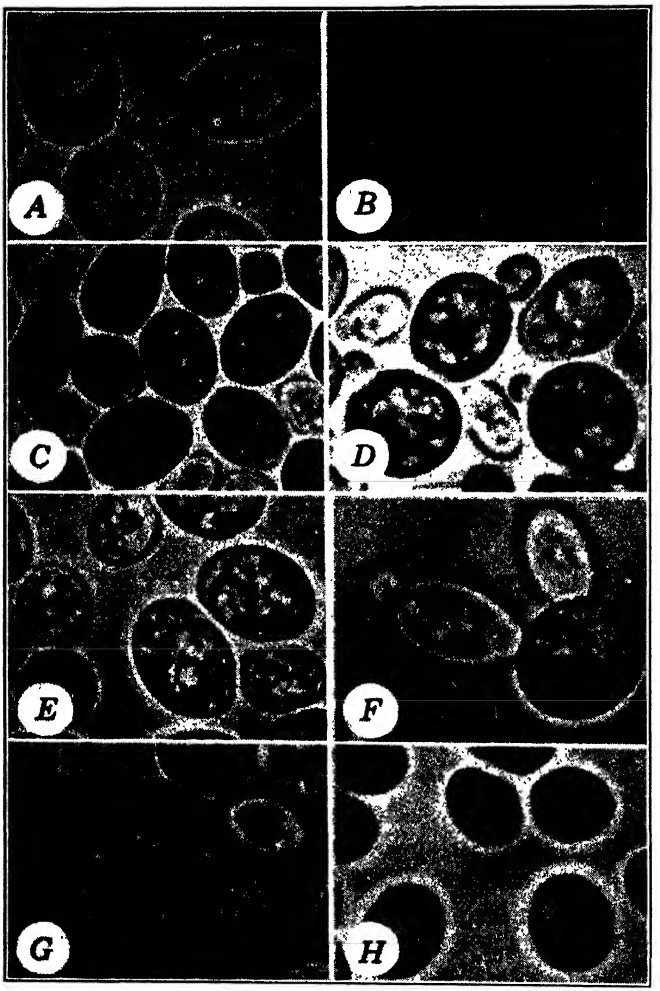


FIG. 17. *a.* Budding vegetative yeast cells at the logarithmic stage of growth showing a single vacuole characteristic of this condition.

b. Budding yeast cell in the lag phase showing the apparently multiple vacuole resulting from deformations of the single vacuole by interference of reserve material.

c. Dormant vegetative yeast cells loaded with fat and glycogen, grown on presporulation agar; the dark color is glycogen stained with iodine.

d. Germinating dormant cells from presporulation agar, showing the vacuole breaking through the enclosing network of fat and glycogen.

e. Cells loaded with fat by growth in aerated sugar solution.

f. Cell of the type shown in *e* germinating.

g. Cell grown in sugar under conditions of reduced oxygen tension loaded with glycogen, a few fat globules surrounding vacuole.

h. Cell of the type shown in *g* stained with iodine, revealing the deformation of the vacuole by the reserve materials.

Beet-leaf extract	10 cc.
Beet-root extract	20 cc.
Apricot juice	35 cc.
Grape juice	16.5 cc.
Dried yeast	2 grams
Glycerin	2.5 cc.
Agar	3 grams
$CaCO_3$	1 gram

Water to make final volume of 100 cc.

The mixture is steamed for 10 minutes and tubed. The tubes are sterilized at 15 pounds for 20 minutes, and slanted. Most strains produce spores on these slants within a few weeks. For rapid sporulation, a mixture of 100 grams of plaster of Paris and 100 cc. of water is poured in test tubes and solidified in a slanting position. These slants are dried at 50° C. for 24 hours and then autoclaved.

From the presporulation medium, the transfer is made by adding 1 cc. of sterile water over a 3-day growth of yeast. After 10 minutes' standing, a suspension is made by stirring the cells in the supernatant water. The yeast suspension is taken up in a pipet and poured over the upper portion of the gypsum slant. About 3 cc. of sterile water, containing sufficient acetic acid to produce a *p*H of 4, is pipetted into the lower half of the gypsum slant. The inoculated slants are kept at 25° C. for 1 to 2 days. The asci may be smeared on a slide from the gypsum slant, fixed by heat, stained in carbol fuchsin for 20 minutes, and rapidly washed in 30 per cent acetic acid. After rinsing with water, methylene blue is applied as a counterstain. This method stains the spores red, and the vegetative cells blue. On this sporulation medium, heterozygous, legitimately diploid cultures produce an excess of viable 4-spored asci. Genetic analysis requires an abundance of such 4-spored asci.

Hybridization of Yeast. About 1900, E. C. Hansen showed that, in yeast, a sexual process takes place which is similar to that in higher plants and in animals. J. Satava (3), Winge and Laustsen (4), and Lindegren (5) have found that, when yeast sporulates, a genetic segregation of spores takes place in such a manner that the spores in any ascus differ genetically; this process is comparable to the formation of gametes in higher plants. When two yeast spores fuse during germination, fertilization takes place. Because of this, we cannot be certain that a pure yeast culture will keep uniform, even if the culture originates from a single yeast cell. If the yeast is permitted to form spores, new types may segregate. This is true, for example, with all species of *Saccharomyces* (bakers', brewers', distillers', and other yeasts). Winge and Laustsen used a microscope and a micromanipulator for the

isolation and fusion of spores. These investigators have shown that the yeasts just mentioned are diploid (having a double set of chromosomes) during most of their life cycle and change to the haploid phase (having a single set of chromosomes) by reduction division just before sporulation. On germination by the conjugation of spores or of descendant cells, the diploid phase is restored again. In *Zygosaccharomyces*, however, the diploid phase is short, lasting only from zygote formation until spore formation, and the vegetative or budding phase is haploid. Lindegren (5) has extensively studied yeast hybridization. He found that, by the method of Winge, copulations occur only rarely. He gave a series of reasons for this and developed a new procedure.

Lindegren's Hybridization Method (5). Different haplophase cultures are mated in 0.5 cc. of broth culture in a culture tube. A large drop of cells from a heavy broth suspension is placed in the tube with a second large drop from a different broth suspension. The test tube is kept at 23° C. in an incubator for 24 hours and examined for copulations. The following medium is used:

Glucose	40.0 grams
Yeast extract powder 3-D (Anheuser-Busch Inc.)	2.0 grams
Bacto peptone	3.0 grams
K_2HPO_4	0.133 gram
KH_2PO_4	1.866 grams
$CaCl_2$	0.25 gram
$MgSO_4$	0.125 gram
Sodium lactate (50 per cent)	1.0 gram

Dilute to 1 liter with distilled water, *p*H 5.9.

Lindegren observed that a considerable number of haplophase cultures, isolated from different strains of bakers' yeasts and from some of his own hybrids, were mated in nearly all possible variations. The experiments of Lindegren and associates have proved that a single pair of genes dominates copulations among the types of yeasts studied. There were only a very few illegitimate matings.

Undesirable Effects. Under normal commercial operating conditions, the " illegitimate " diploid yeast cell exists, and there is little chance for mutation or change. The only possibility would be that, occasionally, yeast cells are allowed to sporulate during fermentation. However, such conditions do not, as a rule, arise in plants. The excessive predominance of the desired types used in industrial processes, as compared to slight quantities of the undesired type that may occur as contaminants, would not produce any harmful effects. In some plants,

however, the stock cultures are frequently changed in order to assure the constancy of the product.

Desirable Applications. Previously, superior yeast types were selected from those found in nature. Now, however, there is evidence that, by proper crossing and combining of desirable characteristics, new industrial types may be developed in the laboratory. Winge and Laustsen (6) found that the function of a yeast to produce a certain enzyme, such as invertase, raffinase, and melibiase, was always a dominant factor. Thus, if a given yeast type with the ability of forming one of these enzymes was hybridized with another type not having this ability, the hybrid always possessed the ability to produce the particular enzyme. A typical top yeast may form a typical bottom yeast from its ascospores. Thus, there is no exact line of demarcation between top yeast and bottom yeast. The brewers' yeast types, which were developed by continuous selection of mutants, have become increasingly suitable to the brewing industry. Owing to mutation, most of these types of yeast have lost the ability to sporulate. Thus, it will be necessary to attain further improvement, to utilize the spore-forming types, and to apply hybridization by making use of their natural tendency to mutation (7). This is Lindegren's (8) view concerning yeast hybridization: "By hybridization, we are able to deal with the factors responsible for each fermentation like separate units, and thus produce yeasts 'made to order.'" Winge and Laustsen (6) were the first to produce a superior baking yeast by hybridization. This yeast is now being used commercially.

Vitamin-synthesizing deficiencies of *S. cerevisiae* are corrected by hybridizing it with a species that is able to synthesize the deficient vitamin. Most strains of *S. cerevisiae* are unable to synthesize biotin, and some cannot produce pantothenic acid. Lindegren and Lindegren (9) have studied a culture of *S. carlsbergensis* which synthesized large amounts of pantothenic acid and biotin but could not produce pyridoxine, which *S. cerevisiae* synthesized well. Haplophase cultures obtained from *S. carlsbergensis* could produce biotin and pantothenic acid but not pyridoxine. These were mated with haplophase cultures of *S. cerevisiae,* which produced pyridoxine but not biotin or pantothenic acid. The resultant hybrid synthesized all three vitamins in large quantities.

There are possibilities also that new types of yeast can be produced by mutations effected by irradiation, X-rays, or radium emanations or by treatment with certain toxic chemicals, such as lithium chloride and cyanide.

THE MANUFACTURE OF COMPRESSED YEAST

In the United States, 230,000,000 pounds of yeast are manufactured per year (10). Most of it is used in the baking, brewing, and alcohol industries. Some of the yeast is employed as a source of vitamins; some, in the manufacture of soluble proteins; some, in the manufacture of glutathione, nucleic acid, enzymes, etc. Extracts of yeast are used in the preparation of media for bacteria.

Commercially, the most important yeasts are beer yeasts, distillers' yeasts, bakers' yeasts, and wine yeasts. The yeast manufacturer is interested only in the type called *Saccharomyces cerevisiae*. This group contains a large number of strains. The isolation and preservation of the proper strain are of great importance.

During the war, great quantities of active dried bakers' yeast were produced for the armed forces. Dried bakers' yeast is about 85 per cent as active on dry basis as fresh bakers' yeast. The dried yeast keeps for several months without refrigeration and is more economical than fresh yeast (11).

The Molasses-Ammonia Process. The molasses-ammonia process originated by Hayduck is the most widely used method for the manufacture of compressed yeast. In this process, molasses (cane or sugar beet), mineral salts, and ammonia are the principal nutrients. The *p*H is continuously controlled. Very dilute wort is employed during the first phase of growth, and the concentrated wort is continuously added at such a rate that any alcohol that may form is assimilated during the growth of yeast. The nutrients are supplied in such amounts that they closely parallel the yeast growth rate. Although only small amounts of alcohol are formed by this process, the yield of yeast is very high.

The most favorable *p*H for yeast growth is between *p*H 3 and *p*H 4.5. If an acid *p*H is maintained, bacterial growth may be completely eliminated. Sulfuric acid and ammonium hydroxide or another alkali are used to adjust the *p*H of the wort. The ammonia is continuously used by the growing yeast, and, as a result, the mash becomes more and more acid and must be neutralized.

The temperature is controlled by cooling coils placed inside the containers. The optimum temperature varies with the strain of yeast employed. When growth is completed, the yeast is separated from the wort by filtration or centrifugation and is washed and pressed. Flour may be added to the yeast to aid in handling.

The following is an improved version of yeast manufacture by the molasses-ammonia process (12).

One thousand three hundred and twenty-three pounds of yeast is brought to an acid content of 2 degrees, and the yeast is allowed to stand for 1 hour. Then 8000 gallons of water is placed in a container of 16,000-gallon capacity. The acidified pitching yeast is added to the water and is well mixed. Twenty cc. of the wort requires 0.35 cc. of a $N/10$ sodium hydroxide solution to become neutral to litmus. The wort, which is still free of molasses, is aerated with 800,000 gallons of air per hour and is then mixed gradually during a period of 11 hours with 7711 pounds of molasses. Care is taken that during the first 9 hours the wort is always slightly acid.

Nitrogen and phosphorus are supplied in the form of 330 pounds of superphosphate, 99 pounds of ammonium persulfate, and 25 gallons of 25 per cent ammonia. After the addition of the total quantity of nitrogen and phosphorus (after 9 hours), 0.4 cc. of a $N/10$ sodium hydroxide solution is required to neutralize 20 cc. of wort, litmus being used as an indicator. Maintenance of strong aeration causes the acidity to drop, and after 10 hours only 0.25 cc. of a $N/10$ sodium hydroxide solution is necessary to neutralize the same volume of wort after all the molasses is added. After 11 hours the wort is neutral to litmus. Aeration is continued for a short time. It is gradually reduced and discontinued after 2½ hours more. The yeast is now matured, and the wort is free of sugar and acid. Eleven thousand ninety-five pounds of wort of 3.4° Balling is obtained. The yield of yeast is 5458 pounds, which, after deducting 1323 pounds used for pitching, corresponds to 53 per cent of the molasses used. The alcohol yield is 132 gallons or 12 per cent of the weight of molasses employed.

Figures 18 and 19 show two main phases of the manufacture of compressed yeast (by courtesy of Dr. C. N. Frey of the Fleischmann Laboratories).

Production of "Galac" Yeast. In order that yeast should be able to grow on a medium containing certain substances such as sulfites or other than the usual sugars, the yeast must first be acclimatized to the abnormal conditions. According to Nilsson (13), bottom yeast may be readily "trained" to ferment galactose when cultivated on the following medium:

Washed and pressed bottom yeast	500 grams
Galactose	100 grams
KH_2PO_4	45 grams
Na_2HPO_4	5 grams
Yeast extract	250 cc.
Water	5000 cc.

The yeast is grown at 25° C. Every day for 5 days 100 grams of

FIG. 18. Fermenting room.

FIG. 19. Filter pressing room.

galactose is added. By the sixth day the galactose-fermenting mechanism is considerably developed and galactose is rapidly fermented by the yeast. This yeast may be dried at room temperature without loss of activity.

Spiegelman and collaborators (14) have demonstrated that in galactose-adapted yeasts the fermentation of galactose was invariably associated with the adapted apoenzyme. Dialyzed Lebedew juice from galactose-adapted yeast fermented galactose readily, provided that codehydrogenase I, hexosediphosphate, and boiled yeast juice were present. Yeast that was not adapted to the galactose, and was used under similar conditions in the fermentation test, did not ferment galactose. Some investigators, however, consider such a process a selection of strains rather than an " adaptation " of enzymes.

Other Methods of Yeast Manufacture. In the Heijkenskjöld process (15) sulfite liquor and a small amount of molasses are used as the raw material. In the Scholler-Tornesch process (16) wood sugar and added mineral salts are employed for the manufacture of fodder yeast.

For a discussion of the large-scale production of compressed yeast as practiced in Germany, see reference 17. Other processes for the manufacture of compressed yeast have been described in detail by Walter (18). A useful monograph on pure yeast culture systems has been published by Laufer and Schwarz (19). See also Chapter II. Table XXVI shows yeast yields obtained when grown in three different media on a laboratory scale (20).

TABLE XXVI

YIELDS OF DRY YEAST GROWN IN VARIOUS MEDIA*

Type of Yeast	Grain Medium %	Molasses-Salts Medium %	Glucose-Salts Medium %
Bakers' yeast A	24.3	34.6	18.0
Bakers' yeast B	42.5	33.6	34.3
Brewers' yeast A	34.6	42.7	29.0
Brewer's yeast A (autoclaved medium)	32.2	...	...
Saccharomyces logos	33.1	28.0	21.4
Willia anomala	21.4	28.6	11.4
Endomyces vernalis	40.9	33.6	30.5

* Dry yeast is based on glucose fermented.

Culture Media for Yeast. Yeast may be grown in all glucose-containing fruit juices, in beer wort, or in the following synthetic media after they have been sterilized at 15 pounds pressure for 15 minutes (18).

A. Dextrin and sugar ...	100.00 gram	$MgSO_4$	0.1 gram		
$(NH_4)_2SO_4$	4.75 grams	$CaSO_4$	0.1 gram		
KH_2PO_3.....	0.75 grams	Water to 1 liter.			
B. Sugar 15.0%	KH_2PO_4	0.2%	$CaCO_3$	0.1%	
Water 84.0%	$MgSO_4$	0.1%	$(NH_4)K_2PO_4$ ..	0.5%	
C. Sugar 15.0%	Water	83.5%	$MgSO_4$	0.2%	
Asparagine 0.7%	KH_2PO_4	0.5%	$CaCO_3$	0.1%	
D. Sugar 15.0%	Water	83.5%	$MgSO_4$	0.1%	
Peptone Witte ... 0.5%	KH_2PO_4	0.5%	$CaCO_3$	0.1%	

If solid transparent media are desired 1 per cent by weight of agar or gelatin should be added to the above solutions before sterilization.

THE RELATION OF GROWTH SUBSTANCES TO YEAST

A number of organic compounds act as food accessories for certain microorganisms. Very small quantities, however, are sufficient when added to their usual carbon source and inorganic salt supply. β-Alanine, for example, is most effective at a concentration of 1:10,000,000, and is still active at a concentration of 1:200,000,000. Large concentrations may have an inhibitory effect.

The requirement for growth substance depend on the type of microorganism, the composition of the medium, and environmental factors. Lesh, Underkofler, and Fulmer (21) showed that magnesium sulfate stimulates bios activity of certain types of *Saccharomyces cerevisiae* yeasts, but not of other strains of the same group. According to Schultz, Atkin, and Frey (22), *thiamin* stimulates only some strains of *S. cerevisiae,* whereas others are inhibited. Schopfer (23) reported that a red yeast (*Rhodotorula rubra*), which is known to require thiamin, in reality needs only *pyrimidine,* whereas *Mucor ramanninanus* requires only *thiazole.*

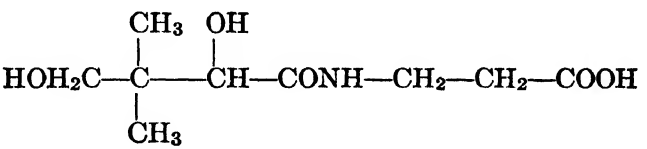
In 1901 Wildier gave the name " bios " to a hypothetical organic substance that stimulated yeast growth. Although Wildier's conception of bios is incorrect, it led the way to important discoveries by others.

The bios complex is soluble in water and 80 per cent alcohol. It is insoluble in absolute alcohol and ether. It dialyzes readily. Bios may be separated into two fractions by treatment with alcoholic barium hydroxide solution. The fraction which forms an insoluble barium salt was named " bios I "; the residual solution, called " bios II " (24), may be further separated into various fractions.

Inositol. It was shown that bios I was identical with the optically inactive inositol (25). Inositol itself is generally without effect, but it

increases the action of bios constituents (26). Not all yeasts respond to inositol, however (27). In plants such as barley, inositol is present as the phosphoric acid ester, phytin.

Pantothenic Acid. Williams and associates (28, 29) have separated bios II with fuller's earth into two fractions: one, which is replaceable by thiamin, and another fraction, named " pantothenic acid," which is not replaceable by thiamin. β-Alanine is a part of the pantothenic acid molecule (30). The growth-stimulating action of this amino acid upon yeast is well established. On the basis of this work Merck research chemists obtained pantothenic acid synthetically (31, 32). It has the following structural formula:



(α,γ-Dihydroxy-β,β-dimethylbutyryl-β-alanide)

Pantothenic acid is formed by condensation of α-hydroxy-β,β-dimethyl-γ-butyrolactone (a known synthetic product), with β-alanine. Thiamin and inositol or a mixture of both increases the action of the acid on certain yeasts (33).

Pantothenic acid is a highly active growth promoter. A concentration of 1:50,000 increases the growth of yeast five times over that of the control experiment (29). Some yeasts are stimulated by pantothenic acid only in the presence of thiamin or β-alanine. Certain yeasts that cannot normally produce this acid can do so when β-alanine is the only nitrogen supply in the medium (34). Liver and rice bran are very good sources of pantothenic acid, and they appear to affect the growth of higher plants and animals (35, 36).

Biotin and Other Growth Factors. This bios factor may be obtained by fractionating bios with charcoal. The charcoal adsorbs bios II. It may be eluted with dilute ammonium hydroxide and acetone (37, 38). The unadsorbed part has been named bios III.

Biotin and pantothenic acid are chick antidermatitis factors. They are not identical, however (27).

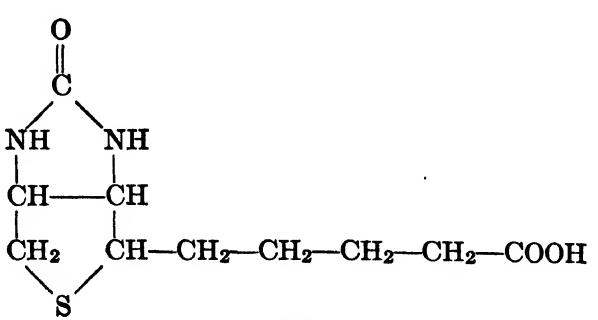
Biotin is the most active biological substance. One part in fifty billion may be distinctly detected in the growth of yeast. Traces of it are present in all important animal organs and body fluids, as well as in many plants (39). Lampen, Bahler, and Peterson (40) analyzed the biotin content of a series of biological materials. Extraction at an acid *p*H was much more effective than water. The following were

found to be the three best sources: beef kidney containing 2500, pork liver 2000, and brewers' yeast 830 millimicrograms of biotin per gram of dry matter. Other good sources are malt rootlets (39) and leaves of the birch (41).

Winzler and associates (42) found that biotin-deficient yeast respired and fermented at rates one-tenth to one-twentieth of normal biotin-rich yeast. Upon biotin addition, in the presence of ammonia, but not in its absence, these metabolic rates rose gradually, fermentation first, then respiration, and finally growth. With yeast that was only partly biotin deficient, added biotin caused an immediate increase in fermentation rate, even without ammonia. This increase was not a function of time. Biotin-deficient yeast rapidly removed from solution its complement of biotin. This biotin uptake was decreased in the absence of phosphate or glucose. The yeast studied was *Saccharomyces cerevisiae,* Fleischmann strain 139.

Chemical Nature of Biotin. Investigators at Cornell University Medical College and at the School of Medicine, Western Reserve University (43), have found in a cooperative study that the three factors known as biotin, *rhizobia* (the growth and respiration factor), and vitamin H (the anti-egg-white-injury factor) are one and the same substance.

The structural formula of biotin has been elucidated by Du Vigneaud and associates (reviewed in reference 44). It contains a five-membered sulfur ring with a valeric acid side chain combined with the carbon α to the sulfur. The sulfur ring is attached to a cyclic urea structure:



Biotin

β-Alanine ($CH_2NH_2CH_2COOH$) and Other Fractions of Bios. Bios II, when treated with charcoal, may be separated into two components (45). The charcoal adsorbate which is liberated by shaking with an aqueous solution of acetone and ammonia has been named

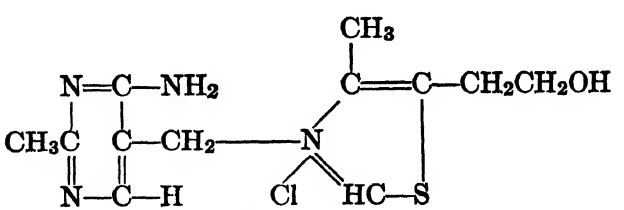
" bios IIB," whereas the unadsorbed fraction has been called " bios IIA." Bios IIA contains β-alanine and leucine (46). In the presence of sugars, inorganic salts, and 5 milligrams of inositol per liter, the growth of some yeasts was stimulated by a concentration of 1:12,000,000 of β-alanine. With the addition of aspartic acid, the effect was increased (47). According to Nielsen and Hartelius (48), β-alanine was toxic in the absence of asparagine or aspartic acid.

Nielsen and Dagys (49) reported that β-alanine, in order to function as a growth factor for yeast, requires biotin, thiamin, asparagine, and glutamic acid. For the last two items may be substituted succinic, citric, tartaric, or malic acid. The action of one of these acids increases the functioning of β-alanine tenfold.

Heating glucose and ammonium tartrate solutions together produced a growth substance for yeast that acted like β-alanine. It is suggested that β-alanine itself may form during the reaction.

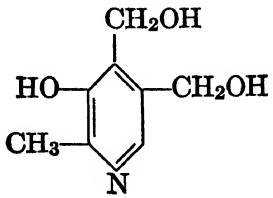
The growth-promoting action is not a property of the amino group in the β position. β-Phenyl-β-alanine, β-aminobutyric acid, glycine, δ-aminovaleric acid, or ε-aminocaproic acids could replace β-alanine (50). Among 31 amino acids studied only β-alanine, lysine, arginine, methionine, aspartic acid, asparagine, and glutamic acid stimulated growth. β-Alanine was the most effective stimulant (51).

Pyridoxine (Vitamin B_6) and Thiamin. Williams, Eakin, and Snell investigated three strains of *S. cerevisiae* as to their pyridoxine requirement in the presence of inositol, pantothenic acid, and biotin, and found that it is relatively unimportant. Schultz, Atkin, and Frey (50, 52), studied forty-four strains of *S. cerevisiae* and *S. carlsbergensis* and divided them into three groups: type *A*, showing growth increase by thiamin and an additional increase by pyridoxine; type *B*, showing 50 per cent decrease in growth by thiamin, and normal growth when pyridoxine and thiamin were present in the media; type *C*, with a decrease of 50 per cent or more by thiamin and an increase above normal by thiamin and pyridoxine.



Thiamin (Vitamin B_1)

(2 Methyl-5-[4 methyl-5-β-hydroxyethyl-thiazolium chloride]methyl-6-amino-pyrimidine)



Pyridoxine

(2 Methyl-3 hydroxy-4,5-di-[hydroxymethyl]-pyridine)

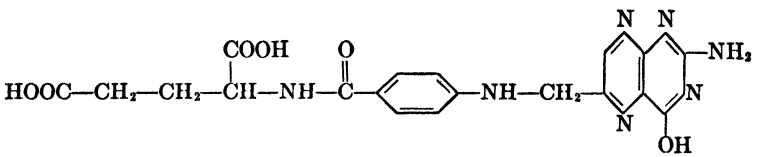
Thus pyridoxine is required by some of the yeasts and acts only in the presence of thiamin. The mechanism of the functions of the pantothenic acid, inositol, biotin, β-alanine, and pyridoxine is not yet known.

FOLIC ACID

Folic acid is one of the more recent members of the vitamin B complex. Like the other members of this group, it was discovered by several groups of investigators and by quite different approaches. At least some of the approaches appear to have arrived at the same destination. In 1940, Snell and Peterson (53) published a paper entitled "Additional Factors Required by Certain Lactic Acid Bacteria," in which they disclosed that certain lactic acid bacteria required extracts of plants or animals for growth. They stated that yeast extract (Bacto) and solubilized liver fractions were both rich sources of the necessary growth factor, and that Norite would adsorb the substance. The eluate was studied, and certain chemical properties were determined. Stokstad (54) prepared a factor, required for the growth of *Lactobacillus casei* from solubilized liver, by adsorption on Norite and purification of the eluate. He concluded that the growth factor is a pyrimidine nucleotide. Many papers have appeared since 1940 describing the isolation and properties of folic acid.

Briggs and collaborators (55) have shown that the Norite eluate concentrate of solubilized liver, prepared according to Hutchings' method, contained at least three factors: folic acid, required for the growth of *L. casei;* vitamin B_{10}, necessary for normal feathering; and vitamin B_{11}, necessary for normal growth of chicks. The fractions high in vitamin B_{10} and B_{11} activity, but low in folic acid, promoted excellent growth and feathering in the chick. Pfiffner and coworkers (56) isolated a crystalline compound from liver. They called it vitamin B_c, and showed that it had both growth-promoting and antianemic properties for the chick. Because vitamin B_c also possessed growth-promoting activity for *L. casei,* these investigators suggested

that vitamin B_c, Hutchings' Norite eluate factor, and Williams' folic acid of animal and plant cells (spinach, yeast, etc.) (57), are probably one and the same factor. Binkley and associates (58) prepared from yeast a crystalline B_c conjugate, which had vitamin B_c potency for the chick, but no activity for *L. casei*. However, B_c conjugate, when it was exposed to an enzyme of hog kidney, was converted to a compound which was active as a source of B_c for the bacterium. Several crystalline substances had been obtained from liver, yeast, and other sources. The synthesis of a compound identical with the *L. casei* factor from liver has been reported (58). It has the following structure:



N—[4—{[(2-amino-4-hydroxy-6-pteridyl)methyl]amino}benzoyl]glutamic acid

This liver factor contains one glutamic acid, while the fermentation factor has three such residues. The name " pteroyl glutamic acid " has been proposed for the liver *L. casei* factor. Folic acid is essential for a number of mammalian and avian species, in addition to being required for the growth of some bacteria. This vitamin appears to be very important to hematopoiesis (blood formation). Synthetic folic acid is being employed in human subjects with pernicious anemia and certain other anemias of the macrocytic type, and in sprue, producing hematologic and subjective responses closely simulating those brought about by potent liver extracts. The dosage, however, appears to be larger than that of potent liver preparations. Owing to this, Berry and Spies (60) and Welch and coworkers (61) conclude that the action of liver preparations cannot be attributed to folic acid per se.

Assay methods for the various growth substances have been described by Williams and coworkers (62).

TABLE XXVII

COMPOSITION OF YEAST

	Per Cent
Crude proteins	46.74
Fat	1.61
Carbohydrates	35.37
Ash	7.87
Crude fiber	8.41

The Composition of Yeast and Its Food Value. Table XXVII shows a typical analysis of beer yeast substance (63). In Table XXVIII the amino acids of yeast are given (64). Yeast is a complete protein, as established by feeding experiments on rats and dogs. Yeast also contains 0.2 per cent glutathione on dry basis.

TABLE XXVIII

AMINO ACIDS OF YEAST PROTEIN

(Calculated to 16 per cent nitrogen)

	Per Cent
Arginine	3.1
Histidine	3.3
Lysine	7.1
Tyrosine	3.8
Tryptophan	1.2
Phenylalanine	4.5
Cystine	1.1
Methionine	2.7
Threonine	5.5
Leucine	7.3
Isoleucine	6.0
Valine	5.3

Phosphorus Compounds of Yeast. Phosphates play a very important role in the metabolism of the yeast cell. Addition of phosphates results in a considerable increase in fat and carbohydrate storage. The following phosphoric acid esters have been isolated: hexosemonophosphate, hexosediphosphate, trehalose monophosphate, phosphoglyceric acid, phosphoglycerol, phosphoglyceraldehyde, dihydroxyacetone phosphate, dihydroxyacetone diphosphate, phosphopyruvic acid, nucleic acid, nucleoproteins, coenzyme I, coenzyme II, riboflavin monophosphates, diphosphothiamin, and phospholipides.

Table XXIX shows the relative concentrations of various phosphorus compounds (65), and Table XXX represents the composition of yeast ash. (For an excellent review concerning the mineral metabolism of yeast see reference 66.)

YEAST AS A FOOD SUPPLEMENT

In addition to its high protein, fat, and mineral salts content, yeast contains large quantities of thiamin, riboflavin, nicotinic acid, provitamin D, pantothenic acid, pyridoxine, biotin, *p*-aminobenzoic acid, and other vitamins of yet obscure identity.

Stone (67) in 1942 suggested a ration of brewers' yeast for the United

TABLE XXIX

PHOSPHORUS COMPOUNDS OF ENGLISH BREWERS' YEAST
(Milligrams of phosphorus per gram of yeast)

Compound	English Brewers' Yeast
Total phosphorus	3.25
Orthophosphate	1.37
Pyrophosphate	0.68
Organic phosphate	1.17
Hexosediphosphate	0.38
Hexosemonophosphate	0.72
Nucleic acid	0.07

TABLE XXX

ANALYSIS OF YEAST ASH (PERCENTAGE)

Ash Constituent	Top Yeast*	Bakers' Yeast†
P_2O_5	52.3	54.5
K_2O	35.4	36.5
Na_2O	0.06	0.7
MgO	4.8	5.2
CaO	1.56	1.4
SiO_2	1.1	1.2
SO_3	0.41	0.5
Cl	....	Trace
FeO	0.43	Trace

* Fulmer *et al.* (1928).
† Frey (1930).

States and British forces. Such rations had been in use by some European troops. In Britain surplus brewers' yeast has been put to many uses. According to Stone, " It goes to make ' meat extracts,' packet gravies and soups. The demand for ' marmite ' and similar yeast products far exceeds the supply. Yeast also is incorporated in poultry foods and has been found suitable to supplement the oat ration of horses if added to the extent of one-eighth of the oat ration, with a small addition of salt. It has been found that irradiated yeast increases the milk production of cows and that the milk from cows so fed has an increased fat and vitamin D content. This diet has been found to effect a cure in the case of cows suffering from tuberculosis." This paper contains some data concerning the use of yeast for animal feeding. See also reference 68.

Carter and Phillips (69) have published an extensive review concerning the nutritive value of yeast for man and animals. These authors conclude: " Although these data are obviously inconclusive, they indicate that yeast may be somewhat inferior to animal proteins in human nutrition." This review contains 64 references.

Figure 20 shows that the addition of moderate quantities of brewers' yeast to white flour enriches the bread with the whole vitamin B complex (70).

Yeast prepared by a large-scale aeration process showed a yield of 150 to 180 parts (or 37.5 to 45.0 per dry weight) per 100 parts of glucose. Molasses was the source of glucose. The protein content of this yeast was 50 to 55 parts. The non-nitrogenous matter, such as cellu-

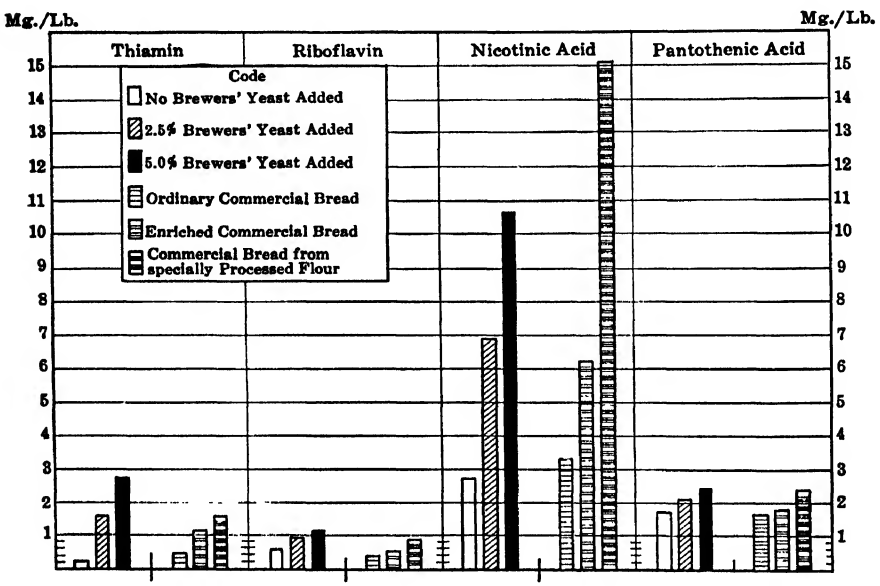


FIG. 20. Thiamin, riboflavin, nicotinic acid, and pantothenic acid contents of experimental and commercial breads.

lose, yeast gum, other carbohydrates, and fats was 37 to 42 parts, whereas the ash was 8 per cent (71). The presence of amino acids, when molasses is the source of sugar, has a beneficial effect on the quality and yield of compressed yeast prepared by the aeration process. Molasses contains 5–6 per cent of amino acids. The principal source of yeast protein nitrogen is ammonia, however.

Fink (72, 73) has extensively studied *Torula utilis* as a source of protein, using a great variety of materials such as wood sugar, sulfite liquor, potatoes, and simple carbon compounds such as acetaldehyde, pyruvic acid, ethyl alcohol, acetic acid, glycerol, and glucose. Glucose was best utilized, and it resulted in the highest yield of yeast. Fink contends that the production of the yeast protein is not practical, since soybeans are less expensive. He considers the production of yeast protein, however, an important potential economic factor in Germany.

In this country, Lewis and coworkers (74) have extensively studied protein and vitamin synthesis by *Torula* yeast. They state that large quantities of feed yeast of the so-called "torula" type are manufactured commercially, utilizing wood sugar, sulfite waste liquor, and molasses as the carbohydrate source. Fruit wastes, such as citrus-waste press juice, may also be used for the production of torula yeast (*Torulopsis utilis*). The protein content of this yeast is fairly uniform, averaging over 50 per cent of the dry yeast substance. It possesses well-developed vitamin-synthesizing functions. When *T. utilis* was grown in fruit juice media, Lewis and coworkers found that the yield in dry yeast was 55 grams per 100 grams of sugar. The addition of certain nutrients and aeration were necessary factors for useful cultivation. Air was supplied at the rate of 1.5 liters per minute per liter of medium. The sugar concentration was about 0.5 per cent. The propagation period of 6 to 8 hours resulted in 10-fold increase in yeast as compared with the inoculum. The temperature was 30° C. The *p*H was kept between 4 and 5. Nitrogen was supplied in the form of ammonia. The content of the vitamin B complex was comparable to that of bakers' and brewers' yeast. Large quantities of the vitamins were found also in the culture fluid.

Stubbs and collaborators (75) conducted pilot-plant experiments concerning the production of torula yeast from fruit wastes. Their conclusions are that the technique employed for bakers' yeast production may also be used for the manufacture of this yeast.

Kurth and Cheldelin (76) have investigated the production of feeding yeasts from wood sugar stillage, using *Torula mycotorula* and *Hansenula*. Their results with the pentose-utilizing yeasts are encouraging.

Composition of Yeast Fat. Täufel, Thaler, and Schreyegg (77) examined the composition of yeast fat (*Saccharomyces* sp.), a commercial product of C. F. Boehringer and Sons (trade name "Cerolin"), obtained by extracting washed brewers' yeast with alcohol. This product had a dark brown color, a somewhat unpleasant odor, and a soft consistency. Before analysis the product was purified by ether extraction. The following values were obtained:

	Per Cent	
Glycerol	5.3	
Volatile acids	5.2	
Palmitic acid	9.5	
Stearic acid	5.9	
Oleic acid	47.6	
Linolic acid	2.9	
Unsaponifiable substances	16.6	of which 3.3 per cent are sterols (ergosterol, cryptosterol)

This analysis shows that, owing to its composition, yeast fat is a suitable food.

Reindel, Niederländer, and Pfundt (78) reported that "fat" production by *Torula* types of yeasts increased with increasing sugar concentrations. The yield was highest when a molasses medium was employed. The main sterol fraction contained ergosterol.

YEAST AS A SOURCE OF VITAMIN D

Steenbock and Black (79) and Hess (80) have shown independently that certain compounds could be made highly antirachitic by ultraviolet light. Later it was shown that yeast is a very good source for the production of vitamin D by irradiation, owing to its ergosterol content. Not all yeasts are suitable for utilization as a source of vitamin D. It was discovered that yeast could be irradiated and then grown in such a manner that four or five multiplications were carried out without the loss of vitamin D potency.

During autolysis the ergosterol content of yeast increases for 9 hours, when it reaches a maximum. After 15 hours it gradually decreases, and after 24 hours it is still greater than it was originally (81).

Yeast contains 0.56 gram of ergosterol per 100 grams of dry substance. One-third of the sterol is converted into vitamin D when irradiated at wavelengths of 280 to 300 $\mu\mu$ in a layer of 1/10 to ½ mm. thickness for 30 minutes (82).

Production of Vitamin D Milk by Feeding Irradiated Yeast to Cows

Wachtel (83) and later Steenbock, Hart, Hanning, and Humphrey (84) found that feeding irradiated dry yeast to cows resulted in milk of increased vitamin D content. The process has been developed on an industrial scale by the Fleischmann Laboratories and the Walker-Gordon Laboratories. When yeast containing 1000 Steenbock units of vitamin D per gram was fed to cows in quantities of 5 ounces daily, the resulting milk contained 160 Steenbock units per quart. Numerous clinical studies have shown that the vitamin D milk produced by feeding irradiated yeast to cows is a satisfactory antirachitic agent.

Preparation of Sterol from Yeast and Other Cellular Material

Goering (85) disclosed the following procedure for the production of sterols: One kilogram of yeast containing 80 per cent moisture

is refluxed for 5 hours with 1500 cc. of 8 per cent sodium hydroxide. The mixture is filtered, and the residue is extracted with 1500 cc. of 95 per cent ethyl alcohol. A second and third extraction is made with 1000 cc. of ethyl alcohol. The combined extract is evaporated to about 10 per cent of its original volume to permit crystallization of the ergosterol. The crude ergosterol may be recrystallized from alcohol, yielding 2.55 grams of pure ergosterol, or 86.1 per cent of the theoretical ergosterol originally present in the yeast. A method for the quantitative determination of ergosterol, based on the measurement of the antimony trichloride reaction product by a spectrophotometer, has been described by Lamb and associates (86).

YEAST AS A SOURCE OF THIAMIN

Pavcek, Peterson, and Elvehjem (20, 87) found that the thiamin content of various yeasts grown on the same medium was about the same but varied to a great extent with different media. The values obtained per gram of dry yeast were 10 I.U. for grain-wort yeast, 3 to 4 I.U. for molasses yeast, and 2.5 to 3.3 I.U. for glucose-salts yeast. With *Endomyces vernalis*, however, the variations were much less; the yield was about 7 I.U. thiamin per gram of dry yeast.

Omission of aeration of the grain medium yielded 90 per cent less yeast but increased the thiamin potency threefold. Thiamin destroyed by autoclaving is resynthesized by yeast. For a process for the preparation of crystalline thiamin from yeast, see reference (88).

PREPARATION OF LIQUID MEDICINAL BREWERS' YEAST

Willstätter and Sobotka (89) found that the flavor of sugar-liquefied yeast may be considerably changed by heating. The flavor so imparted resembles somewhat the flavor of honey.

The following method was employed by these investigators:

Two hundred pounds of washed and pressed brewers' yeast, containing about 30 per cent of dry matter, are placed in a kneading apparatus having a heating appliance, and are rapidly mixed with 300 pounds of ground cane sugar. After 1 hour the mass is slowly brought to 55° C. and is kept at this temperature for 1 hour. In the course of another hour the temperature is brought between 85 and 110° C., and so maintained for 1 hour. Then the thick brown liquid is cooled.

This liquid now contains invert sugar, enzymes, and vitamins. It is recommended for baking purposes, for the improvement of malt extracts, and for therapeutic uses.

PREPARATION OF DRY MEDICINAL BREWERS' YEAST

In the United States 50 million barrels of beer are produced annually; this makes available 150 million pounds of waste yeast. On dry basis this amounts to 30 million pounds. Most breweries are ready to dispose of this waste yeast at a low price to farmers, feed producers, etc., if periodic removal is assured. A considerable amount of waste brewers' yeast finds its way into the public sewer system. Avoidance of dilution and proper drying installations are the most important factors in the economical recovery of waste yeast. With the aid of small drying units wet yeast may now be profitably dried by small breweries (90).

Waste yeast destined as an accessory animal feed does not require pretreatment. It is merely dried to 5 to 10 per cent moisture. Yeast to be used in foods or in the manufacture of medicinal yeasts and concentrates requires purification by washing and debittering with the aid of an alkali.

Bari (91) described the preparation of medicinal yeast from waste bottom-fermentation yeast. The yeast is made into a pulp and sieved. It is washed twice with fresh cold water. The bitter substances are removed by treating the yeast with 4 volumes of cold 2 per cent sodium carbonate solution, or 1 per cent ammonium carbonate solution. The yeast, now containing 75 per cent of water, is dried below 35 to 40° C. The paper also describes the preparation of a yeast extract from bottom yeast, as well as the preparation of pills and ointments containing yeast and yeast extracts.

Siebel, Weber, and Singruen (92) recommend the following improved purification-debittering process: The yeast is washed in cold water and then screened. Working at a temperature near freezing prevents diffusion of vitamins and minerals. The washed yeast is placed in a tank or other suitable container provided with refrigeration and mixing apparatus. An ice-cold 1 to 2 per cent sodium or ammonium carbonate or bicarbonate solution is employed for debittering. The yeast is then run through a separator or over a vibrating screen into another tank containing enough hydrochloric acid to adjust the *p*H of the yeast suspension to *p*H 5.0 to neutralize the residual alkali. The yeast is run into a separator and is ready for drying. If desired, the debittering process may be repeated. The temperature should not be above 2° C. during the whole process.

The purified and debittered yeast may be spray-dried and recovered in the form of powder, or it may be dried with the aid of rolling units that produce a yeast film which may be automatically removed in the form of flakes, or it may be drum-dried (92)

THE USE OF YEAST IN MEDICINE

Yeast, owing to its rich source of various vitamins, glutathione, and probably some hormones, is being extensively used for the treatment of several diseases. Only a few of the more important reports will be briefly reviewed here.

The Effect of Yeast on Cancer. McLeod and Ravenel (93) injected (chiefly intramuscularly) extracts of *Aspergillus niger* and *Saccharomyces cerevisiae* into 150 advanced cancer cases abandoned as hopeless. All the patients showed subjective improvement and a great number showed notable degrees of shrinkage of the tumor masses. Most of the improved patients manifested recurrences; several, however, did not.

Feeding of Boiled Yeast in Experimental Cancer. Maisin, Pourbaix, and Caeymaex (95) observed that boiled yeast contains a compound which counteracts the action of styryl 430 (2-*p*-aminostyryl-6-*p*-acetylaminobenzoylaminoquinoline methyl acetate), a water-soluble cancerogenic substance. Mice that received large amounts of cooked yeast, and that were treated with benzopyrene, showed a great decrease in the incidence of cancer.

Ando (95) reported that the incidence of liver cancer in rats, produced by butter yellow (dimethylaminoazo-benzene) and rice diet, was considerably reduced when yeast was added to the food. Sugiura and Rhoads (96) confirmed the results of Ando. In a more recent paper, Rhoads and associates (97) have shown that rats which were fed casein to which riboflavin was added were substantially protected against experimental liver cancer.

Lewisohn and his associates (98) have been able to prevent tumor growth (carcinoma 2163) in mice by intravenous injections of yeast and riboflavin (non-takes 62 per cent), or yeast and pantothetic acid (non-takes 47 per cent). Yeast alone had an effect on only a small number of animals (non-takes 20 per cent).

Treatment of Diabetes with Yeast. In 60 cases of diabetes, bakers' yeast produced a favorable influence on metabolism. The urinary sugar disappeared and the blood sugar decreased. The beneficial results were attributed to the high vitamin B, nucleic acid, and glutathione contents of yeast (99, 100). These reports are in harmony with the earlier animal experiments of Nasset, Pierce, and Murlin (101), who studied the effect of yeast ingestion on normal and depancreatized dogs. See also section on cocarboxylase concerning the blood sugar lowering action of this coenzyme (Part I of this book).

THE ENZYMES OF YEAST

Yeast contains large quantities of a number of enzymes. Some are present, however, only in small amounts. Several of the enzymes have been found to be a mixture of two or more specific enzymes and co-enzymes. The yeast enzymes are discussed in the first part of this book.

ADSORPTION OF ENZYMES BY YEAST CELLS

Kursanov and Issaeva (102) reported that *S. Steinberg* cells adsorb from aqueous solution the enzymes invertase, peroxidase, trypsin, and β-glucosidase. The degree of adsorption varies for each enzyme and depends on the *p*H of the solution and the age and the vitality of the yeast. Some enzymes are bound more firmly than others and may be released from the yeast cells by such enzymes that become more firmly bound. β-Glucosidase removes all previously adsorbed enzymes. Invertase is only very lightly bound to the cells. Various substances had been tried, but only gelatin was able to liberate the adsorbed enzymes. Dead yeast cells do not adsorb enzymes. The authors believe that the protein enzymes permeate through the cell membrane and are fixed on the element of the protoplasm.

FERMENTATION APPARATUS OF SCHULTZ, ATKIN, AND FREY (103)

This fermentation apparatus is used with a known atmosphere (see Fig. 21). The reaction bottles (volume, 250 cc.) are provided with glass hooks designed to hold oversize Keilin tubes containing the yeast suspension until the system has reached equilibrium. The system is filled with nitrogen or oxygen by evacuating the prepared reaction bottle and repeatedly flushing the gasometer with the gas by means of the stopcock on top and the inlet below. The evacuated reaction bottle is quickly connected with the filled gasometer, the connections are flushed out by means of the three-way stopcock, and the gas is admitted to the bottle. Excess gas is discharged through the top of the gasometer. The temperature is 30° C., and the bottles are shaken at 100 oscillations per minute. The gas evolved is measured

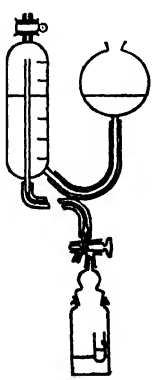


FIG. 21. Apparatus for the measurement of fermentation under various gases.

every half hour for 3 hours. See also the fermentometer given in Chapter XXXII.

Materials. The fermenting solution has a final volume of 80 cc. and it contains 0.8 gram of moist (compressed) bakers' yeast, a phosphate-citrate buffer of *p*H 5.4, 3 grams of the sugar under study, nicotinic acid (1 milligram), thiamine (0.05 milligram), and mineral salts, including ammonium ions (200 milligrams of ammonium sulfate).

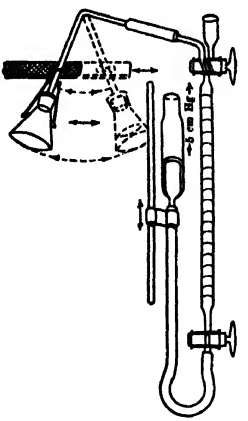


FIG. 22. Euler-Nielson fermentation apparatus.

THE EULER-NIELSON FERMENTATION

Figure 22 shows the Euler-Nielson fermentation apparatus as described by Funk (104). The fermentation mixture is placed in a small Erlenmeyer flask, using the following proportions: 0.2 gram dried yeast, suspended in 0.5 cc. 20 per cent glucose solution, plus 1 cc. of *M*/15 phosphate of *p*H 7.3, and 1 cc. of water. The buret is filled with mercury which is gradually displaced by the carbon dioxide produced during fermentation. The carbon dioxide may then be read off in cubic centimeters at the desired periods, usually after 1 to 1½ hours, by lifting the mercury level in the reservoir to the gas level within the buret. A series of six of these burets is run simultaneously. The small flask is connected with a shaking mechanism. An experiment requires several hours, depending on the nature of the experiment and the yeast.

PREPARATION OF SOME COMPOUNDS WITH THE AID OF YEAST

The Synthesis of *l*-Ephedrine. The Chinese plant, *ma huang*, has been in use for medicinal purposes in China since 3000 B.C. Its active ingredient, *l*-ephedrine, was introduced into this country in 1925. It is widely used as a nasal astringent and in the treatment of asthma and other ailments. In 1939 the production of ephedrine in the United States amounted to 403,237 ounces, valued at $593,778 (105).

In 1904, Fourneau described a method for the synthesis of ephedrine, and the publication of numerous other methods soon followed. The synthetic product, however, is a racemic mixture, and the dextro isomer has little pharmacological importance. For the isolation of the levo isomer from the optically inactive mixture, several procedures are available, but they are all very costly.

Hildebrand and Klavehn (106) have patented a method based on the biological condensation of benzaldehyde and acetaldehyde to *l*-acetylphenyl carbinol, according to the procedure of Neuberg (107, 108). This reaction cannot be carried out by chemical means, although similar syntheses are well known. The resultant carbinol is allowed to react with methylamine, and the condensation product is reduced to *l*-ephedrine with activated aluminum or hydrogen in the presence of colloidal platinum as a catalyst:

$$\underset{\text{Benzaldehyde}}{C_6H_5CHO} + \underset{\text{Acetaldehyde}}{CH_3CHO} \longrightarrow \underset{\text{l-Acetylphenylcarbinol}}{C_6H_5CHOHCOCH_3} + \underset{\text{Methylamine}}{CH_3NH_2} \longrightarrow$$

$$C_6H_5CHOHC(CH_3)=NCH_3 + H_2 \xrightarrow{\text{Catalyst}} \underset{\text{l-Ephedrine}}{C_6H_5CHOHCH(CH_3)NHCH_3}$$

Neuberg's Biological Synthesis of *l*-Acetylphenylcarbinol. One kilogram of top yeast is added to 25 liters of a solution containing 1.25 kilograms of glucose syrup; the mixture is then allowed to ferment for 30 minutes. Then 100 grams of benzoic acid-free benzaldehyde is added, in small amounts, with vigorous stirring. After 3 days of fermentation the yeast is filtered off and the filtrate is extracted with ether. On evaporation of the ether extract, 91 grams of an oil remains, containing 25 grams of *l*-acetylphenylcarbinol, 45 grams of benzyl alcohol, and 4.5 grams of benzoic acid.

The Hildebrandt-Klavehn Process. One hundred and twenty grams of the oil fermentation product of Neuberg is added in the course of 2 hours to a solution of 10 grams of methylamine in 500 cc. of ether in the presence of 20 grams of activated aluminum (i.e., aluminum superficially amalgamated with mercury). At the same time 20 to 30 grams of water is added drop by drop. The sudden, vigorous reaction is slowed down by occasional cooling. After completion of the reaction, the mixture is filtered and the *l*-ephedrine is extracted from the ethereal solution with dilute hydrochloric acid. The yield is 25 to 45 grams of *l*-ephedrine hydrochloride. This product is in every respect identical with the natural *ma huang*, *l*-ephedrine.

PREPARATION OF GALACTOSE FROM MILK SUGAR (110)

Galactose for medical purposes may be prepared from milk sugar by the following fermentation method: The sterilized solution of 2 kilograms of lactose and 88 cc. of concentrated sulfuric acid in 10.8 liters of water is neutralized with calcium carbonate, decolorized with

charcoal, filtered, and diluted to 20 liters. Fermentation with 100 grams of bakers' yeast is carried out at 35° C. until $\alpha_D = 9°$, and, after addition of another 100 grams of yeast, until $\alpha_D = 7.2°$. The filtered and acidified solution is evaporated *in vacuo*. To a 40 per cent solution of the solid residue, 75 per cent ethyl alcohol is added until a slight turbidity results. The crystals, which form after a week in the cold, are washed with ethyl alcohol. A second crop may be obtained from the mother liquor. The yield is 625 grams of galactose.

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CHAPTER XVII

PRODUCTION OF ETHYL ALCOHOL BY FERMENTATION

Gay-Lussac and Thénard, in 1810, and Saussure, in 1815, were the first who published exact quantitative reports on the fermentation of sugar. Their experiments were based on the earlier work of Lavoisier. The quantitative studies showed that 100 parts of sugar formed 51.34 parts of alcohol and 48.66 parts of carbon dioxide. In 1828 Dumas and Boullay published a modification of Gay-Lussac's equation. It included the preliminary hydrolysis of cane sugar.

The original equation of Gay-Lussac, which he proposed for the fermentation of grape sugar, $C_6H_{12}O_6 = 2CO_2 + 2C_2H_5OH$, still holds for the main fermentation products. Gvaladze (1) found that, when evaporation and entrainment during alcoholic fermentation were prevented, the yield of alcohol and carbon dioxide was best, e.g., alcohol yield 47.8 to 48.1 per cent and carbon dioxide yield 47.0 to 47.6 per cent. The average alcohol-carbon dioxide ratio is 1 to 1.

The main products of alcoholic fermentation account for 95 per cent of the sugar changed. Alcoholic fermentation with the aid of yeast yields about 10 per cent less than that expected. In scientific experiments, however, the loss is only 2 per cent. This amount of sugar is used up by the growing yeast in its various metabolic functions, during which these non-fermentable products form.

Highest yields in industrial ethyl alcohol production were obtained by fermenting in closed vessels under carbon dioxide pressure. Aeration of the worts during fermentation should be omitted, and the quantity of yeast employed for seeding should be high (2).

Table XXXI shows the amount of alcohol producible per ton of some raw materials (3).

Whereas the manufacturer is mainly interested in the yield of alcohol per ton, to the farmer the yield per acre is most important. In this country molasses is the principal material used in the manufacture of industrial alcohol. In Germany, however, the main source of the raw material is potatoes, and, in France, sugar beets.

In the production of ethyl alcohol by fermentation, the following operations are employed: (*a*) saccharification of the raw materials; (*b*) fermentation; (*c*) distillation.

TABLE XXXI

ALCOHOL PRODUCTION PER TON OF RAW MATERIAL

	Gal./acre	Gal./ton
Sugar beet	287.0	22.1
Sugar cane (La.)	268.0	15.2
Jerusalem artichokes	180.0	20.0
Potatoes, white	178.0	22.9
Potatoes, sweet	141.0	34.2
Apples	140.0	14.4
Raisins	102.0	81.4
Grapes	90.4	15.1
Corn	88.8	84.0
Rice (rough)	65.6	79.5
Molasses (blackstrap)	45.0	70.4
Grain sorghum	35.5	79.5
Wheat	33.0	85.0

The Raw Materials. Higher carbohydrate materials, such as corn, malt, barley, wheat, oats, rye, potatoes, sweet potatoes, cassava (manioc), rice, and Jerusalem artichokes, must be hydrolyzed by malt or acid before they can be fermented. Sugar-containing raw materials, however, such as blackstrap molasses, sugar cane, sugar beets, and fruit juices, do not have to be saccharified.

In normal times, blackstrap molasses is a convenient and economical source of alcohol. However, in the lack of sufficient supply of molasses, the establishment of a large number of Fischer-Tropsch gasoline plants producing alcohol as by-products may become a serious competition in alcohol manufacture. In this process, the ethylene, which is formed during cracking of petroleum gases, is converted to ethyl alcohol.

Table XXXII shows the yields from the most important sources of raw materials employed in the production of ethyl alcohol in the United States for the fiscal year 1947.

Cultivation of Starter. About 10 cc. of sterile wort is inoculated from a pure culture of *Saccharomyces cerevisiae* or other suitable yeast. This tube is kept at 25° to 30° C., and after sufficient growth has taken place it is used to inoculate a 200-cc. sample of sterile mash. After incubation this culture may be employed to inoculate several liters of sterile mash, and in due time the latter cultures may be used to " pitch " a volume of sterile mash ten times larger. Aeration accelerates yeast growth.

By using pure yeast propagators, starters may be cultivated with more safety and less effort, and reinoculation is less often required.

TABLE XXXII

ETHYL ALCOHOL PRODUCTION, BY KINDS OF MATERIALS USED IN THE UNITED STATES AND TERRITORIES FOR THE FISCAL YEAR 1947

	Quantity	Unit
Ethyl sulfate	106,456,844	Gal.
Molasses*	70,305,871	Gal.
Products used in redistillation	50,082,760	Proof gal.
Grain and grain products*	509,569,387	Lb.
Potatoes and potato products*	526,455,909	Lb.
	3,579,619	Gal.
Sulfite liquors	205,336,430	Gal.
Cellulose pulp, chemical, and crude alcohol mixtures:		
Crude alcohol mixtures	1,198,710	Gal.
Cellulose pulp and chemical mixtures	2,501,412	Gal.
Cassava and cassava products*	5,961,324	Lb.
Whey	6,473,805	Gal.
	496,890	Lb.
Pineapple juice	2,880,198	Gal.
Wood sugar liquors	2,880,871	Gal.
Orange juice	437,777	Gal.
Wine	4,991	Gal.
Hydrol	103	Gal.
Other mixtures:		
Potatoes and potato products	39,622,374	Lb.
Grain and grain products	9,010,894	Lb.
Syrup	810,921	Lb.
Cassava and cassava products	799,102	Lb.
Malt syrup	517,619	Lb.
Maltose	34,344	Gal.
Syrup	33,000	Gal.
Molasses	4,381	Gal.

* Additional amounts used in combination with other materials included under "Other Mixtures."

Net production, that is, the gross production minus products used in redistillation, was 248,798,639 proof gallons.

Alcohol Tax Unit, U. S. Treasury Department.

GENERAL PRINCIPLES FOR PRODUCTION OF ETHYL ALCOHOL FROM MOLASSES (4, 5)

The least expensive raw material of fermentation alcohol is blackstrap sugar-cane molasses, which is the waste liquor remaining after the removal of crystallized sugar from concentrated sugar-cane juice. Most of the blackstrap molasses is imported from Cuba, and a small quantity from Puerto Rico.

"High-test" molasses has been used in large amounts. This is evaporated raw sugar-cane juice which contains all the original sugar and in which the sucrose has been hydrolyzed with acid. This syrup

is made in order to find a market for sugar-cane juice produced by Cuban and other growers. High-test molasses contains up to 78 per cent of total sugar, whereas blackstrap molasses contains 50 to 55 per cent of sugar, mainly sucrose. A sugar concentration of 12 per cent is most frequently employed. Molasses does not always contain all the substances required for proper fermentation. Ammonium phosphate or sulfate is sometimes added to correct phosphorus or nitrogen deficiencies. The *p*H of the mash is adjusted to 4.0 to 4.5 with sulfuric acid. The starter represents 4 to 6 per cent of the main mash. Instead of acid, the mash may be inoculated with lactic acid bacteria, preceding alcohol fermentation.

Oxygen Requirements. Oxygen is required only during the first phase of yeast growth. Fermentation proceeds under anaerobic conditions which set in when carbon dioxide forms in large quantities. The temperature, which rises as a result of fermentation, is maintained below 30° C. by cooling the tanks with coils and sprays. Fermentation is completed in 20 to 50 hours, depending on the temperature.

Distillation. The fermented mash (" beer ") is pumped to stills and separated into 60 to 90 per cent alcohol and the " slop," which is usually discarded but sometimes is sold for various uses. A second distillation results in 95 per cent alcohol and fusel oil (amyl alcohol, isobutyl alcohol, etc.). The 95 per cent alcohol may be denatured or it may be dehydrated to yield 99.5 per cent alcohol. If continuous distillation is employed, the several fractionations may be carried out in one operation, resulting in the saving of steam.

CONTINUOUS PROCESS FOR ALCOHOLIC FERMENTATION OF MOLASSES

This process was developed by Bilford and associates (5). It is based on the use of a single-vessel continuous fermentation system and a storage tank. With this process molasses mashes containing 12 to 13 grams of sugar per 100 cc. can be fermented to completion in a 5- to 7-hour cycle, and yeast inoculation is necessary only at the beginning of a run. One of the principal values resulting from this process is the extensive reduction in equipment requirements.

The Continuous Fermentation System. Figure 23 shows a diagram (5) of the continuous fermentation system. It consists of a wide-mouthed Pyrex flask. Carbon dioxide is introduced through a glass tube and dispersed by 1-inch Aloxite spheres. Another glass tube provides an outlet for the gas, which is passed through scrubbers to remove

the alcohol vapors. A third tube is used to remove samples for analysis during the run and to add ammonia for *p*H control. The feed medium is introduced through a storage flask and connecting tube. The fermented material is removed by a draw-off tube.

During the initial phase of fermentation the sugar in the mash is fermented in 5 to 6 hours, depending on the initial cell count and other conditions. Continuous operation is started by feeding fresh (uninoculated) fermentation medium of high sugar concentration at a constant rate from the storage flask into the fermenter. The fermented material is removed at the same rate. For successful operation a pure culture system must be maintained.

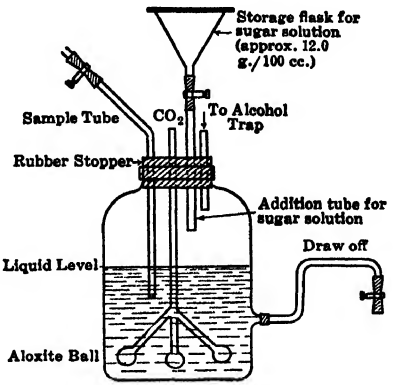

FIG. 23. Laboratory unit for continuous fermentation.

Conditions and Medium. Approximately 18° Balling molasses mash (suitably fortified if necessary), containing about 12–13 grams per 100 cc. of sugar, is employed for growth of inoculum and fermentation. The initial yeast inoculum of approximately 350 million cells per cubic centimeter is obtained by aerating large flasks of the molasses mash at 28.8° C. for 18 hours. The strain should be a suitable variety of *Saccharomyces cerevisiae*. The cells are separated by centrifugation and resuspended in the fermentation mash. The inoculated mash is then placed in the vessel and agitated with 4.8 liters of carbon dioxide per minute per liter of mash. The temperature is held at 32.2° C. The *p*H is maintained at 4.5 to 5.0 by adjustment with 3 *N* ammonium hydroxide. Under these conditions the sugar is 92 to 94 per cent fermented in 5 to 6 hours. At this stage the cell count is about 500 million per cubic centimeter. The stationary phase is thus completed and the continuous phase commences. The fermented material is removed from the fermenter at a rate of 15 to 20 per cent, and new 18° Balling mash is introduced at an equal rate. The process is now in continuous operation.

Fortification of Molasses. Cuban blackstrap molasses requires no supplement; refined molasses requires the addition of 75 milligrams of $(NH_4)_2SO_4$ per 100 grams of molasses, and beet molasses requires 100 milligrams of $(NH_4)_2HPO_4$ per 100 grams of molasses, for successful continuous fermentation.

PRODUCTION OF ETHYL ALCOHOL FROM STARCHY RAW MATERIALS

Yeast does not contain amylases. The starches must be saccharified by enzymic hydrolysis or by acid hydrolysis.

The enzymic hydrolysis may be carried out by employing malt, molds, or mold amylases. The use of malt in the production of industrial alcohol is identical with the methods employed in beer making. However, there the mash is boiled before yeasting.

In the amylo process the starches are saccharified by the use of molds such as *Mucor rouxii, Rhizopus japonicus,* and *R. tonkinensis.* Purified amylase preparations *per se* are not employed in the United States for saccharification before fermentation.

RAPID CONTINUOUS PROCESS FOR THE PRODUCTION OF ALCOHOL FROM CORNSTARCH AND MALT

Cooking and Conversion of Corn. The continuous cooking process originated in Russia (6) and was introduced in this country by Gallagher and coworkers (7). Corn is mixed with water, the slurry is continuously cooked by a steam jet, the cooked material is continuously cooled, and malt is continuously added to the moving material. This process is simpler in design, easier to operate, and much less costly in construction. Gallagher and collaborators (7) suggested the following procedure: The corn is cooked for 1 minute at 182.2° C. instead of the usual 2.5- to 3.0-hour batch cycle. The cook is cooled to the conversion temperature of 62.8° C., and barley malt slurried in water is added to the cook. The *p*H of the mash is kept at 5.4 to 5.8. The malt is in contact with the mash for 40 seconds at 62.8° C. before cooling to fermenter temperature. Although the iodine test indicates the presence of an excess of starch, sugar analysis shows that there is over 70 per cent conversion of the cooked starch to maltose by this rapid process.

Conversion Equipment. A newly devised, very simple conversion equipment (7) (Fig. 24) is employed for the conversion of the cornstarch. It consists of a small proportioning pump which continuously injects the malt slurry into the stream of cooked grain just ahead of the mash pump; the malt slurry is then passed through a section of 4-inch pipe 105 feet long, through which the mass flows. The capacity of this unit permits the conversion of 5000 bushels of grain per day. From this unit the discharge passes into mash coolers, where it is cooled to fermentation temperatures of 21.1° to 23.9° C. in 1½ minutes.

Fermentation of the Mash. The cooled converted grain mash is inoculated with a suitable strain of *Saccharomyces cerevisiae*. The mash is then made up to the final volume of 38 gallons per bushel of grain and the *p*H is adjusted to 4.8 to 5.0. Fermentation is then allowed to proceed to completion.

The alcohol yields on quickly converted mashes are 2 per cent higher than those obtained with the standard practice of 30 to 60 minutes conversion time at 62.8° C. The apparent increase in yield may be due to the fact that the short holding period results in little destruction of the amylase system during conversion. Thus the balance of the amylase is available for use in the fermenter.

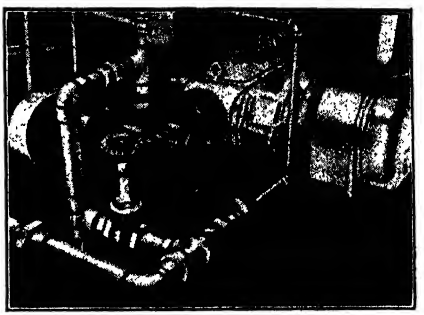

FIG. 24. In the new method, malt is metered out through the pump and mixed with the mash in a pipe line approximately 105 feet long; the malt is in contact with the mash for 40 seconds at 62.8° C. before cooling.

Detailed procedures for laboratory cooking, mashing, and fermentation, as well as methods for the calculations of yields, are given in the article by Stark and associates (8).

RAPID CONTINUOUS AEROBIC PROCESS FOR DISTILLERS' YEAST

Owing to the large capacity equipment and long over-all processing time required by the present methods employed in the production of distillers' yeast, Unger, Stark, Scalf, and Kolachov (9) proposed the following improvements.

Procedure for Laboratory-Scale Operation. A mash bill containing 40 per cent corn, 30 per cent barley malt, and 30 per cent long-fiber malt sprouts is employed to prepare the wort, or 85 per cent barley malt and 15 per cent long-fiber malt sprouts may be used. Five hundred cubic centimeters of sterile 10° Balling wort at *p*H 4.5 are inoculated and aerated with 0.4 cubic foot of sterile air per minute through two spherical Aloxite (electrically fused alumina) spargers. This method produces a pure culture yeast at a concentration of 500,000,000 cells per cubic centimeter in comparison with the usual 150,000,000 cells per cubic centimeter obtained by the sour-mash anaerobic process. This yeast compares well with the one obtained by the old methods in its fermenting activity.

Optimum Conditions for Yeast Growth. The optimum conditions are: (*a*) 20 minutes of sterilization at 121° C. followed by immedi-

ate cooling; (*b*) 3 square inches of sparger area per gallon of working capacity, using a number 60 teardrop-shaped carbon sparger (National Carbon Company); (*c*) ¼ cubic foot of air per minute per gallon; (*d*) only ammonia and ammonium sulfate required as nutrients in concentrations to supply 40 to 45 milligrams of amino nitrogen per 100 cc. in the propagator; (*e*) optimum temperature, 28.3° C.; (*f*) maintenance of the *p*H at 4.0 to 4.2 by the addition of ammonia.

The strained wort is dropped into a tank, and 1 to 2 per cent of filter aid is added to it. The mixture is then filtered to yield a 10° Balling clarified wort and is transferred through a storage vessel and sterilized. Meanwhile a yeast inoculum is prepared in a pure culture vessel (propagator) using an 18° Balling medium prepared from diamalt (barley malt syrup) containing maltose, dextrine, and amylase. This inoculum is added to a part of the clarified wort in the propagator, and sterile air is sparged into vessel. When the yeast count increases, an additional amount of wort is passed into the propagator to increase the volume to 300 gallons. At this stage the count becomes 400,000,000 cells per cubic centimeter. Now the operation becomes continuous with a wort flow to the vessel of 75 gallons per hour and an equal removal of yeast.

Ammonium sulfate and ammonia must be added to the wort at such a rate to maintain the level of amino nitrogen at 40 to 45 milligrams per 100 cc., and occasionally an antifoam agent of Vegefat and lard must be added.

Proposed Procedure for Plant-Scale Operation (for Distilling 5000 Bushels per Day). A mash bill consisting of 85 per cent barley malt and 15 per cent malt sprouts is prepared. The barley malt is obtained in the state of a stream of mash of 35 gallons per bushel and at 60° C. from a continuous cooker system. Seven and a half bushels of malt sprouts, enough for 24 hours, is charged into a scale hopper. From here it is fed into a malt-mixing chamber of 10-gallon capacity. A centrifugal pump circulates the combined mixture. Steam sparged into the vessel keeps the mash at 63° C. The mash is pumped into the infusion tube where it is kept at this temperature for 30 minutes. The mash is transferred to a rotary Oliver filter 3 feet in diameter and 1 foot wide, where it is strained through a 40-mesh screen and the cake is washed to recover all available maltose. The cake is repulped and pumped back into the continuous cooker. The strained wort is pumped to a Paraflow plate-type heat exchanger where its temperature is brought to 100–121° C. The wort is maintained for 5 minutes at this temperature in the protein-coagulation tube which is 8 inches in diameter and 6 feet long. The wort is then cooled to 49–65.5° C. and it is centrifuged. The clarified wort is pumped into the yeasting system

and the solids are returned to the cooker. The over-all recovery of sugar by this process is 75 to 77 per cent.

The wort is transferred to a vessel of 60-gallon capacity. It is jacketed and cooled to 7° C. to prevent bacterial growth. The vessel is maintained under air pressure in order to transfer it to the sterilizer and propagator.

The wort is sterilized by passing it into a heat exchanger, then into a jet heater where its temperature is brought to 121° C. It is kept so in a tube for 20 to 30 minutes, and then the wort is cooled and transferred to the propagator of 530-gallon capacity and of 225- to 250-gallon working capacity. Into this vessel sterile air is sparged at a rate of 60 cubic feet per minute through porous carbon diffusers. This air at a pressure of 40 pounds per square inch is filtered and then sterilized by being passed through a chamber fitted with Westinghouse Sterilamps. In pilot-plant practice cotton pads serve as good bacteria filters.

The level in the propagator is kept at 240 gallons, and sterile wort is sprayed into it at the rate of 60 gallons per hour. This allows 4 hours to grow the yeast and to deplete the medium of sugar. The completed yeast wort has an alcohol concentration of 1.75 per cent. This yeast wort is removed continuously at the bottom of the vessel to a centrifuge where the yeast cell concentration is increased from 500,000,000 to 2,000,000,000 per cubic centimeter. The supernatant fluid is returned to the distillery, and the yeast is transferred to refrigerated storage and kept there until needed.

Unger and associates believe that this rapid process can readily be adapted to the manufacture of bakers' and brewers' yeast and that their principles are adaptable for the construction of central yeast plants for distilleries and industrial alcohol factories. Figure 25 shows a diagram of a continuous aeration process yeast plant (9).

WHITE POTATOES AS A SOURCE OF ALCOHOL

In the United States, the normal requirements of white potatoes average between 40,000,000 and 45,000,000 bushels annually. Before the second World War, potatoes were not used for alcohol production in this country to any great extent. In Germany, in 1939, over 2000 distilleries produced industrial alcohol from potatoes. In European countries, farmers employ specially made alcohol engines which operate entirely on potato alcohol. In 1946, however, this country produced 75,000,000 bushels of potatoes, and the excess was converted into beverage alcohol (neutral spirits for the blending of whiskey). The average yield of 95 per cent alcohol from an acre of Irish potatoes

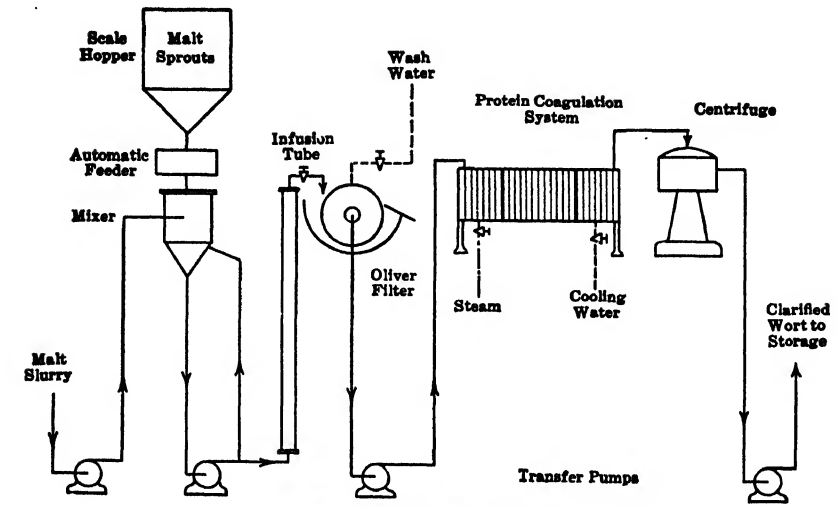

FIG. 25. Proposed yeast plant.

is 178 gallons. This yield is higher than that obtainable from sweet potatoes, corn, wheat, or rye (see Table XXXI). Thus, the potentialities of potatoes as a source of beverage alcohol are considerable. However, industrial ethyl alcohol plants must depend on blackstrap molasses, since the present price of potatoes is much too high for such use.

Processing of Potatoes (10, 11). The potatoes are washed, and may or may not be ground. Then, they are placed in a specially constructed cooker (Fig. 26). Cookers of such type are in use in Germany. They have a 5-ton capacity and contain two perforated steam coils. One is located in the base, through which steam is blown into the potatoes; the other, under the dome of the cooker, is employed to force the batch out when cooking is completed. The potatoes are introduced at the top. In the base is a perforated steel plate through which the potatoes are forced by steam pressure from the upper coil. The starch is first gelatinized for 20 minutes by introducing steam through the bottom coil; the vapors are released through the wide aperture at the top. Now, the pressure is brought to 30 pounds per square inch and maintained there for a short period. The cooked potatoes are immediately forced through the perforated plate into a mashing tank, where they are cooled to the conversion temperature (65°) by the addition of water and the use of cooling coils. The potatoes are now converted, using 15 per cent of dried malt on dry basis. Saccharification is carried out at 65° for 5 to 15 minutes. Then the mash is pumped through a cooling coil to the fermenter. The yeast inoculum (2 to 5 per cent per final volume of the mash) is added at about 29°, and the maximum temperature should not rise above 32°. Fermentation requires from 36 to 72 hours. The finished beer is pumped to the beer still, where it is processed in accordance with its use.

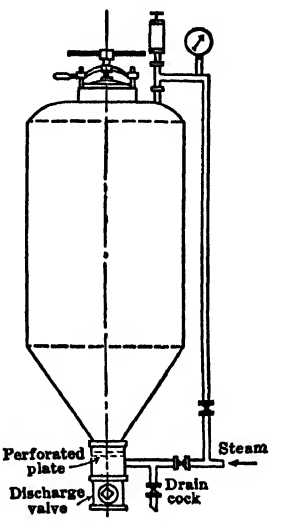

FIG. 26. Potato cooker.

THE USE OF MOLDY BRAN IN ALCOHOLIC FERMENTATION

In oriental countries molds have been used for alcoholic fermentation for hundreds of years. In France, Calmette and Bodin applied *Amylomyces* in the production of alcohol as early as 1891. This method is

known as the amylo process. In this country, about 1914, Takamine (12) conducted successful large-scale experiments with moldy bran, suggesting the replacement of the more expensive malt in the fermentation industry. His method was not adopted because there were slight odors in the alcohol. For the production of industrial alcohol, however, this would be a negligible factor.

Recently Schoene, Fulmer, and Underkofler (13) have shown that the use of moldy bran in the saccharification of starchy grain mashes for the manufacture of industrial alcohol is of economic interest. Of the eight species of bacteria and twenty-two species of strains of molds investigated, two strains of *Aspergillus oryzae* gave the best results. The mold was grown on wheat bran in small rotary drums, dried in air, and ground. The optimum conditions for saccharification with moldy bran were found to be identical with those practiced with malt. There was an average of 12 per cent higher ethanol yield with moldy bran as compared to that obtained with a good dried barley malt.

In practice, it is best to use 4 per cent moldy bran alone or in combination with malt. Thus, 2 per cent dry moldy bran and 4 per cent malt may be employed, depending on the activity of both amylase sources. The weight of the converting agent is based on the weight of the corn or other starch source used.

Underkofler and associates (14) proposed a rapid (24-hour) fermentation procedure for evaluating different samples of moldy bran as to their usefulness in alcohol production, since other factors than α-amylase are involved. The method is said to be applicable also to the evaluation of malts. Thorne and coworkers (15) have likewise been interested in the evaluation of malt for the production of alcohol. For further information concerning moldy bran see Chapter XXII.

The Sulfite Conversion Process of Balls and Tucker. During the second World War immense quantities of barley malt were saved by the distilling industry, owing to the discovery of Balls and Tucker (16) that a solution of 0.05 per cent sodium sulfite caused liberation of the β-amylase of wheat. This sulfite slurry required only a small percentage of malt in order to function as an efficient converting agent of grain mashes. From unmalted granular flour, the quantity of diastase liberated by the dilute sodium sulfite solution was about the same as that obtained from a medium grade of barley malt (Lintner value 130). However, there was damage to the distilling equipment, owing to the various sulfur compounds that formed from the sulfite. The process was gradually abandoned by the industry.

JERUSALEM ARTICHOKES AS A SOURCE OF ALCOHOL

The Jerusalem artichoke or girasole (*Helianthus tuberosus*), a plant native to the United States, has been considered a new farm crop. This plant has high yields, and it is a good source of fructosan. The polysaccharide is readily hydrolyzed by mild acids. The plant has been suggested as a source of fructose and of industrial alcohol. (For an extensive bibliography concerning this problem see reference 17.)

TABLE XXXIII

YIELD DATA FOR SEVERAL VARIETIES OF JERUSALEM ARTICHOKE

Variety or Accession Number	Yield per Acre				Average Total Sugar		Average Alcohol	Corn Equivalent
	Urbana, Ill. tons	Corvallis, Wash. tons	Washington, D. C. tons	Mean tons	per Ton pounds	per Acre pounds	per Acre gallons	per Acre bushels
Blanc Ameliore	6.43	18.42	7.90	10.92	313.8	3426	238.0	91.2
Chicago	4.98	19.96	8.48	11.14	356.4	3969	275.7	105.6
26,944	9.28	21.51	8.88	13.22	315.4	4170	289.7	111.0
26,984	6.36	20.00	6.32	10.90	336.8	3670	256.0	97.7
27,007	8.11	18.62	9.55	12.10	318.4	3853	267.7	102.6
27,079	8.81	15.52	8.90	11.08	368.0	4076	283.1	108.5
27,095	7.67	18.08	11.13	12.29	358.6	4407	306.1	117.3
27,574	10.95	20.78	11.40	14.38	380.2	5467	379.7	145.5
28,098	7.74	19.08	7.53	11.75	307.2	3610	250.8	96.1

Table XXXIII shows data as presented by Boswell and associates (18). Twenty varieties of the plant were studied. Those that gave an average sugar yield of 10.69 tons per acre or better are tabulated, as well as the pounds of sugar yield per acre. In the last column of the table, corn equivalent per acre is given for comparison (yields are on the basis of 90 per cent of theoretical anhydrous alcohol). The 20 varieties of Jerusalem artichokes examined by Boswell and his coworkers contained an average of 13.33 per cent of levulose and 16.38 per cent of total sugar. No practical use of this plant has yet been made by the alcohol-producing industry of this country.

Preparation of the Material for Fermentation (17)

The dried or fresh tuber chips are extracted with water in a diffusion battery at 80° C. The extract is concentrated under reduced pressure to a concentration greater than 70 per cent total solids. Whereas

bacteria and yeast do not grow in this syrup, mold growth can be prevented only if it is stored in an atmosphere of carbon dioxide.

Fermentation of the Syrup. The syrup is diluted to contain 12 per cent reducing sugar after acid hydrolysis. Acidification of the polysaccharides to a *p*H of 1.75 with hydrochloric acid and heating for 1 hour at 80° C. effect complete hydrolysis. The syrup is then sterilized, cooled, and inoculated with *Saccharomyces cerevisiae, S. anamensis,* or *S. pombe.* The yield of alcohol is 90 per cent or more. The syrup requires no further hydrolysis before fermentation, and no nutrients have to be added. The fresh tubers and their dilute extracts do not keep well. Some methods for storage, however, have been suggested (19).

According to Underkofler and his associates (17), who studied this problem extensively, the fermentation of artichoke syrup offers no difficulty.

Sulfite Liquor as the Carbohydrate Source. The June 1945 issue of *Pulp and Paper Industry* (20) contains an excellent article concerning the production of ethyl alcohol from sulfite liquor. It describes the alcohol plant at the Puget Sound Pulp and Timber Company, which is the world's largest plant of this type. The sulfite pulp effluent (sulfite liquor) obtained at this plant contains 1 per cent fermentable sugar and is concentrated to contain 12 to 13 per cent sugar. The sulfur dioxide is removed, and the *p*H is adjusted with lime. Urea is added to serve as nutrient for the yeast. From the fermenting mash, the yeast is collected by centrifuging and employed as an inoculum for the adjusted effluent. Fermentation is completed in 20 hours at 30° C. The plant has a manufacturing capacity of 5000 gallons of ethyl alcohol per day. Investment in the plant is $1,000,000. Sulfite liquor is extensively used for yeast manufacture.

ETHYL ALCOHOL FROM CELLULOSE-CONTAINING MATERIALS

Fermentation of Wood Sugar during the War, in Germany. According to the report of the Joint Objectives Agency, Office of Military Government for Germany, Fiat. No. 499 (21) during the second World War, Germany manufactured significant quantities of alcohol by the Scholler process and from sulfite liquors, totaling 10,000,000 and 25,000,000 liters per year, respectively. However, the Scholler process was not considerably expanded during the war, nor has there been any important progress in the production in wood sugar. The fermentation of wood sugar to alcohol was carried out by a rapid, continuous

yeast reuse method requiring only 5 hours. The two Bergius wood sugar plants in Germany were using the well-known methods, as given in the earlier literature. Owing to the chemical requirements, this process is impracticable. Almost all sulfite liquor in Germany was employed to produce yeast and alcohol. According to the report, German alcohol-manufacturing methods did not improve during the war.

ETHYL ALCOHOL FROM WOOD WASTE

Several processes have been described dealing with wood saccharification. The Bergius-Rheinau (22) process is based on the discovery of Willstätter that 40 per cent hydrochloric acid solution completely hydrolyzes cellulose to glucose at room temperature. Wood is used as the source of cellulose; lignin, however, is not attacked by the acid. After hydrolysis, the mixture is neutralyzed with lime, filtered, and fermented with a suitable strain of yeast. In the Scholler (23) process, dilute sulfuric acid, high temperature, and steam under pressure are used for the hydrolysis of the wood cellulose.

The Madison Wood Sugar Process. Scientists of the U. S. Forest Products Laboratory (24) have developed a process for the production of wood sugar and its conversion to ethyl alcohol. This process is superior in many respects to the German Scholler method. Hydrolysis is accomplished in shorter time, and heating is at a lower rate, so that less sugar is destroyed and fewer yeast inhibitory by-products are produced and therefore fermentation is more rapid. The sugar formed in 2.8 hours from a ton of dry, barkfree, Douglas fir wood waste yielded 64.5 gallons of 95 per cent alcohol, as compared to 13 to 20 hours for 55 gallons by the Scholler process carried out in Germany. In the new modification, the wood waste is hydrolyzed with 0.5 to 0.6 per cent sulfuric acid at 150° to 180° C. Then, the mixture is adjusted to the desired *p*H and fermented. Figure 27 shows the pilot-plant equipment employed in the production and fermentation of wood sugar (glucose from wood) in the Madison laboratory. It is expected that this process will produce 5,950,000 gallons of alcohol per year at the wood hydrolysis plant in Springfield, Oregon. A method for the determination of fermentable sugar has been published by Saeman and associates (25).

ETHYL ALCOHOL FROM THE FERMENTATION OF LACTOSE IN WHEY

Browne (26), with a view to finding a use for cheese whey, studied the fermentation of lactose to ethyl alcohol by specific yeasts. The

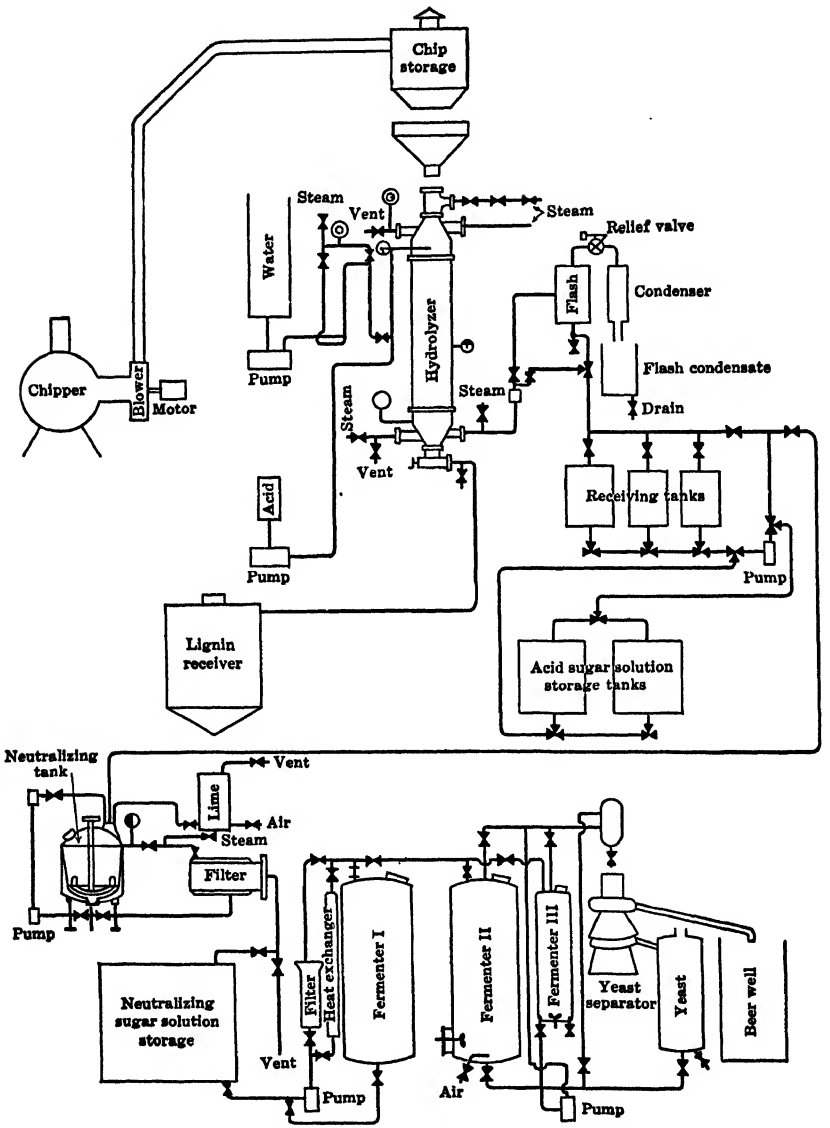

FIG. 27. Equipment for saccharification of wood and fermentation of wood sugar solutions.

TABLE XXXIV

Fermentation Data on Lactose Alcohol Mashes

Expt. No.	Organism	Whey Used		Weight of Pitching Yeast	Final Distillate				Weight of Separated Yeast	Fermentation Time	Fermentation Temperature
		Weight pounds (gal.)	Lactose Hydrate Content %	grams (pounds)	Volume cc.	Alcohol by Volume %	100% alcohol equivalent cc.	Theoretical Yield %	grams	hours	° C.
1*	*Torula cremoris* A.T.C. 2512	169 (20)	4.62	500 (1.1)	4160	44.0	1830	80.3	720	21.7	30 to 32
2†	*Torula cremoris* A.T.C. 2512	165 (20)	4.95	500 (1.1)	4280	41.7	1785	75.0	...	48.0	.....
3‡	*Torula lactosa* A.T.C. 7014	168 (20)	5.31	570 (1.25)	3820	46.37	1771	68	770	24.0	35 to 36
4§	*Torula lactosa* A.T.C. 7014	259 (30)	4.91	500 (1.1)	5660	44.68	2525	68	...	40.8	32 to 37
5‖	Yeast A.T.C. 2702	171 (20)	5.12	750 (1.65)	4470	43.5	1945	75.8	...	24.0	.....

* Filtered whey; *p*H 4.5; fermented in closed milk cans; initial specific gravity 6.8°, final 1.2° Brix; specific gravity of slops 3.3° Brix.

† Unfiltered whey; *p*H 5.3; initial specific gravity 7.3°, final 2.3° Brix. Yeast not separated.

‡ Filtered whey; *p*H 4.5; initial specific gravity 7.5°, final 1.2° Brix; some mycoderma; distilled without fat; riboflavin in slops 1.3 micrograms per cubic centimeter.

§ Unfiltered Roqueford cheese whey; *p*H 5.35.

‖ Filtered Swiss cheese whey; initial specific gravity 7.2°, final 1.2° Brix.

following organisms were used: *Torula cremoris,* American Type Culture Collection (A.T.C.) 2512; *Torulopsis sphaerica,* A.T.C. 2504; *Torula lactosa,* A.T.C. 7014; yeast, A.T.C. 2702; and kefir yeasts KY2, KY6, KY8, and KY10.

First Culture Medium. Alcohol yield was first tested in a medium containing 100 grams of lactose hydrate, 1.0 gram of monoammonium phosphate, and 0.7 gram of ammonium sulfate per liter. The medium was warmed to dissolve the lactose and autoclaved. The *p*H was 5.1, and the Brix was 16°.

Yeast 2504 showed an alcohol yield of 25.3 per cent, and the others 76.3 to 83.6 per cent, after 11 to 17 days of fermentation.

Large Medium. This was prepared by using 5 liters of whey to inoculate larger mashes of the aerated, " feed-in " type. The pitching (seeding) yeast was separated by centrifuging, filtering, and acrating on large Büchner funnels.

The Whey Mash. The mash employed was a filtered or unfiltered sweet rennet cheese whey. The whey was heated to boiling, filtered through a filter press with Filter-Cel, adjusted to *p*H 4.5 with sulfuric acid, and cooled. Then 0.013 per cent of ammonium sulfate was added, and the whey was pitched with the organism.

Fermentation was complete after 21.7 to 48.0 hours at 30° to 37° C., when the attenuation attained 1.3° Brix. Lactose was determined polarimetrically. The spent mash was run through a centrifuge to separate the yeast before distillation. The large-scale experiments are summarized in Table XXXIV.

RECOVERY OF FERMENTATION STILLAGE AS FEED

The grain distillers and brewers have developed equipment and an attractive market for their recovered fermentation residues. Manufacturers of butanol and acetone are producing a dried stillage that is high in vitamin content, especially in riboflavin. However, the molasses distillers are still comfronted with a serious recovery-disposal problem. Stillage (residue from the stills) is the only waste from a distillery operating on cereal grains. Stillage contains 5 to 7 per cent total solids, of which about half is suspended. This stillage has a 5-day biochemical oxygen demand (B.O.D.) of 15,000 to 20,000 parts per million. Before drying, the stillage is passed through screens (1-mm. opening). The removed screenings are then passed through presses and rotary driers. This product is called *distillers' grains.* The screened stillage is sent to multiple-effect evaporators and concentrated to 25 to 35 per cent solids. This syrup is then dried in drum

driers to yield *distillers' dried solubles*. This product was originated in order to replace expensive dried milk products used in poultry and other livestock rations. Boruff and associates (27) use centrifuges prior to multiple-effect evaporation. This process is said to throw more of the drying load on the rotary driers, thus reducing steam consumption per unit of stillage produced. The protein, fat, vitamin content and the bulk of the dried grains make them valuable in dairy rations. The dried solubles containing large amounts of soluble proteins and of vitamins serve as valuable additions in poultry and swine rations. The vitamins in these products originate from the grains used as well as the yeast developed in the course of fermentation. Boruff's review (27) contains many important details concerning distillers' grains and solubles and a bibliography of recent articles.

LITERATURE REVIEWING THE PRODUCTION OF ALCOHOL AND ALCOHOLIC BEVERAGES

Wilkie and Kolachov (28) described the production of power alcohol, plant construction, and raw materials. Jacobs (29) has given an extensive presentation of all important factors concerning the distilling industries, such as production, consumption, organization, sources, financial aspects, and uses. Arroyo (30) has written a very useful monograph dealing with the production of rum. In the book by Hirsch (31) may be found procedures for the manufacture of whiskey, brandy, and cordials. De Becze and Rosenblatt (32) published a critical review concerning the various processes employed in continuous fermentation.

BY-PRODUCTS OF ALCOHOLIC FERMENTATION

In 1860 Pasteur proved that, besides the alcohol and carbon dioxide, some glycerol and succinic acid are also formed from the sugar by the yeast. Only a very small amount of sugar is utilized by the yeast cells during its growth. Much of the vanished glucose may be isolated in the form of an unfermentable polysaccharide which yields reducing sugars on hydrolysis (Guillemet and Leroux).

A number of products form in small quantity during alcoholic fermentation. These add aroma and flavor to the final product. Ehrlich (33, 34) published a number of papers on this subject. He has shown that phenylalanine was changed to phenylethyl alcohol, tryptophane to indolethyl alcohol, histidine to β-imidazolethyl alcohol, valine to isobutyl alcohol, leucine into isoamyl alcohol, and tyrosine into para-

hydroxy-β-phenylethyl alcohol. Thus are formed the higher alcohols, amyl-, propyl- and isobutyl alcohol or fusel oil. Phenylethyl alcohol, for instance, is a fraction of oil of roses. Other higher alcohols have a slightly bitter taste. These higher alcohols are believed to play a role in determining the typical flavor of beers, originating in the differences in cereal proteins. Ehrlich has also shown that glutamic acid might be transformed to succinic acid by fermenting yeast.

Numerous investigators (Embden, Euler, Kluyver, Meyerhof, Neuberg, Van Laer, Warburg, and others) have made important contributions to the chemistry of fermentation. The number of compounds which form and take part in alcoholic fermentation is very large. For quantitative data see Table XXXV. All the compounds and enzymes interacting in this highly interesting and complicated process are not known as yet, however.

Joślyn and Dunn (35) studied the formation and utilization of *volatile acids* during the alcoholic fermentation of grape juices. The most important fixed acids formed are lactic and succinic acids, whereas pyruvic and glyceric acid form only in traces (36).

GLYCEROL PRODUCTION

Neuberg and his associates (37–39) were the first to explain the mechanism of the formation of glycerol from sugar when the fermentation medium contained neutral sulfite or alkali. In the presence of sulfite, sugar is fermented to one molecule of glycerol per molecule of acetaldehyde formed:

$$C_6H_{12}O_6 = \underset{\text{Glycerol}}{CH_2OHCHOHCH_2OH} + CO_2 + \underset{\text{Acetaldehyde}}{CH_3CHO}$$

The acetaldehyde combines with the sulfite: $CH_3CHO + Na_2SO_3 + H_2O = CH_3CHO{\cdot}HSO_3Na + NaOH$. Thus enzymic reduction of acetaldehyde to alcohol is prevented. A triose produced from the hexose functions as the hydrogen acceptor and becomes reduced to glycerol. Pyruvic acid, which is also produced during this process, is fermented. This is called the fixation method. It was extensively used in the industrial production of glycerol during the first World War. As much as 1,000,000 kilograms of glycerol was produced per month with a yield of 15 to 20 per cent of the sugar fermented.

The quantity of glycerol produced depends on the type of yeast employed and the composition of the medium (40). Top yeast is best because of its resistance to sulfites. Glycerol production varies inversely with the activity of the yeast. The ration of alcohol to glycerol is 100:6 to 100:14; in beer it is 100:1.5 to 100:5.

TABLE XXXV

FERMENTATION PRODUCTS AS FOUND BY GVALADZE

Culture	*Champagne*	*Rkatsiteli*	*Steinberg*	*Rkatsiteli*	*Champagne*	*Steinberg*	*Rkatsiteli*
Medium	Grape Juice	Grape Juice	Grape Juice	Grape Juice	Grape Juice	Sugar Salts	Sugar Salts
Sugar (grams):							
Fermented	180.3	180.0	176.6	72.0	72.1	85.5	34.0
Remaining	1.1	1.36	4.8	0.74	0.4	1.25	1.24
Fermentation products (grams):							
Carbon dioxide	84.76	85.7	84.2	33.7	34.1	39.3	14.0
Alcohol	86.3	86.8	84.9	34.3	35.1	40.3	13.6
Glycerin	5.38	4.69	4.85	2.18	1.87	3.18	4.27
Acetic acid	1.1	0.9	1.15	0.38	0.52	0.79	1.21
Acetaldehyde	0.020	0.068	0.030	0.022	0.013	0.091	0.149
2,3-Butylene glycol	0.099	0.160	0.181	0.051	0.027		
Lactic acid	0.72	0.51	0.71	0.26	0.35	0.20	0.20
Yeast (dry weight)	1.0	1.0	1.0	0.5	0.5	0.4	0.4
Alcohol, % obtained*	93.7	94.3	94.1	93.2	95.2	92.2	78.2

* Calculated on the basis of Gay-Lussac's equation.

Lees (41) produced glycerol by controlling the fermentation of sucrose, dextrose, or molasses, with the addition of $MgSO_3$, as well as Na_2SO_3. For the substrates, products obtained from starchy materials and maltose were used. A solution of 5 per cent maltose and 4 per cent Na_2SO_3 required 15 days to complete its fermentation and produced 20 per cent of the theoretical glycerol. Using dextrose under similar conditions, 3 days was required to produce the same results. Employing $MgSO_3$ and acid-hydrolyzed starch or starch corn products, 23 per cent of the theoretical glycerol was obtained. Large yeast inocula and high concentrations of sulfite were necessary for yields above 25 per cent. Enzyme-converted starch material fermented very slowly.

The Sulfite Process. The following is an example of the sulfite process (42).

Medium.	Sucrose	1	kilogram
	Ammonium nitrate	50	grams
	Dipotassium phosphate	7.5	grams
	Sodium sulfite	400	grams
	Dissolved in 10 liters of water.		

This medium is inoculated with 100 grams of fresh yeast and incubated at 30° C. for 2 to 2.5 days.

The sulfite is precipitated as a calcium salt by the addition of calcium oxide, hydroxide, or chloride. After filtration and removal of the calcium ions by precipitation with sodium carbonate, the glycerol is obtained by distillation under reduced pressure (43).

The Eoff Process (44). *Medium.* Blackstrap molasses, corn sugar, and malt sprouts, or sucrose with nutrient salts, may be used in this process. The optimum sugar concentration is 17.5 to 20 grams per 100 cc. of the medium. Ammonium chloride increases the yield of glycerol. The medium is kept alkaline.

Highest yields were obtained with *Saccharomyces ellipsoideus* (var. *Steinberg*) and *S. ellipsoideus* (var. *California*) (40).

The medium is inoculated with the selected strain of yeast that has been previously accustomed (trained) to the alkaline *p*H. The alkaline compounds used are about 5 per cent. The fermentation is continued for 5 to 7 days at 30° to 32° C. The training of the yeast is best carried out in small flasks by adding 0.5 to 1 per cent of sodium carbonate to the medium.

According to Neuberg and his associates, for the production of glycerol in an alkaline medium, 1 molecule of acetic acid is formed

with each two molecules of glycerol:

$$2C_6H_{12}O_6 + H_2O = CH_3COOH + 2CH_2OH—CHOH—CH_2OH + C_2H_5OH + 2CO_2$$

For an extensive discussion concerning glycerol production by the fixation method see reference 45.

THE COURSE OF ALCOHOLIC FERMENTATION

Alcoholic fermentation may be defined as the conversion of sugar into ethyl alcohol without the interaction of atmospheric oxygen. It consists of a series of oxidations which bring about a series of simultaneous reductions. These are called oxidation-reduction systems. They include various substrates, enzymes, coenzymes, and activators (see Part I of this book). The yeast cell also contains enzyme systems which catalyze the interaction of oxygen, such as the cytochrome and hemin systems. This function is called respiration. It is an important mechanism of the yeast cell but does not directly concern alcoholic fermentation.

In the course of fermentation, during the stepwise breakdown of sugar, each oxidation is followed by phosphorylation. This is a mechanism for the storing of energy which is made available by the oxidations. The stored energy is used in biosynthesis, a process which we usually call growth and life.

On the basis of present findings, sugar fermentation by yeast proceeds as follows. From glycogen, which is always present in yeast, glucose-1-phosphate (Cori ester) is formed by phosphorylase. This ester is transphosphorylated to glucose-6-monophosphate (Robison's ester) by phosphoglucomutase. From glucose-6-monophosphate, the isomeric fructose-6-phosphate (Neuberg's ester) forms. This reaction is catalyzed by phosphohexose isomerase. Fructose-6-phosphate takes up a second molecule of phosphoric acid, changing into fructose-1,6-diphosphate (Harden-Young's ester) by the action of phosphatase. Here, adenylpyrophosphoric acid supplies the phosphate (46). Fructosediphosphate is changed to dihydroxyacetone phosphate and to *d*-3-phosphoglyceric aldehyde by aldolase (47). The *d*-3-phosphoglyceric aldehyde is oxidized to 3-phosphoglyceric acid by triosephosphate dehydrogenase. This reaction requires codehydrogenase I, adenosine diphosphate, and inorganic phosphate (48). However, this oxidation has been divided into two separate reactions. First, phosphoglyceric aldehyde is oxidized to some intermediary compound, which has not yet been isolated. This unknown compound is oxidized to *d*-3-phospho-

glyceric acid (49), which is changed to *d*-2-phosphoglyceric acid by phosphoglyceromutase. *d*-2-phosphoglyceric acid is converted into enol-phosphopyruvic acid by the enzyme enolase (50). Phosphopyruvic acid interacts with adenylic acid, yielding adenosinetriphosphate and pyruvic acid (51).

The various reactions just listed are the same in yeast and muscle. The final reactions, however, from pyruvic acid on, are different. In yeast, pyruvic acid is changed to acetaldehyde by carboxylase. The acetaldehyde is reduced to *ethyl alcohol.* This reaction is catalyzed by alcohol dehydrogenase. The reduced codehydrogenase I (reduced cozymase) necessary for this reaction is furnished during the oxidation of 1,3-diphosphoglyceric aldehyde. In muscle, pyruvic acid is reduced to *lactic acid* by reduced cozymase. This reaction is catalyzed by lactic dehydrogenase. The exact conditions necessary for these reactions, using the isolated enzyme systems, have been described in the first part of this book. The mechanism of carbohydrate metabolism has been excellently reviewed by Barron (52). Figure 28 is a scheme of reactions as they appear to take place in alcoholic fermentation and in muscle glycolysis. See also Part I of this volume.

THE DISACCHARIDES, SUCROSE, MALTOSE, AND LACTOSE, DIRECTLY FERMENTED BY YEAST

Willstätter and associates, and others, have shown that the fermentable disaccharides (sucrose, maltose, and lactose) are directly fermented by yeast and do not require the preliminary action of the invertases. For instance, Stark and Somogyi (53) have found that the half fermentation of maltose at *p*H 6.4 (optimum *p*H of maltase) was 93 minutes, whereas it was only 27 minutes at *p*H 4.8, at which maltase action was almost negligible, and 47 minutes at *p*H 3.6, at which maltase was inactive. This indiates that the action of maltase is not required for the fermentation of maltose by yeast.

INHIBITORS OF ALCOHOLIC FERMENTATION

The addition of substances that produce changes in the *p*H of the fermentation medium will invariably affect the rate of fermentation. In order to observe the effect of inhibitors or activators on the rate and course of fermentation, it is best to add the substance to be tested after fermentation is well in progress.

Elimination of Induction Period. If sugar is added to fresh yeast, fermentation commences immediately. Dried yeast, however, sometimes displays a short induction period, and maceration juice (Lebedev

juice) and activator-free zymase preparations show an induction period of several hours. Some zymase preparations do not ferment at all, unless a small quantity of sodium-hexosediphosphate is first added to

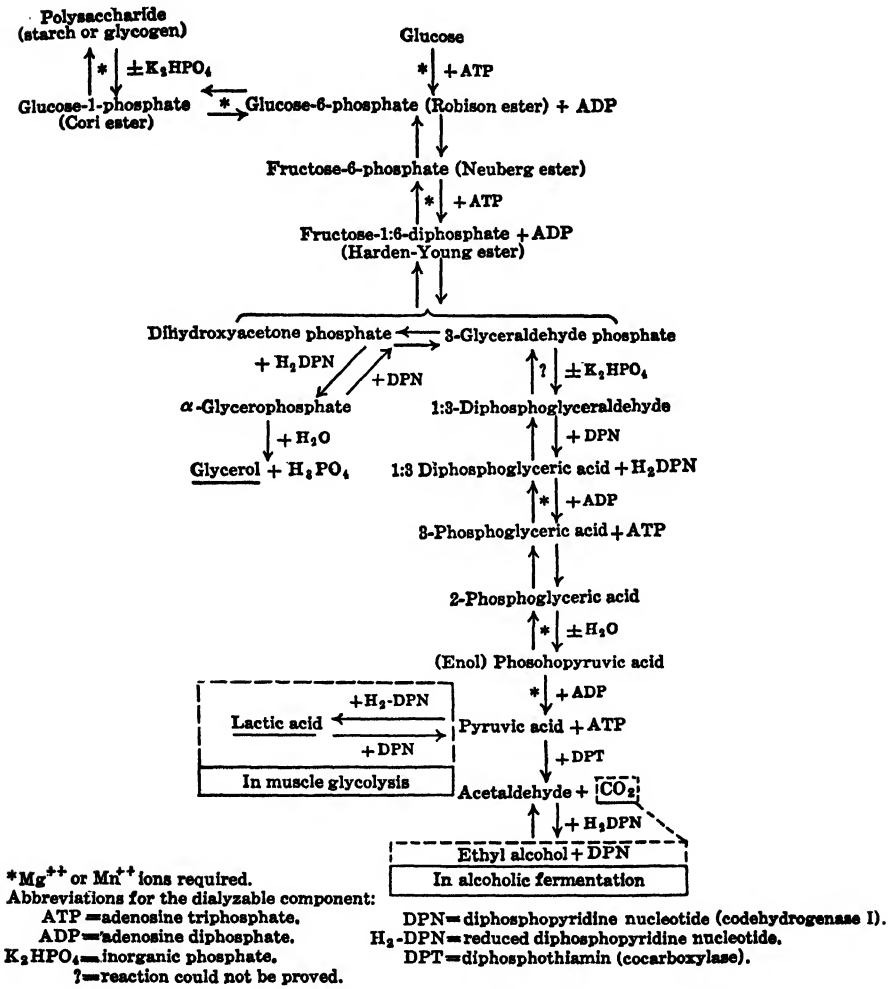

FIG. 28. Scheme of alcoholic fermentation and muscle glycolysis.

the fermentation mixture. Addition of a small amount of acetaldehyde together with hexosediphosphate accelerates the elimination of the induction period.

Inhibitory Substances. Although the action of fresh yeast is not affected by excessive amounts of phosphate, various zymase prepara-

tions are inhibited by them and prolonged induction time results. Very high sugar concentrations have no effect on the induction time but have an inhibitory action on the rate of fermentation.

Selective Inhibitors. Some substances, such as toluene, fluorides, monoiodoacetic acid, and sulfites, selectively inhibit certain phases of sugar breakdown by fresh yeast. Toluene, however, does not inhibit fermentation by yeast-maceration juice, provided that a small amount of acetaldehyde is present.

Sodium fluoride inhibits carbon dioxide formation as well as the phosphorylation of sugar. Yeast glycogen, however, is phosphorylated in the presence of fluoride, and glucose-6-phosphate is formed.

If hexosediphosphate is added to fresh yeast that has been poisoned with sodium fluoride, hexosemonophosphate, phosphoglyceric acid, and acetaldehyde are formed. The reaction is governed by the concentration of hexosediphosphate. The phosphoglyceric acid is gradually converted to acetaldehyde, which in turn is reduced to ethyl alcohol. Monoiodoacetate affects the course of alcoholic fermentation at an earlier stage than fluorides (Meyerhof). The various intermediary reactions of alcoholic fermentation were extensively studied by Nilsson, Embden, Meyerhof, and Neuberg. This subject has been reviewed by Nilsson (54).

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CHAPTER XVIII

THE ROLE OF ENZYMES IN BREWING

Successful beer making depends on the proper control of two series of isolated enzymic reactions (1, 2). The first reactions occur during malting and mashing and are carried out by the malt diastases and other malt enzymes. When the first series of reactions is completed, these enzymes are destroyed by boiling the wort. A second group of

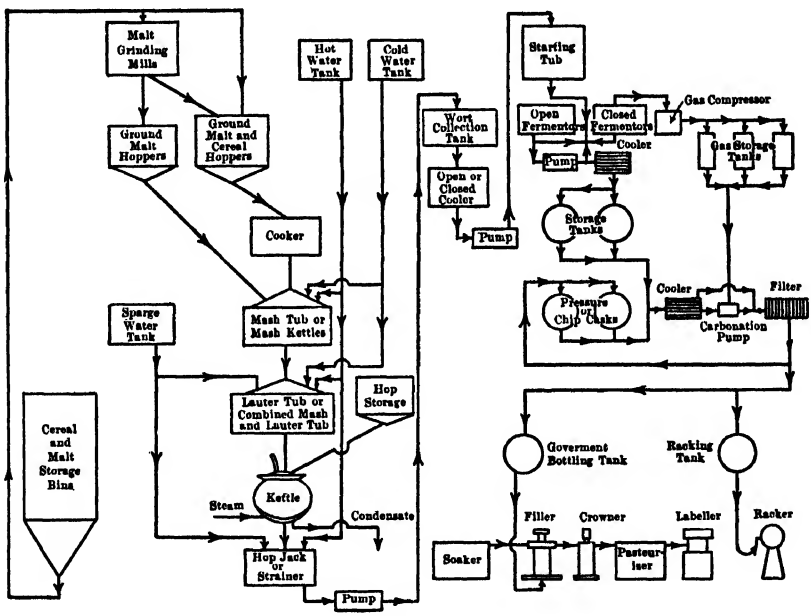

FIG. 29. Flow sheet of brewery process.

enzymes is introduced by the addition of yeast to the cooled wort. These enzymes act during fermentation, storage, and maturing of the beer. To be able to regulate enzyme activity in these successive operations, a properly balanced wort must be obtained. Carbohydrates as well as nitrogenous compounds must be correctly distributed. Figure 29 represents a flow outline of brewing (1).

The Malting Process. Malting is the operation that effects the softening of the cell walls of the barley kernel by the action of "cytase" and a partial hydrolysis of the proteins of the grain. Starch

hydrolysis is only slight during malting. The malting process must be conducted in such a manner as to obtain maximum enzyme formation and minimum loss of metabolites. Usually barley is employed in the production of malt. Other cereal grains, however, may also be used.

The following are the main phases of malting:

1. *Washing and Steeping.* The clean grain is stored in large cylinders, barley malt elevators, until needed. In large steel tanks washing and soaking (steeping) of the barley take place.

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FIG. 30. Germinating drums.

2. *Germination.* After the water has been drained off, the barley is transferred to either germinating drums (Fig. 30), floors, or compartments. A stream of temperate humid air is supplied through the grain as it is being turned over. Within a few days, germination of the barley is complete.

3. *Kilning.* Germination is arrested by slow artificial heat in drying kilns where special machinery continually turns the barley over, to expose each individual grain uniformly to the heat. The rootlets are removed from the dried barley, and the malt is stored and aged. Later the malt is crushed in special grinding mills when needed for the brewing process.

The temperature during kilning is low at first and is then gradually increased. Care must be taken, however, not to destroy the enzymes.

The chemistry of malting has been admirably discussed by Hopkins and Krause (3).

ENZYMES DURING MALTING

Germinating seeds show increase in the activity of most enzymes. The following enzyme systems react during malting: cytases, desmolases, carbohydrases, proteolytic enzymes, and esterases.

Cytase. Within the barley kernel the starch granules are enclosed in a protein film embedded in a complex carbohydrate framework. Proteases and carbohydrases form in large quantities as soon as growth commences. These enzymes cannot act, however, until the protein and complex carbohydrate cell walls are broken down and the hardness of the endosperm is softened. The enzyme mixture cytase is important in this reaction.

The enzyme known as cytase consists of a group of several enzymes. Oppenheimer (4) lists under cytase the several polyases that hydrolyze mannan, galactan, xylan, and araban. The last two polysaccharides are probably hydrolyzed by the same enzyme.

During malting the soluble pentosans increase from 0.25 per cent to 1.1 per cent (5). The exact nature of these pentosans is not known. Though it has been stated that pectic enzymes have an important role in the disintegration of the barley kernal, it has been shown that neither barley nor malt contains any pectin (6).

Cytase activity tests using a pentosan as a substrate might be a useful tool in the evaluation of barley, since this activity probably precedes that of other enzymes.

Malt lichenase acting on a cellulose-like hexosan called lichenin, converting it to cellobiose, has been extensively investigated. The activity of the enzyme increases considerably during germination (7–10).

Desmolases. Germinating seeds contain independent fermentation and respiration enzyme systems. The absence or presence of oxygen is the main directive factor. Carbon dioxide formation, however, does not depend on the presence of oxygen. It is well known, for instance, that the carboxylase enzyme system decarboxylates pyruvic acid also in the absence of oxygen.

During steeping a constant supply of oxygen, brought about by proper ventilation, is important. If the oxygen supply is depressed, considerable amounts of esters, detectible by their characteristic odor, are formed, and germination is inhibited. Excessive ventilation causes excessive respiration, which means loss of metabolites. Malting without any respiration is not possible, however. Complete exclusion of

oxygen during malting increases the alcohol formation (fermentation) from 0.0155 per cent to 1.7 per cent, whereas, by inhibition of carboxylase action in an atmosphere of carbon dioxide, oxygen consumption continues (11).

Barley respiration during germination is increased by aeration during the steep. This also improves the resultant malt and the brightness of the resultant wort. A floor malt displayed greater respiration and had better properties when processing was completed than a well-steep-aerated drum malt (12, 13).

De Clerck and Cloetens (14) state that inadequate aeration during steeping may be detrimental to enzyme formation. During germination of barley there is an increase in the flavin-dehydrase system (15). This enzyme has probably an important function in the respiration of barley. Oxygen consumption increases during germination and may be measured with the aid of the Warburg respirometer (16–17).

Oxidases. Malt contains peroxidase, phenolases, and catalase. According to Lüers (11), darkening of wort is caused by phenolases. Just (18) reported, however, that the darkening of wort is due to non-enzymic oxidation by contact with air. He observed that moderately kilned malts did not darken more than highly kilned types. Moreover, wort darkens even after destruction of the oxidases. During malting, catalase activity increased fourfold, whereas peroxidase activity remains unchanged (19).

Malt Amylase. Fletcher and Westwood (20) examined the amylase activity of barley during 10 days of germination and found that the saccharifying power was at a maximum after 7 days and the liquefying power after 9 days of germination. It is not known whether the increase in amylase activity is brought about by the formation of new enzymes or by an activation of inhibited enzymes or by a destruction of inhibitors. Ford and Gutrie had shown as early as 1908 that, after treatment with papain, barley released much more diastase than when extracted with water alone. β-Amylase in barley is partly bound to insoluble proteins and is, as such, inactive. By the action of proteolytic enzymes such as papain, the enzyme is liberated (21). The case during germination may be similar, owing to the considerable increase in barley papainase. The chemistry and technology of malt amylases are discussed in various chapters of this volume.

ENZYMES IN THE MASHING PROCESS

From the malt mills the crushed grain is transferred to mashing tanks (cooker, mash tub) where adjuncts and water are added. When con-

version of the starches into maltose and proteolysis, under proper control, is complete, the mash is drained in the same tank or is pumped to other tanks with false slotted bottoms. Here the amber wort is drained off, leaving the spent grains behind. The strained wort is then pumped through copper containers called "grants" into copper kettles where the hops are added. After the wort and hops have been boiled together, the liquid passes through another straining device to free it from the hops. The boiling hot liquid is cooled in special equipment and then conducted to the starting cellar for beginning of fermentation.

American beers are manufactured from mixture of 65 to 80 per cent of malt with 20 to 35 per cent of malt adjuncts, such as rice, corn grits, corn meal, refined grits, or corn flakes, and various sugars and syrups (2, 22). By the proper selection of these unmalted adjuncts, typical beers may be developed.

The Conversion Temperature. This is the temperature at which the mash is kept to allow the diastase to produce the desired ratio of sugars and dextrins. This is the most important enzymic reaction at this stage of beer making. The optimum temperature for diastase action is 56° to 68° C. When unmalted cereals are employed as adjuncts, however, mashing must commence at a lower temperature such as 45° to 50° C. At lower temperatures more maltose is formed, whereas if the temperature is immediately raised to 75° C. the iodine color becomes negative almost at once, less maltose having been formed (22, 23).

TABLE XXXVI

THE EFFECT OF TEMPERATURE ON THE RATIO OF SUGAR TO NON-SUGAR IN MASHING

Conversion Temperature, ° C.	Ratio of Sugar to Non-Sugar
64	1:0.37
66	1:0.40
68	1:0.48
70	1:0.52
72	1:0.57

Table XXXVI shows the effect of temperature on the ratio of sugar to non-sugar in mashing operations, brought about by diastatic action (24).

For low-alcohol beers high in dextrins, high conversion temperatures are used; and for high-alcohol beers low in dextrins, low conversion temperatures are employed.

Thermal Effects on Amylases during Mashing. Swanson and Kruzic (25) reviewed the thermal effects on α- and β-amylase during mashing, using the infusion system. Maximum α-amylase and

β-amylase activity occurred close to 56° C. Both amylase activities showed a rapid decrease between 56° and 68° C. The activity decrease was more extensive for β-amylase than for α-amylase. After the optimum temperature for maltose formation was reached, the rate of thermal inactivation was considerably lessened by the protective action of maltose on the enzymes.

β-Amylase was completely inactivated during the customary mashing off temperature of 78° C.; α-amylase was more stable; only 0.55 per cent was still active after 84° C. was reached.

The Use of Wheat Malt. Collett and Green (26) found that the use of 10 to 12 per cent wheat malt in the grist increases the permanently soluble nitrogen and the amino nitrogen. There is a slight increase in the *p*H and a decrease in the buffer coefficient. This results in the production of a better yeast when wheat malt is used with barley malt of low nitrogen content, and adds to the stability, flavor, and head retention. Although wheat malt results in 4 to 5 per cent more extract than barley malt, the malting operation is more difficult. The wheat malt retains excessive moisture, which causes moldiness at the kiln. Table XXXVII shows average data on chemical changes during malting (27).

TABLE XXXVII

CHANGES IN MALTING (27)

	Barley	Malt
Starch	62.5	57.0
Reducing sugars (as invert)	1.0	3.0
Sucrose	1.5	5.0
Pentosans (furfural × 1.71)	9.5	10.5
Cellulose	5.5	6.0
Protein (nitrogen × 6.25)	10.0	9.5
Fat (ether extract)	2.5	2.0
Ash	2.5	2.2
Other constituents (lignin, tannin, etc.)	5.0	4.8

This is an average analysis of a two-rowed barley and its malt. The figures represent percentage of dry matter. Similar results were obtained with six-rowed barleys.

Proteolysis during Malting and Mashing. Barley malt contains a proteinase which is very similar to papain. Most of the enzyme is readily extracted with water. It has an optimum *p*H at 4.6 to 4.9, and it becomes about 50 per cent more active when treated with hydrocyanic acid, hydrogen sulfide, or glutathione. There are also petidases in barley malt. Barley itself, however, contains only traces of these proteolytic enzymes. The increase during germination is very considerable (28).

It is important to the brewmaster to obtain enough soluble protein

breakdown products during malting and mashing to give body, flavor, and character to the beer. The nitrogenous products of proteolysis are also necessary to furnish the yeast with nutrients during fermentation. Proteolysis, however, should not be too extensive. A certain amount of correctly dispersed colloidal protein is essential for best results in body and foam production and stabilization.

In the evaluation of nitrogen distribution Lüers and Nishimura (29) differentiate between total nitrogen, soluble nitrogen, and coagulable nitrogen. Kolbach (30) follows proteolysis by estimating the permanently soluble nitrogen, which he found to be 20 per cent of the total nitrogen of light malt.

The proper solubilization of proteins is dependent on correct malting. In brewery practice it is often found advantageous to determine the soluble nitrogen of the laboratory wort. According to Kolbach, on the basis of malt nitrogen, 41 per cent soluble nitrogen indicates very good solubilization; 35 to 41 per cent indicates good; and below 35 per cent solubilization is fair.

Oliver (31) reported that plant-scale mashing gives at least 25 per cent more soluble nitrogen than thin laboratory mashing. The difference is said to be caused by the greater mash concentration and longer reaction time of the plant process. Hind and Bishop estimated a 12 per cent difference between the two operations. Oliver furnished six experiments which showed that the figure varied from about 31 to 46 per cent and depended on the size of the mash and the adjuncts employed.

The proteolytic enzymes can be estimated fairly closely by the method of Lüers and Loibl (32). They determined proteolytic activity by estimating formol nitrogen formed in a mash kept for 3 hours at 50° C. A control mash was prepared by destroying the enzymes with alcohol. This method had been modified by Kolbach and Simon (33). Laufer (34) published an extensive study on the proteolytic activity of barley malt, using several methods and a series of substrates.

More recently Laufer (35) conducted mashes on an American malt at various constant temperatures (7° to 75° C.) and *p*H ranges (4.5 to 6.6) for 1 hour, and at normal *p*H of 5.5 for 2 hours. The extracted soluble nitrogen, permanently soluble nitrogen, and formol nitrogen were determined. The optima were at *p*H 4.5 to 5.0 at 45° to 50° C. for soluble nitrogen, 45° to 55° C. for permanently soluble nitrogen, and 45° to 50° C. for formol nitrogen. Different malts, however, show different degrees of proteolysis.

Esterases of Malt. No particular significance can be attached to the *lipase* of malt (36). *Phosphatases* of malt, however, are important

(37). Barley contains 1 per cent of phosphorus, of which 80 per cent is organically bound. Most of the organic phosphate is in the form of inositol hexaphosphate (phytin). These esters are hydrolyzed during malting and mashing, thereby furnishing a desirable acid phosphate buffer.

The *p*H of the Mash. The *p*H of the mash is an important factor. With neutral mash water the natural acid phosphates furnish usually an acid *p*H, varying from 5.5 to 6.0. Thus the *p*H of the mash is not always that which is optimum for malt diastase (about 5.0) and for total proteolysis (about 4.3 to 5.0). The *p*H of the mash may be adjusted somewhat but the adjustment must be made at the temperature of mashing, since the *p*H decreases with increase in temperature; otherwise a correction is necessary.

The Rest Period and Sparging. The last phase of the mashing operation consists of a rest period. The grains and coagulated proteins are allowed to precipitate. Enzyme action, however, is still proceeding. The wort is permitted to pass to a layer of grains which functions as a filter and is released through the false bottom of the mash tub. If enzyme action was correctly regulated during mashing, a rapidly filtering, clear wort is obtained. The wort is now pumped into the kettle. The spent grains are extracted several times with hot water. This process is called sparging. A more finely ground malt may require a separate settling tank (Lauter tub) or a mash filter. This operation yields more extractives. The brewery yield from the malt is 64 to 65 per cent with the normal mashing method. With certain methods, however, the yield may be as high as 70 per cent or more, which is the yield obtained by laboratory mashing.

When beer is made by the high-temperature short-mashing process, clarification and filtration difficulties often arise. Although an extended rest period sometimes proves helpful, it may result in the solubilization of excessive proteins. Verbrugghen (38) suggested the addition of proteolytic enzymes, such as trypsin, to the cooled wort. Proteolytic action is allowed to continue until proper digestion of the proteins takes place.

Boiling the Wort. The wort and spargings are transferred to a brew kettle, which is usually made of copper. For each beer barrel of 31 gallons 0.55 to 0.9 pound of hops is added.

The wort is now boiled for several reasons, namely, to concentrate it to destroy the enzymes, to sterilize it, to extract the hops, to coagulate proteins, and to caramelize some of the sugar for color formation. Destruction of the malt enzymes is necessary for maintenance of a constant sugar-dextrin ratio for the fermentation process. Yeast con-

tains very little amylase. The hops have a coagulating effect on proteins. In addition, the tannin content of hops has an antiseptic effect, whereas the resins and aromatic ingredients of hops give beer its typical characteristics.

After boiling, the wort is filtered through a hop strainer and cooled by passing through or over coolers. In most breweries, great care is taken to eliminate bacterial infection in all phases of beer making. Quick cooling is most beneficial for the precipitation of the protein-tannin complexes.

FERMENTATION

The cooled wort is run into the starting cellar. Here yeast is added to the brew and fermentation begins. From the starting cellar the brew is soon transferred to special fermenting cellars supplied with purified refrigerated air.

The yeasts are selected strains of the genus *Saccharomyces cerevisiae*. The strain of yeast employed in fermentation is important, for it determines the character of the beer. Yeasts used in the manufacture of lager beers are called bottom yeasts. These types of yeasts collect on the bottom of the fermenter when fermentation is about to be completed. *Saccharomyces carlsbergensis* and *S. monacensis* are typical bottom yeasts. For the manufacture of ales, top-fermenting yeasts are employed.

Fermentation is governed by the type and nature of the yeast and by the composition, *p*H, and temperature of the wort.

The optimum *p*H for the proliferation of yeast is at 5.4 to 6.8 (39), and the optimum temperature is 28° to 30° C. However, in brewery practice bottom fermentation is conducted at 5° to 14° C., and top-yeast fermentation at 13° to 22° C (40). Alcohol inhibits the growth of yeast, even in small amounts (0.7 per cent) (41). A concentration of 2 per cent has a considerable inhibiting action, and one of 5 to 7 per cent stops yeast growth completely. Fermentation, however, is not much affected at low alcohol concentrations. At 4 to 5 per cent concentrations there is considerable inhibition, however, and at an alcohol concentration of about 12 per cent fermentation is halted completely. Some wine yeasts, however, withstand even higher alcohol concentrations.

Oxygen (aeration) and extensive mixing accelerate fermentation (42), in general, and result in more complete enzyme activity. It has been stated that aerated fermentation results in a low-protein beer of higher chill resistance (43). This is important in countries where proteolytic enzymes are not permitted for chillproofing.

In some breweries the fermenting wort is pumped to another fermentation tank as soon as the krausen stage is reached; this is indicated by the appearance of white foam on the surface of the wort. This effects a separation of precipitated proteins and hop resins. Larger breweries collect the carbon dioxide that forms during fermentation by means of closed fermenters and use this gas for carbonation of the beer.

Fermentation effects an increase in temperature. Modern breweries use cooled fermenters to avoid too high temperatures. The fermenting action of yeast ceases as soon as the fermentable sugar is used up. This requires from 5 to 10 days. The product at the end of this period is called " young " or " green " beer. This beer still contains suspended material and the final quality of the beer has to be developed. For this purpose the brew is transferred to large storage vats, which may be glass lined, where it remains for several weeks, slowly aging to perfection. During this period a slow after-fermentation or krausening takes place. From the storage tanks the beer is pumped through filters, carbonated, and finally bottled or racked into kegs.

Most breweries pasteurize their mottled beer at about 63° C. for 20 or 30 minutes.

After-Fermented Beer for Diabetics. According to the process of Silbereisen (44), a highly fermented beer may be prepared by the addition to the wort of fresh diastase or a yeast which has been liquefied in toluene. Such beer contains little or no residual sugar and is suitable for diabetics.

BEER DEFECTS

Proteins, protein-tannin compounds, resins, starch and microorganisms produce undesirable turbidities in beer.

Starch Turbidities. These are caused by insufficient amylolytic action during mashing or by a destruction of the diastase during kilning. The addition of diastase to the storage vats removes starch turbidity.

Nissen and Estes (45) observed a new kind of turbidity when beer was subjected to freeze-thaw cycles. The precipitate was produced by stable dextrin which had changed to the insoluble modification. Warming the beer to 37° to 60° C. dissolved the precipitate and it did not reappear even when the beer was cooled to a temperature of —4° C.

Turbidities caused by pitch or calcium oxalate are rare and may be prevented by proper filtration.

The most frequent bacterial turbidities are produced by *sarcinae*. These may be prevented by aseptic technique, by the use of pure yeast cultures, and by the improvement of sanitary conditions in the brewery. The same principles hold for the prevention of wild-yeast turbidities.

Protein Turbidities. Protein turbidities are frequent. All colloidal solutions have the property of throwing down a precipitate. Since beer is a colloidal solution it does not keep for more than a few weeks after pasteurization and transportation.

According to Hartong (46) the more unstable the colloids of a beer, the better is its organoleptic property (*Vollmündigkeit*). Hartong and others have found that the proteins which affect rapid aging and instability are the natural proteins of barley that have been carried unchanged through the entire brewing process.

The proteins of barley are: insoluble glutelin, alcohol-soluble hordein, salt-soluble globulin, and water-soluble albumin. Wort and beer contain two natural proteins, one an albumin and another a protein called *globulose* by Hartong. Albumin is readily coagulated by heat; globulose, however, is more resistant to heat, is salt-soluble, and has other globulin properties. Its molecular weight is much smaller than that of a globulin.

These two proteins are the cause of two different kinds of turbidities, although only traces of these proteins find their way into the beer. They appear after a shorter or longer time interval as a flocculent precipitate or as a haze. These turbidities are hastened by shaking of beer containers during transportation.

Helm (47) reported that chill-haze-producing proteins are of the order of a few milligrams per liter of beer. According to this author, chill haze may be prevented (*a*) by sharp filtration, using filter mass mixed with asbestos, or by filtration through kieselguhr at low temperatures; (*b*) by tannin precipitation at various stages of brewing; and (*c*) by the use of proteolytic enzymes.

CHILLPROOFING BEER WITH PROTEOLYTIC ENZYMES

For the chillproofing of beer proteolytic enzymes have been successfully used since 1911. Small quantities of a proteolytic enzyme are added at any stage of brewing, after boiling the wort. The enzyme acting at the *p*H of the beer (4.0 to 4.5) renders it stable to cold by digesting the proteins. This important contribution was made in 1911 by Wallerstein (48). The enzymes papain, bromelin, pepsin, and those originating from fungi have been used extensively. Wallerstein (49) applied the tannase of *A. niger* for the hydrolyzing of tannin-protein complexes of beer. It is claimed that a lighter-colored and clearer product is thus obtained.

Enders, Saji, and Hartong (50) described the following *method for chillproofing*. One gram of papain is added per hectoliter of beer.

One hour is allowed for action at 60° C., then the beer is pasteurized for 45 minutes and placed for 1 week in the refrigerator. Now a turbidity test is carried out.

The Turbidity Tests. Hartong (51) suggests the following methods for testing the chillproofness of beer.

1. Sufficient saturated ammonium sulfate is added to the beer to produce turbidity, and the degree of turbidity is measured with the aid of a nephelometer.

2. Ammonium sulfate is added, but not enough to produce turbidity, and the time for turbidity to occur is determined.

3. The beer is titrated with saturated ammonium sulfate until turbidity is shown in direct light. The turbidity may be measured photoelectrically.

Precipitation of the beer proteins by ammonium sulfate is constant below *p*H 3.5, increases to *p*H 5.0, and above this value becomes again constant (52).

Chillproofness may also be tested by shaking in special containers and cooling for definite time, and then estimating the increase of turbidity in a nephelometer. A haze may also form without shaking if the beer is allowed to stand for a sufficient period. Only if the haze forms too quickly is the beer abnormal.

TABLE XXXVIII

ACTION OF PROTEINASE ON THE CHILL HAZE

(Pilsener beer, gravity 10.95 per cent, pasteurized)

Enzyme Added, gram per hectoliter	Bottled under	Chill Haze Determined Nephelometrically after 2 Hours in Ice Water, the Beer having Been Kept at 20° for				Head Retention
		0 days	4 days	12 days	30 days	
0	Air	175	285	450	..	5.29
	CO_2	175	225	350	..	5.05
0.2 pepsin	Air	40	10	16	14	4.88
	CO_2	30	10	5	3	4.74
0.2 papain	Air	0	0	7	20	5.10
	CO_2	2	0	5	10	4.83

Table XXXVIII shows chillproofing experiments by Helm (53). They demonstrate the favorable action of proteolytic enzymes on the chill haze of beer. In the presence of oxygen the turbidity is much greater than in the presence of carbon dioxide.

For details concerning brewing practice a book by Hind (54) is recommended.

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CHAPTER XIX

MOLD FERMENTATIONS

The selection of the proper organism in industrial fermentation is apparently the most important factor. Small-scale laboratory experiments must be conducted which show that a specific organism has the ability to convert a certain substrate to the desired substance with good yields. The most economical media must be selected. Optimum conditions for oxygen supply (in the case of an aerobe), of temperature, and of speed of agitation must be determined. The production of various compounds with the aid of microorganisms offers the most fascinating problems. Wells and Ward (1) state: " Indeed the nature of these problems is such that no process can be considered beyond improvement."

The conversion of agricultural surpluses and wastes into desirable chemical products is important from the standpoint of national economy. The probability of competition by new chemical methods to biological processes, however, must always be considered.

CITRIC ACID PRODUCTION BY FERMENTATION

Citric acid may be prepared synthetically but not industrially. Some is manufactured from citrous fruits in California and some from pineapple in Hawaii; however, the most important method of manufacture of citric acid is by the fermentation of sucrose by molds. The first successful industrial application was conducted in this country; see Table XXXIX (2). Whereas before 1927 much citric acid was imported (Table XL) from Italy and other European countries, during the 1930's large quantities of the acid were exported to Europe (2). Herrick and May (3) are of the opinion that it is unlikely that the mold fermentation processes will be displaced by any method of synthesis.

Wehmer (1892) was first to describe a group of fungi which produced citric acid from glucose. He named them *Citromyces*. These fungi were green *Penicillia*. Later it was found that strains of *Aspergillus niger, A. aureus, A. clavatus, P. luteum, P. citrinum, Ustulina vulgaris,* and several other molds are also citric acid producers. Citric acid may be produced from 3-, 4-, 5-, 6-, and 12-carbon sugars. Maximum yields were obtained from sucrose (4–6).

TABLE XXXIX

UNITED STATES PRODUCTION OF CITRIC ACID FOR SALE, 1914–1935 (2)
(From the U. S. Bureau of the Census)

Year	Pounds	Value	Average Price per Pound*
1914	2,657,840	$1,516,326	$0.53
1919	3,163,676	3,047,371	1.10
1921	3,849,789	1,913,774	0.49
1923	5,689,473	2,829,306	0.50
1925	7,589,213	3,469,740	0.46
1927	7,058,215	3,150,976	0.44
1929	10,755,789	4,832,984	0.46
1931	8,361,441	3,060,185	0.36
1933	5,695,793	1,795,382	0.32
1935	10,493,068	2,768,377	0.28

* Current price, $0.24 per pound; average price figures from trade journals.

TABLE XL

U. S. IMPORTS OF CALCIUM CITRATE AND CITRIC ACID FOR CONSUMPTION, 1910–1936 (2)
(Figures from U. S. Bureau of Foreign Commerce and Navigation)

	Calcium Citrate			Citric Acid		
Year	Duty per Pound	Pounds	Value	Duty per Pound	Pounds	Value
1910*	Free	4,114,256	$ 568,175	$0.07	142,001	$ 40,967
1915*	$0.01	6,242,244	1,109,629	0.05	722,434	447,131
1919	0.01	3,865,294	1,583,806	0.05	1,224,591	1,187,267
1920	0.01	12,490,196	3,027,823	0.05	1,317,467	1,142,842
1921	0.01	988,969	151,811	0.05	922,737	490,084
1922†	0.01	16,000,692	2,223,506	0.05	1,325,366	477,568
1923	0.07	1,672,604	200,143	0.17	757,864	233,665
1924	0.07	1,938,647	199,620	0.17	673,114	186,512
1925	0.07	3,475,964	376,694	0.17	288,574	79,634
1926	0.07	3,039,319	347,073	0.17	284,897	77,525
1927	0.07	416,045	46,864	0.17	71,291	18,515
1928	0.07	.....	.....	0.17	1,338	524
1929	0.07	None	None	0.17	None	None
1930	0.07	None	None	0.17	6,726	1,987
1931	0.07	None	None	0.17	90,850	19,641
1932	0.07	704	34	0.17	134,521	19,746
1933	0.07	55,272	2,367	0.17	9,784	1,213
1934	0.07	None	None	0.17	5,275	748
1935	0.07	None	None	0.17	575	79
1936	0.07	None	None	0.17	40	12

* Figures for 1910–1915 are for the fiscal year; 1919–1936 figures are for the calendar year.
† New law went into effect Sept. 22, 1922.

When sucrose is fermented 1 molecule of the cane or beet sugar produces 2 molecules of citric acid, or 1 gram of sucrose produces 1.12 grams of citric acid,

$$\underset{\text{Sucrose}}{C_{12}H_{22}O_{11}} + H_2O + 6O = \underset{\text{Citric acid}}{2C_6H_8O_7} + 4H_2O$$

Not all the theoretical amount of sucrose is converted to citric acid. Some is utilized in the formation of mycelium, and some is converted to carbon dioxide owing to respiration of the organism.

Doelger and Prescott (7) have extensively studied citric acid fermentation, using liquid media in aluminum pans.

Medium. Doelger and Prescott (7) recommended the following medium which, with a certain strain of *A. niger,* produced good citric acid yields with only 2 per cent of oxalic acid:

	Grams per Liter
Sucrose	140
NH_4NO_3	2.23
K_2HPO_4	1.00
$MgSO_4 \cdot 7H_2O$	0.23

This medium is adjusted to *p*H 2.20 to 1.60 with *N* hydrochloric acid and sterilized at 8 to 10 pounds' steam pressure per square inch for 30 minutes.

Optimum Conditions for Shallow-Pan Fermentation. Shallow aluminum pans (25 by 33 cm.) of 99.8 per cent purity, which allow large surface fermentation, yield best results. Copper, iron, and other metals are harmful to mold growth. The optimum temperature is 26 to 28° C., and fermentation requires 7 to 10 days (7). Agitation retards growth and should not be used, nor is an extensive air supply required. Air, however, is necessary for the oxidation. The omission of calcium carbonate results in higher yields and shortens the fermentation period (8). Usually the yield of citric acid is about 60 per cent of the sugar employed. A yield close to 100 per cent has been reported by Bulkewich and Gaewskaya (9).

Production of Citric Acid by Submerged Fermentation

Until recently, the general opinion was that it is impossible to produce citric acid by the submerged fermentation method on commercial scale. However, Szücs (10) disclosed a procedure for the production of citric acid by submerged fermentation using *A. niger* as the organism. The medium should be free of assimilable phosphorus compounds and should be supplied with finely dispersed oxygen or an oxygen-contain-

ing gas. The inoculum is grown for 3 to 4 days at 25° C. in the following medium:

	Grams per Liter
Sucrose	25 to 50
$MgSO_4 \cdot 7H_2O$	0.25
KH_2PO_4	0.30
NH_4NO_3	2.25
HCl (*N*)	10 cc. (*p*H 2.0)

The solution is agitated while oxygen is passed through. The mycelium is transferred into a fermentation solution of the following composition:

	Grams per Liter
Sucrose	200
NH_4NO_3	1.10
KCl	0.15
$MgSO_4 \cdot 7H_2O$	0.25
HCl (*N*)	10 cc. (*p*H 1.91)

Fermentation is carried out under vigorous stirring at 25° C. Finely dispersed oxygen is passed through 2 liters of solution per minute. The presence of oxygen under an increased pressure of 1 to 4 atmospheres is claimed to have an accelerating effect on citric acid production. In about 4 days, 70 to 75 per cent of the sugar consumed is claimed to have been converted to citric acid. Other carbohydrates such as fructose, glucose, purified molasses, corn syrup, and corn sugar may also be used in this process.

Waksman and Karow (11) found that, by employing *A. wentii,* but not *A. niger,* as the fermenting organism, citric acid may be produced by the submerged procedure. *A. wentii,* according to these investigators, grows rapidly under submerged conditions as well as under surface conditions. It produces citric acid abundantly but little oxalic and gluconic acids.

Suggestions for Improvements in Citric Acid Production

Gerhardt and collaborators (12) purified beet molasses with potassium ferrocyanide and diatomaceous earth in order to make it of use for citric acid production by *A. niger.* The shallow-pan method was employed, simulating conditions in industry. Yields of 50 per cent of the available sugar (calculated as sucrose) were obtained. It was suggested that beet-sugar impurities that are inhibitory to citric acid production are probably an excess of heavy-metal ions, which are removed by the reagents just mentioned.

Prolodyakonov (13) suggested improvements for the production of citric acid by *A. niger* under plant conditions. By replacing ammonium nitrate with ammonium chloride in the nutritive medium it was possible to grow the mold in the presence of *Bact. coli* and *B. lactis aerogenes* without affecting the yield of citric acid. These bacteria convert HNO_3 to HNO_2 and thus inhibit citric acid formation by the mold. *B. subtilis, Oidium lact.,* and *A. fumigatus* did not change the acid-forming capacity of the mold; other organisms, however, almost completely destroyed the acid-forming micelles. The citric acid was separated as the calcium salt. Washing the containers and the air in 1 per cent formalin is suggested.

Ammonium sulfate inhibits the formation of oxalic acid by various molds (14). Quaternary ammonium or cyclic ammonium compounds were recommended as accelerators in the fermentation of molasses by *Aspergillus, Citromyces, Mucor,* etc., for citric acid production. The amount of accelerator necessary is 1 to 2 per cent of the carbohydrate in the medium (15).

Isolation of Citric Acid. When fermentation is complete, the solution is drained off and the mycelium is pressed free of the absorbed citric acid. From the hot solution the acid is precipitated as the calcium salt. The citric acid is liberated by the addition of an equivalent of sulfuric acid and separated from the calcium sulfate. If desired the unchanged sugar may be fermented by yeast before precipitation of the citric acid.

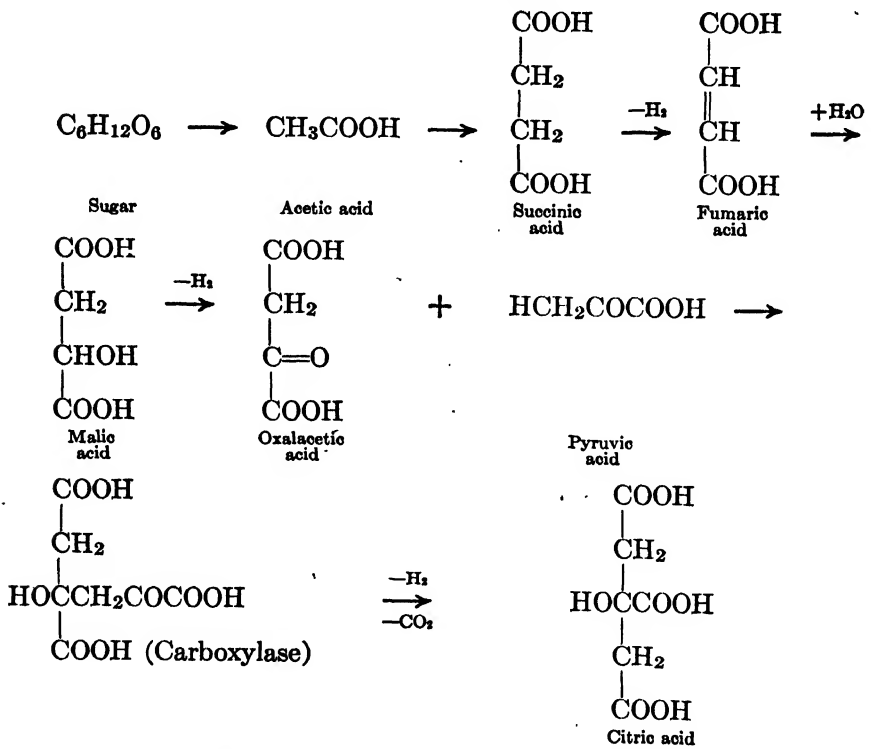
Industrial Production and Uses. At present 26,000,000 pounds of citric acid are produced annually in the United States by one large company. This quantity appears to be insufficient to meet the demand (16). Probably all countries now produce citric acid by the fermentation process. About 65 per cent of the acid produced in this country is used for pharmaceutical purposes (citrates, etc.), 15 per cent is employed in foods, and 9 per cent in candies. The remainder is used in the preparation of inks, in dyeing, silvering, calico printing, engraving, etc. (3).

The Chemistry of Citric Acid Fermentation. Nothing is definitely known concerning the sequence of citric acid formation, although numerous investigations of this interesting problem have been made.

Citric acid was found to be produced from the following carbon sources: glucose, fructose, mannose, galactose, sucrose, arabinose, xylose, glycerol, trioses, glyceric acid, mannit, gluconic acid, saccharic acid, adipic acid, acetic acid, glycolic acid, and alcohol (5, 17–24). On the basis of this work, numerous theories for citric acid fermentation have been proposed.

Knoop and Martius (25) synthesized citric acid *in vitro* by treating at room temperature an equimolecular mixture of oxalacetic acid and pyruvic acid with alkali carbonate. Then the mixture was oxidized with hydrogen peroxide. This step removed the carbon dioxide from the intermediary keto acid. After 30 hours as much as 35 per cent calcium citrate was isolated. They suggest that this reaction may also take place during citric acid fermentation.

On the basis of the work of Knoop and Martius citric acid production, *in vivo*, could take place in the following manner:



This is one of a number of schemes proposed for the formation of citric acid. The enzymes present in molds are numerous. The elucidation of these enzymic reactions *in vitro* offers highly interesting studies.

GLUCONIC ACID PRODUCTION BY SUBMERGED MOLD GROWTH

Gluconic acid as a product of fermentation was first observed by Boutroux in 1878, when glucose was changed to gluconic acid by *Mycoderma aceti* (*Acetobacter aceti*).

In 1922 Molliard identified gluconic acid, citric acid, and oxalic acid as products of fermentation by *Aspergillus niger*. Two years later Bernhauer isolated a strain of *A. niger* which produced predominantly gluconic acid when calcium carbonate was added to the glucose-containing media. He has shown that low temperature and low nitrogen concentration favored gluconic acid production, whereas high temperature and high nitrogen content resulted in high yields of citric acid.

For some years members of the United States Department of Agriculture have conducted intensive and successful research concerning

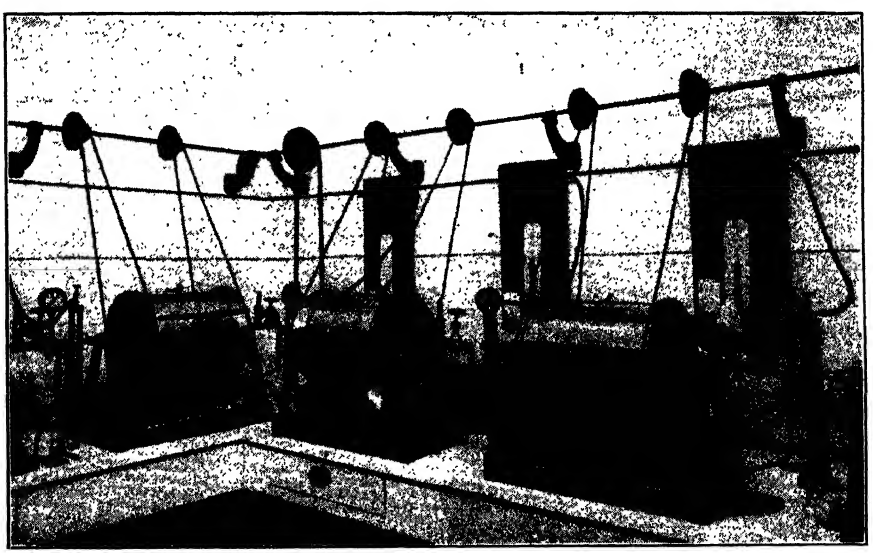


FIG. 31. Laboratory-scale rotary drum.

the production of gluconic acid and other products by microorganisms. Several molds from the collection of Dr. Thom have been found to be excellent gluconic acid producers. Moyer, May, and Herrick (26) found *P. chrysogenum,* culture 5,034,11, the most suitable organism for the production of gluconic acid when grown on 20 to 25 per cent commercial glucose, 3.00 grams $NaNO_3$, 0.300 gram KH_2PO_4, and 0.250 gram $MgSO_4 \cdot 7H_2O$ per liter. Sixty per cent of the glucose was oxidized in 8 to 10 days at 30° C. Ferric chloride stimulated growth when pure salts were employed. These cultures were grown in *shallow pans* of high-purity aluminum.

The Rotary Drum Process. In 1935 Herrick, Hellbach, and May developed the laboratory rotary drum (see Fig. 31) for the production of gluconic acid by submerged mold growth. This drum can be ster-

ilized with steam. A definite amount of air which is necessary for the oxidation processes passes through the rotating drum during fermentation (27–29).

By using the rotating laboratory drum process the time required for conversion of glucose was reduced from 11 days to 2.2 days. The yield of gluconic acid was increased from 57.4 per cent to 80.0 per cent.

Media and Process. *Aspergillus niger*, strain 67, is cultured on slants of medium *A* (Table XLI) for 7 days at 30° C. These cultures are employed to inoculate 20 or more 1-liter Erlenmeyer flasks, each containing 150 cc. of medium *B* and incubated at 30° C. for 7 days. The mycelium with its heavy crop of spores is transferred aseptically to 17 liters of medium *C* and macerated. This is divided into 7- and 10-liter portions; proportionate amounts of calcium carbonate are added and are aseptically transferred to the laboratory aluminum drums. The charge is one-third of the total volume of the drum.

During germination the following conditions are maintained:

Air pressure	30	pounds per square inch gauge reading
Air flow	375	cc. per liter per minute
Speed of rotation	5.8	rpm.
Temperature	30° C.	
Time	24	hours

The Pilot-Plant Drum. With the solution just described, the large pilot-plant rotary aluminum drum (Fig. 32), containing medium *D* (Table XLI), was inoculated by blowing the solution directly from the small drums to the large fermenter via a hose connection.

TABLE XLI

SUMMARY OF MEDIA USED FOR GLUCONIC ACID PRODUCTION BY *Aspergillus niger*

Ingredient	*A* Culture	*B* Sporulation	*C* Germination	*D* Fermentation
Grams per liter:				
Refined corn sugar	30.0	50.0	100.0	Varies
$MgSO_4 \cdot 7H_2O$	0.10	0.12	0.25	0.156
KH_2PO_4	0.12	0.144	0.30	0.188
$(NH_4)_2HPO_4$	None	0.56	0.80	0.388
NH_4NO_3	0.225	None	None	None
Peptone	0.25	0.20	0.02	None
Potatoes	200	None	None	None
Agar	20.0	1.5	None	None
$CaCO_3$	4.0	None	37.5*	26.0*
Cc. per liter:				
Beer	None	45	40	None
Kind of water	Distilled	Distilled	Tap	Tap

* Separately sterilized.

The pilot-plant drum is 3 by 6 feet, of ⅜-inch high-purity aluminum sheet containing less than 0.1 per cent of copper, iron, and manganese. The end castings are of aluminum-silicon alloy (28). The drum holds 420 gallons and is charged with 140 gallons of medium. The general principles of the large fermenter are identical with those of the laboratory drum. Proper facilities are provided for sterilizing, filling, and emptying, and for passing humid, sterile air under pressure.

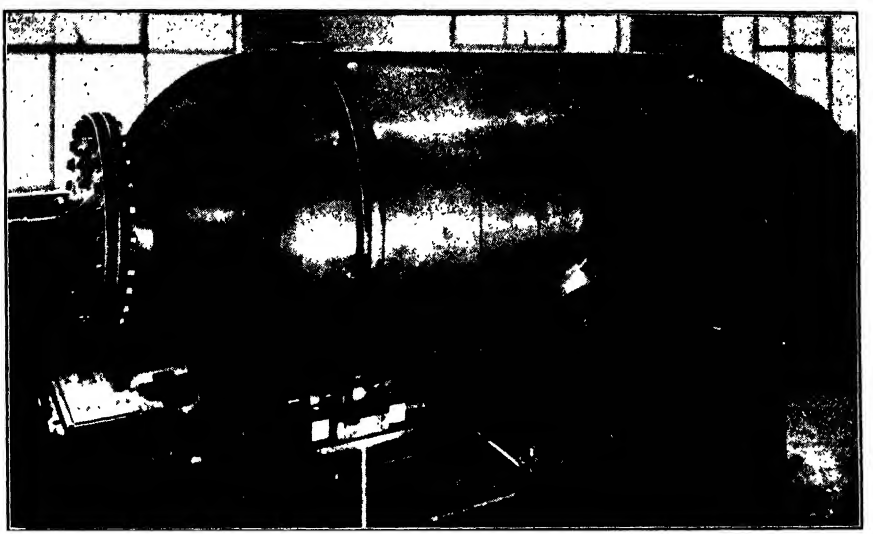


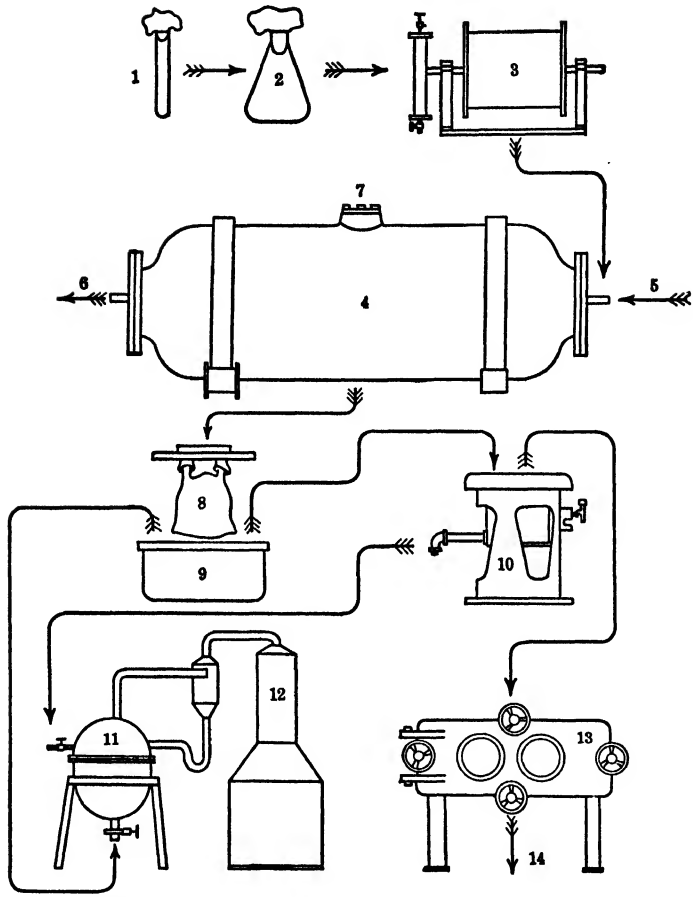
FIG. 32. Large-scale rotary drum.

Optimum Conditions. An air flow of 375 cc. per minute per liter of solution is required at an optimum *p*H of 5.0. The *p*H is readily maintained by the addition of 2.6 grams of calcium carbonate per 100 cc. of medium. Owing to the exothermic nature of the fermentation on a pilot-plant scale, the constant temperature (30° C.) is obtained by using a thermostatically controlled water spray applied to the exterior of the fermenter. The optimum glucose concentration varies between 15 and 20 grams per 100 cc.

Germinated spores are superior to ungerminated spores. When the same mycelial growth is used for repeated fermentations, the over-all fermentation time is decreased.

Yield. A charge of 91 kilograms of refined corn sugar in 530 liters of medium is fermented in less than 24 hours, yielding gluconic acid equal to more than 95 per cent of the sugar originally present.

Figure 33 represents a flow sheet of gluconic acid production (29).



1. Tube culture.
2. Sporulation flasks.
3. Small rotating aluminum germination drums (17-liter volume).
4. Large rotating aluminum drum fermenter (540-liter volume).
5. Air inlet; water for fermentation solution and inoculum from 3 are also introduced here.
6. Air outlet.
7. Hand hole; commercial dextrose, nutrients, and sterile calcium carbonate are added here.
8. Bag filter.
9. Aluminum tank for neutralization and crystallization; calcium hydroxide milk is added here.
10. Centrifuge (stainless steel basket, aluminum-lined curb).
11. Vacuum evaporator for mother liquors.
12. Condenser.
13. Vacuum dryer.
14. To calcium gluconate storage.

FIG. 33. Flow sheet of gluconic acid production.

Improvements in Gluconic Acid Production

Fermentation of Concentrated Solutions of Glucose to Gluconic Acid. Free gluconic acid and calcium gluconate precipitate on the mycelium, are injurious, and inhibit fermentation. This defect is overcome by the use of boron compounds, such as boric acid or borax with an excess of calcium carbonate. The boron salts form soluble complexes with calcium gluconate.

Moyer, Umberger, and Stubbs (30) have shown that the precipitation of calcium gluconate during the normal fermentation of 20, 25, 30, and 35 grams of glucose per 100 cc. could be prevented by 500, 1000, 1500, and 2500 p.p.m. boron, respectively.

Medium. The medium consists of 0.388 gram diammonium phosphate, 0.500 gram sodium nitrate, 0.500 gram monopotassium phosphate, 5 grams calcium carbonate, and 2 to 3 grams corn seep liquor (yeast compound) per liter.

The organism used is *A. niger.*

It is claimed that under these conditions and with the reuse of the mycelium it is possible to ferment 25 per cent glucose solutions to gluconic acid in 24 hours.

Porges, Clark, and Aronovsky (31) described a semi-continuous method for gluconic acid production. The mycelia were recovered by pressure filtration and reused in nine successive fermentations. With this method, higher glucose concentrations were fermented by the flotation procedure than previously.

Gluconic Acid Production by Bacteria. Bacteria, in general, are slow gluconic acid producers and as yet have found no application in the industry. Under definite conditions gluconic acid from glucose is produced by the following acetic acid bacteria: *Acetobacter aceti, A. acetosum, A. oxydans, A. industrium.*

Pervozvanskii and Iwashkemain found that the main product of glucose oxidation by *Bact. putidum* L et N was gluconic acid. The optimum conditions for the oxidation were a glucose concentration of 2 to 6 per cent (up to 10 per cent with air blowing), the presence of calcium carbonate, a source of nitrogen, a temperature of 30° C., and periodic mixing. Under the optimum conditions, the yield of gluconic acid was 87.6 per cent. The Russian investigators report that this method of glucose oxidation by means of fluorescing bacteria is applicable to large-scale calcium gluconate production (32).

Calcium gluconate is extensively used in supplying calcium to children and to lactating and pregnant women.

Mechanism of Gluconic Acid Fermentation. In this process, glucose $\left(RC\begin{matrix}O\\ \diagdown\\ H\end{matrix} + \tfrac{1}{2}O_2\right)$ is oxidized to gluconic acid (RCOOH) by glucose oxidase. The enzyme is readily isolated from the pressed juice of *A. niger* or *P. glaucum* by alcohol precipitation of the aqueous extract of the mycelium. The enzymic reaction may be studied in the Warburg apparatus by measuring the oxygen absorption aerobically, using glucose as the substrate. The disappearance of reducing power of glucose solutions by any of the sugar methods may also be used. Von Müller (33) has extensively studied glucose oxidase.

Besides *d*-glucose only *d*-mannose and *d*-galactose are oxidized by this enzyme. This enzyme preparation also contains a maltose oxidase. Mannose and galactose, however, are only slowly oxidized by it and are not changed to the corresponding acids by the mycelium itself (34).

Franke and Lorenz (35) showed that glucose oxidase also acts in the absence of oxygen, provided that certain hydrogen acceptors, such as indophenol dyes or quinone, are present. Hydrogen peroxide has been shown to be formed under aerobic conditions. These properties classify the enzyme as an aeroglucose dehydrase rather than an oxidase. This is a flavin enzyme (36). See also Part I of this volume.

GALLIC ACID PRODUCTION

Gallic acid is produced by modifications of the Calmette process (37). A clear tannin extract is prepared from plants, and the sterilized material is inoculated with a pure culture of a suitable strain of *Aspergilli*. To facilitate hydrolysis of the tannin, air is blown through the agitated solution. From time to time samples are tested, and when all the tannin has disappeared fermentation is discontinued. The gallic acid is recovered by the usual procedures.

Fang (38) found that the best yield of gallic acid was 20.4 grams per 100 cc., using a concentration of 20° Baumé of tannin solution. For cultivation of *Aspergillus niger* the following medium was employed: 250 grams of rice was cooked with water; the excess water was removed, and 180 grams of wheat bran was added. After mixing and sterilizing, the medium was inoculated with the pure culture and kept at 30° C. for 2 days. The culture so prepared was dried at a low temperature and sieved. This sieved portion, containing most of the spores, was used as the pure culture in the fermentation experiments.

One hundred parts of the rice-wheat bran mixture yielded 7–8 parts of the pure culture after sieving.

Gallic acid is used in the production of certain dyes, of inks, and of skin remedies. When condensed with the aid of sulfuric acid it forms hexahydroxyanthraquinone.

DEXTROLACTIC ACID PRODUCTION BY MOLDS

Ward, Lockwood, Tabenkin and Wells (39) described an efficient and simple process for the preparation of dextrolactic acid (sarcolactic acid or *l*(+)lactic acid) by using the mold *Rhizopus oryzae* (Went and Geerlings 395) in a submerged condition. Thirteen per cent glucose solutions are fermented in 30 to 35 hours with a yield of 70 to 75 per cent of *d*-lactic acid. The fermentation is carried out in the rotary aluminum fermenters at 30° C. (see "Gluconic Acid Production" above).

The germination medium has the following composition:

Commercial glucose	110.0 grams
Urea	2.0 grams
$MgSO_4 \cdot 7H_2O$	0.25 gram
KH_2PO_4	0.60 gram
$ZnSO_4 \cdot 7H_2O$	0.088 gram
$CaCO_3$	10.00 grams
Distilled water to make 1 liter	

The commercial glucose contained 91.5 per cent glucose, 8.0 per cent moisture, and 0.4 per cent dextrin.

The spores are germinated in a 4-liter bottle for 24 hours at 30° C. The bottle is provided with an outlet tube and is mechanically shaken. Aliquots of 250 cc. each are employed to inoculate 3-liter portions of nutrient solution of the following composition:

Commercial glucose	150 grams
Urea	2.0 grams
$MgSO_4 \cdot 7H_2O$	0.25 gram
KH_2PO_3	0.60 gram
$ZnSO_4 \cdot 7H_2O$	0.044 gram
Octadecyl alcohol	0.03 gram in 1.7 cc. ethyl alcohol
Distilled water to make 1000 cc.	

Urea as the nitrogen source allows the preparation of a colorless medium which is clear and pure. The fermented liquors have an agreeable odor and contain no volatile acids. Bacteria require large quantities of crude nitrogenous material. The *d*-lactic acid is readily isolated by this process.

Two hundred grams of calcium carbonate is sterilized separately and added to the 3-liter portions of the solution just before inoculation. The octadecyl alcohol is added to prevent foaming during fermentation. The inoculated medium is then fermented in the rotary aluminum fermenters.

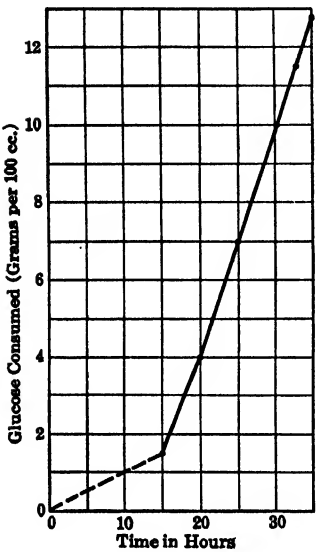


FIG. 34. Course of a typical *d*-lactic acid submerged fermentation induced by *Rhizopus oryzae*.

The rate of fermentation is observed by determining the reducing-sugar concentration. Figure 34 represents a typical experiment. During the first few hours sugar utilization is slight; but after 15 hours fermentation progresses rapidly and is complete after 35 hours. The following are the analytical data for this experiment per 100 cc.:

Original glucose concentration	13.3
Glucose consumed	12.8
Ethyl alcohol produced	0.62
Calcium in solution	2.245
Lactic acid equivalent to dissolved calcium	10.12
Lactic acid found by analysis	9.66
Acidity due to lactic acid, per cent	95.4
Yield of *d*-lactic acid, based on glucose consumed, per cent	75.5

For the production of *l*-lactic acid, the common form, see Chapter XX.

Production of Itaconic Acid. This metabolic product of *Aspergilli* is a substituted metacrylic acid. Its esters polymerize in the same manner as the metacrylates. If itaconic acid could be produced at a low price, it could find application in the plastic and detergent industries. At present, itaconic acid is made by the pyrolysis of citric acid at a price that is much too high for industrial purposes. Moyer and Coghill (40) have reinvestigated the earlier findings of Kinoshita concerning the production of itaconic acid by *Aspergilli*. They found that 28 to 29 grams of the acid are produced from 100 grams of glucose in 10 to 12 days by *A. terreus* NRRL 265, when the mold is grown at 30° C. on the surface of shallow layers of the following medium:

	GRAMS PER LITER
Glucose	250
$MgSO_4 \cdot 7H_2O$	0.250
KCl	0.050
NH_4NO_3	2
$ZnSO_4 \cdot 7H_2O$	0.044

To this mixture the following solutions are added:

Corn steep liquor	4 cc.
HNO_3 $N/2$	50 cc.

Ninety per cent of the itaconic acid may be recovered from the fermentation liquid.

More recently, Lockwood and Nelson (41) investigated the production of itaconic acid by agitated cultures using the mold *A. terreus* 1960. They found that rigid *p*H control in the range of 1.8 to 1.9 is necessary and that the $MgSO_4 \cdot 7H_2O$ concentration should be 0.75 gram per liter of medium. The addition of $ZnSO_4$ did not improve the yield, and in the presence of NaCl less itaconic acid was produced. Maximum efficiency was obtained with 6 per cent glucose concentration and a very small inoculum.

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CHAPTER XX

BACTERIAL FERMENTATIONS

ACETONE-BUTANOL FERMENTATION

Pasteur was the first to identify butyric acid as a product of fermentation. Later Fitz studied the fermentation products of several organisms such as *Bacillus butylicus*. These spore formers were found to produce butyl alcohol, butyric acid, and small amounts of ethyl alcohol. In 1887 Gruber described several strains of *B. amylobacter* (*Clostridium butyricum*) which produced butyl alcohol and butyric acid from carbohydrates. The formation of acetone by microorganisms was first reported by Schardinger in 1905. He showed that the same organism (*B. macerans*) also produced ethyl alcohol, acetic acid, and formic acid. Potatoes, or potato starch with peptone and calcium carbonate, were described as best for acetone production (1).

In 1911 Fernbach and Weizmann were the first to report on bacteria that produce amyl alcohol, butyl alcohol, ethyl alcohol, and acetone as the end products. They employed potato starch as the substrate. One year later Weizmann isolated an organism that produced four times more acetone than the previous one and fermented all kinds of starches. This organism was first named *B. granulobacter* and later *Cl. acetobutylicum* (2). During the first World War, the manufacture of acetone by the Weizmann process played an important role, in several Allied countries, in the production of explosives. After the war, when acetone was not required for the manufacture of explosives, *n*-butyl alcohol was found to be of use in the manufacture of *n*-butyl acetate for lacquers. In 1919 a number of papers appeared which described the production of acetone and acetone-butanol (3–5).

Industrially, corn is extensively employed as the raw material. The germ is removed and further processed for its oil content. The kernels are ground and mixed with water to make a concentration of 6 to 8 per cent. The mash is then cooked under 30 pounds' steam pressure for 2 hours. After the temperature is lowered to 37° C., the mash is inoculated with *Cl. acetobutylicum*. Fermentation proceeds for 48 to 72 hours.

A great variety of carbohydrate-containing raw materials, such as prehydrolyzed starches, disaccharides, hexoses, pentoses, and molasses,

have been used (6). Wood sugar may be fermented to butanol, acetone, and ethanol by *Cl. felseneum* and *Cl. butylicum* (7).

Proteins are not necessary for the growth of *Cl. acetobutylicum* or for the normal production of acetone and butanol (8).

Izsak and Funk (9) reported the isolation of a new organism, *Clostridium saccharobutylicum* (*gamma*), for fermenting sugar and forming isopropyl alcohol and acetone in different proportions. The β type of the organism is said to form from five to ten times more isopropyl alcohol than acetone (10), whereas the γ type produces ten to twenty times as much acetone as isopropyl alcohol. This new organism by itself does not ferment cereal starch but ferments molasses at 20° to 40° C.

Underkofler, Fulmer, and Rayman (11) hydrolyzed oat hulls, a typical agricultural cellulose waste, with dilute mineral acids, and on addition of corn mash they were able to ferment xylose completely with *Cl. acetobutylicum.*

Brown, Wood, and Werkman (12) have obtained normal amounts of the solvents by growing acetone-butanol organisms in a medium containing glucose, hydrolyzed casein, tryptophan, mineral salts, ammonium sulfate, and an acidic ether-soluble fraction of Difco yeast extract.

Optimum Conditions (13–16). Optimum temperature for acetone-butanol fermentation is at 37° to 42° C. Proper precautions must be taken to avoid loss due to evaporation of the solvents produced. The highest yields are obtained under anaerobic conditions. A *p*H between 5 and 7 is most favorable.

The following are examples of two modifications of acetone and butyl alcohol processes. Both methods utilize molasses in combination with other materials.

A. The Mass Inoculation Process (17). One hundred parts of rice bran in 900 cc. of water is inoculated with *Clostridium acetobutylicum,* and, after 24 hours, this is added to 60 parts of a sugar mash (6 per cent solution prepared from blackstrap molasses). After 48 hours, fermentation is complete. The fermentation products are 47.2 liters of gas, 28.8 parts of neutral solvents, and 4.6 parts of non-fermentable sugar remains. The maximum concentration of neutral solvents obtained by this process is 2.1 per cent, consisting of 8 per cent ethyl alcohol, 28 to 32 per cent acetone, and the remainder butyl alcohol. The yield is more than the theoretical amount, since some of the substrate is derived from the cellulosic and hemicellulosic materials. This process overcomes a lack of degradable proteins (furnished by the bran)

and the action of inhibitory toxic substances in molasses. The yield of solvents is, according to Weitzmann, higher than usual, being as high as 33 per cent of the sum of the starch, sucrose, and monosaccharide. The spent mash, owing to the production of riboflavin and the presence of thiamin supplied by the rice bran, is an important food.

B. Two-Phase Fermentation of Molasses and Corn (18). In this process, 4.5 per cent corn meal and up to 6.2 per cent molasses of any type are employed. However, only 20 to 50 per cent of the molasses to be used is added at the beginning, and the remainder at the twelfth to eighteenth hour of fermentation. Since molasses has a strong buffering capacity it should be acidified before mashing, in order to prevent a decrease in the yield of neutral volvents. The optimal concentration of molasses to be used is governed by the amino nitrogen content: the higher the amino nitrogen, the lower should be the molasses concentration. Too high molasses concentrations lower the yield of fermentation products.

Acetone-Butanol Fermentation of Waste Sulfite Liquor

The utilization of waste sulfite liquor from pulp and paper mills is a problem of considerable importance. Patents for the utilization of the sugars of waste sulfite liquor by acetone-butyl alcohol fermentation give no details concerning this problem.

Wiley and associates (19) have published an extensive study concerning the industrial utilization of waste sulfite liquor by acetone-butyl alcohol fermentation.

The sugars in waste sulfite liquor result from the acid hydrolysis of the hexosans and pentosans of the wood. In a waste liquor with 10 per cent solids, the total reducing sugars (calculated as glucose) amount to 1.3 to 3 per cent and may be as high as 750 pounds per ton of pulp.

Selection of Organism. Forty-eight different cultures of the genus *Clostridium* were studied, of which *Cl. butylicum* (Fitz) was found to be most suitable for the production of the solvents.

Pretreatment of Waste Sulfite Liquor. Because waste sulfite liquor is too acid (*p*H 2.5 to 3.5) and contains free and loosely bound sulfur dioxide and small amounts of furfural and formic and oxalic acids, it must be treated before it is suitable for fermentation. Lignin must also be removed. Fractional precipitation with calcium hydroxide was found most suitable for pretreating the waste sulfite liquor (see Fig. 35).

Medium. Wiley and associates (19) suggested the following medium for commercial use (waste liquor treated to remove sulfur dioxide and lignin):

$(NH_4)_2HPO_4$	0.05 per cent
Molasses	0.10 per cent
$CaCO_3$	0.10 per cent
Temperature	34° to 37° C.
Optimum *p*H	5.8
Incubation time	10 to 18 hours

Yield. Waste liquor containing 1 to 3 per cent of reducing substances was fermented 70 to 80 per cent. Of the sugars, 25 to 30 per

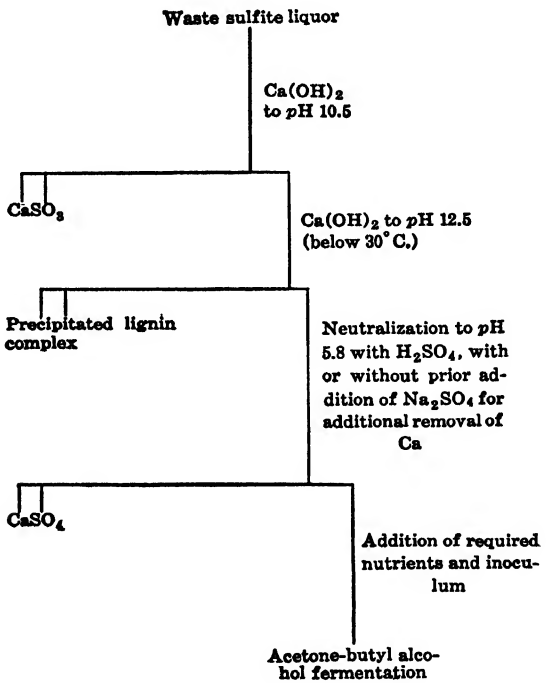


FIG. 35. Flow sheet for pretreatment of waste sulfite liquor prior to fermentation.

cent were converted to volatile products having a ratio of 75 parts of butyl alcohol to 20 parts of acetone and 5 parts of ethyl alcohol.

ACETONE-BUTANOL FERMENTATION OF JERUSALEM ARTICHOKES

The Jerusalem artichoke or girasole (*Helianthus tuberosus*) is a native plant of the United States. This plant is rich in levulan and may be readily hydrolyzed to levulose. Attempts to utilize this carbo-

hydrate are not lacking. In 1941 Wendland, Fulmer, and Underkofler (20) reviewed the literature concerning commercial outlets of the plant and studied the conversion of its levulans into butanol and acetone by fermentation with *Clostridium acetobutylicum.*

Procedure. Dried artichokes are hydrolyzed, at *p*H 1.75 with hydrochloric acid or at *p*H 1.50 with sulfuric acid, by heating for 1 hour at 80° C. After cooling the hydrolyzate is adjusted to *p*H 5.5 to 6.0 with strong sodium hydroxide and diluted to give a sugar concentration of 4 per cent. This liquid does not require sterilization. The other constituents are sterilized by autoclaving. Maximum yields of total solvents are obtained when 0.95 to 2.50 per cent of soybean meal is present in the hydrolyzate. Corn meal or corn gluten meal is also a satisfactory nutrient.

Seventy-five per cent of the total solvent is recovered in the usual ratio of 2 parts of butanol to 1 part of acetone.

Mechanism of Acetone-Butanol Fermentation. Bernhauer (21, 22) was of the opinion that butanol is produced from butyric acid, which in turn is formed from butyric aldehyde, and that acetone is produced from acetic acid or from acetoacetic acid. In the presence of calcium carbonate, more butyric acid and butanol and less acetic acid and acetone, respectively, are formed.

Janke and Siedler (23) found that the addition of acetaldehyde to sugar-containing yeast water shifted the ratio between acetone and butanol from 1:1.7 to 1:4.7. On the addition of calcium carbonate the ratio shifted to 1:22. Cultures suspended in phosphate of *p*H 6.3 produced butyric acid but no acetone or butanol. Thus the authors concluded that butyric acid is an intermediary of butanol formation in acetone-butanol fermentation. Simon and Weizmann (24), however, on the basis of their experiments with calcium carbonate, could not confirm that conclusion. Attempts to isolate a cell-free enzyme system effecting acetone-butanol, fermentation failed. See also the later studies of Wood and coworkers (25).

Bacteriophage. Normally the solvent-producing organisms grow rapidly, forming acids which they then convert to solvents. The fermentation terminates with a low sugar and low acid content. However, in a phage-contaminated fermentation the acids are not converted to solvents. Fermentation concludes with a high residual sugar and a high acid content. McCoy and coworkers (26) published immunizing procedures for the elimination of the phage strains.

ACETONE-ETHYL ALCOHOL FERMENTATION

The bacterial production of acetone was discovered by Schardinger (27). His studies were carried out on *Bacillus macerans,* which he has isolated. Potato starch or potatoes in the presence of peptone and calcium carbonate produce acetone, ethanol, acetic acid, and formic acid. In 1919 Northrop and associates (28) isolated an organism from old potatoes which they called *B. acetoethylicus.* This organism is not identical with *B. macerans.* It is a spore-forming, facultative anaerobe, growing well on a 2 per cent corn medium and calcium carbonate. It grows best at 40° to 43° C. and at *p*H 8 to 9. It ferments best at *p*H 6 to 8. The end products are ethyl, propyl, and butyl alcohol. Under certain conditions acetone and formic acid are produced.

Materials. Corn, potatoes, molasses, hydrolyzed corn cobs, and oat and peanut hulls may be used as the raw material (29, 30).

Mechanism of Fermentation. According to Bakonyi (31–32), when starch is fermented by *B. macerans* glucose is not an intermediary product. This author claims that, in a neutral medium in the presence of calcium carbonate, the organism produces from starch 2 molecules of ethyl alcohol and 1 molecule of acetone and hydrogen gas:

$$\underset{\text{Starch}}{2C_6H_{10}O_5} + 3H_2O = \underset{\text{Ethyl alcohol}}{2CH_3CH_2OH} + \underset{\text{Acetone}}{CH_3COCH_3} + 5CO_2 + 4H_2$$

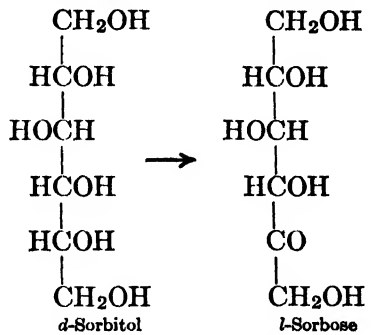
Bakonyi has obtained acetaldehyde by the fixation method. Much of the aldehyde is reduced to ethyl alcohol. Most, however, is condensed to aldol, which in turn is changed to alcohol and acetic acid by dismutation. The acetic acid is further fermented to acetone, according to this scheme. Although this process has not yet been used in large-scale production, Bakonyi predicts its adoption in plants engaged in ethyl alcohol fermentation where slops could be utilized for the process just discussed.

Tilden, Adams, and Hudson (34) recommended a method for the preparation of *macerans* amylase.

PRODUCTION OF SORBOSE FROM SORBITOL

Bertrand (1896) was the first to show that *l*-sorbose may be formed from *d*-sorbitol by the action of a bacterium now called *Acetobacter xylinum.* Numerous investigations on this problem have appeared. *l*-Sorbose is now employed in the chemical synthesis of vitamin C (*l*-ascorbic acid).

The following reaction takes place when *d*-sorbitol is fermented by *A. xylinum* or by *A. suboxydans* or certain other species of *Acetobacter:*



Wells, Stubbs, Lockwood, and Roe (35) found that by using a 15 per cent sorbitol solution and 0.5 per cent Difco yeast extract a yield of more than 93 per cent of *l*-sorbose may be obtained within 24 hours after inoculation. Highly aerobic conditions at 30° C are best. The organism used was *Acetobacter suboxidans.*

Semi-Plant-Scale Production of *l*-Sorbose by the Submerged-Growth Method. On the basis of the work just mentioned by employing the rotary drum the following semi-plant-scale method for the production of sorbose from sorbitol had been developed by Wells and coworkers (36).

Preparation of Inoculum. Test-tube slants are prepared by growing the bacterium for 48 hours on 2 per cent agar, 5 per cent sorbitol, and 0.5 per cent yeast extract. Suspensions of several test-tube slants are used for the inoculation of 50-cc. Kolle flask cultures. After 3 days of growth at 30° C., 50 cc. of sterile water is added and the agar surface is removed by scraping. The suspension from one Kolle flask is transferred to a wide-mouth 9-liter Pyrex bottle containing 6 liters of a sterile culture solution containing per liter 100 grams of sorbitol, 5 grams of Difco yeast extract, and 0.5 gram of octadecyl alcohol. The 9-liter Pyrex bottle is connected with a sintered-glass distribution tube (Jena, type 33c porosity No. 0). The outer end is connected to a source of sterile air having a needle valve for flow control. A second tube, inserted through the rubber stopper which holds the distribution tube, provides an outlet for the gas. The outcoming gas is measured by means of a flow meter. After inoculation sterile air is passed through the culture solution at the rate of 12 liters per minute. This is important in order to provide proper aeration and agitation of the solution. Analysis of the sorbose content and *p*H is made on samples removed through a glass tube inserted through the stopper. The medium, at the

end of 48 hours, is employed to inoculate the fermentation solution. At this time the sorbose content ranges from 6 to 7 per cent. Twenty-four liters of inoculum is used to inoculate a 530-liter batch of culture medium.

Semi-Plant-Scale Medium. Wells and associates employed a commercial sorbitol consisting of 74.9 per cent sorbitol, 2.8 per cent glucose, 0.9 per cent sodium sulfate, and 21.4 per cent water. Corn steep liquor (A. E. Staley Manufacturing Co.) is recommended as a nutrient substitute. This product contains acid, however, that must be neutralized with calcium carbonate. The *p*H should be 4.2 to 6.4. After 10 minutes of mixing, the medium and the apparatus are sterilized at 110° C. for 75 minutes. A slight excess of calcium carbonate is necessary in order to neutralize gluconic acid that forms from glucose.

The air flow is 375 cc. per minute per liter of solution. The gauge pressure is 30 pounds per square inch. The drum rotation is 13 r.p.m. The temperature is 30° C. It requires about 33 hours to ferment a 20 per cent solution of sorbitol under these conditions.

Sorbose Recovery. The fermented solution is decolorized with carbon and filtered with the aid of Filter-Cel. The clear and colorless filtrate is concentrated in a copper still under a vacuum of 22 to 28 inches (560–710 mm.) pressure at about 60° C. Some of the sorbose crystallizes during concentration. Further crystallization is effected when the solution is allowed to stand overnight at 15° C. The mass is centrifuged, washed with ice water, and dried. From the mother liquid and washings further quantities of sorbose may be obtained on concentration. The yield is 70 per cent sorbose.

Sorbose in Vitamin C Production. Reichstein and Grüssner (37) prepared *l*-ascorbic acid as follows:

$$\text{Glucose} \xrightarrow{H_2 + Pt} \text{sorbitol} \xrightarrow{A.\ xylinum} \text{sorbose} \xrightarrow{\text{acetone}} \text{diacetone sorbose} \xrightarrow{KMnO_4} \text{diacetone sorbonic acid} \xrightarrow{HCl} l\text{-sorbonic acid} \xrightarrow{HCl} l\text{-ascorbic acid}$$

PRODUCTION OF DIHYDROXYACETONE BY THE ACTION OF *Acetobacter suboxydans* UPON GLYCEROL

Bertrand was the first to describe the formation of dihydroxyacetone from glycerol by the action of *Acetobacter xylinum.*

Recently Underkofler and Fulmer (38) modified the method of Neuberg and Hofmann for the crystallization of dihydroxyacetone from a fermented medium using *Acetobacter suboxydans* (American Type Culture Collection No. 621).

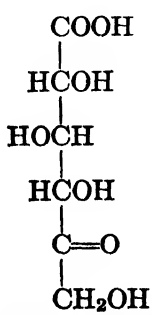
Medium. Stock cultures are kept on malt-extract agar slants. Active cultures are prepared from these by transfer every 48 hours into a medium containing, per 100 cc., 0.5 gram of yeast extract (Difco powder) and 6 grams of glycerol; 100-cc. portions of the medium are placed in 300-cc. Erlenmeyer flasks. The temperature is 28° C.

Optimum yields are obtained in 7 days at 28° C. with 6 per cent glycerol, 0.5 per cent yeast extract, 0.10 to 0.30 per cent KH_2PO_4, at an optimum *p*H of 5.5 to 7.0.

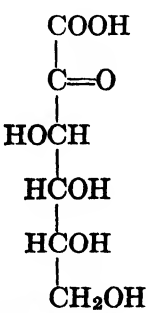
Isolation of Crystalline Dihydroxyacetone. To 1000 cc. of the fermented medium is added 10 grams each of Norite, calcium carbonate, and diatomaceous earth. The mixture is shaken and filtered. The clear filtrate is concentrated *in vacuo* to 150 cc., 3 to 4 volumes of absolute alcohol is added, with stirring, and the mixture is filtered. Most of the alcohol is evaporated *in vacuo*, and the syrup is poured into 10 volumes of acetone with stirring. The turbid solution is allowed to stand overnight. The mixture is then shaken with Norite and filtered. The clear filtrate is concentrated *in vacuo* to a very thick syrup. The last amounts of water and acetone are removed in a vacuum desiccator over sulfuric acid. The syrup crystallizes spontaneously. Washing with cold absolute alcohol results in a pure white crystalline product. The crystals are dried in a vacuum desiccator. The yield is 80 per cent of the pure product on the basis of the crude substance used.

PRODUCTION OF KETOGLUCONIC ACIDS BY BACTERIAL FERMENTATION

Stubbs, Lockwood, Roe, Tabenkin, and Ward (39) studied the formation of ketogluconic acid from glucose by bacterial fermentation. They obtained 90 per cent yields of 5-ketogluconic acid from a 10 per cent glucose solution in 33 hours by using *Acetobacter suboxydans*. Eighty-two per cent yields of 2-ketogluconic acid were obtained by the action of an unnamed bacterium from a 10 per cent glucose solution in 25 hours.



d-5-Ketogluconic acid



d-2-Ketogluconic acid

Both aluminum rotary drum fermenters (see "Gluconic Acid Production" in Chapter XIX), and vertical vat fermenters were employed for these studies; a nutrient solution, such as those shown in Table XLII, was used.

TABLE XLII

COMPOSITION OF NUTRIENT SOLUTIONS* FOR PRODUCTION OF KETOGLUCONIC ACIDS

Components	Grams for 5-Ketogluconic Acid	Grams for 2-Ketogluconic Acid
Commercial glucose	118†	118†
Corn steep liquor	5	5
Octadecyl alcohol (antifoam agent)	0.3	0.3
Urea (sterilized separately)	...	2
$MgSO_4 \cdot 7H_2O$	...	0.25
KH_2PO_4	...	0.60
$CaCO_3$ (sterilized separately)	27	27
Distilled water	To make 1000 cc. in both cases	

* The solutions were sterilized by autoclaving at 20 pounds' pressure for 45 minutes.
† This quantity of commercial glucose gave a medium of 10 per cent glucose concentration.

PRODUCTION OF *l*-2,3-BUTYLENE GLYCOL BY FERMENTATION

The production of *l*-2,3-butylene glycol by fermentation is of considerable industrial importance owing to its use in the synthesis of rubber, for this compound may be readily converted to butadiene. However, 2,3-butylene glycol was not produced industrially during the second World War.

Fulmer and associates (40) recommended the following medium and conditions for the production of 2,3-butylene glycol from sucrose:

Ammonium chloride	0.250 gram	per 100 cc. of medium
Potassium monophosphate	0.150 gram	
Calcium chloride	0.150 gram	
Magnesium sulfate	0.200 gram	

The optimum *p*H is at 6.0, and the optimum temperature is 37.5° C. A concentration up to 8 per cent is fermented to about 100 per cent in 18 days with 47 per cent glycol formation. At higher concentrations such as 8 to 12 per cent the quantity of sucrose fermented is 85 per cent. The organisms studied by Fulmer and coworkers were *Aerobacter faeni, Aerobacter motorium,* and *Aerobacter pectinovorum.* The last organism is most suitable for the production of the glycol. After com-

pletion of the fermentation the glycol may be recovered by vacuum distillation.

Kluyver and Scheffer (41) suggest the use of malted potatoes or molasses mash containing ammonium sulfate, a phosphate, and a carbonate such as calcium carbonate for the production of 2,3-butylene glycol. Air is to be blown through the mash during fermentation. These investigators used *Aerobacter aerogenes* as the organism.

Ward and associates (42) found that certain strains of *Aerobacillus polymyxa,* such as those isolated from soil and from spoiled starch, grow well on substrates like grain mashes, sweet-potato mashes, or sugar solutions without aeration and without additional nutrients, producing *l*-2,3-butylene glycol. During fermentation the mash is kept quiescent and at a *p*H above 5.2 but below 7.0.

Production of Industrial *l*-2,3-Butylene Glycol from Molasses. Torres and Frías (43) described the following process for the production of *l*-2,3-butylene glycol:

Sterilized molasses is diluted to 13° to 14° Brix, brought to *p*H 6.0 to 6.2, cooled to 30° C., inoculated with a culture (5 per cent by volume of the mash), and fermented for 36 hours at 31° to 32° in closed vessels provided with cooling and heating means. During the first 24 hours, the mash is aerated with sterile air at the rate of 6.6 cubic feet per minute per 1000 gallons of mash; if mechanical agitation is provided, the amount of air can be reduced to 3 cubic feet, and the yield of glycol is thereby increased. The aeration is discontinued when the rate of the decrease of sugar content reaches 0.1 per cent per hour. During fermentation, the *p*H decreases slowly; the amount of reducing sugars should also decrease gradually (this necessitates frequent tests); otherwise, large amounts of acetylmethylcarbinol form. The fermented mash contains 4 per cent of the glycol, 0.45 per cent of ethyl alcohol, and 0.55 per cent of acetylmethylcarbinol. The glycol can be separated by countercurrent extraction with butanol or by flash distillation with kerosene. In flash distillation, kerosene vapor at 150° is bubbled into the closed container under pressure; the kerosene, saturated with the volatile components of the wash, is flashed from the top; this leaves the solids in water. The paper (43) contains a flow diagram of the process.

ACETIC ACID FERMENTATION—THE PRODUCTION OF VINEGAR

Acetic acid was first manufactured by the distillation of wood. The demand, however, soon exceeded the quantity made by wood distil-

lation, and other sources were sought and found. The biochemical and the synthetic processes are now in use industrially.

Vinegar is a dilute solution of acetic acid made by the fermentation process. It contains salts and extractives. Any aqueous solution of a fermentable sugar may be converted into vinegar. Many fruit juices are suitable for this purpose.

All vinegar is made by two series of fermentative reactions. The first reaction is brought about by yeasts which ferment the sugar to ethyl alcohol and carbon dioxide. The second phase consists of the oxidation of ethyl alcohol to acetic acid. This second process is called acetic fermentation and is carried out by a widely distributed group of bacteria which belong to the genus *Acetobacter.*

Bact. schuezenbachii or *Bact. curvum* is employed to produce vinegar from ethyl alcohol by the quick vinegar process. *Bact. orleanse* is used in both the quick vinegar process and the Orleans process.

Raw Materials. Apples, grapes, oranges, peaches, pears, berries, solutions of sugar syrup or molasses, cornstarch, beer, and wine may be employed for the production of vinegar.

Yeast Fermentation. For the fermentation of the sugars, *Saccharomyces ellipsoideus,* a selected wine yeast, is most suitable, owing to the desirable flavor which it imparts to the finished products. Compressed yeast may also be used as a starter. The optimum temperature is 23.9° to 26.7° C. (44). The course of sugar fermentation is followed by the use of hydrometers. When fermentation is complete, the yeast and other solids must be removed. This is accomplished by allowing 2 to 3 weeks for sedimentation.

To assure proper oxidation of the alcohol, a concentration of 10 to 13 per cent is best. Greater concentrations must be diluted.

The Oxidation Phase. The oxidation process by the acetic acid bacteria requires an abundant supply of oxygen. Large casks or generators with truncated cones are used. Perforated scaffolds at top and bottom and air inlets near the bottom provide a large surface for the bacterial film to carry out the oxygen transfer. A temperature of 26.7° to 29.4° C. is most favorable, depending on the organism employed. When all the alcohol is oxidized, the action of the acetic acid bacteria is stopped. At this stage air must be completely excluded by storing the vinegar in closed barrels or tanks; otherwise bacteria and their enzymes will destroy the vinegar by oxidation. The vinegar is next aged, clarified if necessary, and bottled and pasteurized at 60° to 66° C. for 30 minutes.

Vinegar is made in homes and on farms by allowing cider or wine to ferment spontaneously. The product obtained by this method is not always of best quality.

Pressing of Fruits and Conversion of the Fruit Juices into Alcohol. Fresh fruits such as grapes, apples, and oranges are crushed and pressed. Apples are usually fermented by first allowing the pomace to ferment for 2 to 3 days before pressing. Ten to twenty gallons of actively fermenting cider is added per ton of pomace. This method is favorable to yeast fermentation in that it inhibits acetification before completion of fermentation. A medium that is too acid inhibits alcoholic fermentation and results in an inferior vinegar. The pomace press juice should not be added directly to the whole-apple juice since it is of inferior quality.

Any fruit containing more than 9 per cent sugar may be fermented to yield more than the legally required 4 grams of acetic acid per 100 cc.

Fifty gallons of actively fermenting liquid is required for the inoculation of 500 gallons of fresh juice. In 4 to 5 days this juice may be employed to inoculate 5000 gallons of fresh juice, which, in turn, may be used to inoculate 50,000 gallons of juice. Further fermentation may be conducted by using 10 per cent by volume of the actively fermenting juice per volume of fresh juice. Pure yeast cultures must be employed in the fermentation.

The addition of 6 to 8 ounces of potassium metabisulfite or 3 to 4 ounces of sulfur dioxide per ton of crushed juice results in clearer fermentation and higher alcohol yields (45).

The commercial methods for the production of vinegar are the slow or Orleans process and the quick process.

The Orleans Process. The Orleans or the French process is the oldest and the best, producing fine-quality vinegar. *Bact. orleanse* is employed. Barrels of 200-liter capacity are filled one-third with vinegar, which acts as the starter, and to this 10 to 15 liters of wine is added per barrel. Every week for 4 weeks the same volume of wine is added. After 5 weeks about 15 liters of vinegar is removed and the same volume of wine replaced. By repeating the operation the process becomes continuous. Air is introduced through holes on the top of the barrel. Several modifications of this process are known.

The Quick or Generator Process. This process, which is used by the industry, is much faster than the above mentioned, owing to the greater circulation of air in the tank (Fig. 36) (46). It requires greater care, however. The generator may be constructed in any desired size. The large generators have a perforated shelf about halfway between the top and the bottom of the tank, supporting beechwood shavings or other materials which serve to allow a large surface for the acetic acid bacteria. Above the shavings there is a rotating sprinkler which provides a uniform distribution of the vinegar and alcohol-containing substrate. The substrate may be continuously

passed through the same generator, or it may be transferred through other tanks until the desired acid concentration is obtained. A generator 10 feet in diameter and 20 feet high yields about 100 gallons of vinegar per day (47).

Effect of Environment (48). In the following study, the culture was introduced into a generator containing beechwood shavings or short sections of porcelain tubing impregnated with the nutritive medium. After standing 1 to 2 days, the apparatus was fed the

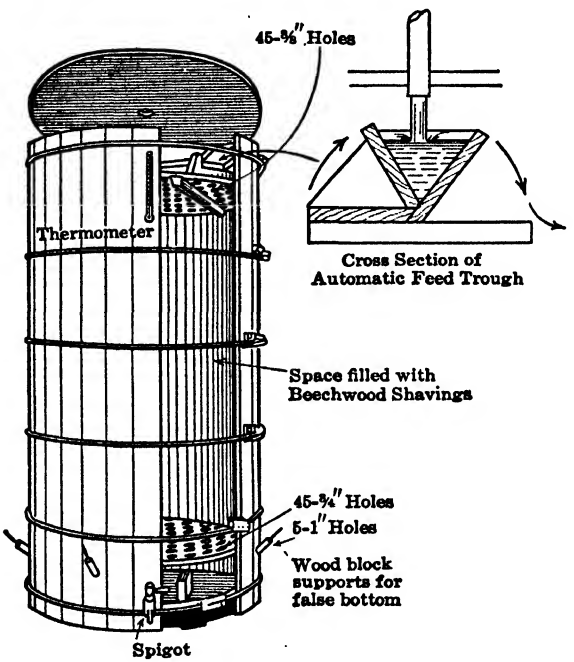


FIG. 36. Generator for quick or generator process.

substrate continuously, under aeration, at 28° to 30° C. Usually the bacteria spread rapidly on the shavings, and acidification of alcohol began in 3 to 4 days. On porcelain, the process began after 40 days. The substrate contained 0.1 gram each of $(NH_4)_2SO_4$, $MgSO_4$, and K_2HPO_4; 0.2 gram $(NH_4)_2HPO_4$; about 54 grams of acetic acid; 45 cc. of alcohol; about 25 cc. of must; and 1 liter of tap water. The total sugar content was 0.3 to 0.5 per cent. Abrupt change in aeration inhibited bacterial activity for several days, and the bacteria were swept off the shavings into the receiver. They did not adhere to the porcelain under any conditions. When substrate was added at the rate of 60 to 70 cc. per 24 hours, the optimal concentration of

alcohol was 5 per cent and that of acetic acid was 6 per cent. Addition of sucrose greatly increases bacterial activity. The bacteria utilize 5 to 20 milligrams of sucrose to produce 1 gram of acetic acid. Must had no advantages over glucose when ammonium salts were present. Abrupt changes in the concentration of must did not affect activity. The bacteria in the generator dissociate into R and S forms, but these do not show any essential difference in acid production.

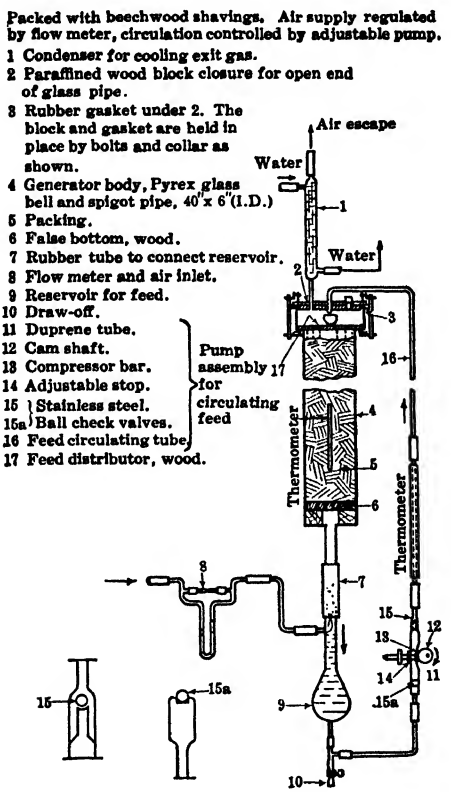


FIG. 37. Sketch of experimental vinegar generator.

Aging. Freshly prepared vinegar is harsh in flavor and odor. This defect can be eliminated by placing the vinegar in well-filled containers for 6 to 12 months. During this storage it acquires a pleasant flavor and odor. The odor or the so-called " bouquet " formation is probably a result of the synthesis of esters during aging. In the slow vinegar process, however, a considerable aging takes place during the course of manufacture.

Filtration and Pasteurization. Various clarifiers and filter aids are employed in order to obtain a clear product. The clarified vinegar

must be pasteurized at 59.5° C. for a few seconds; otherwise vinegar bacteria continue to grow and thus produce cloudiness.

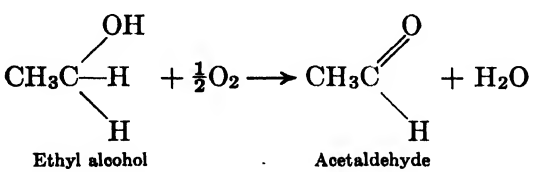
Laboratory Vinegar Generator. Figure 37 is a sketch of an experimental vinegar generator (quick vinegar process) and the course followed by the ingredients during operation, as described by Hildebrandt (49). By this method ethyl alcohol may be completely oxidized and there is very little loss. The total volume of packing is 1018 cubic inches. The rate of air circulation is 140 cc., and the charge is circulated at a rate of 2.6 cc. per minute. In contrast to the plant-size equipment the laboratory-size apparatus allows duplication of results. Nutrients and various other factors effecting fermentation (oxidation) may be studied. The generator may also be used in control work in vinegar manufacture.

MECHANISM OF ETHYL ALCOHOL OXIDATION

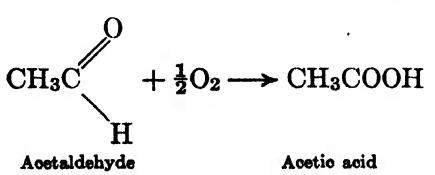
Acetic Acid Fermentation

In 1899 Hoyer showed that acetaldehyde is an intermediary product in the oxidation of ethyl alcohol. Neuberg and Nord (50) supported this view by applying calcium sulfite fixation. It has long been known that when there is lack of oxygen in the generators acetaldehyde may accumulate, owing to incomplete oxidation of the ethyl alcohol.

In normal acetic acid fermentation ethyl alcohol is dehydrogenated in the following manner:



Acetaldehyde is then further oxidized to acetic acid:



Contrary to earlier theories, evidence now indicates that both ethyl alcohol and acetaldehyde are probably oxidized by the same enzyme system.

Wieland (51) showed as early as 1913 that ethyl alcohol and acetaldehyde may be oxidized by acetic bacteria anaerobically in the presence

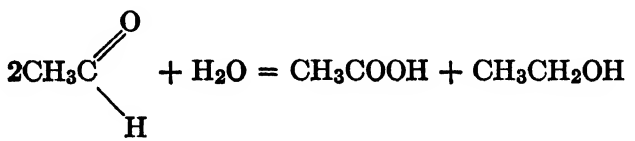
TABLE XLIII

DIFFERENTIAL CHARACTERISTICS OF *Acetobacter* SPECIES

Acetobacter species	Film Formation: Character	Film Formation: Cellulose Reaction	Optimum Temp. (°C.)	Oxidation of Ethanol	Compounds Commonly Oxidized	Differential Characteristics
A. aceti	Many variations	−	30–35	Complete	Ethanol, propanol, glycol, glucose	Ability to utilize ammonium salts as sole source of N
A. xylinum	Thick, leathery	+	30–35	Complete	Ethanol, propanol, arabinose, glucose, galactose, sucrose, maltose, glycerol, sorbitol	Production on surface of liquids of thick, leathery membrane giving cellulose reaction
A. rancens	Dry, folded, ascending	−	30–35	Complete	Ethanol, propanol, butanol, glycol, glucose, adonitol, mannitol, sorbitol	Unable to utilize ammonium salts as sole source of N
A. melanogenum	Generally only a ring	−	30–35	To acetic acid	Ethanol, propanol, glucose, maltose, mannitol, sorbitol	Production of dark brown pigment in glucose media
A. roseum	Thin, ascending	−	30–35	To acetic acid	Ethanol, propanol, arabinose, glucose, fructose, galactose, mannitol, glycerol	Production of rose pigment in glucose agar; enhanced by addition of $CaCO_3$
A. suboxydans	Thin	−	30	To acetic acid	Ethanol, propanol, glucose, glycerol, sorbitol	Ketogenic; produces 5-ketogluconic acid from glucose or gluconic acid
A. oxydans	Thin	−	20	To acetic acid	Ethanol, propanol, glycol, arabinose, glucose, fructose, galactose, sucrose, maltose, raffinose, dextrin, erythritol, mannitol, glycerol	Low optimum temperature

of methylene blue or quinone. This showed that it is not the free oxygen which acts as a direct hydrogen acceptor, but that the hydrogen atoms of ethyl alcohol and of aldehyde hydrate are activated by some specific intermediary catalytic system.

It had been suggested that bacterial acetic acid formation from alcohol requires a two-step dehydrogenation *via* acetaldehyde catalyzed by a dehydrogenase (50, 52). This enzyme has not been isolated, however. Neuberg and Nord (50) described an anaerobically acting mutase which acted on two molecules of acetaldehyde in the following manner:



This enzyme, however, is not the major factor in the oxidation of acetaldehyde. Acetone powder of the bacteria oxidizes alcohol only slightly and acetaldehyde not at all (52). Probably cytochrome and cytochrome-oxidase or indophenol oxidase are responsible for the oxidation of both ethyl alcohol and acetaldehyde (53–55). This phase of enzymology is still in a state of flux and offers a highly interesting avenue of research.

Under certain conditions acetic acid may be an intermediary product of metabolism of acetic acid bacteria. In large-scale operations, if oxidation is permitted to proceed until all the alcohol is oxidized, up to 20 per cent of the acetic acid is oxidized to carbon dioxide (56). In laboratory experiments, however, the oxidation of acetic acid by *Acetobacter* appears to vary considerably with the species (52, 57). Some show only negligible carbon dioxide formation, whereas others are able to metabolize acetic acid to carbon dioxide completely (57). See Table XLIII.

Determination of Oxidative Ability of *Acetobacter*. Vaughn (57) suggested the following basal medium for the determination of the oxidative ability of cultures of *Acetobacter:* 0.5 per cent tryptone (Difco), 0.2 per cent yeast extract powder (Difco), and 0.1 per cent K_2HPO_4. This medium is adjusted to *p*H 6.8 to 7.0, the compound to be studied is added, and the whole is sterilized at 15 pounds' pressure for 15 minutes. The formation of organic acids (relative oxidizing power) may be determined by titration with 0.1 *N* alkali. Study of the formation of compounds other than acids requires chemical analysis of the culture medium.

PRODUCTION OF LACTIC ACID BY BACTERIAL FERMENTATION

The optically active as well as the racemic mixture of lactic acid is produced by bacteria. However, commercial lactic acid is usually the racemic mixture. *Lactobacillus delbrueckii* and *Streptococcus lactis* produce *d*-lactic acid; *Leuconostic mesenteroides* var. Sake and *L. leichmannii* usually form *l*-lactic acid, whereas some bacteria such as *Lactobacillus pentoaceticus* produce *i*-lactic acid. These bacteria are most active at 50° C. At this temperature contamination is reduced to the minimum. It has been suggested that certain organisms introduced into the cultures as contaminants contain an enzyme (racemiase) that converts optically active lactic acids into inactive lactic acid (58).

Commercial Production. The organisms employed commercially are those which produce lactic acid as the main end product (homofermentative). They are *L. delbrueckii, L. casei, L. bulgaricus, L. leichmannii,* and *Streptococcus lactis.*

Media. Glucose, sucrose, or lactose may be used. Polysaccharides such as corn and potato starch must first be hydrolyzed to maltose or glucose by the action of amylases or acids.

Growth Substances. Riboflavin (59, 60), pantothenic acid (61), and nicotinic acid (62) were found to be essential for some lactic acid bacteria.

A New Activator of Lactic Acid Fermentation. Virtanen, Karstrom, and Kahra (63) reported the presence of an activator in milk that vigorously stimulated lactic acid fermentation.

B. casei epsilon (*Thermobacterium helveticum*) was cultivated in 5 liters of whey. The organism was collected by centrifuging, washed with water, and suspended in 100 cc. of water. The following test was used.

To 5 cc. of the aqueous suspension of the organism was added 200 milligrams of glucose in 5 cc. of *M*/2 phosphate buffer of *p*H 6.34. The ash had no effect, indicating that the catalyst is an organic compound. Yeast also contains this factor. Thiamin has no similar effect on lactic acid fermentation, and the factor does not stimulate alcoholic fermentation by yeast. These growth substances are contained in the so-called accessory nutrients such as steep water, malt sprouts, and thin grain residue (64).

LACTIC ACID PRODUCTION FROM WHEY (65, 66)

A quart of sterile skimmed milk is inoculated with a culture of *L. bulgaricus* containing a yeast to increase the rate of fermentation.

After 24 hours at 43° C. the culture is placed in a 40-quart jug which contains pasteurized milk. After 24 hours at 43° C. the contents of the jug are added to 500 gallons of pasteurized whey. This starter is allowed to incubate for 24 hours at 43° C. and is added to the main fermentation tank of 5000-gallon capacity. The mash is kept at 43° C. Lime is added to the mash every 6 hours. Fermentation is complete in about 42 hours. The lactalbumin is removed by heat coagulation at 96° C. and is allowed to settle. The calcium lactate is decanted, decolorized with carbon, purified, dried, and sold as calcium lactate or converted to the desired grade of lactic acid with sulfuric acid.

White Calcium Lactate Production. Daly, Walsh, and Needle (67) employ milk containing undenatured proteins. Whole milk, buttermilk, or skimmed milk in the liquid or in the dry state may be used. The following is a typical mash:

	Pounds
Sugar (dextrose)	6600
Milk powder	150
Diammonium acid phosphate	50
Calcium carbonate	4600
Water to 7000 gallons	

This mash is inoculated with 300 gallons of a 24-hour culture of *L. delbrueckii* and incubated at 49° C. for 5 to 7 days. During fermentation the sugar decreases to 0.1 per cent. The microorganisms are destroyed by heating the mash to 82° C. From 100 to 200 pounds of lime is added to adjust the *p*H to 11. Proteins and other insoluble material are removed by filtration. The filtrate is adjusted to a *p*H of 6 to 7 lactic acid. The filtrate is decolorized with a vegetable carbon, yielding a water-white solution. The solution is concentrated to 20 to 21° Baumé and then spray dried. The resultant powder is glasslike and homogeneous, containing 98 to 99 per cent calcium lactate. If lactic acid is desired the decolorized filtrate may be treated with a definite quantity of sulfuric acid and the calcium sulfate may be removed by filtration. The filtrate is decolorized and concentrated to desired strength.

Tatum and Peterson (68) developed a small-scale method by which *d*-lactic acid may be produced by *L. casei, S. lactis, R,* and *L. delbrueckii, 3.*

Lactic Acid Production by Molds. In 1894 Eijkman showed that *Mucor rouxii* produced lactic acid. Since then, lactic acid has been found to be produced by the molds *Rhizopus arrhizus, R. chinensis, R. pseudochinensis, R. elegans, R. oryzae, R. tritici,* and others.

Mechanism of Lactic Acid Fermentation. *Thermobacterium delbruckii, B. lactis acidi,* and certain molds produce almost pure *d*(-) lactic acid according to the equation:

$$C_6H_{12}O_6 = 2CH_3CHOHCOOH$$

Other organisms, in addition to producing lactic acid, yield ethyl alcohol, glycerol, acetic acid, and mannit. Mannit, however, is among the fermentation products only when fructose is employed as the substrate.

There are two kinds of enzyme systems by which lactic acid may be produced. In one, having the glycolytic enzyme system, methylglyoxal is an intermediary product. In the other, having the zymase complex, pyruvic acid is an intermediary. All organisms producing lactic acid appear to possess both enzyme systems.

Lactic Acid Uses. In the United States approximately 10 million pounds of various grades of lactic acid is produced annually. The leather industry is the principal user of lactic acid, consuming over 80 per cent of the entire production in the United States. The solubility of calcium lactate and the mild action of the acid on the hide make it an ideal deliming agent. Lactic acid is also used in the dyeing and textile industries. Some is converted into ethyl lactate, which is a good solvent for nitrocellulose, used in the manufacture of pyroxylin lacquers. The edible and U.S.P. lactic acids are used in the production of foods such as pickles and sauerkraut, where it acts as a preservative. It is used to adjust the *p*H of worts in beer manufacture and to prevent the growth of the butyric acid bacteria in yeast manufacture. Since lactic acid is readily metabolized, its uses in the various food industries are unlimited.

Production of Other Organic Acids by Fermentation. Other organic acids such as formic, fumaric, malic, oxalic, succinic, and butyric may also be prepared by fermentation. At present, however, chemical methods are preferred by the industries. For an excellent review of the literature concerning these products see reference 69.

PRODUCTION OF RIBOFLAVIN BY MICROORGANISMS

Certain microorganisms, when grown on liquid media, are capable of synthesizing 300 or more gamma per cubic centimeter of riboflavin. Such high-riboflavin-producing organisms were found to be the yeasts of the *Candida* genus, *Ashbya gossypii* Guilliermond, *Eremothecium ashbyii,* and, in somewhat lower levels, by organisms such as *Clostridium acetobutylicum* (70). The riboflavin may be precipitated in

the form of a reduced precursor, which is formed by the action of certain types of reducing bacteria, particularly a group of generally avirulent streptococci (71).

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CHAPTER XXI

PRODUCTION OF ANTIBIOTICS

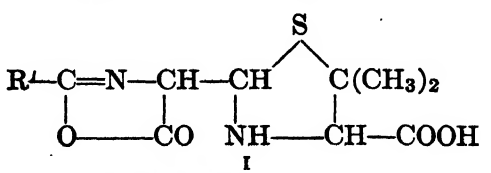
PENICILLIN

Several powerfully antibacterial substances produced by microorganisms have been studied. Two of these, penicillin and streptomycin, have become very valuable in the treatment of a number of diseases. Penicillin was discovered by Fleming in 1929 (1). Dr. A. Fleming, of London University, and Drs. E. Chain and H. W. Florey, of Oxford University, were awarded the 1945 Nobel Prize for physiology and medicine in recognition of their discovery of penicillin and its healing effects. As shown by Fleming in his original publication, penicillin is highly active against Gram-positive bacteria and is active to some extent against certain Gram-negative bacilli, especially *Salmonella enteritidis*.

In 1947 the production of penicillin in the United States was about 20 tons; however, a shortage of the substance still existed in that year.

Chemical Properties

The term "penicillin," as originally used, designated the total antibacterial potency produced by a certain mold. It is now known, owing to the joint efforts of a number of groups of American and British chemists (2, 3), that several unrelated compounds may be produced by the same organism, and the term "penicillin" really means a number of specific substances to which the main antibacterial activity is due. They all have the basic structure, shown in structure I.



I

Proposed general structure for penicillins

At least six penicillins are known, differing only in variations of the R-grouping (Fig. 38). These chemically different penicillins probably also differ in their biological properties. Owing to lack of sufficient material, this problem has not been fully investigated.

It should be noted that experiments involving the production of synthetic penicillin were carried out in several laboratories in which *d*-penicillinamine hydrochloride (structure II) and 2-benzyl-4-meth-

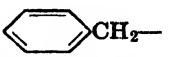
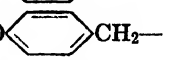
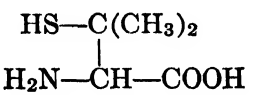
NAME	SOURCE	R—
F	*P. chrysogenum-notatum*	$CH_3CH_2CH{=}CHCH_2$—
Dihydro F	*A. giganteus*	$CH_3CH_2CH_2CH_2CH_2$—
Flavacidin	*A. flavus*	C_5—
G	*P. chrysogenum-notatum*	
X	*P. chrysogenum-notatum*	
K	*P. chrysogenum-notatum*	$CH_3CH_2CH_2CH_2CH_2CH_2CH_2$—

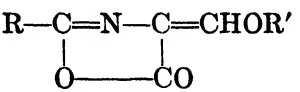
FIG. 38. Types of natural penicillins.

oxymethylene-5(4) oxazolone (see general structure III) were condensed.



II

d-Penicillinamine



III

General structure of oxazolones

Concentrates of this reaction mixture contained only a small fraction of the activity of the natural product. However, du Vigneaud and associates (2) found that, when the condensation was carried out in pyridine-containing triethylamine, followed by heating the final product in pyridine-containing pyridinium chloride, a crystalline compound (triethylammonium salt of benzylpenicillin) was obtained in a very small quantity. This product was in every respect identical with the natural triethylammonium salt of benzylpenicillin. The synthetic mixture, however, contained only 0.1 per cent active substance, and the isolation of the pure compound was very difficult.

The type of penicillin produced by a certain mold depends on the nature of the culturing method. According to Rake and Richardson (3), it is impossible now to give any definite data concerning the relative composition of a given penicillin sample. Surface cultures produce mainly the F type, whereas deep fermentations form predominantly the G type. Commercial products available now contain a mixture of F, K, and G penicillin. The X type is present only in traces in these samples. The original penicillin preparations assayed 50 units per milligram. This activity was gradually increased. The minimum

requirements of the Food and Drug Administration have progressively been raised, and, on September 1, 1945, they were increased from 300 to 500 units per milligram. However, even the best commercial clinical-grade penicillin preparations still contain 25 per cent impurities. Crystalline penicillin G has now been placed on the market by several concerns for clinical use.

The free penicillins are strong acids, having *p*H's close to 2.8. They form salts with various cations. Usually the sodium and calcium salts are prepared. Penicillin and its salts are very soluble in water, acetone, dioxane, ether, esters and in chloroform. The solubility, however, varies with the *p*H's of the solutions. This property permits a convenient isolation of the substance. The free carboxyl group may be esterified without impairment of the basic structure of the compound. The methyl, ethyl, butyl, benzyl, and benzhydryl esters of penicillin have been prepared (4, 5). These esters are insoluble in water but soluble in fats. They are inactive until hydrolyzed. Penicillin decomposes readily at *p*H 8.5 or higher. The basic structure is destroyed by alcohols, such as methyl alcohol, by sulfhydryl compounds, by heat, and by repeated freezing and thawing. It is also destroyed by the enzyme penicillinase, which is produced by many microorganisms (see section on penicillinase in this chapter).

Synthetic Media for Penicillin Production

According to Stone and Farrell (6), who investigated the use of synthetic media for penicillin production at the Pennsylvania State College for the Office of Production Research and Development of the War Production Board, the following media are suitable for commercial preparation of penicillin. These media have been used at several plants for short periods. Mold growth on these is slower than on corn steep media, and the yield is slightly lower. This fact, however, is offset by the greater ease of purification of the resulting product. Stone and Farrell especially recommend these media for research on the production of various penicillins.

Many workers found that it is necessary for the medium to contain phosphate, sulfate, iron, potassium, magnesium, zinc, and about 1 p.p.m. of copper. Most cultures gave best results when the potassium-sodium ratio was greater than 1. Some source of sugar or starch was required. No carbohydrate was found superior to lactose, which has been generally employed, together with corn steep solids, in industrial penicillin production. Some amino or ammonia nitrogen was necessary,

but too high concentrations of either decreased penicillin yield. All the successful synthetic media contained some organic acid, acetic acid giving the best results. It was found that the *p*H must be kept between 6.5 and 8.2.

Surface Growth Conditions. Culture 1259.B21NRRL was grown in 1.5 liter round milk bottles, plugged with cotton, and incubated at 23° to 25° C. in a horizontal position. About 250 cc. of the following basal medium was used:

	Per Cent
Lactose	0.4
Glacial acetic acid	0.5
NH_4NO_3	0.5
KH_2PO_4	0.1
$NaNO_3$	0.5
$MgSO_4 \cdot 7H_2O$	0.025
$FeSO_4 \cdot 7H_2O$	0.02
$ZnSO_4 \cdot 7H_2O$	0.004
$CuSO_4 \cdot 5H_2O$	0.0005

The *p*H was adjusted with potassium hydroxide to 6.1 before sterilization, so that the final acidity was above *p*H 5.6. An equivalent quantity of potassium acetate may be substituted for acetic acid, eliminating the potassium hydroxide. The addition of phenylacetic acid up to 0.4 per cent increased the broth assay from 50 Oxford units to 100 units per cc., while the addition of both 0.4 per cent phenylacetic acid and 0.1 per cent cysteine or cystine gave 120 to 150 units. The chief difficulty in surface culture is that penicillin formation is readily influenced by slight changes in environment. Such changes cause differences in the utilization of sugar, effecting changes in the *p*H of the medium.

Submerged Growth. Cultures X-1612 and NRRL 1951.B25 were used in shake flasks or tanks. In these types of fermentations, employing the above medium, it is important that the *p*H be kept below 8.2 at the end of the first stage of fermentation. For this reason, some free acid may be added. During the second stage, the *p*H must be above 6.5. Acid or alkali may be employed, if necessary.

Shake Flask Culture. The following medium is satisfactory for 1-liter, cotton-stoppered Erlenmeyer flasks, containing from 100 to 150 cc. of medium: 3.0 per cent lactose, 0.6 per cent glacial acetic acid, 0.5 per cent glucose, 0.5 per cent NH_4NO_3, 0.2 per cent KH_2PO_4, 0.05 per cent $MgSO_4 \cdot 7H_2O$, 0.02 per cent $FeSO_4 \cdot 7H_2O$, 0.002 per cent $ZnSO_4 \cdot 7H_2O$, and 0.0005 per cent $CuSO_4 \cdot 5H_2O$. The *p*H is adjusted

to 6.1 with KOH. The shaker should move a distance of 2.75 inches at 90 r.p.m. With culture X-1612, under proper aeration, this medium produces from 100 to 150 units of penicillin per cubic centimeter in 6 to 8 days. The addition of 0.04 per cent phenylacetamide or β-phenylethylamine increases the yield by 50 units per cc.

Stirred Bottle and Tank Culture. In this type of work, 20-liter Pyrex glass bottles mounted in a water bath and stirred with a 5-inch straight-blade stirrer at speeds of 300 to 500 r.p.m. was employed. The air was introduced by means of $\frac{1}{32}$-inch holes drilled in a small-diameter aluminum tube placed across the bottom of the bottle. Twelve liters of medium was used per bottle. The rate of aeration was 1 to 1.5 liters of air per liter of medium per minute. Two per cent octadecanol in lard oil (Swift's Mellocrust) was employed as an antifoam. Culture X-1612 was used. The medium was similar to the one used for shake flasks, with the only exception that 1.5 per cent lactose, 0.4 per cent glacial acetic acid, and 0.35 per cent KNO_3 were also included. In 90 hours, 164 Oxford units of penicillin was produced per cubic centimeter.

Submerged Production. Stefanik and coworkers (7) studied pilot-plant equipment for the submerged production of penicillin and described a 100-gallon tank, air filters, and agitators. With this equipment, penicillin yields of more than 200 units per cc. were obtained with culture 1951-B25(NRRL) and more than 400 units per cc. with culture X-1612 (Carnegie) in 72 hours. An aeration rate of 1 volume of air per minute per volume of culture medium was found optimal. Agitation was necessary. Aluminum and Allegheny metal were nontoxic, whereas iron caused slight toxicity. The paper contains diagrams of the equipment and detailed directions for operating procedures.

Recovery from Medium. Whitmore and collaborators (8) described the carbon process and the solvent process as developed at the Pennsylvania State College. By the carbon process, the penicillin adsorbate is collected in the filter and eluted with aqueous acetone. The acetone eluate is flash-evaporated to yield aqueous penicillin solution. This method gives 80 to 90 per cent recovery and volume reduction 5 to 1. By the second procedure, the penicillin is continuously extracted from the culture by an organic solvent (amyl acetate), from which it is transferred to a buffer solution by a continuous countercurrent extraction procedure.

Berger (9) recommends the following procedure for the isolation of penicillin: The culture fluid is adjusted to *p*H 6.4 with 20 per cent phosphoric acid. Inactive material is precipitated by adding ammo-

nium sulfate to 40 per cent concentration at 0° C. After filtering, 5 volumes of the filtrate are extracted with 1 volume butyl alcohol in a separatory funnel. To the butyl extract is added 1 volume light petroleum ether and ½ volume 2 per cent sodium bicarbonate. After separation, the petroleum ether-butyl alcohol layer is again extracted with bicarbonate. The penicillin in the bicarbonate extracts is further purified. Complete details are given in the article.

All-Electronic Penicillin Drying System. The Radio Corp. of America developed an all-electronic penicillin drying system which is manufactured by the F. J. Stokes Co., Philadelphia. This system contains three separate units: a radio heat bulk-reducer, which completes in 30 minutes a bulk-dehydrating operation requiring 24 hours by existing methods; an electronic vacuum drier, which in 3 minutes reduces 1-cc. quantities of concentrated penicillin solution to a dry film in vials; and vacuum heating chambers, which remove the remainder of moisture from the vials, completing the drying process (for details see reference 10).

Suggestions Concerning Improvements. Foster and associates (11) found that cottonseed meal is at least as good as corn steep liquor for penicillin production by *P. chrysogenum,* strains Demerec X1612 and Wisconsin Q176, and that it is not necessary to add chemical precursors using the cottonseed meal. *P. chrysogenum* required an adaptation to lactose for the most rapid and efficient utilization of lactose. Foster and coworkers (12) observed that active strains of *P. notatum* tend to degenerate or lose their capacity to produce penicillin, especially after continued serial transfer on laboratory media. The degeneration can be eliminated by reducing vegetative transfer. A method for doing this is given by the authors. Zinc catalyzes the rapid oxidation and utilization of glucose by the mold, thus preventing the accumulation of gluconic acid, which is responsible for the fall in *p*H of the medium- and low-penicillin yields. When *P. notatum* is the mold used, cottonseed meal, soybean meal, and corn meal are as good supplements as corn steep liquor, in submerged cultures (13). Backus and coworkers (14) exposed spores from a monoconidial isolate of strain X-1612 of *P. chrysogenum* to ultraviolet irradiation. A new strain, Wisconsin Q176, was obtained, which has consistently surpassed its parent in submerged penicillin production in pilot-plant experiments.

Assays. Foster and Woodruff (15) described complete details of the cup assay for penicillin and included discussions of the principles involved. Higuchi and Peterson (16) published an extensive study concerning the microbiological estimation of types of penicillin in

broths and finished products. Dorfman and collaborators (17) proposed a new physical method. When penicillin interacts with susceptible bacteria, the ζ potential of the mixture rises within the first minutes. The ζ effect of penicillin is a function of its concentration. The method is said to be applicable also to other antibiotics.

Color Tests for Penicillin. DiFonzo (18) published the following color test for penicillin. An aqueous solution of the sodium salt of penicillin is acidified with hydrochloric acid, and cold sodium nitrate solution is added drop by drop. The mixture becomes red-violet and then gives a violet precipitate.

Staab, Ragan, and Binkley (19) described the following colorimetric method: To 1 cc. penicillin solution are added 1 cc. of neutral 6 per cent hydroxylamine solution and 1 cc. of 0.1 *M* 5.4 acetate buffer. After 5 minutes at room temperature, 1 cc. of 0.8 *N* hydrochloric acid and 1 cc. of 10 per cent ferric chloride in 0.1 *N* hydrochloric acid are added. The color is read 5 minutes after ferric chloride addition, using a Klett-Summerson colorimeter and a Klett 54 filter. Readings of ± 5 per cent are easily obtained with 500 to 1500 units of penicillin per cubic centimeter. See also Ford (20).

PENICILLINASE

The penicillin-destroying enzyme penicillinase was first described by Abraham and Chain (21), who prepared it from *Escherichia coli.* Many other investigators obtained this enzyme from various organisms such as bacteria, yeast, and fungi (22). Good sources appear to be aerobic spore-forming bacteria and certain actinomycetes (22). Sodium azide, iodoacetate, and ferrous chloride are strongly inhibitory to penicillinase. Sulfhydryl groups destroy penicillin but activate penicillinase. Benedict and coworkers (23) described a method for the preparation of a very active soluble penicillinase from *Bacillus cereus* NRRLB-569. One milligram of the purified enzyme completely destroyed 165,000 units of crystalline penicillin in 3 hours at *p*H 7.0 and 30° C. Papain, peptidases, polidase, takadiastase, ficin, trypsin, urease, lysozyme, carbonic anhydrase, α- and β-amylase, and emulsin did not destroy penicillin (23). Bondi and Dietz (24) found that 16 of 115 strains of staphylococci examined were resistant to penicillin. The resistant strains produced penicillinase but not the susceptible strains. Foster (25) observed that, when cell-free bacterial penicillinase acted on penicillin in the presence of bicarbonate, carbon dioxide was liberated. This would indicate that a new acidic group is formed

by the action of the enzyme on penicillin. Benedict and associates confirmed this finding. However, the reaction product or products were not identified.

STREPTOMYCIN

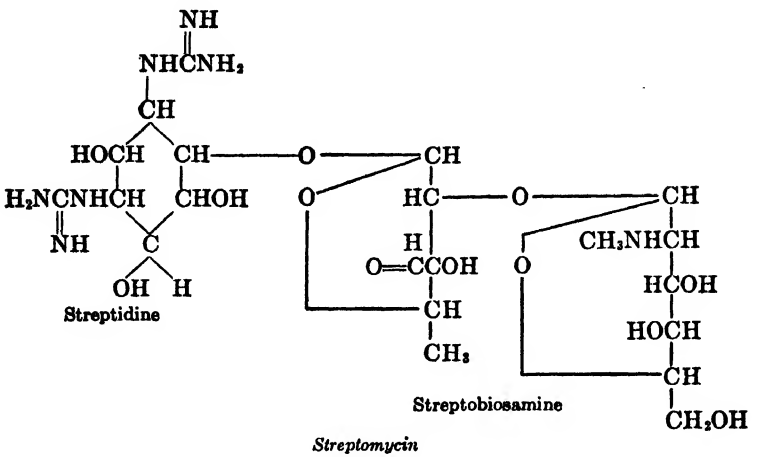
Streptomycin was discovered in 1944 by Waksman (26). This substance is produced by the moldlike soil organism *Actinomyces griseus*, which had been described about 1925 by Waksman. This organism sporulates and produces aerial mycelia. It has the generic name *Streptomyces*. Streptomycin displays great activity against various Gram-negative bacteria (26). Clinically, streptomycin has been found to be valuable in the treatment of several diseases, the most significant of which are certain types of tuberculosis.

Isolation and Chemical Properties (26). Streptomycin is an organic base, soluble in water, but not in ether, chloroform, or acetone. From the cell-free culture filtrate, the substance is prepared by adsorption on active charcoal, such as Norite. The adsorbate is washed with alcohol to remove impurities and then with dilute acid-alcohol to elute the streptomycin. From the clear eluate, the substance is removed by the addition of 10 volumes of ether. The ether removes the alcohol and leaves the streptomycin in the aqueous fraction, from which it is precipitated with acetone. Further purification and crystallization are obtained by precipitation with phosphotungstic acid and by converting the liberated base into the crude picrate. Then the picrate is fractionated by chromatographic procedures. When the picric acid is removed, highly active fractions are obtained. The addition of Reinecke salt yields crystalline precipitates. From the insoluble reineckate, soluble salts, such as the hydrochloride and the sulfate, may be obtained. The addition of methyl orange, the sodium salt of helianthine, results in the crystallization of the slightly soluble streptomycin-helianthate, which can be readily converted to the salts of streptomycin (27).

The Structure of Streptomycin (28, 29)

The acid degradation of streptomycin yielded streptidine and a disaccharide streptobiosamine. Streptidine has been found to be a 1,3-diguanidino-2,4,5,6-tetrahydroxycyclohexane. Degradation of streptomycin with methanol and hydrogen chloride yielded streptidine and a derivative of streptobiosamine. Further hydrolysis of a

streptobiosamine derivative resulted in the isolation of *N*-methyl-*l*-glucosamine. Further work by Kuehl and associates (30) led to the following structure of streptomycin:



Streptomycin

Medium. Waksman and associates (26) employed the following medium for the production of streptomycin:

Glucose	10 grams
Peptone	5 grams
Meat extract	5 grams
NaCl	5 grams
Tap water	1000 cc.
Final *p*H	6.5 to 7.0

Portions of this medium are sterilized and inoculated with streptomycin-forming strains of *S. griseus*, and shaken for 7 days aerobically.

Submerged Pilot-Plant Production. Brook and coworkers (31) produced streptomycin in iron tanks containing 100 liters of the following medium:

Glucose (cerelose)	10 grams
Curbay B-G (soluble butanol fermentation products, U. S. Industrial Chemicals, Inc.)	1 gram
NaCl	5 grams
Na_2PO_4	1 gram
$(NH_4)_2SO_4$	2.5 grams
$MgSO_4 \cdot 7H_2O$	0.25 gram
$CaCO_3$ (sterilized separately)	3.5 grams
Tap water to make 1 liter.	

The culture medium is sterilized, cooled, and seeded with a 5 per cent volume of a 48-hour vegetative inoculum. The following extraction method was employed for the isolation of the sulfate.

One hundred liters of culture liquor assaying 120 units per cc. is acidified with sulfuric acid to *p*H 2 and stirred for 30 minutes with 250 grams of Nucher C-190-N. The mixture is filtered on a filter press, using 2000 grams of Celite 545, and the cake is washed with 10 liters of water. The filtrates and washings are adjusted with sodium hydroxide to *p*H 7.0 and stirred with 1000 grams of Darco G-60 for 30 minutes. The carbon containing the streptomycin is collected on a filter press, using 500 grams of Celite, and is washed twice with 10 liters of water. The streptomycin is eluted from the carbon by extracting three times with 3.5 liters of 5 to 10 per cent acetone. Then it is acidified and kept at *p*H 2.5 with sulfuric acid. The combined eluates are stirred with 3 volumes (28.5 liters) of acetone, which precipitates the streptomycin sulfate. After being kept overnight at 40° C. the precipitate is collected by decanting and filtering. The precipitate is dissolved in 250 cc. pyrogen-free distilled water, and the insoluble impurities are removed by filtration. The filtrate is adjusted to *p*H 7.0 with sodium hydroxide. The insoluble material is discarded.

Brook and associates (31) clarified this solution of streptomycin sulfate, then froze it and dried it in vacuum at 200 to 600 μ. The yield was 12 grams of a white powder assaying 600 units per milligram. This represented an over-all recovery of 7.2 million units, or 60 per cent. Their paper contains numerous useful data concerning the purification of streptomycin.

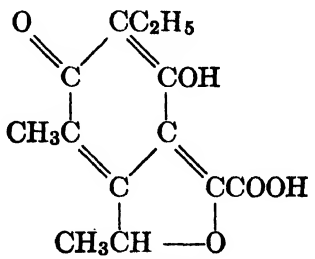
Assays. Waksman and Reilly (32) developed an agar-streak method for assaying streptomycin and other antibiotic substances, and Loo and coworkers (33) described an assay of streptomycin by the paper-disc plate method.

A Color Test for Streptomycin. Sullivan and Hilmer (34) found that aqueous solutions of streptomycin react with oxidized nitroprusside giving a striking red color in very dilute solution. Streptothrycine does not give this reaction.

CITRININ

In 1931, Coyne and coworkers (35) isolated the pigment citrinin from a culture of *Penicillium citrinum* Thom and described its prop-

erties, its derivatives, and its breakdown products. They suggested the following structural formula for this compound:



Citrinin

Method for the Preparation of Citrinin. Raistrick and Smith (36) recommended the following Czapeck-Dox solution for the production of citrinin:

$NaNO_3$	2	grams
KH_2PO_4	1	gram
KCl	0.5	gram
$MgSO_4 \cdot 7H_2O$	0.5	gram
$FeSO_4 \cdot 7H_2O$	0.01	gram
Glucose	50	grams
Distilled water 1 liter		

This medium is placed in 350-cc. portions in liter flasks, plugged with cotton, sterilized, inoculated with *Penicillium citrinum,* and incubated at 24° C. for 14 days. Tauber, Laufer, and Goll (37) obtained the citrinin from the yellow solution by adjusting the clear filtrate (1500 cc.) to *p*H 3.0 to 2.5 with *N* hydrochloric acid. After 5 minutes, the crystals of crude citrinin are centrifuged off and dissolved in a small volume of dioxan (20 to 30 cc.) at room temperature. The solution is centrifuged, and the small amount of insoluble material is discarded. To the clear supernatant fluid, 1 volume of distilled water is added. Citrinin crystallizes out immediately. The citrinin is centrifuged off and dried *in vacuo* at room temperature. The yield is 1.5 grams. The melting point is between 163° and 166° C. Ethyl alcohol may also be used for the recrystallization of the citrinin (36). This method is slower, however. Tauber, Laufer, and Goll (37) confirmed the action of citrinin on *Streptococcus aureus* as reported by Raistrick and associates.

Some Properties of Citrinin. In contrast to penicillin, citrinin is a very stable compound, and its method of preparation is simple. The yield of citrinin is very much higher than that of penicillin.

Penicillin is much more active, however. Citrinin gives the phenol color test with ferric chloride.

A Color Test for Citrinin. Tauber, Laufer, and Goll (37) described the following test for citrinin. To 1 milligram of citrinin dissolved in 0.5 cc. of 95 per cent alcohol, 0.3 cc. of 3 per cent hydrogen peroxide is added. The mixture is agitated for 1 minute. The intense yellow solution becomes first colorless and then light brown. Then 0.3 cc. of 0.2 *N* sodium hydroxide is added. A wine-red color forms at once. On the addition of 0.3 cc. of 0.2 *N* sulfuric acid, the wine-red color turns orange-yellow, and on the addition of another 0.3 cc. of 0.2 *N* sodium hydroxide the wine-red color reappears. In a control tube containing water instead of hydrogen peroxide, an orange-yellow color develops. Citrinin solutions that had been treated with sodium hydroxide and readjusted to the original *p*H did not give the hydrogen peroxide-sodium hydroxide reaction. Alcoholic citrinin solutions continuously exposed to air undergo certain changes which are not identical with the reaction just described. Continued exposure of citrinin to solutions of dioxane results in a hydrogen peroxidelike reaction.

This reaction is not given by penicillin. Anthocyanins and related natural pigments, however, and polyphenols containing phenolic groups in positions 3 and 5 also give typical colored oxidation products under similar conditions (38).

Citrinin in *Aspergillus* sp. Timonin (39) reported that one culture of *Aspergillus* sp. of the *Candidus* group had antibacterial properties similar to those of citrinin.

The Toxicity of Citrinin. Timonin and Rouatt (40) reported that 2 milligrams of citrinin proved to be lethal to 20-gram mice, whereas 50 milligrams per kilogram body weight in rats resulted in no ill effects to the animals. Ambrose and DeEds (41) have extensively studied the acute and subacute toxicity of citrinin, using rats, guinea pigs, and rabbits in their experiments. They concluded that, owing to the tissue changes produced and the fact that the administration of citrinin may result in delayed deaths up to 14 days, a statement concerning an LD_{50} dose would be misleading.

Therapeutic Properties of Citrinin in Surface Infections. Though citrinin was found to be toxic when used internally in animal experiments, Wang and coworkers (42) reported very encouraging results when citrinin was employed on rabbits and human beings having surface infections. Patients were those with staphylococcal and streptococcal local infections. Citrinin-sodium bicarbonate powder was applied locally to the infected region, and the indurated base was infiltrated with a 1 per cent solution of sodium citrinin. This was followed

by dressing with citrinin-soaked gauze. The results according to the authors were "dramatic." "The infected area dried up in 6 to 18 hours, the crust formation being followed by rapid healing."

EFFECTS OF AN ANTIBIOTIC FROM *Aspergillus fumigatus* ON TUMOR CELLS *in Vitro*

Kidd (43) has made the remarkable observation that *A. fumigatus* Fresenius produces an antibiotic that after a few minutes' contact renders the cells of various animal tumors incapable of further prolification *in vitro*. The antibiotic has been prepared in crystalline form. It resembles gliotoxin chemically and in its high activity against tumor cells *in vitro*. However, the two substances do not appear to be identical.

The mold was cultivated on the surface of a modified Czapek-Dox medium for 7 to 12 days at 28 to 30° C. The *p*H which was generally 3.5 to 4.5 was adjusted to 7.4 with sodium hydroxide. The culture filtrate was diluted with buffered Ringer's solution (*p*H 7.4) containing 250 milligrams per cent glucose. The tumor types employed were a lymphosarcoma of C_3H mice (Gardner tumor), a sarcoma (RSI), and the Brown-Pearce carcinoma of rabbits. Dilutions of culture filtrates of *A. fumigatus* from 1:10 to 1:80 prevented entirely the growth of tumor cells upon implantation of the mixture in suitable situations in susceptible hosts.

ANTIBIOTICS PRODUCED BY AEROBIC SPORE-FORMING BACTERIA

Several antibiotics that are produced by aerobic spore-forming bacteria have been described in the literature. Most of these antibiotics are very toxic when administered internally. Some of them, however, may become important aids in the treatment of surface infections. Thus, general use is being made of tyrothricin in Band-aids and in preparations for the treatment of mastitis.

TYROTHRICIN, GRAMICIDIN, AND TYROCIDIN

These antibiotics are produced by *Bacillus brevis* B.G. Peptone cultures of the bacillus are adjusted to *p*H 4.8. The precipitate which forms is extracted with ethyl alcohol. The alcohol-soluble fraction is called "tyrothricin." On the addition of sodium chloride, a precipitate forms. This may be separated into two components by

extraction with a mixture of acetone and ether. The soluble fraction consists of gramicidin and may be crystallized from acetone. The insoluble fraction contains tyrocidin. Both substances have antibiotic properties. It is believed that tyrothricin contains from 10 to 20 per cent gramicidin and 40 to 60 per cent tyrocidin-HCl. *B. brevis* B.G. produces about 0.5 gram tyrothricin per liter of medium.

Gramicidin is effective against Gram-positive forms. It is toxic on intraperitoneal and intravascular injection. It is stable in alcoholic solution but not when dissolved in water or when its solutions are heated.

Tyrocidin is active *in vitro* against Gram-positive coccal forms and some Gram-negative organisms. It is toxic when injected intravenously and intraperitoneally. This antibiotic is stable in alcoholic solution even at high temperature.

Gramicidin and tyrocidin are neutral compounds containing carbon, hydrogen, nitrogen, and oxygen. Acid hydrolysis indicates that both are polypeptides. Trypsin, pepsin, papain, bacterial proteinase, and "erepsin" do not destroy the biological potency of these compounds, nor is any increase in free amino or carboxyl groups noticeable. Apparently the structure of the antibiotics is different from the usual linkages found in proteins and their products of hydrolysis. Tyrothricin, gramicidin, and tyrocidin have been discussed in a very interesting review by Hotchkiss (44); this article contains 171 references.

SUBTILIN

Considerable interest has been shown in the antibiotics produced by various strains of *Bacillus subtilis*. Some strains of this bacillus produce an antibiotic of peptide nature which is called subtilin. It is being studied at the Western Regional Laboratory of the U. S. Department of Agriculture and at the University of California. This antibiotic protects mice against *D. pneumoneae* (III), *Strep. pyogenes, Staph. aureus;* it protects guinea pigs against *B. anthracis;* and it has a suppressive effect on *M. tuberculosis* in guinea pigs. Subtilin is not toxic to animals. A detailed description concerning the production and properties of subtilin may be found in reference 45.

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CHAPTER XXII

THE PRODUCTION OF ENZYMES USING MICROORGANISMS

THE PRODUCTION OF ENZYMES FROM BACTERIA

Boidin and Effront (1, 2) were among the first to produce enzymes from bacteria on a commercial scale for the industries. They used strains from the groups *Bacterium subtilis* and *B. mesentericus*.

Contrary to the general belief bacteria do not secrete the maximum amount of amylase when placed in a starch-rich medium. Boidin and Effront (1) claim that maximum liquefying activity was developed by repeated subculturing in media rich in nitrogenous substances. These authors used soybean cake and peanut cake with added salts as the medium. For best yields the bacteria must be grown in thin layers to allow the formation of a film. Strong aeration at the beginning of growth is essential.

Boidin and Effront (1) developed a special culture apparatus suitable for industrial use. To permit the formation of a film the apparatus is equipped with a large number of trays. Provision is made for the control of temperature and aeration. For each cycle of sterilization and propagation a routine of cleansing and sterilization is applied. Continuous internal pressure is maintained to eliminate contamination. One culture apparatus holds 500 to 1000 gallons of medium.

Figure 39 shows the culture vessel of Boidin and Effront as improved by Wallerstein (3).

Large-Scale Preparation of Bacterial Diastase in Shallow Culture

Schultz and Átkin (4) described the following large-scale method for the preparation of bacterial diastase. *Bacillus mesentericus* is grown on a medium consisting of 1 part of soy meal (or cotton seed) in 5 parts of water to which sulfuric acid is added in an amount equal to 5 per cent by weight of the soy meal. The mixture is heated for 4 hours at 20 pounds of steam pressure. The mash is adjusted to *p*H 6.5 to 7.0 while hot. The hot filtrate is placed in shallow containers and sterilized in flowing steam for 3 minutes on two successive

days. The sterilization is carried out by keeping the *p*H always at 5.85. This wort is inoculated with a "pony" of a 48-hour growth of the organism of the type of *Bacillus mesentericus*. The "pony" is added to an extent of 5 per cent of the final volume of the medium. The medium is of a depth of 0.5 cm. and the temperature is 30° C. Care is taken that a sufficient amount of oxygen is supplied. Under

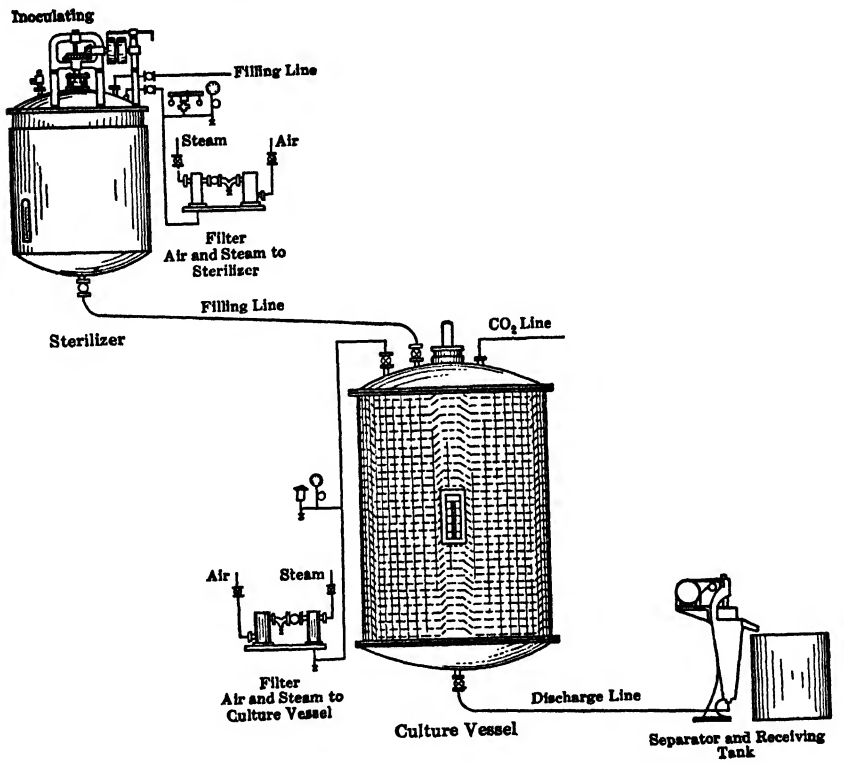

FIG. 39. Diagram of culture method for bacterial enzyme production.

these conditions diastase concentration is maximum after 72 hours of incubation. The liquid when separated from the bacteria has high diastatic activity. The optimum *p*H for starch-liquefying power is 5.85.

Large-Scale Preparation of Bacterial Diastase in Submerged Culture

Imshenetskii and Solntseva (5) employ thermophilic bacteria and a potato medium for the large-scale production of diastase. The organism used is *B. diastaticus*. To each 100 liters of water, 5 kilo-

grams of chopped potatoes and 10 grams of chalk are added. The mixture is sterilized in the autoclave for 30 minutes at 22 to 75 pounds of pressure. Then the mixture is cooled to 65° C., and the liquid portion is decanted for use as the medium. Sulfur- and chlorine-free water is recommended. The culture vessels should be of aluminum, or tinned or enameled iron. The standard strain of *B. diastaticus* can be kept in a medium containing 0.5 gram of peptone, 0.1 gram of chalk, 2 grams of agar, and 100 cc. of 20 per cent potato infusion, provided that transfers are made every 20 to 25 days. Good aeration of the culture is important for efficient yields. The optimum temperature for growth is at 60° C. The amylase (superbiolase) accumulates directly in the culture vessel and, when tapped, must be kept at 2° to 4° C. In order to obtain a dry product the filtrate is evaporated in vacuum at 60° C., or it is precipitated with ammonium sulfate (60 kilograms per 100 liters of culture) and the precipitate is dried at 70° C. The yield from 100 liters of culture is 400 to 500 grams of dry amylase. Drying and storage have no effect on the activity of the preparation. However, products obtained by ammonium sulfate precipitation contain large amounts of this salt.

Beckord (6) and coworkers described a method for the production of bacterial amylase from thin stillage, an alcohol fermentation by-product. The organism employed was *Bacillus subtilis*. Total amylase formation was the greatest when the liquid layer of the medium was 2.5 to 3.8 cm. and the temperature was 37° C. Shaking did not increase the amylase yield. Application of the drip method, in which thin stillage trickled over wood chips covered with pellicle growth, resulted in amylase formation after a short incubation period. The thin stillage, which had an original *p*H at about 4.0, was adjusted to 7 to 8.

Bacterial Proteinase Production

Kline and associates (7) suggest the use of the press juice from waste asparagus butts and trimmings as a constituent of industrial microbiological media, for the production of bacterial proteinase. The activity of the culture filtrates was comparable to that obtained in commercial procedures. Major raw materials, according to the authors, may be replaced by asparagus-butt juice. Contrary to the concept of enzyme "adaptation," the medium does not have to be rich in protein or protein-derived nitrogen and poor in carbohydrate for the formation of high yields of proteinases. A synthetic medium, high in carbohydrate, containing citric acid, and having more than 98 per cent of its nitrogen in the inorganic form, produced nearly as

much proteinase as a medium containing protein-derived nitrogen exclusively. The organisms used were *Bacillus brevis* and *B. subtilis scaber*, respectively. The bacteria were cultivated in Erlenmeyer flasks of 250-cc. capacity, each containing 50 cc. of medium. For efficient enzyme production it was necessary to supplement the glucose content of the asparagus-butt press juice to meet a 6 per cent glucose level and to adjust its *p*H to 6.8. In 3 to 4 days at 35° C., enzyme formation was at a maximum on synthetic as well as natural media. Table XLIV shows proteinase production by *B. subtilis scaber* grown on various media. The symbol B.P.U. $\frac{Hb}{ml.}$ indicates bacterial proteolytic units per milliliter as determined by the hemoglobin method and B.P.U. $\frac{M}{ml.}$ means units per milliliter as determined by the milk clot method.

TABLE XLIV

PROTEINASE PRODUCTION BY *B. subtilis scaber* GROWN ON VARIOUS MEDIA

Medium*	$[B.P.U.]^{Hb}_{ml.} \times 10^4$	$[B.P.U.]^{M}_{ml.}$
Asparagus-butt juice†	99	6.1
Soybean meal hydrolyzate‡	101	6.0
Difco bacto-tryptone	82	5.9
$(NH_4)_2SO_4$ + citric acid + bacto tryptone§	83	5.6
$(NH_4)_2SO_4$ + citric acid + glutamic acid§	81	4.0
$(NH_4)_2SO_4$ + citric acid§	69	3.0
l-(+) glutamic acid	15	...
l-(+) aspartic acid	9	...
l-(+) asparagine	5	...
Glycine	0.3	...
Sodium nitrate	2	...
Ammonium sulfate	0.0	...

* All media contained 1.20 mg. N/ml., 0.28 mg. Ca/ml., 6% glucose; with the exception of asparagus-butt juice, they were supplemented with mineral nutrients (0.005 *M* $MgSO_4$, 0.007 *M* $CaCl_2$, 0.006 *M* KH_2PO_4, 1 p.p.m. Fe, 1 p.p.m. Mn, 0.2 p.p.m. Zn, 0.1 p.p.m. Cu, and NaOH to *p*H 6.8) and growth factors (inositol 5.0 mg./liter, pyridoxine·HCl 0.5, thiamine Cl·HCl 0.5, nicotinic acid 0.5, riboflavin 0.2, Ca pantothenate 0.1, *p*-aminobenzoic acid 0.01, and biotin 0.001).

† Results are averages obtained from 9 different juices.

‡ A commercial, solvent-extracted, soybean meal hydrolyzed with a commercial trypsin.

§ 0.6% citric acid used. Where organic N compounds were used in conjunction with $(NH_4)_2SO_4$ and citric acid, relative amounts were 1.18 mg. ammonia N and 0.02 mg. organic N per ml.

The enzyme may be obtained in dry form by precipitation with 2 volumes of cold methyl alcohol followed by vacuum drying. This product contains 83 per cent of the original activity. Consecutive precipitation with two-thirds saturated ammonium sulfate and cold methyl alcohol and drying in vacuum yields a product as active as the crystalline proteinases.

Ramon and collaborators (8) reported the production of a very active proteinase using a medium containing 80 grams of bran, 4 grams of yeast, and 6 grams of dried malt extract per liter. Growing

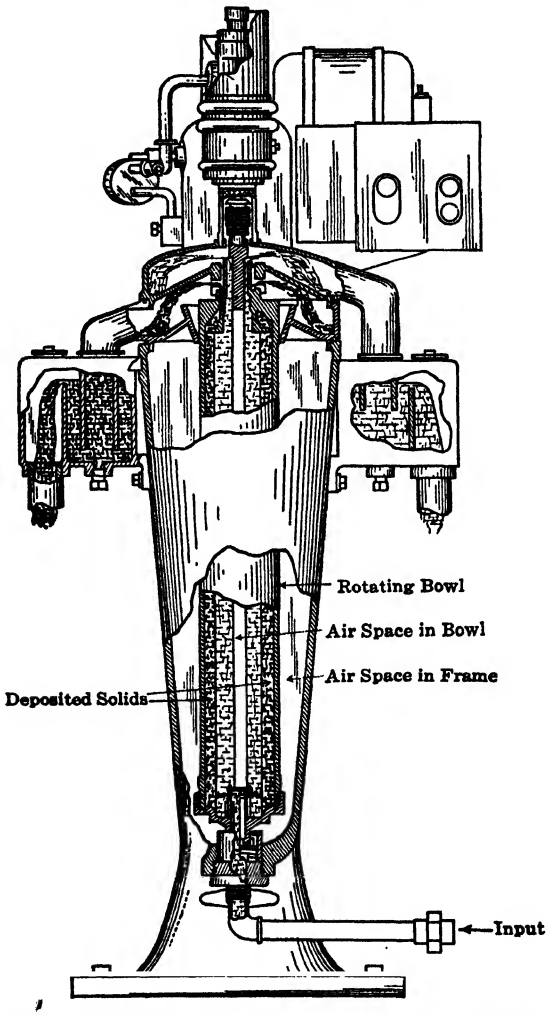

FIG. 40. In this Sharples Super-Centrifuge the material to be centrifuged enters the machine at the bottom; the liquid passes continuously through the bowl. The solids precipitate along the wall of the bowl.

in this medium, *B. subtilis* produced as much as 10,000 gelatinolytic units per cubic centimeter. Cultivation on ordinary horsemeat broth yielded only 20 to 100 units of the proteinase. Here, a unit is the least amount of culture filtrate which will liquefy 1 cc. of 3 per cent gelatin in 4 hours at 45° C.

Processing of Enzyme Solutions. As soon as it is found that the enzyme content of the liquid no longer increases, the liquid is removed from the apparatus, cooled, and passed through a Sharples Super-Centrifuge (Fig. 40). Centrifuging removes most of the bacteria from the liquid. The enzyme solution may now be stored at low temperatures and preserved with a suitable antiseptic, or it may be filtered through a filter press and stored in sterile containers. If desired the enzyme solution may be concentrated *in vacuo*.

For purification the salting-out method or precipitation with organic solvents such as alcohol or acetone may be employed.

Concentration of Enzymes by Dialysis. Youngburgh (9) found that by dialyzing enzyme solutions against a concentrated dextrin solution the enzyme solution may be concentrated to $\frac{1}{10}$ to $\frac{1}{50}$ of its original volume within 4 to 48 hours. The dialysis may be carried out in a refrigerator.

THE PRODUCTION OF ENZYMES FROM MOLDS

For the production of enzymes from molds the selected organism is grown in trays or in cabinet incubators (Fig. 41) (10, 11) or in rotating drums similar to those used in the production of gluconic acid. In the production of enzymes from molds solid media must be employed, however.

Qualitatively and quantitatively the enzyme content of even related molds differs considerably. Typical examples are given in Tables XLV and XLVI. As may be seen molds contain a great number of

TABLE XLV

THE ENZYME SYSTEMS OF TWO IMPORTANT MOLDS

Aspergillus oryzae		*Aspergillus niger*	
α-Amylase	Rennin	α-Amylase	Rennin
β-Amylase	Lipase	β-Amylase	Lipase
Maltase	Amidase	Maltase	Amidase
Lactase	Cellulase	Lactase	Cellulase
Pentosanase	Cytase	Trehalase	Glucose oxidase
Pectinase	Phytase	Tannase	Glucose dehydrogenase
Trehalase	Nucleases	Catalase	" Zymase " (trace)
Tannase	Sulfatase	Proteinase	Urease
Catalase	Pyrophosphatase	Polypeptidase	Inulase
Proteinase	Phosphodiasterase	Dipeptidase	Melibiase
Polypeptidase	Phosphomonoesterase		
Dipeptidase	(acid phosphatase)		

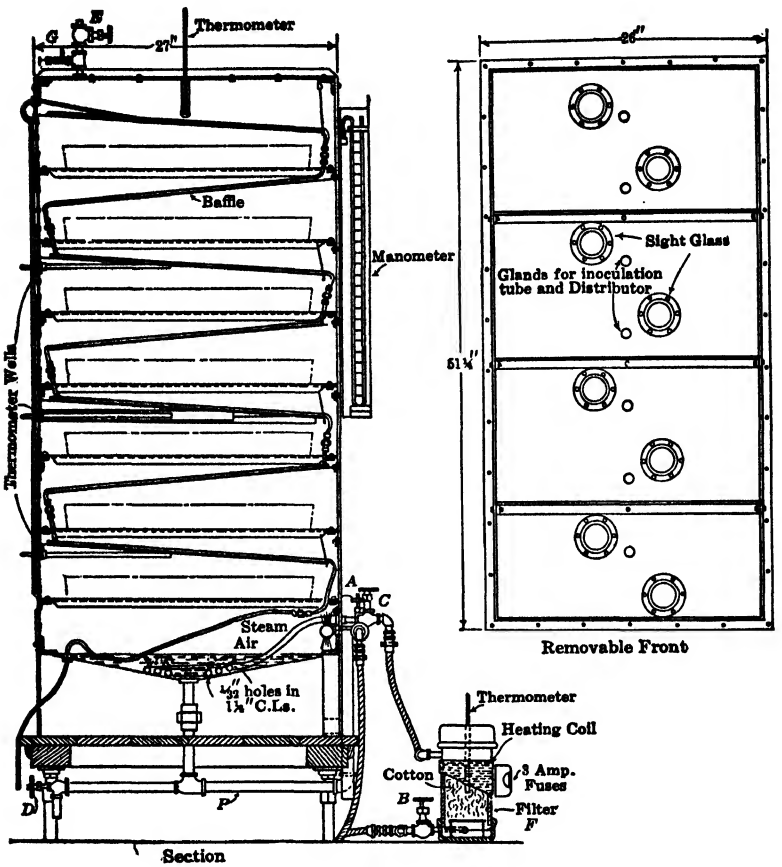

FIG. 41. Cabinet incubator.

TABLE XLVI

VARIATIONS IN THE QUANTITY OF DIASTASE AND PROTEASE PRODUCED BY A NUMBER OF MOLDS (11)

Name of Mold	Culture No.	Diastase Value	Protease Value
Penicillium roqueforti Thom	18	15	6
Aspergillus parasiticus Speare	3509	25	130
Aspergillus tamarii Kita	4235.X.I.	21	112
Aspergillus effusus type	APb	13	260
Aspergillus oryzae (Ahlb) Cohn	113	100	8*
Aspergillus flavus Link	108	25	56
Aspergillus ochraceous	AO5b	305	208
Aspergillus ochraceous	AOK	40	200

* Before sporing.

enzymes. It is interesting to note that peroxidase is not present in molds.

For the production of diastase the mold *Aspergillus oryzae* is most often employed. This organism plays an important role in the national economy of oriental countries. There, many foods and a number of beverages require the use of this mold. *A. oryzae* is to the Orient what malt is to the occidental countries.

Carbon and Nitrogen as Sources. The following substances were found to be good carbon sources for *A. oryzae:* various carbohydrates such as polysaccharides, hexoses, pentoses, and trioses, glycerol, erythritol, adonitol, mannitol, sorbitol, dulcitol, styracitol, quercitol, ethyl alcohol, succinic acid, azelaic acid, sebacic acid, pyroracemic acid, lactic acid, malic acid, citric acid, tartaric acid, gluconic acid, saccharic acid, mucic acid, quinic acid, salicylic acid, gallic acid, kojic acid, glycine, *l*-alanine, *l*-serine, *l*-valine, *l*-leucine, *l*-isoleucine, *l*-aspartic acid, *l*-glutamic acid, *l*-phenylalanine, *l*-proline, *l*-histidine, *l*-tryptophane, and *l*-arginine (12).

Aspergillus is able to utilize 127 different inorganic and organic substances as nitrogen sources, ranging from ammonium salts and nitrates to purines and pyrimidines. Some organic compounds function simultaneously as the nitrogen and the carbon source (hippuric acid, uric acid, and a variety of amino acids) (12).

The molds require small quantities of inorganic salts for growth. Most of them thrive well on Czapek's solution. See Table XLVII.

TABLE XLVII

CZAPEK'S SOLUTION AGAR (15)

(Modification not affecting *Aspergilli* and most molds)

Water	1000	cc.
Sodium nitrate	2.0	grams
Potassium phosphate (K_2HPO_4)	1.0	gram
Magnesium sulfate	0.5	gram
Potassium chloride	0.5	gram
Ferrous sulfate	0.01	gram or trace
Sucrose	30.0	grams
Agar-agar	15.0	grams

Growing *Aspergillus oryzae* for Enzyme Production (13, 14). Wheat bran or other solid medium is moistened to contain 60 per cent water, then is steamed for 1 hour to sterilize and gelatinize the starch. After cooling to 40° C., a small quantity of the spores of the mold is mixed with it. Now the bran is placed in trays having wood or metal frames provided with false-bottom wire nets. Thus air may be ob-

tained from top to bottom. The trays are placed on racks one above the other and 2 inches apart. The temperature of the room should be at about 30° C. at first. This temperature may be attained by opening steam jets into the space, which also keeps the room moist. After about 18 hours the mold multiplies and there is a gradual rise in temperature in the room. Now the steam is turned off gradually. After 24 hours, growth is so vigorous that the room must be cooled by a fresh cool-air draft. The room must be kept moist. The temperature in the room may rise as high as 42° C. This is not harmful if water is added occasionally to the cultures. Optimum temperature is between 30° and 35° C. The room should be kept at this limit. Growth is complete after 30 to 60 hours.

For the production of other enzymes see the first part of this book.

Plant-Scale Production of Moldy Bran in Shallow Trays

Apparently, the rotating drum procedure is not suitable for the large-scale production of moldy bran. According to Underkofler and collaborators (16, 17), the Mold Bran Co. at Eagle Grove, Iowa, produced, in 1946, 10 tons of moldy bran per day, employing shallow trays. Wheat bran is mixed with dilute hydrochloric acid, and the mixture is heated for 30 minutes at 100° C. by direct steam injection while being continuously agitated. The mixture is cooled to 32° C. by a stream of air, and spores of *Aspergillus oryzae* are mixed with the bran. The mixture now contains about 50 per cent water, has been sterilized, and has a *p*H of 3.5. The inoculated bran is spread out on swinging trays in an incubator room. Humidified air at controlled temperature is passed over and under the trays until maximum growth takes place, which requires about 36 hours. The product is then dropped from the trays and is air dried to a moisture content of about 12 per cent. The dried moldy bran keeps for long periods, but the moist product decomposes readily. Figure 42 (18) shows the production of moldy bran at Mold Bran Co. At that grain

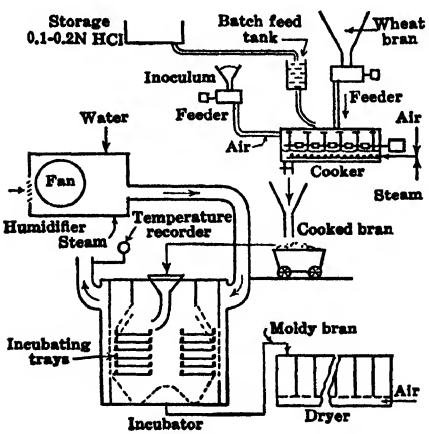

FIG. 42. Flow diagram for production of moldy bran (a culture of *Aspergillus oryzae* on wheat bran) at Eagle Grove, Iowa.

alcohol plant, it was found that the use of 2000 pounds of moldy bran per day instead of 7200 pounds of malt in plant yeast culture mashes resulted in better and more vigorous cultures and in considerable time-saving. Four pounds of moldy bran per 100 pounds of grain in the mash resulted in the same saccharification as 10 pounds of barley malt usually employed. Yields of alcohol were about 2 per cent higher. Saccharification and fermentation in the 150,000-gallon fermenters in the alcohol plant was completed in about 36 hours with mold bran as against 48 hours with barley malt (16–18).

Moldy Bran Supplement to Poultry Feeds. Hastings (19) reported a net gain of 6 ounces and a saving of 0.5 pound of feed per pound of

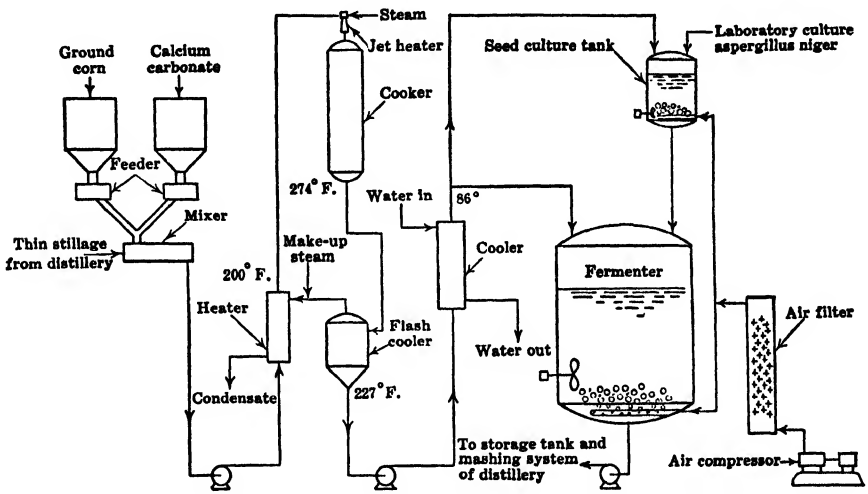

FIG. 43. Mold amylase liquor flow sheet. Northern Regional Research Laboratory.

chicken with 2.5 per cent moldy bran added to high-fiber mash. No advantage was observed by employing 2.5 per cent moldy bran with a low-fiber mash, or a high-fiber mash with 1 per cent moldy bran. It is suggested that the moldy bran contains fiber-splitting enzymes which enable the chicken to utilize some nutrients from the fiber. It is well known that mold enzyme products contain powerful cellulases and related enzymes.

Production of Mold Amylase by Submerged Culture

The strain *Aspergillus niger* of the Northern Regional Research Laboratory No. NRRL 337, when grown on thin stillage, produces appreciable quantities of amylase, useful in the conversion of grain mashes. LeMense and associates (20) propagated mold cultures with

continuous aeration and agitation using a medium composed of thin stillage, 1 per cent corn meal, and 0.5 per cent calcium carbonate. Amylase formation was complete in approximately 60 hours. The enzyme solution so obtained was then used to saccharify grain mashes which had been cooked under pressure and cooled to 59–60° C. The alcohol yields were comparable to those obtained with malt when mold-culture liquor equivalent to 6 to 10 per cent of the final mash volume was employed. As many as five consecutive fermentations were conducted satisfactorily with the stillage from the preceding fermentation as the medium on which to grow the mold. The price of this amylase is said to be considerably less than that of malt. Figure 43 is a diagram for the production of mold amylase by submerged culture.

Soluble Mold Enzymes

Dry soluble mold enzymes may be obtained by percolating the moldy bran with water and by adding alcohol to the aqueous extract to obtain an alcohol concentration of 70 per cent. The alcohol precipitate is dried *in vacuo* at 30° C. or in a blast of warm air. The alcohol precipitate contains 15 per cent ash.

For the preservation of the mold extracts chloroform, toluene, phenol, thymol, or sodium flouride may be used. A 20 per cent sodium chloride concentration is also satisfactory (21).

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CHAPTER XXIII

ENZYMES FOR MEDICINAL USE

A number of enzymes have been used therapeutically for many years. Some work, however, has not progressed beyond the experimental phase as yet.

Emmerich and associates (1) suggested as early as 1902 that bacterial enzymes be used clinically. These investigators found that filtrates of *Bacillus pyocyaenus* digested staphylococci and *Bacillus diphtheriae in vitro* and had favorably affected experimental anthrax infections in animals. They thought that the enzyme nuclease was the active principle.

In 1931 Avery and Dubos (2) showed that certain bacterial enzymes were capable of decapsulating type 3 pneumococci both *in vitro* and *in vivo*. These enzymes (polysaccharidases) were found to be neither bacteriolytic nor bactericidal. They decompose the capsular matter of the pneumococci by hydrolysis, thereby exposing the bacteria to the action of the cells of the infected host (3). The bacterial enzymes protected mice against very large lethal doses of type 3 infection. Tyrosinase and amine oxidase preparations had been found to lower blood pressure.

Some enzymes, however, are very toxic. Intravenous injections of urease into animals, for instance, results in death from ammonia poisoning within a few minutes. A tolerance is acquired by the animals, however, if quantities below the lethal dose are injected first (4).

Therapeutic Use of Proteolytic Enzymes

Enzyme preparations such as pancreatin, pepsin, papain, bromelin, malt diastase, and mold enzymes have been successfully used in digestive diseases and other disorders for many years (5–7). (See also Chapter I.) In China *Aspergilli koji* has been employed for generations for the treatment of gastric disturbances. In 1912 Wohlgemuth (8) tested the tryptic action of taka-diastase and found that 1 gram of it equaled 100 cc. of human pancreatic juice. Owing to its high proteolytic power he recommended it in gastrointestinal deficiencies. Beazell (9) has reported that the amylase of taka-diastase was very effective in salivary deficiencies.

Nemetz (10) reported favorable clinical results with a new enzyme product marketed by " Norgine A. G. Prague " under the trade name Enzypan. This product contains an outer shell that dissolves in the stomach, releasing gastric enzymes, while the pancreatic enzymes trypsin, lipase, and amylase are incorporated in the inner shell of the tablet, which releases its content into the duodenum.

Kaufmann (11) stated that the most important factors in enzyme therapy of gastrointestinal disturbances are adequate enzyme activity of the preparation, the dosage, and, in pancreatic preparations, sufficient acid protection of the trypsin. He tested ten German products, and only Enzypan filled these requirements adequately, from both the chemical and the clinical points of view.

Beguet (12) reported that he treated 522 cases of enteritis successfully with lactic enzyme cultures. Similar results were reported by Zanca (13) on 30 cases of gastric enteritis.

Enzymes and Experimental Pancreatic Achylia. Beazell, Schmidt, and Ivy (14) produced absolute pancreatic achylia by separating the pancreas from the duodenum in six dogs. The dogs were fed a high-starch diet (62 per cent). The experimental achylia resulted in 18 to 39 per cent of starch excretion in the dogs' feces. Administration of diastatic enzymes in large amounts showed a very definite reduction in starch loss. The vegetable diastases taka-diastase, malt diastase, and Ovaltine were more effective than pancreatin, probably owing to the pancreatin's being more sensitive to the gastric acidity than the vegetable enzymes. Enteric coating improved the pancreatin, which was then as effective as the vegetable enzymes. Some of the enzyme activity, however, was lost because of the coating. The doses given were: 25 grams taka-diastase; 25 grams malt extract; 50 grams Ovaltine; 25 grams pancreatin; 25 grams enteric-coated pancreatin.

Schmidt and associates (15) found, in experiments using seven dogs with pancreatic achylia, that 25 grams per day of pancreatin (two times U.S.P. potency) in the form of enteric-coated tablets was very effective in reducing nitrogen and fat loss in the dogs' feces.

Treatment of Achylia Pancreatica with Pancreatin. Beazell, Schmidt, and Ivy (16) studied each of four cases of achylia pancreatica with pancreatin as the therapeutic agent. Diagnosis was established by proving the absence of pancreatic enzymes in the duodenal drainage, an excess of fat and nitrogen in the feces, and an increased absorption of fat and nitrogen when enzyme therapy was applied.

The enzyme therapy (oral administration of pancreatin) decreased the fat content in the feces by an average of 63.3 per cent and the nitrogen content by an average of 62 per cent. Beazell and associates re-

ported that: "Two of the patients who had been reduced to a state of semi-invalidism by loss of weight and inanition were able to resume their normal activities within a few weeks after treatment was instituted. The response of the other two patents was equally satisfactory but less dramatic, because the initial symptoms were less severe."

The pancreatin used by these investigators was somewhat better than the usual commercial U.S.P. products. The dose was 24 grams of pancreatin per day in the form of enteric-coated tablets. Their observation that, of eight pancreatin products purchased at local drug stores (in Illinois), two were inactive is important from the standpoint of enzyme technology.

Similar observations concerning pepsin had been made in Germany by Dultz (17). He reported that commercial pepsin preparations became completely inactive because of their acid or alkali content.

In this connection it should be noted that there exists an incompatibility between enzymes. Stratton (18) described an elixir with digestive properties which contained the enzymes of pepsin, rennin, and papain together in solution. It is well known, however, that these enzymes digest each other, the destructive agent being that enzyme which happens to be most active at the *p*H of the solution. The presence of rennin in this preparation is superfluous, since pepsin itself possesses considerable milk-clotting power.

Treatment of Sloughing Wounds with Proteolytic Enzymes. Ammon (19) reported that pancreatin in ointment form has a healing action on wounds, said to be effected by the digestion of matter interfering with the healing process. The following conditions were treated successfully: decubital necrosis, furuncles, carbuncles, ringworm, panaris, superficial suppurative skin diseases, scars, lupus erythermatosis, certain types of ulcerations, and lupus vulgaris. Torantil, an enzyme preparation from the mucous membrane of the small intestine, was recommended by Ammon for local application in colitis ulcerosa.

Glasser (20) described a new treatment for sloughing wounds. He uses a mixture of papain, oleic acid, stearic acid, and mineral oil and adds to this, just before use, a 5 per cent solution of triethanolamine which serves as an activator. The papain effected the therapeutic results. It digested only dead tissue, and hastened healing as a result of this action. In concentrated solution it had some bactericidal action on organisms that produce putrefaction.

According to Glasser, the results obtained in 58 cases had been excellent. "In no case did the enzyme mixture fail to remove slough." The average number of applications necessary was 2.34. With some foul-smelling wounds the effect was dramatic.

Treatment of Scleroderma and Sclerodactylia with Enzymes. Robinson (21) recommends the treatment of these diseases with pancreatic enzymes given before each meal in tablet form. Instead of tablets, 100 to 200 grams of raw pancreas per day may be given. The pancreatic enzymes may be supplemented by "gastric-duodenal" enzyme tablets. Months to years of continuous treatment with these enzymes are required for beneficial results in scleroderma.

Therapeutic Use of Tyrosinase

Destruction by Tyrosinase of the Toxicants of Poison Ivy and Other Plants. Poison ivy is found in nearly all parts of the United States and Canada. This plant produces skin irritation, which, if neglected, may become so serious that hospitalization is necessary. Similar toxic reactions are produced by poison oak and poison sumac, and, in Japan, China, Yunnan, Formosa, and Indo-China, by various members of the lac tree family. It has been found that these poisons are phenols or catechols having long hydrocarbon side chains attached to the ring structure. Sizer and Prokesch (22) found that purified mushroom tyrosinase oxidizes the toxic principles of many different commercial poison ivy concentrates as well as extracts from poison oak, Japanese lac, and liquid from the shell of the cashew nut. The enzyme had similar action on the purified toxicants. The oxidation of these poisons by tyrosinase was followed by an appreciable decrease in the dermatitis-producing properties, as shown by patch tests on human and guinea-pig skin. The enzyme was effective a few hours after the toxicants penetrated into the skin. The authors recommend the use of tyrosinase in the treatment of poison-ivy dermatitis in its early stages.

"Blood-Pressure-Lowering Action of Tyrosinase." Schroeder and Adams (23) studied the effect of tyrosinase of the common mushroom, *Psalliota campestris,* prepared according to Ludwig and Nelson (24), on arterial hypertension, using rats and dogs made hypertensive by various methods.

They found that the intravenous injection of tyrosinase consistently lowered the blood pressure of the hypertensive animals, but on the average it did not affect normal animals. The renal function of the hypertensive dogs was not affected by the treatment with tyrosinase. *In vitro* renin, angiotonin, Victor's pressor substance, adrenalin, and tyramine were inactivated by tyrosinase; this alters their action on blood pressure. These experiments indicate that *in vivo* some phenolic substance responsible for hypertension is changed by the tyrosinase.

Schroeder (25) has extended these studies to human hypertension.

Seventeen patients suffering from arterial hypertension were treated daily with subcutaneous injections of tyrosinase for 3 to 4 weeks. In most patients the blood pressure fell considerably, and symptoms, when present, were relieved. The kidneys' ability to concentrate urine was unchanged. When injections of tyrosinase were discontinued for 3 to 6 days, the blood pressure returned to its original level. "Symptomatic improvements, as well as the improvement in the ocular fundi, lasted for several weeks or months."

Sometimes a moderate degree of pyrexia occurred after the injection. One patient received a small amount of tyrosinase intravenously. A severe reaction, with nausea, vomiting, signs of increased peristalsis, fall of blood pressure, and bradycardia resulted. The blood pressure was low for 24 hours after the injection. Large doses of the enzyme were required when given subcutaneously.

Prinzmetal and associates (26) showed that heat-inactivated mushroom tyrosinase preparations can produce considerable lowering of blood pressure and remission of other symptoms of arterial hypertension in man. These effects were as marked as those described by Schroeder, who used active tyrosinase preparations. Thus it is obvious that the effects produced by mushroom preparations upon symptoms of arterial hypertension are of non-enzymic nature.

Schroeder (27) also reported that a preparation of hog-liver amine oxidase consistently lowered the blood pressure of hypertensive rats and dogs when injected intravenously. Rocha e Silva (28) found that trypsin injected intravenously into dogs leads to a marked drop in blood pressure. According to Van Goor (29), carbonic acid anhydrase accelerated tissue oxidation and the activity of the frog heart. Inactivated enzymes were not used as controls in these experiments; thus they are of questionable value.

Angiotonin and Its Enzyme System. Renal hypertension is believed to be brought about by a change in a vasopressor system which has its origin in the kidneys. Renin, an enzyme liberated from the kidneys, reacts with a globulin fraction from the blood (renin activator), producing a pressor substance, angiotonin. Neither renin nor the blood globulins show pressor activity. Angiotonin, however, is physiologically very active. It can be inactivated by angiotonase (hypertensinase), an enzyme present in the kidneys and other tissues. A disturbance in the balance of this system may produce hypo- or hypertension (30, 31).

Croxatto (32) examined the hypertensinase activity of kidney *l*-amino acid oxidase and that of the venom of the South American

snake *Bothrops neuwidii*. He concludes that the hypertensinase action of the oxidase is due to some other enzyme impurity, and that the similar activity of the snake venom is probably due to a proteolytic enzyme. This author showed earlier that hypertensin (angiotonin) is hydrolyzed by aminopolypeptidase.

"Therapeutic Value" of Histaminase

Clinically histaminase has been recommended for a great variety of diseases: allergies, asthma, vasomotor rhinitis, urticaria, skin disorders, serum sickness, etc. (33). Serious doubt, however, has been cast concerning the oral therapeutic use of histaminase. Zeller and Schär (34) stated that histaminase administered by mouth is destroyed by 0.05 *N* hydrochloric acid, pepsin, and trypsin and that therapeutic results obtained with histaminase preparations given by mouth cannot be attributed to histaminase. In view of this statement it should be noted that a number of clinical studies have appeared which show that the oral administration of histaminase has brought marked relief to a great number of patients in various disorders (35–39). These beneficial results may have been brought about by some other substance and not by histaminase.

Lemley and Laskowski (40) found that the injection of histaminase protected at least one-third of the guinea pigs which simultaneously received one-and-one-half times the lethal dose of histamine. They conclude, however, that histaminase at the present state of purity is highly toxic, and the dose cannot be increased to the level that will give safe protection.

The earlier statement by Atkinson, Ivy, and Bass (41) should also be quoted: "Therapeutic studies of histaminase action to date are characterized by a failure to use inactivated histaminase as a control. Such a control would seem to be essential, particularly in view of the fact that all preparations of histaminase are very impure."

The Use of Cholinesterase in Shock

According to Schachter (42), dogs having hemorrhagic shock responded well to beef plasma, administered in appropriate volumes, by recovering from the shock. Dogs in traumatic shock were benefited by plasma only temporarily. This investigator found that, when dogs in traumatic shock were given intravenous injections of cholinesterase,

the blood pressure usually returned to normal and remained there for the duration of the experiment.

Antidiabetic Action of Artichoke Oxidase

Risi (43) described the preparation of an oxidase from the leaves of the artichoke (*Cynara scolymus*). It had antidiabetic action. Good results (clinical and laboratory) were obtained with 105 patients having mild and severe diabetes. Large doses of extract in glycerol solution were well tolerated.

Enteric Coatings of Medicaments

Medicaments Coated with a Water-Insoluble Polymerization Product. Hagedorn, Bockmühl, and Gorr (44) reported that enzyme preparation may be coated with a water-insoluble organic polymerization product containing acid groups; for example, 100 grams of a polymer obtained from a mixture of acrylic acid butyl ester (90 parts) and styrene (10 parts) is swelled in 2 liters of acetone and 200 cc. of methyl alcohol, and the mixture is heated for 45 minutes at 50° C. with 0.5 liter of 2 *N* potassium hydroxide in methyl alcohol. The ester group is saponified, and the salt precipitate is dissolved in water. The salt is converted with diluted acetic acid into free acid. A solution of the acid in acetone may then be used to form a film which is insoluble in water but soluble in a weakly alkaline medium. The coating material is capable of passing the stomach unaffected and dissolving in the intestine.

Medicaments Coated with Cellulose Derivatives. This coating for the medicaments is composed of a derivative of cellulose which contains free carboxyl groups. They may be mixed esters or ethers. To make these cellulose derivatives, cellulose or a cellulose derivative containing free and available OH groups is caused to react with a polycarboxylic acylating agent, such as an anhydride of a polycarboxylic acid or phthalic, succinic, diglycolic, and maleic anhydrides. Cellulose derivatives, which may be used to react with these, are cellulose acetate, propionate, butyrate, and ethers such as ethyl, butyl, and benzyl (45).

Hodge and associates (46) conducted clinical tests of cellulose acetate phthalate as an enteric coating. They observed that tablets and capsules coated with this substance possessed satisfactory enteric characteristics. In 79 to 100 per cent of trials in human subjects, disintegration occurred in the intestine sometime in the 8 hours after ingestion.

Surgical Catgut and Enzymes

The technical aspects of the preparation of surgical catgut have been discussed by a number of authors. Bulloch, Lampitt, and Bushill (47) have published an excellent treatise on the subject. Their book contains 440 references, most of which concern catgut.

Surgical catgut is made from sheep's intestines. The methods of manufacture vary somewhat from plant to plant. Catgut is composed of collagen. It is closely associated with enzymes from the time the raw material leaves the packing house until the finished catgut has served its purpose in the human body. It is indeed an interesting object of practical enzymology. In the United States the sheep's intestines destined to be made into catgut are called " green gut." In England they are known as " runners." Although much scientific work has been conducted over a period of years, the present methods of catgut manufacture are still being improved.

Basic Principles in Catgut Making. Bulloch and associates state, " To prevent rapid putrefaction and softening [of green gut] which is very liable to set in as a result of enzyme action or bacterial growth, the runners are either salted or frozen." Prevention of autolysis and bacterial decomposition, resulting from the hydrolysis of the intestinal collagen, is obviously important.

Sheep's intestines are also used in tennis rackets, sausage skins, and musical instruments. Although the basic principles of manufacture are the same for these products as for surgical catgut, surgical catgut making requires extreme cleanliness. For all purposes the mucosa and the circular muscular coat have to be removed.

The green guts are thoroughly washed in water. Then they are split into two or three pieces (ribbons), depending on the construction of the splitting mechanism (a small piece of razor blade attached at the proper angle to a razor). The cut ribbons are then washed in water and are scraped by machines or by hand. The purpose of scraping is to remove the mucosa and the muscular wall from the catgut ribbons. When the catgut scraping is finished, only the *tunica submucosa* remains, and this constitutes the finished catgut ribbon. During and after scraping the ribbons are subjected to various baths. This problem has been extensively studied by Bulloch et al.

After completion of these stages the ends of the ribbons are attached to loops of cord. Then the ribbons, one or more, according to desired size, are spun into catgut. Electrically driven wheels or hand wheels are employed for this purpose. Now the catgut is dried under proper tension, polished, cut in the required length, and sterilized. Good ten-

sile strength, proper digestion time, and absolute sterility are the most desired characteristics of an acceptable product.

Various grades of surgical catgut are sold. It is classified according to its digestion time or its resistance to absorption. To lengthen its digestion time surgical catgut is subjected to hardening processes similar in many respects to the tanning of skins in the manufacture of leather.

Attempts have been made in Germany to produce a synthetic, absorbable suture from polyvinyl alcohol. This product, however, could not be obtained in a suitable quality, according to the German medical literature (48, 49). However, in the United States enzyme-resistant (non-absorbable) sutures are being manufactured from synthetic fibers such as nylon and vinyon.

Determination of Digestion Time *in Vitro*. Serious attention has been given to the study of the digestion time of catgut *in vitro*. It is obvious that too rapid absorption of catgut *in vivo* may cause serious complications. On the other hand complete absorption within the proper time is desirable.

Kraissl (50) developed a simple and useful method for the determination of catgut digestion time *in vitro*. This procedure may readily be carried out in any laboratory. Ten centimeters of catgut is subjected to a solution of 2 per cent (Difco) trypsin in a buffer of *p*H 8 (94.5 cc. of *M*/15 Na_2HPO_4 and 5.5 cc. *M*/15 KH_2PO_4). The apparatus (Fig. 44) employed in the test consists of centrifuge tubes with hooks fused in their lower ends. Ten-centimeter samples of catgut are measured on the tubes. The beam is balanced on a frictionless knife edge, and the clips are counterbalanced by the switch trip. The switches are of mercury and therefore free of friction. The clips are connected to switches and electric (24-hour) clocks. The apparatus is kept at 37.5° C. in an incubator. When the catgut is digested the circuit is broken and the time is automatically recorded. The addition of 2 cc. of a 1:1000 solution of merthiolate per 10 cc. of trypsin solution eliminates bacterial growth, and the placing of 2 cc. of sterile albolene on top of the enzyme solution prevents evaporation. See also reference 51.

This method is more accurate than that employed when animals were used to determine the digestion time of catgut.

Absorption of Catgut in Human Beings. Wolff and Priestley (52) of the Mayo Clinic studied the absorption of catgut in 164 surgical cases. They tested 358 strands of catgut and found that small sizes of catgut lasted as long as or longer than large sizes. These authors stated that "Labels which indicate the length of time necessary for absorption of catgut are entirely fallacious in so far as the human being

is concerned." They found that drainage and suppurating wounds did not accelerate absorption of catgut, that absorption of catgut varied

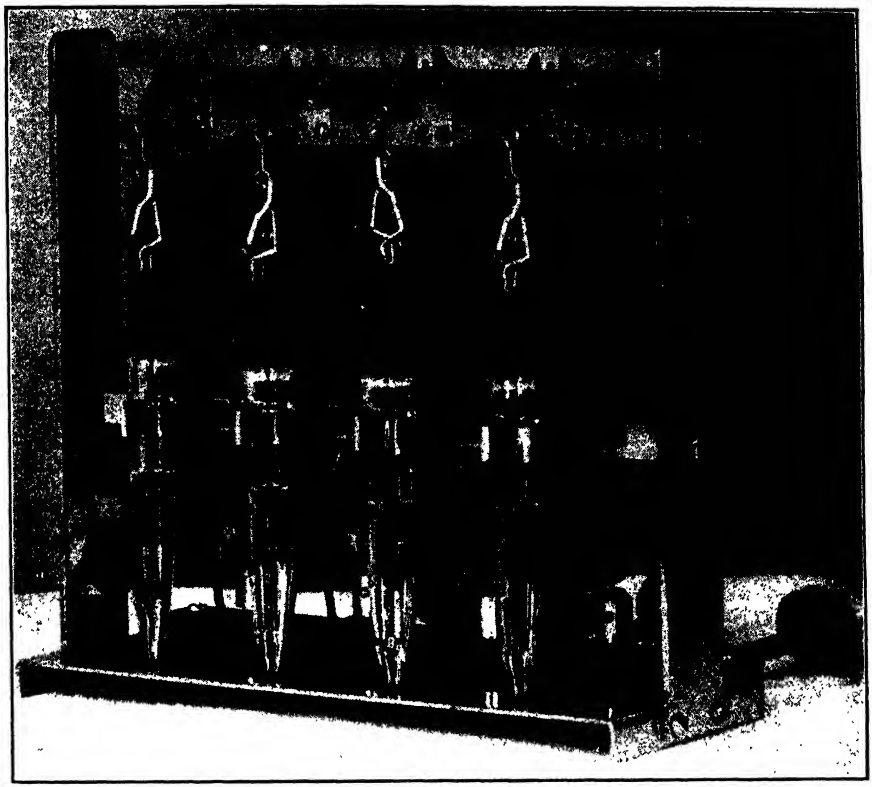

FIG. 44. Digestion apparatus for testing surgical catgut.

among individuals, and that some brands of catgut lasted longer than others.

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CHAPTER XXIV

THE ROLE OF AMYLASES AND PROTEINASES IN BREAD MAKING AND THE PRODUCTION OF MALT SYRUP

AMYLASES AND PROTEINASES IN BREAD MAKING

Fermentation in dough is brought about by the action of the enzymes of flour and those of yeast on the carbohydrates and proteins in dough. Flour contains diastases (mainly β-amylase) and a fairly potent papainase; yeast furnishes the fermentation enzymes. The effect on dough of the flour proteinase and of the added α-amylase may be considerable. The main changes under proper proportions of water, salt, sugar, and flour are the formation of the leavening gas carbon dioxide, alcohol, some organic acids, and flavoring compounds (1). Saccharogenic activity is influenced by the degree of digestibility of the starch and by the enzyme content. Often the diastase content is inadequate to supply the yeast with sugar for normal fermentation. The metabolic state of the yeast employed is also important. This subject has been reviewed by Landis and Frey (1), who also give an extensive bibliography.

Flour contains a considerable amount of β-amylase but little or no α-amylase, and the amount of fermentable sugars in dough is inadequate for the best baking results. To correct this, baking aids, such as malt extracts containing diastases and maltose, are added to the flour. Usually 20 to 40 grams of the diastatic baking aid is added per liter of dough. As will be seen, it is the α-amylase that produces the desired beneficial effect on the dough.

Flour contains 72 to 75 per cent starch, 11 to 13 per cent protein, and 0.4 to 0.7 per cent ash. Flour proteins readily swell when mixed with water, and they clot when acted upon by proteolytic enzymes. Owing to its constituents, flour when mixed with water forms a characteristic viscous material called dough. Flour, although it is a dead substance, undergoes a constant desirable oxidation during storage. For this reason, white flour is allowed to age before it is used. It is believed that the oxidation inactivates the papainlike proteinase contained in the flour. The beneficial effects of oxidizing-bleaching agents are said to be based on the inactivation of the flour papainase.

The following characteristics of flour, as summarized by Blish (2), are important in judging *quality of flour.* For specifications of baking properties, characteristics of flour are expressed in terms of dough behavior in the bake shop. The following terms are used: strength, stability, fermentation tolerance, tolerance for mechanical treatment, and tolerance for oxidizing (artificial maturing) agents. Strength is the capacity for making a large, bold-looking loaf, and is governed by the protein content. Stability is resistance to slackening or stickiness during fermentation. Fermentation tolerance means the extent of the fermentation time-range over which good bread can be made; it is a function of the diastatic or " gassing " power of the flour. Tolerance to mechanical treatment, largely a varietal characteristic, indicates comparative resistance or response to the operations of mixing, molding, etc. By tolerance to oxidizing agents is meant the response or tolerance to certain chemical maturing agents used by the miller (in bleaching) and by the baker (in " yeast foods "). This is a very important property, and the difference in responses shown by different flours necessitates careful control in the use of oxidizing agents if uniformity of baking behavior is desired.

The quality of bread may be determined by its volume, its water content, the nature of its crust, and its keeping quality.

Thus, the making of good bread is influenced by many factors. In this chapter, only those connected with enzymes will be discussed, however. For the discussion of bread technology, see references 3–6.

THE ROLE OF PROTEINASE

The presence of a proteolytic enzyme in flour was observed by Balland (7) as early as 1884. More recently, several authors have studied the proteinases and other enzymes of flour. Balls and Hale (8) and Jorgensen (9) simultaneously and independently showed that the wheat proteinase is a papainlike enzyme, being activated by reduced glutathione and inactivated by oxidizing agents and by the usual bread improvers. Practically all authors agree that flour contains a powerful protease, becoming fully active on the addition of reducing agents like cysteine, ascorbic acid, and other papain activators. The proteinases have an undesirable clot-producing action on flour proteins (10, 11), and their inactivation is important in bread making. Owing to the oxidation of natural activators and probably the sulfhydryl group of the proteinase, the oxidizing agents used for bleaching flour eliminate the action of the proteinases; hence the improvement in the appearance of bread.

Aging of flour to improve its quality has been practiced for many years. It has been shown that aging of flour greatly reduces protease activity (3).

The Effect of Activating and Inactivating Agents on Wheat Proteinase. Balls and Hale (8) published some very convincing baking experiments which dealt with the effect on wheat proteinase of added activating and inactivating agents in bread dough. Figure 45 shows the marked effect of added potassium persulfate, an oxidizing inhibitor, and of glutathione, an activator of wheat proteinase. In experiments

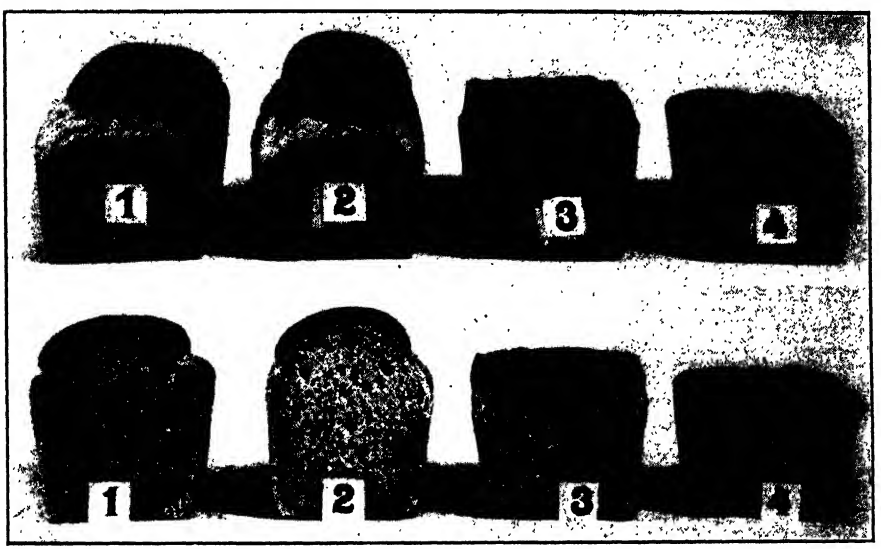

FIG. 45. One-pound loaves of bread containing the additions shown.

with papain and glutathione, the increase in proteinase activity caused an almost complete liquefaction of the dough and no rising of the loaf. Oxidizing agents had the opposite effect.

Extensive bleaching with chlorine, or continued storage, completely destroys the proteinase, since it cannot be activated at this stage. If the oxidation is mild, however, the reaction is reversible (10).

Potassium bromate inactivates the proteinase of wheat flour, just as it does papain, bromelin, and cathepsin. According to Jorgensen (9), ascorbic acid, an inhibitor of papain-type enzymes, is a good improver of the baking strength of wheat flour. Since yeast is one of the best sources of reduced glutathione, Jorgensen (12) used yeast water to make the dough. In another experiment he employed pure

glutathione. The loaves prepared with glutathione (or yeast water) and tap water showed striking differences. The loaves containing glutathione had bad crumb and small volume, and they exhibited a general weakening effect on the gluten.

Harries and Johnson (13) found that addition of papain and pepsin to dough decreased its viscosity. Potassium bromate retarded dispersion in the papain-treated dough. Melville and Shattock (14) confirmed Jorgensen's results (15) concerning the bread-improving qualities of ascorbic acid. The New Zealand investigators claim that dehydroascorbic acid is more potent than ascorbic acid and that this form of the vitamin was equivalent to bromate as an improver. Read and Haas (16) and several other investigators (17), however, do not believe that the flour proteinase has a very important role in bread making.

DIASTATIC BAKING AIDS

Lintner, as early as 1890, observed that wheat diastase interacts in dough making. Since then, many papers dealing with this problem have appeared. The effect of various extracts of flour and germinated grain on starch was extensively studied.

Flour must be able to produce and retain carbon dioxide. There are only small amounts of sugars (0.1 to 0.4 per cent calculated as sucrose) in flour. These amounts are insufficient for rapid gassing (18). The natural diastases of flour produce an additional and adequate quantity of sugar. This sugar production, however, is very slow. The addition of malt extract or malt flour (containing a good source of diastase) to dough furnishes the yeast at once with available maltose, resulting in vigorous and continuous fermentation. Not only does fast fermentation result in an enormous saving of time, but also the resulting bread is superior in volume, texture, and grain (19, 20). Tissue and Bailey (21) found that a constant rather than a large sugar supply is required. According to Rumsey (22), American bakers in 1922 used 30 million pounds of malt extract valued at 2½ million dollars.

Larmour and Brockington (23), using a concentration of 0.001 per cent potassium bromate together with 0.5 per cent malt extract in their baking tests, obtained satisfactory results.

The Effect of Added α- and β-Amylase on Dough. The effect of added diastases, wheat flours, and various commercial malt preparations on dough has been extensively studied, and their desired qualities have been commercially applied (for review see reference 1). These preparations contain both α- and β-amylase in varying amounts.

Blish, Sandstedt, and Kneen (24) showed that α-amylase is the most important agent when malt flour is industrially employed, the β-amylase being of little effect. Stamberg and Bailey (25) published some highly interesting and valuable investigations on the effect of added α- and β-amylase, respectively, on dough. Instead of using preparations containing both enzymes, they prepared α-amylase by Ohlsson's (25) method of heating germinated wheat extract to 70° C. for 15 minutes. This destroyed the β-amylase. The β-amylase was prepared by acidifying an extract of normal wheat with hydrochloric acid to a *p*H of 3.3, keeping the extract for 15 minutes at 0° C., and

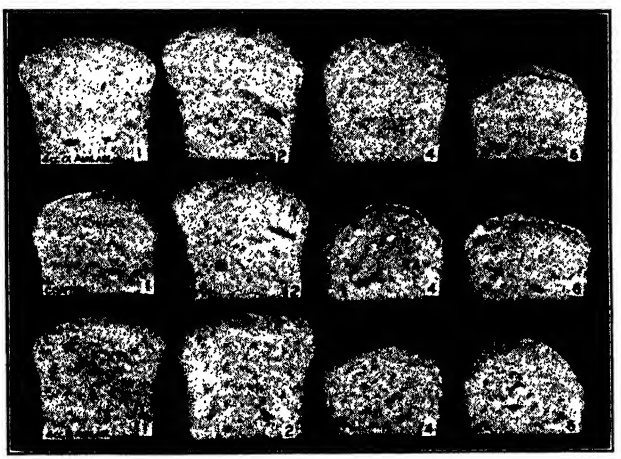

FIG. 46. Interior of bread made with 4 cc. of α- and β-amylase in doughs with 1, 2, 4 and 6 hours of fermentation.

then adjusting the *p*H to 6.0 with secondary sodium phosphate, as described by Ohlsson. The α-amylase had high dextrinizing activity on soluble starch. The β-amylase showed no dextrinizing activity but had strong saccharifying action.

The flour was of medium strength; it contained 11 per cent protein. It had a diastatic activity of 220 Rumsey units. Each loaf contained: 100 grams flour; 1 per cent salt; 3 per cent yeast; 62 per cent adsorption (including enzyme solution); no sugar; no shortening.

The experiments of Stamberg and Bailey (25) indicate that normal flour contains sufficient β-amylase. The addition of small quantities of α-amylase, however, brought about a very marked improvement on the loaf volume at the various fermentation times. (See Fig. 46.) Too much α-amylase, not shown in the figure, resulted in slightly more loaf volume, but grain and texture were inferior. The authors state

that " overdiastating " dough might be one of the causes of sogginess of the crumb of some commercial bread found in certain markets.

Sandstedt, Jolitz, and Blish (27) also state that the improving action of added malt on many flours is brought about by the α-amylase on the starch. Damage of the starch in milling and hypochlorite treatment may be the cause of certain undesirable baking characteristics. The hypochlorite damage may be repaired by treatment with large amounts of malt.

Loaves of bread baked from an unbleached, 85 per cent bakers' patent flour, with a protein content of 12.5 per cent, showed very marked improvement with both oxidation and malt α-amylase. Thus the destruction of the flour proteinase is not essential to obtain the α-amylase response. Sandstedt and associates point out, however, that all flours do not respond equally to added malt or malt α-amylase. See, also, the earlier experiments of Geddes and McCalla (28).

Factors influencing the baking test have been discussed by Blish (29), Markley and Bailey (30), Kent-Jones (31), Geddes, Larmour, and Mangels (32), Schultz and Landis (33), Landis and Frey (34), and others.

Kneen and Sandstedt (35) warn against the use of bacterial amylase in baking. Owing to their high heat stability, not only is starch breakdown in the oven too extensive, but also some of the amylase may remain active throughout the baking period and cause liquefaction of the gelatinized starch after removal of the bread from the oven. This type of heat-stable amylases produces undesirable breadcrumb characteristics similar to those associated with " ropy bread." Mold amylase (of the *Aspergillus oryzae* type), however, which has a thermostability level similar to or lower than that of malt amylase, is to be recommended as a baking aid. Mold amylase is an α-amylase. The concentrated dry precipitates are water soluble and highly active. They may be diluted with fillers, such as starch or wheat flour. All authors agree that the Lintner value of a malt is not related to the action of malt for flour supplementation. Malts that have a high saccharifying action do not necessarily have a high α-amylase activity.

Catalase. Catalase activity in flours varies with the regional and climatic origin. According to the findings of Blish and Bode (35), catalase activity should not be employed for evaluating flour.

MALTOSE SYRUP PRODUCTION

General Consideration Concerning Conversion Methods for Syrup Production. Kerr and Schink (37) have examined new claims con-

cerning improvements for increasing the fermentability of starchy mashes by the use of mold enzyme products after preliminary acid conversion of starch. These investigators conclude that their " attempts have failed to obtain a more substantial reduction in the resistant residues of starch remaining after a preliminary acid conversion by the addition of enzyme-containing products which would increase either the α- or β-amylase ratio above that existing in malt diastase." Nor could Kerr and Schink find that extensive acid conversion would be a more efficient method for increasing the fermentability of a syrup above the diastatic conversion of cornstarch. The percentage of nonfermentable dextrins left after the various conversion procedures studied appears to be the same. It was also found that, by increasing the dextrose equivalent of a maltose syrup from 46 to 69 per cent, the fermentability of the product was left unchanged. This would indicate that dextrose was formed from maltose and not from dextrins. Both sugars are fermentable to ethyl alcohol by yeast. Whatever the method, it is more desirable to produce syrups of high maltose content than of high dextrose content. Syrups that contain large amounts of dextrose produced by acid hydrolysis or by mold enzymes (maltase and α-amylase converted) are sweeter, but crystallize readily on standing and are unsuitable for certain uses because of the economics of handling (38).

The Preparation of Maltose Syrup. Weichherz (39) recommends the following method for the preparation of a syrup containing 35 to 40 per cent maltose: A paste is made by mixing 5000 liters of hot water and 500 kilograms of potato, wheat, or manioca starch. The mixture is cooled to 70° C. To this is added a malt extract prepared by extracting, for 12 hours, 150 kilograms of malt with 450 liters of water at 35° C. The mash is kept at 70° C. until the iodine color has completely disappeared. The *p*H is adjusted to neutrality with sodium bicarbonate or calcium carbonate, the temperature is raised, and the mash is filtered with the aid of a filter press. The proteins may be precipitated with tannin. The filtrate is concentrated in vacuum to a syrup of 80 per cent solid content. The dextrins may be removed from the filtrate by repeated additions of alcohol during the concentration process, and the maltose is then obtained in pure crystalline state (40).

Baker (41) prepares solid crystalline maltose, or a maltose syrup, by adding to liquefied starch 10 to 15 per cent of air-dried malt per weight of starch. The conversion is carried out at 52° to 55° C. at a *p*H of 5.00 for 48 hours. The mixture is then filtered, boiled with decolorizing charcoal, again filtered, and evaporated under diminished

pressure to a syrup containing about 80 per cent solids. The syrup can be solidified by seeding it with a small amount of crystalline maltose.

Malt syrup may be prepared with or without diastatic activity, depending on the temperature used for the concentration of the syrup.

Kerr and Schink (37) published a fermentation test for the determination of fermentable extract in corn syrups. For further details concerning maltose syrup see reference 42.

Uses of Sugar Syrups. Sugar syrups are used as bread and cake improvers, and in the manufacture of soft drinks and candy. Tracy (43) reported some very remarkable results in connection with the replacement of sucrose by enzyme-converted corn syrup in ice-cream making. There was an improvement in the body and texture of the ice cream, and certain fruit flavors appeared to be accented when sucrose was replaced up to 30 per cent. In sherbets and ices, up to 50 per cent of the sucrose was successfully replaced with the enzymatically hydrolyzed corn syrup. The resultant products were superior in appearance and quality as compared with products that contained all sucrose.

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CHAPTER XXV

ENZYMES IN DAIRY PRODUCTS

THEIR USE IN TESTS FOR EFFECTIVE PASTEURIZATION OF MILK AND DAIRY PRODUCTS

Enzymes and Milk. The inactivation (destruction) of individual enzymes of milk has been taken as the basis for tests which indicate efficiency of pasteurization. Some milk enzymes, such as aldehyde-reductase, catalase, and amylase, vary to a great degree from sample to sample and are often only partly destroyed during pasteurization (1–3).

A pasteurization efficiency test which was based on the gasometric estimation of residual catalase was suggested, but it was not adopted because of its inaccuracy (4). For instance, *catalase* is only partly destroyed when heated at 90° to 92° C. for 30 minutes; nor is *aldehyde-reductase* completely destroyed at pasteurization temperatures. The test was based on the reduction of a methylene blue-formaldehyde solution by the enzyme in the milk. For a number of reasons in addition to the one mentioned the test had to be abandoned (2). Similarly, the *amylase* test, which was based on the starch-iodine reaction, and the peroxidase test (5, 6) were also shown to be inadequate for the control of effective pasteurization.

The role of pasteurization is to destroy completely the pathogenic microorganisms which the milk may contain. The test for effective pasteurization must be based on the complete inactivation of an enzyme somewhat more stable than the most resistant pathogenic microorganism (*Myco. tuberculosis*). The enzyme in such a case is still active, if the temperature and heating time are below the heat inactivation of the enzyme. A method based on complete destruction of an enzyme is the most reliable, if the addition of raw milk to pasteurized milk can be readily detected.

A test based on these principles was the ingeniously selected phosphatase method, by Kay and Graham in 1933 (7). This enzyme, called phosphomonoesterase A_1 by Folley and Kay, has an optimum *p*H of 9 to 10 and hydrolyzes phosphoric acid esters such as hexosephosphates, glycerophosphates, nucleotides, and phenylphosphate. Kay and Graham employed Na-β-glycerophosphate, buffered with glycine, as

the substrate. Later, Kay and Graham revised their original method using disodium phenylphosphate as the substrate. The new substrate furnished greater accuracy than the phosphate test, owing to the greater sensitivity with which the liberated phenol could be estimated by its reaction with Folin-Ciocalteu's reagent.

When the test was first applied to determine efficiency of pasteurization, 35 per cent of milk was found to be incorrectly pasteurized. Now it is rare to detect an incorrectly pasteurized milk sample (8).

The New York City Department of Health, using Scharer's modification of the phosphatase method, made more than 150,000 tests in two years between 1938 and 1940. A report of a given milk sample can be made ready within a few minutes if necessary. One-tenth of 1 per cent raw milk or a drop of 1° F. in pasteurization temperature is said to be detectable by Scharer's modification (9).

Application of the Phosphatase Test to Sour Cream and Sour-Cream Butter. According to Parfitt and Brown (10) testing adequate pasteurization of sour cream and sour-cream butter is more difficult than testing sweet products. Some of the difficulties are: the concentration of the enzyme in the cream; the inactivation of the enzyme, due to the age of the product and the temperature of storage; the emulsifying action of the milk fat; the presence of phenol-producing bacteria; the possibility of the presence of phenol in the wash water; the use of chlorinated water, and the concentration of the enzyme by the butter-making process. In general, according to Parfitt and Brown, the phosphatase test is a good tool for controlling the pasteurization of cream for butter making and for detecting butter made from inadequately pasteurized cream. Owing to the problems mentioned, however, the test remains in this instance a tool and not a control instrument.

Wiley, Newman, and Whitehead (11) reported that the phosphatase test cannot be employed on butter to detect whether the cream from which the butter was made was correctly flash-pasteurized.

Detailed methods of the various pasteurization tests are given in references 12 and 13.

A Test for Distinguishing Human Milk from Cow's Milk. Human milk is bought and distributed for infant feeding by many institutions. The price paid to mothers for their milk is many times that of an equal amount of cow's milk. Thus, it appears necessary to guard against dilution of human milk with cow's milk. Rodkey and Ball (14) proposed that the xanthine oxidase test be used as an index to determine the origin of a milk sample. Xanthine oxidase is not present in human milk, but there are considerable quantities of the enzyme

even in pasteurized cow's milk though not in boiled cow's milk. In the test, 2 cc. of milk and 1 cc. of 0.1 *M* phosphate buffer, *p*H 7.2, are placed in the main compartment of a Warburg manometer flask. The side arm of the flask contains 0.2 cc. of a 0.05 *M* solution of hypoxanthine dissolved in 0.05 *M* sodium hydroxide. After temperature equilibrium has been established at 37° C., readings are taken during 5 minutes, in order to be certain that no oxygen is consumed in the absence of the substrate. The substrate is then added, and readings are taken every 10 minutes for a total period of ½ hour.

The simpler Thunberg test, using the reduction of methylene blue by the oxidase substrate system as the basis, may also be used for assaying the enzyme. As little as 10 per cent of admixture of cow's milk with human milk may be detected.

FLAVOR DEFECTS IN DAIRY PRODUCTS

The most important flavor defect in dairy products is the so-called "oxidized flavor" produced by the oxidation of fatty compounds. This phenomenon is common to all industries using fats and oils. In the dairy industry, great care is taken not to have the products come into contact with metals such as copper, or be exposed to sunlight; this eliminates great losses due to off-flavors. Ascorbic acid inhibits the formation of copper-induced oxidized flavor in milk; and, when fresh tomato juice or fresh orange juice is added, the protection is greater than might be expected from their ascorbic acid content (15). Oat flour, crude sugar, and very small amounts of trypsin and lipase act in a similar fashion. Pepsin, however, has no antioxidant properties, indicating that trypsin and lipase probably produce the effect as a result of their action on fatty substances.

Corbett, Tracy, and Hansen (16) studied a cereal antioxidant which preserves butter flavor. These investigators reported that this cereal concentrate is effective in retarding flavor development and in improving the keeping quality of butter. The cereal antioxidant may be used in three ways: in cream, in salt, and on the wrapper. This product is manufactured by the Quaker Oats Co., Chicago.

THE ROLE OF ENZYMES IN CHEESE MAKING

Most of the 600,000,000 pounds of cheese manufactured annually in America is made with rennin, or rennet, as the enzyme is called by the cheese maker. The enzyme rennin coagulates the casein of the milk, thus forming insoluble calcium paracaseinate. (For the properties of

rennin and its chemistry see Part 1 of this book and reference 17.) It is the formation and finishing of this curd, based on physical and chemical details in processing, that determine the final nature of the cheese. The physical appearance is brought about by the temperature during curd formation, cooking, ripening, and pressing (18).

CLASSIFICATION OF CHEESES (17)

Soft	Hard
Unripened	Semi-hard
Cottage	Ripened by molds
Cream	Gorgonzola
Neufchatel	Roquefort
Ripened by molds	Stilton
Camembert	Ripened by bacteria
Brie	Brick
Ripened by bacteria	Münster
Limburger	Very hard
Liederkranz	Without gas holes
	Cheddar
	Edam
	Gouda
	With gas holes
	Emmentaler
	Swiss
	Parmesan

Chemical Changes in Cheese Ripening (17–19). The following are the main chemical changes:

1. Fermentation of lactose to lactic acid and to small quantities of acetic and propionic acids and carbon dioxide.
2. Proteolysis.
3. Some lipolysis.

The enzyme bringing about these reactions originates from the lactic acid bacteria employed in the starter and the lactobacilli in the ripening of the cheese, the various bacteria carried by the milk, the action of microorganisms in the air of the cheese factory, the rennin employed to produce the curd, and, finally, from the enzymes of the milk itself. Table XLVIII shows the enzymes in cheese and their functions (19).

Starters in Cheese Making. The role of starters in cheese making is to furnish a rapid source of acid. The acid inhibits the growth of undesired organisms; it furnishes a favorable acid *p*H for rennet action; it effects a proper shrinkage of the curd and a subsequent expulsion of whey. These factors in turn effect the formation of a good body, permit the development of desirable flavor and aroma, and promote mellowing (20).

TABLE XLVIII

ENZYMES IN CHEESE

Source	Enzyme	Substrate	Products formed	Practical significance
1. Milk	Lipase	Fat	Butyric, caproic, etc., acids and condensation-oxidation compounds	Flavor (pungent)
2. Rennet	Proteinase ("galactase")	Protein	Proteoses, peptones, etc.	Syneresis of coagulum — hardness of body
	Rennin	Casein complex	"Paracasein compounds"	Mellowing of green cheese
	Rennin-papainase	Casein complex	Peptones, etc.	Mellowing of green cheese
	Rennin-peptidase (?)	Casein complex	Peptides, etc.	Mellowing of green cheese
	Pepsin and other enzymes	Protein	General proteolysis	Mellowing of green cheese (? bitterness)
3. Lactic acid bacteria	Lactic acid system	Lactose	Lactic acid, etc.	Flavor and syneresis
	Proteinases, etc.	Proteins	General proteolysis	Mellowing
(*Streptococci*)	Various	Lactose and lactic acid	Volatile acids, alcohols, ketones, etc.	Flavor in quick-ripening cheese
(*Lactobacilli*)	Various	Lactic acid (and ? bound sugar in proteins)	Volatile acids, alcohols, ketones, esters, etc.	Flavor in show-ripening cheese
4. Miscellaneous bacteria	Proteinases	Protein	Peptones, etc.	General ripening and flavor. May cause characteristic taints.
	Lipases	Fat	Butyric, etc., acids	

General Considerations Concerning Curd Formation. The curd as formed by the action of rennet is exceedingly elastic and has, under proper conditions, great shrinking ability. The coagulum produced by acid, however, is gummy and is not elastic. The rennin-produced curd precipitates calcium and other insoluble salts, whereas the acid-produced curd releases these salts and they are lost in the whey. By proper control of the clotting time and temperature, the hardness of the curd can be determined. Hard cheeses contain only 30 to 40 per cent moisture, and their keeping quality is much greater. Soft cheeses, owing to their high moisture content (up to 75 per cent), do not keep for long.

The following example will illustrate how the action of rennet determines the physical appearance of certain cheeses. For the production of hard cheeses the amount of rennin used is such that curdling should take place within 25 to 45 minutes. Then the curd is cut, and after separation of the whey the curd is heated to varying temperatures depending on the type of cheese desired.

When soft cheeses are to be obtained the temperature of milk is kept below 30° C. and only small quantities of rennin are employed in order to delay curd formation. The clotting time then varies from 1 to 2 hours or even longer. The elasticity of the curd increases in proportion with the increase in the temperature up to 41° C. (21).

Low calcium content of milk may cause undue delay in curd formation. This may be obviated by the addition of small amounts of calcium chloride to the milk.

Babel and Hammer (22) state that certain strains of propionic acid organisms (genus *Propionibacterium*), when added to pasteurized milk, are very beneficial for the development of the desired sweet flavor when ripening Swiss-type cheese. Added calcium propionate also produces the typical flavor of Swiss-type cheese.

The Ripening Process. For cheddar cheese the most favorable temperature for ripening is 4° C. To speed up ripening, temperatures at 12° to 15° C. are applied, however. The finished product is then stored at slightly above freezing. A highly dehydrated coagulum does not ripen well, since a proper amount of moisture is necessary for the growth of microorganisms (23). Rennin continues to act on casein and paracasein. Rennin has been shown to be present in a number of cheeses which have ripened for many months. No rennin was found in Emmentaler because the high temperature employed inactivates it (24).

Ripening as Influenced by Various Added Enzymes. According to Doan and Freeman (25), addition of supplementary amounts of rennin

hastened the ripening of cheddar cheese. A mixture of pepsin and rennin produced similar results. A marked degree of ripening was obtained when small amounts of trypsin were employed.

Camembert-Brie Type of Cheeses. The Camembert-Brie type of cheeses which originated in the northern part of France owes its texture to the proteolytic activity of *Penicillium camemberti.* These cheeses are prepared as thin cakes, about 1¼ to 1½ inches in thickness and varying in diameters. The fresh curd is salted on the surface and inoculated with the mold or placed in a room in which the spores of the mold are abundant. In about a week the entire surface becomes white with the mold, about 1 to 2 mm. deep. In 10 days or so the mold becomes blue or green from developing conidia. After 10 days the mold softens the curd to a smooth buttery texture which gradually extends from all sides to the center of the mass. Different molds develop different textures.

Roquefort Cheese. Roquefort cheese has been made in the south of France for hundreds of years from the milk of ewes. Most of this cheese is made in the Department of Aveyron, a calcareous plateau at an elevation of 2500 to 3500 feet in the southwestern portion of the Cévennes Mountains. The temperature here ranges from 2° to 4.5° C. in winter and 17° to 20° C. in summer. The cheese is made in a number of factories of this region and is shipped to the caves of Roquefort where it is ripened.

The ripening agent is *Penicillium roqueforti,* a velvety green mold indigenous to the caves. The caves have a temperature of 4.5° to 10° C. and a humidity close to 100 per cent. Natural ventilation changes the air three times a day.

P. roqueforti invades the loose-textured cheeses. In preparing the curd cracks are left or holes are punched to allow the mold to grow. Such cheeses show green marbling on the cut surface. The usual period of ripening is at least 6 months.

Thom and Currie found that among a variety of molds only *P. roqueforti* has the ability to grow in an atmosphere having very little oxygen, and that only this mold can grow in the low oxygen tension which occurs in cheese. Currie showed that the characteristic flavor of these cheeses was due to the hydrolysis of the butterfat by this mold to form a different mixture of fatty acids from those produced by bacteria (26).

Dattilo-Rubo (27) extensively studied the taxonomy of the molds of eight varieties of blue-veined cheese and found that *P. roqueforti* Thom was the dominant strain. This type could be subdivided in accordance with colony characteristics into three classes, each typical of certain kinds of cheese.

Bryant (28) showed that each of the eight strains of *P. roqueforti* destroyed fatty acids such as caproic, caprylic, and capric acids in culture media at concentrations of 0.05 per cent. Concentrations of 0.1 per cent, however, were toxic. Hammer and Bryant (29) found that methyl-*n*-amyl ketone was formed from caprylic acid by mold action. This ketone is an important flavor constituent of blue cheese.

American "Blue" Cheese. There is a considerable demand for "blue" cheese in the United States and in Canada, and reports concerning successful attempts to manufacture this type of cheese have appeared in the literature. The United States Department of Agriculture (30) issued the following standards for Roquefort cheese.

"The cheese is made by the Roquefort process from unheated, unpressed curd obtained by the action of rennet on the whole milk of sheep, with or without the addition of a small proportion of the milk of goats. The curd is inoculated with a special mold (*Penicillium roqueforti*) and ripens with the growth of the mold. The fully ripened cheese is friable and has a mottled or marbled appearance in section."

The flavor of Roquefort cheese is due to the ewe's milk, the ripening process, and specific atmospheric conditions.

Various countries now produce, under a variety of names, cheese from cow's milk which is similar to Roquefort. In the United States this type of cheese is known in the trade as "blue" cheese. Owing to the lower price of cow's milk blue cheese costs much less.

Distinction between blue cheese and Roquefort cheese became necessary. Garard and associates (31) employed a standardized procedure for determining Polenske numbers by which Roquefort cheese may be distinguished from substitutes. Polenske numbers from 3.6 to 5.95 were obtained for Roquefort cheese, whereas cow's milk cheeses did not have values over 2.9 and were usually 1.8.

The ripening of blue cheese may be considerably accelerated by the addition of *steapsin* to the milk or to the curd. Competent judges, however, consider this cheese inferior to normal cheese because of its bitter flavor. The addition of steapsin alone to the cheese is not sufficient, and *Penicillium roqueforti* must be added to obtain the characteristic flavor during ripening (32).

Effect of Bacteria on the Ripening of Cheese. Lactic acid bacteria such as *Streptococcus lactis* and *Lactobacillus casei* accelerate the ripening process and the development of the flavor of cheese (33, 34). For further details concerning bacteria in cheese and an extensive bibliography see reference 35.

The Manufacture of Cheddar Cheese. The following is a typical method for the manufacture of cheddar cheese, a hard cheese made by

rennet action on whole milk. This cheese is a favorite in the United States and other English-speaking countries. The method to be described is typical for the manufacture of cheese.

The milk is brought to 30° C. (87° F.). A suitable starter of lactic-acid-producing bacteria is added. The milk is mixed, and, when acidified, from 0.17 to 0.2 per cent rennet is added. In 20 to 40 minutes curd formation takes place. After satisfactory clotting the curd is cut into small cubes to allow the whey to separate. The curd is now heated to 37.8° C. and agitated until acidity and body are of satisfactory nature. The whey is drained off as soon as the curd acquires the desired texture. Then the curd is cut into large pieces which are turned frequently and placed one on top of the other. This procedure, called cheddaring, removes the whey.

The cheddared curd is now cut and milled into small pieces. One and a half pounds of salt is added to each 1000 pounds of curd. After mixing, the product is cooled at 26.7° C., packed in cheesecloth linings, and pressed to the desired shape. Then the cheese is ripened.

During ripening, microorganisms and enzymes develop characteristic changes in the cheese. *Streptococcus lactis* and *Lactobacillus casei* have an important role in cheddar cheese ripening. Mold growth is prevented by the salting of the surface of the cheese. This is an important step, since mold growth must be prevented in this type of cheese. Ten pounds of milk yields 1 pound of cheese by this process (36).

Miller (37) reported that the immersion of cuts of cheddar cheese in 8 per cent propionic acid solutions increased the mold-free life at 15.6° C. from the usual 3 to 5 days to 12 to 38 days. Solutions of 14 to 16 per cent of calcium or sodium chloride were necessary to obtain a similar protection. Benzoic acid and sodium benzoate in the allowed strength were less effective than an 8 per cent propionic acid solution.

THE MANUFACTURE OF RENNIN

In some countries plant rennin, obtained from *Galium verum* (lady's bedstraw), *Pinguicula vulgaris* (butterwort), and the seeds of *Withania coagulans*, is employed for cheese making. The best source for the production of rennin, however, is the fourth or true stomach of the calf.

Liquid Rennin. Holwerda (38, 39) described the following method for the preparation of liquid rennin. The mucosa of the cardiac end of the stomach (this portion being richest in rennin) is extracted with

a solution containing 2 per cent boric acid and 10 per cent sodium chloride for 24 hours. The mixture is filtered and adjusted to *p*H 4.8 to 5. After 3 to 4 days at 25° C. the extract is adjusted to *p*H 5.3 to 6.3.

This method is still in use by some manufacturers of Emmental cheese.

Solid Rennin. For the manufacture of most cheeses other products are employed, however. Methods for the preparation of these products are all based on the early process of Blumenthal (40), which follows.

Two thousand dry calf stomachs are finely ground and placed in a container holding about 2000 liters. Fifty kilograms of sodium chloride and 40 kilograms of boric acid are dissolved in 1000 liters of water and added to the ground calf stomachs. Instead of boric acid, glycerol, alcohol, thymol, etc., may be used as an antiseptic. The mixture is slowly stirred for several days until there is no more increase in rennin activity. The extract is then decanted. It contains a considerable amount of mucin and some pepsin. The mucin is removed by the addition of a small amount of hydrochloric acid and by passage through a filter press. Then the extract is saturated with sodium chloride. The precipitate is now spread into thin layers and dried at room temperature. The mass is ground to a fine powder and standardized by the addition of sodium chloride.

Desirable Properties of a Good Rennet Product. A rennet of good quality must show good keeping quality, it must be free of interfering microorganisms, and it must be free of other enzymes. Price is another important factor to be considered.

Bacteria rapidly destroy the enzyme rennin. For this reason, preservatives such as boric acid, benzoic acid, or often a 15 to 20 per cent concentration of sodium chloride are employed.

Davis (41) suggested that a rennet intended for cheese making should be free of coliform bacteria, of *Clostridium*, of yeasts, and of molds. This investigator claims that traces of pepsin have no effect but appreciable quantities of pepsin in rennet products produce bitter-flavored cheese. For best results cheese makers should use pure rennin preparations.

A Test for Rennet Activity

Standard Buffered Milk (Ege-Thygesen). Forty-two grams of sodium hydroxide is dissolved in about 500 cc. of distilled water, 115 cc. of 80 per cent acetic acid is added, and the mixture is diluted with distilled water to 1000 cc. Fresh cows' milk is diluted with an

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CHAPTER XXVI

ENZYMES IN THE MEAT, EGG, VEGETABLE, AND FRUIT INDUSTRIES

Typical examples of the roles that enzymes play during storage of foods are the sweetening of grain, potatoes, and cheese, due to enzymic hydrolysis. Most fruits become soft during storage because of pectolysis. The hydrolysis of meats by tissue-bound enzymes is a desirable process and is greatly accelerated by the treatment of meat with various enzyme preparations. Enzyme action in vegetables and eggs during storage, however, is not desirable. The lower the temperature, the lower the rate of enzyme action; and any method that can inhibit enzyme action can probably increase the keeping quality of foods. Toxic substances cannot, of course, be employed to preserve foodstuffs.

ENZYMES IN THE MEAT INDUSTRY

After death, animal tissues undergo a gradual self-digestion effected by autolytic enzymes such as cathepsin, lipase, nucleases, and others present in tissues. It is generally known that tissues frozen and then thawed decompose much faster than tissues that have not been frozen at all. Disruption of cells brings the substrate and the enzyme closer together. Experiments of Balls and Lineweaver (1) indicate that it is important in the rapid autolysis of thawed-out material to know how far enzyme action has already progressed during the frozen state. These authors, using crude enzyme preparations as well as crystalline enzymes, found that lipase action is considerable at low temperatures, whereas other enzyme action is slight. But even this slight action is important, owing to the fact that the first phase of enzymic attack, which has taken place during freezing, considerably hastens enzyme action when the materials are brought to ordinary temperature. Interesting low-temperature studies were published by Nord (2, 3).

The value of meat is judged by its flavor and tenderness. In the United States the general procedure for tenderizing meat is to keep it at 1° to 3° C. from two to several weeks. Autolysis lessens somewhat the original desirable flavor of meat. Tenderness, however, is much more desired in good-quality meats. Thus all high-class meat is aged for some weeks.

Plank (4) found that meat kept at a temperature of —1.5° C. becomes very tender and shows good qualities even after 7 weeks. Tressler and Murray (5) showed that "sirloin steaks aged 4 days at 1° to 3° C., and then cut, packed, quick frozen, and stored at —18° C. for a month or longer, when thawed are as tender as and of better flavor than adjacent steaks aged 6 or 7 days at 1° to 3° C. and then tested immediately without freezing." These authors devised a mechanically operated gauge for determining the relative tenderness of meat.

Smorodinzew and Laskowskaja (6) suggested a method for determining the quality of meat by measuring its digestibility by pancreatin. By this method meats of better quality are digested 20 to 30 per cent faster than are meats of poorer quality.

Meat Tenderizing by Controlled Ultraviolet Radiation

Coulter (7) developed a tenderization process based on controlled ultraviolet radiation. The principle of the process is to keep meat, after slaughtering, dressing, and short chilling, at a comparatively high (15° C) temperature for 3 days to allow increased enzyme action. Enzyme activity at 15° C. is 19 times more than at 1° C. and is the cause of increased tenderness. Whereas it takes weeks to obtain the desired tenderness at 1° C., with the new process uniform tenderness is obtained in 5 days at 12½° C., in 2 days at 18° C., and in a few hours at 29° C. The atmosphere must be kept constantly at 85 to 90 per cent humidity to prevent the meat from drying out. Ultraviolet radiation with the Westinghouse Sterilamp, which has a wavelength of 2537 Å., protects the meat from decomposition by bacteria and molds. When the desired tenderness is obtained, quick chilling is applied at the customary refrigeration temperature of 1° to 2° C. It is said that this meat "met with immediate customers' acceptance," and now several millions of pounds of the tenderized meat are produced per week by a number of plants.

Meat Tenderizing by the Use of Fruit Enzymes

Papain. The tenderizing of meat with the aid of papaya juice has been practiced for many years by natives of the tropical regions of Central and South America. A great portion of the papain imported by the United States is employed for tenderizing meat. For this purpose, commercial products are available which are suspensions of the enzyme in some bacteriostatic fluid such as dilute alcohol. The preparations are smeared on the meat a few minutes before it is cooked. Sometimes provision is made to allow the enzyme to penetrate into the

meat by applying deep cuts; then the meat is cooked in the usual manner. This method produces the best results. There is slight but definite digestion of connective tissue and muscle fibers, resulting in softening of the flesh (8).

Papain is more resistant to heat than other proteases; thus most of the hydrolysis takes place during cooking. The use of too much enzyme must be avoided; otherwise excessive disintegration of the meat takes place. Too high a temperature destroys the enzyme. It is best to cook at 70° C.

Gottschall (9) made the interesting observation that papain, which is inactive by the usual tests, may be very active in the digestion of meat or other products containing sulfhydryl groups or capable of producing them during proteolysis. Activation increases as digestion progresses. Preliminary activation of papain for use of such substrates is not necessary. Tests of the original activity of the enzyme preparation give little indication of its potency in such use. Peptic digests of meat also bring about rapid activation of chymotrypsin.

Bromelin. Bromelin, the pineapple proteinase, is as effective as papain in tenderizing meat, and it has a more desirable odor.

Other fruit enzymes used for tenderizing meat are fig juices, which contain ficin, and the protease of Osage orange. These enzymes are also used to tenderize sausage casings (10).

The Use of Silver for the Prevention of Molding. The use of silver preparations for combatting molds in meats in cold storage has been reported by Moiseev and Ivanova (11). "Silver water" was used to prevent molding of meats in cold storage. The best results were obtained when the meat was washed before freezing with silver water containing 3×10^{-6} gram of silver per cubic centimeter. The silver water was prepared by allowing the water to stand in contact with silver sand. The product is said to be harmless to the consumer.

THE ACTION OF ENZYMES IN EGGS

Eggs in the shell, unlike meat and vegetables, cannot be frozen because freezing causes the shell to break. A temperature just above freezing is required for preservation because eggs decompose rapidly if not brought to a low temperature soon after laying. Proper care increases the commercial value of eggs considerably. Deterioration of eggs during storage, accompanied by a decrease in the proportion of thick white to thin white, may be caused by enzymes present in the eggs which weaken the membranes around the yolk.

Balls and Swenson (12) reported experimental data which indicate

that a tryptic proteinase is responsible for this change. They found that this enzyme is identical with pancreatic trypsin since it also is activated by intestinal enterokinase. The egg trypsin is present only in the thick white of the egg, whereas the thin white contains an antitrypsin. (See also reference 13.)

There is some evidence that microorganisms such as those of the *proteus* and *mesentericus* type may also effect a deterioration of eggs (14, 15).

Eggs release carbon dioxide but do not take up oxygen. This is said to be a certain form of deterioration. Deterioration is inhibited by the presence of carbon dioxide surrounding the eggs in storage. Accumulation of carbon dioxide in the egg inhibits further decomposition. This can be accomplished by closing up the egg pores to prevent the escape of further carbon dioxide. The United States Department of Agriculture (16) developed a process for the preservation of eggs based on coating eggs with thick mineral oil. The eggs are dipped in oil, then the " air is pumped out of them by a vacuum pump, and finally the oil is pushed into the pores of the shell by releasing the vacuum." Eggs treated by this method are said to " grade higher " after storage when subjected to the candling test. Oiling the eggs also helps to minimize evaporation of water.

Methods for the determination of egg quality have been discussed and a simple method has been described by Hoover (17).

ENZYMES IN THE VEGETABLE AND FRUIT INDUSTRIES

The Action of Enzymes in Potatoes

During storage at room temperature, respiration takes place constantly in potatoes. Diastases and maltase form glucose from starch, and the glucose is further metabolized. At low temperatures (3° C. or so), however, respiration ceases and sugars accumulate; this results in a sweetening of potatoes. Freezing is very harmful as it ruptures the plant cells, resulting in higher enzyme activity. Temperatures at or slightly above 3° C. and good ventilation are essential for maintaining high-quality potatoes. The careful handling of potatoes is required at all times.

The darkening of the cut surfaces of potatoes is due to the oxidation of tyrosine by the enzyme tyrosinase (18, 19). This reaction, called melanin formation, is undesirable in industrial processes. Boiling the potatoes and excluding air eliminates the darkening effect. Heavy-metal salts accelerate melanin formation.

Enzymes in Dehydrated Vegetables

Mallette and coworkers (20) stored commercially dehydrated cabbage, Irish potatoes, and sweet potatoes for one year under controlled conditions of temperature, moisture, and atmosphere. Fresh blanched and dehydrated samples were assayed for vitamin, oxidative enzymes, available iron, total copper, and moisture content. The dehydrated cabbage and potatoes deteriorated rapidly, as evidenced by loss of ascorbic acid, discoloration, and development of off-odor, when kept above 20 to 27° C. Irish potatoes deteriorated at moisture levels above 7 per cent. At lower temperatures, however, the vegetables were more stable. No correlation was found between this deterioration and the oxidative enzyme activities (peroxidase, catalase, ascorbic acid oxidase, catacholase, cresolase, and laccase) or iron and copper content of the dehydrated vegetables. All the vitamins assayed except ascorbic acid were fairly stable during storage. The employment of sulfite in the blanch reduced the ascorbic acid losses but resulted in the destruction of thiamin in dehydrated cabbage. There was no regeneration of enzymes during storage. The data of Mallette and coworkers do not support the contention that the storage deterioration of commercially dehydrated cabbage and potatoes is brought about by oxidizing enzymes whose presence arises from inadequate blanching or regeneration during storage. These investigators obtained analytical data at various stages of processing and compared them with those of the unprocessed fresh vegetables. The enzyme units are expressed per 100 grams of dry matter, and the vitamins as milligrams per 100 grams of dry matter. Physical characteristics, such as color and odor, are tabulated, and precise processing methods are also described.

Concerning the peroxidase test in dehydrated potatoes, Cruess and associates (21) came to the following conclusion: "Our experiments indicate that some peroxidase activity may be present without impairing keeping quality. On the other hand excessive peroxidase activity indicates inadequate blanching and probably impaired keeping quality. As a general principle it is better that such tests be unduly severe than unduly lenient."

The Western Regional Research Laboratory (22) has described a peroxidase test for dehydrated and undehydrated potatoes to indicate adequacy of blanching. This procedure minimizes the subjective factor in color estimation by the use of permanent color standards consisting of potassium dichromate and cobalt nitrate. The reagents employed in the peroxidase test are a 10 per cent solution of guaiacol in 95 per cent undenatured ethyl alcohol, 30 per cent hydrogen peroxide,

and 5 *M* ammonium acetate. The information sheet contains detailed instructions concerning the test as well as a discussion concerning its interpretation in control work.

The Action of Enzymes during Quick Freezing of Fruits and Vegetables

Over 85,000,000 pounds of vegetables are frozen per year by the frozen-pack industry in the United States. The freezing is important because frozen food can be transported and stored more readily and for longer periods than fresh foods. Commercial freezing was initiated and developed in this country. Extensive researches are available, and preferred lists of fruits and vegetables for freezing have been compiled.

Perhaps the most important step in freezing vegetables is the scalding process. If the enzymes are not destroyed or inhibited, their action continues even below —18° C. In addition to their autolytic action, bad odors and flavors, as well as color changes, are produced by them. Heat was found to be the best way to destroy the enzymes. Live steam or hot water is used.

Frozen fruits, however, are not scalded. They contain active enzymes, etc., and, once thawed, must be looked upon as perishable. Sugar or sugar syrup, added to certain fruits before freezing, preserves the thawed fruit for not more than 24 hours.

Frozen fruits have an excellent appearance and lose none of their vitamins during freezing. For an extensive bibliography concerning quick-frozen fruits and vegetables a paper by Diel (23) should be consulted. Here also many valuable details may be found concerning quick-freezing methods.

According to Joslyn and Marsh (24), the phenolase test is the most definite index for proper blanching of vegetables and fruits that discolor. Table XLIX, compiled by Joslyn and Marsh, illustrates the scalding times proposed by various authors.

Bedford and Joslyn (25) studied the activity of catalase, peroxidase, and ascorbic acid oxidase of string beans scalded under various conditions in relation to flavor retention, when stored at 17° C. The qualitative peroxidase test was found to be the best index for proper scalding of string beans. Not all the enzyme must be destroyed. The same authors (26) also found that flavor retention in scalded asparagus is closely related to peroxidase activity, using gum guaiac as the substrate. Scalding in water for 4 minutes at 92° C. or 3 minutes at 100° C. was satisfactory for retention of flavor.

Campbell (27) found a qualitative peroxidase test applicable in the

TABLE XLIX

Blanching Periods Used in Preparing Vegetables for Freezing

Authority	Asparagus	Green and wax beans	Lima beans	Broccoli	Cauliflower	Peas	Spinach	Kale	Sweet corn	Mushrooms
Joslyn and Cruess (1929)	2–5 min. in boiling water	2–5 min. in steam				2–5 min. in boiling water or steam	2–5 min. in steam or boiling water		2–5 min. in steam	
Joslyn and Marsh (1933)	1 min. in steam	1 min. in steam for cut beans				1 min. in steam				
Diehl *et al.* (1934)	2–3 min. in boiling water	2–4 min. in boiling water or flowing steam		3–5 min. in boiling water or flowing steam	2½–3½ min. in boiling water	½–1½ min. in boiling water	2–3 min. in steam or boiling water		6 min. in boiling water; 8 in steam with husks; 3–4 min. in water or 6 in steam	2 min. small; 3–4 min. large
Arighi *et al.* (1936)						2 min. at 176–194° F.				
H. H. Moon *et al.* (1936)		2–4 min. in boiling water (about 203° F.)	2–4 min. in boiling water			1–3 min. in boiling water (206° F.) depending on size and maturity			4 min. in boiling water (average temperature 206° F.)	
Smart and Brunstetter (1936)			3 min. in boiling water				3 min. in steam	5 min.		
Wiegand (1936)	2–3 min. in boiling water	2–3 min. in boiling water	2–3 min. in boiling water	3–4 min. in boiling water	2–3 min. in boiling water	½–1½ min. in boiling water	2–2½ min. in boiling water		6 min. with husks; 3–4 without	2–4 min. in boiling water
Goodale (1937)	2–3 min. in boiling water	5 min. in boiling water			2–3 min. in boiling water	2–3 min. in boiling water	2 min. in boiling water		None.	2–3 min.
Wiegand (1937)	2–3 min. in boiling water	2–3 min. in boiling water	2–3 min. in boiling water	3–4 min. in boiling water	2–3 min. in boiling water	½–1½ min. in boiling water	2–2½ min. in boiling water		6–8 min. with husks, 6 without	3–4 min. in boiling water

determination of adequacy of scalding of cut corn for freezing. The peroxidase test was carried out by adding 10 drops of 3 per cent hydrogen peroxide and 10 drops of an alcoholic solution of gum guaiac to 5 cc. of the filtered extract. The extract was prepared by pulping 25 kernels of scalded corn in 15 cc. of distilled water. A greenish blue color ½ inch in height on top of the test solution indicates active peroxidase and insufficient scalding. Table L shows how the peroxidase test becomes gradually negative when the scalding period is increased from 30 to 45 seconds.

TABLE L

COMPARISON OF SCALDING, PEROXIDASE ACTIVITY, AND THE QUALITY OF CUT CORN HELD AT −15° C. FOR 11 MONTHS (27)

Scalding Treatment	Peroxidase	Quality
None	Strongly positive	Poor color; sour odor; bitter flavor
15 sec. flowing steam	Strongly positive	Fair color; good odor; slight bitter flavor
30 sec. flowing steam	Slightly positive	Good color; good odor; good flavor
45 sec. flowing steam	Negative	Good in all respects
60 sec. flowing steam	Negative	Good in all respects

CATALASE TEST FOR PROCESSED VEGETABLES

Vegetables deteriorate rapidly in cold storage unless they have been blanched sufficiently to destroy the enzymes. It is important to test whether or not they contain appreciable quantities of catalase, because this is also a reliable indication of the degree of destruction of the other enzymes. The United States government requires that in some vegetables the enzyme catalase be destroyed after the blanching process as a control of quality of dehydrated products. Other government specifications, however, require that most dehydrated vegetables prepared for army use show a negative peroxidase reaction. See section on the action of enzymes in potatoes.

Thompson (28) recommended the following rapid and simple method for the estimation of catalase in vegetables after blanching and before dehydration or freezing.

Procedure. A 1.0-gram sample is ground in a mortar with 0.6 gram of calcium carbonate and 1.0 gram of fine sand. Ten cubic centimeters of water is added and the grinding is continued for 2 minutes. One cubic centimeter of the mixture is pipetted into one half of the divided flask (see Fig. 47), and 2 cc. of 3 per cent hydrogen peroxide is placed in the other half. The flask is attached to the manometer, and the whole apparatus is suspended in a constant-temperature water bath at 20° C. When the apparatus attains the bath temperature, the stop-

cock is closed (water level in U-tube set at 0 cc.) and the apparatus is shaken for 2 minutes. A reading of the pipet is then made to estimate the volume of oxygen liberated. The catalase may be reported as cubic

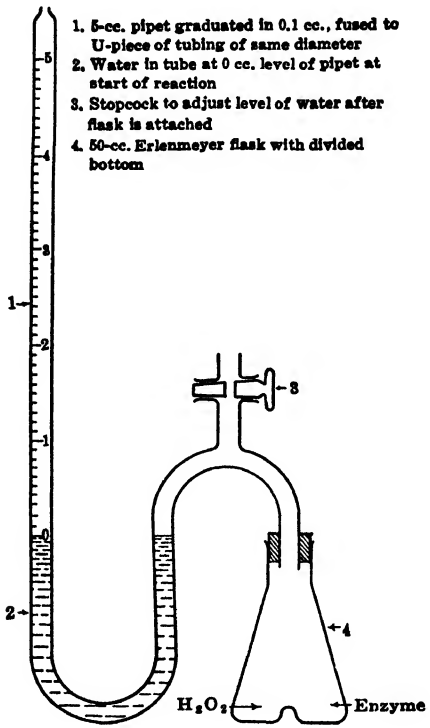

FIG. 47. Catalase apparatus.

centimeters of oxygen liberated by 0.1 gram in 2 minutes. The calculation suggested by Tressler and Evers (29) may also be used:

$$100 - \left(\frac{B - A}{C - A} \times 100\right) = \text{percentage of catalase inactivated}$$

in which A = inactivated sample (heat treated) } = always 0.0 cc.
B = blanched sample to be tested } = cubic centimeters of O_2 liberated by reaction
C = raw untreated sample }

THE PEROXIDASE CONTENT OF SOME PLANT PRODUCTS

The peroxidase content of a series of agricultural products is listed in Table LI (30). The materials marked with an asterisk contain appreciable quantities of inhibitors and the values are somewhat less exact.

TABLE LI

APPROXIMATE PEROXIDASE CONTENT OF SOME PLANT PRODUCTS

	Peroxidase Units per Kilogram		Peroxidase Units per Kilogram
Malt sprouts*	550	Beets	11
Horseradish	403	Wheat*	10
Turnips	110	Barley	9
White potatoes	36	Barley with hulls	3
Sweet potatoes*	30	Oats	3
Radishes	30	Onions	3
Rye	14	Soybeans	2

The grains were dry, the vegetables not dried. Except radishes and onions, the plants had been stored through the winter.

ENZMYES IN TOMATOES

Heat Inactivation of Tomato Pectase. The preservation of the pectic materials which give "body" to the tomato products is of utmost importance to the canning industry. It was known for many years that the enzymes in tomatoes had to be destroyed for best results before the so-called "hot break" method to inactivate the enzymes by heat was introduced. In crushed, unheated tomatoes 70 per cent of the natural pectin is destroyed in 10 minutes (31, 32). Kertesz has shown that cold-pressed tomato juice does not contain enzymes of the pectinase group. The enzyme pectase (esterase) causes the decomposition by rapid demethoxylation of the pectin. This enzyme may be completely destroyed by heating the tomato juice to 80° C. for 45 seconds (33). Kertesz suggests the following simple test for the control of effectiveness of heating: To 25 cc. of only slightly acid pectin solution 1 cc. of the heated tomato juice is added and the mixture is titrated with 0.1 *N* alkali in the presence of methyl red until the mixture just loses its last pink tint. If the pectase is destroyed, the mixture should show no color change after being kept at room temperature for 1 hour.

THE EFFECT OF CALCIUM ON CANNED TOMATOES

The quality of canned tomatoes is determined by the solidity of the pack. Much is lost in quality and flavor at the end of the season when whole tomatoes often fall apart during canning. Kertesz very appropriately suggested that calcium chloride be added to canned tomatoes. Pectase or pectin methoxylase converts soluble pectin, in the presence of calcium salts, into a gel. With 0.61 gram of anhydrous calcium chloride per No. 2 can, the solidity of the pack can be greatly improved.

The addition of calcium chloride, however, is not desirable when the fruit is sufficiently firm. It is with fully ripened fruits that the effect is most beneficial. Figure 48 shows calcium chloride-canned tomatoes 10 minutes after removal from the can (34).

The Action of Enzymes in Fruits. Fruit intended for storage is practically always collected green and allowed to ripen during storage. Freezing or keeping fruit too close to freezing impairs its quality. The optimum storage temperature varies with the type of fruit, but it is usually a few degrees above freezing. Fruits and most vegetables respire. During this process the plants take up oxygen and release carbon dioxide. In apples diastases form sugar from starch and a

FIG. 48. Canned tomatoes 10 minutes after opening the can. Left, control; right, canned with the permitted amount (0.07 per cent) of calcium chloride.

part of the sugar is changed to carbon dioxide. In apples, for instance, during the ripening stage, carbon dioxide production is at the maximum (16); it decreases gradually as the fruit becomes old. When all the starch is used up the apple dies. There is a final increase in physiological activity which suddenly reaches zero and is followed by decomposition of the fruit. This metabolic process is, of course, carried out by the enzymes within the cells of the fruit and is greatly accelerated by the presence of molds and bacteria.

Factors to be Considered during Storage Ripening of Fruits. Respiration, being necessary for ripening of fruits, necessitates a constant oxygen supply. Oxidation, however, produces heat. The heat of oxidation may be so great during transportation or storage as to cause the decomposition of much of the fruit. To eliminate this situation enzyme action must be decreased. This can be accomplished by lowering the temperature. Thus it is best to cool fruit before it is

shipped and so eliminate the danger of overtaxing the refrigerating equipment.

Another method of decreasing respiration is to place fruit in a dark place in an atmosphere containing an increased quantity of carbon dioxide (35). Apples respire and ripen very slowly when stored in air containing about 10 per cent of carbon dioxide. The storage time of apples in carbon dioxide-enriched air may be increased twice without affecting the quality of the fruit. For a review see reference (16).

Sometimes it is desirable that the ripening of fruits be hastened. This can be accomplished by an increase in temperature. Lemons are stored for 2 to 3 weeks at 50° C. Lemons and bananas are readily ripened when placed in air containing a small amount of ethylene gas. Ripening apples also give off ethylene gas, which has the opposite effect from carbon dioxide. Ethylene speeds up ripening. Thus ventilation is required. Inasmuch as ventilation results in evaporation and drying of the fruit, the humidity is kept at 75 to 80 per cent.

Denny (36) showed that ethylene has a marked effect in producing the desired ripe color in green apples. He exposed green lemons to ethylene gas and found that a dilution as low as 1/1,000,000 resulted in more rapid carbon dioxide formation and a rapid appearance of yellow color on the lemons. Chace and Sober (37, 38) observed a rapid ripening of pears, with resultant amylolysis and sugar formation, when the fruit was exposed to ethylene gas during ripening. Other investigators have also reported results on treating various fruits and vegetables with the gas (39–44).

Gane (45, 46) found that one apple (200 grams) emanates 1 cc. of ethylene gas during its lifetime. Niederl, Brenner, and Kelley (47) found that 0.1 to 0.2 cc. of ethylene is given off per 100 pounds of fruit during the ripening period.

Swanson (48) found that combined wheat is better for bread making after it has been aged for several weeks. To accelerate the ripening, Balls and Hale (49) treated freshly harvested combined wheat with air containing 1 part per 1000 of ethylene. The ethylene-treated wheat and a proper control were then subjected to a baking test. The ethylene-treated wheat furnished bread that had excellent texture and color and a normal general appearance. The control had a smaller volume and was soggy and greenish (see Fig. 49). The untreated wheat, after a month's aging, had attained all the properties of wheat exposed to ethylene. Nord and Franke (50, 51) have shown that ethylene increases the permeability of the cells, and this results in an increase in enzyme activity.

According to Haller (52) and Emmett (53) the softening of pears and apples is caused by the enzymic decomposition of pectin.

Some Metabolic Studies. Archbold and Haynes (54, 55) found complete hydrolysis of the starch in apples stored for a period of 50 days. Kokin (56) reported on his observation of the tree ripening of apricots and apples. First glucose is produced, which is later

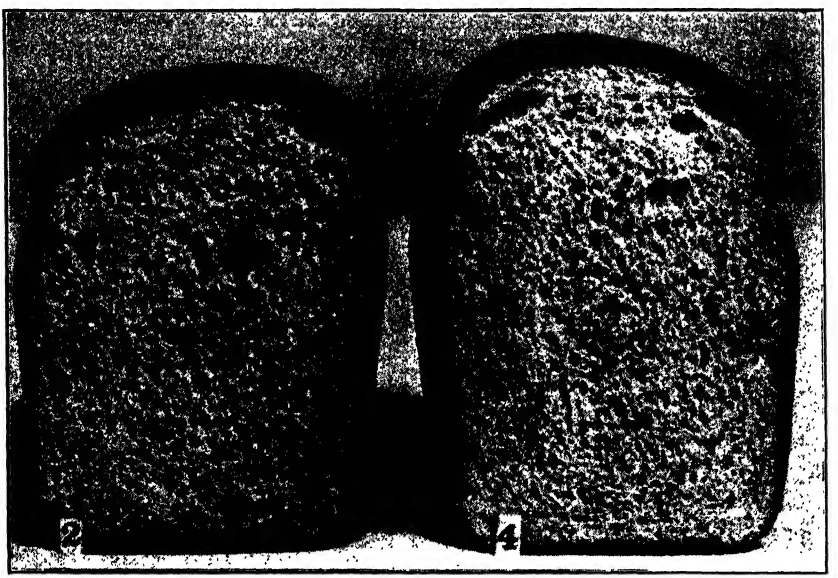

FIG. 49. Effect of ethylene on wheat: No. 2, untreated; No. 4, treated—after 19 days.

changed to fructose. Finally sucrose is formed from the monosaccharides. There is a decrease in the citric acid and malic acid content of apples during storage (57).

Poland and associates (58) studied the quantitative relations of the sugars of the Gros Michel banana, the commercial type in this country, at various stages of ripeness. Total sugars increased from 2 per cent in the green fruit to 20 per cent in the ripe fruit. Total reducing sugars in the partly ripe fruit were 3.69 per cent; in the fully ripe banana they were 7.45 per cent. The sucrose increased from 7.95 to 12.08 per cent. The ratio of glucose and of fructose to total reducing sugar was almost constant. Glucose was about 58 per cent and fructose about 42 per cent of total reducing sugars at any stage of ripeness. Figure 50 represents tests of total sugar changes in samples from practically all Central American banana-producing countries,

using more than 100 cargoes. The changes were recorded from the arrival of the bananas at the coast to senescence.

Color Changes in Fruits

Color changes in fruits and fruit products, such as take place on injury or over-ripening, may be caused by the enzymic oxidation of pigments or precursors of pigments. The color formation, however, may also be produced by non-enzymic oxidation. The mechanism of the darkening is as yet not completely known. According to Bach and Chodat a substance is autoxidized to an organic peroxide which, acti-

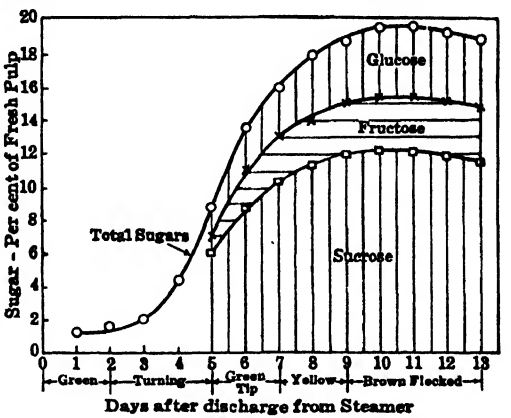

Fig. 50. Variation in sugar content of the banana during ripening.

vated by the enzyme peroxidase, oxidizes chromogens and tannins. Some colors are produced by phenol oxidases acting on certain phenolic compounds (59).

Nagai (60) found that certain flavanols and flavones are oxidized by oxidizing enzymes to brownish substances. Sand (61) showed that quercetin is enzymatically oxidized to deep red products which rapidly change to brown pigments. This reaction takes place at the expense of flavanols. Apples, pears, and other fruits are said to contain *o*-dihydroxyphenylalanine (dopa) which is oxidized to a brown pigment by the enzyme dopa oxidase (62), which is also present in the fruits.

According to Balls and Hale (63, 64), darkening of injured apple tissue is hastened by horseradish peroxidase and therefore apple tissue discoloration is a reaction produced by peroxidase. They believe that hydrogen peroxide formation from molecular oxygen by a respiratory enzyme is a required intermediary step in the browning of apple tissue.

Darkening of most fruits is due to the oxidation of catechol tannins containing orthodihydroxy groupings (65). Peroxidase is inhibited by cysteine, glutathione, and other sulfhydryl compounds. These substances prevent browning by their action on peroxidase. The available data indicate that polyphenols are probably oxidized by phenolases and peroxidases. Balls and Hale (66) found that, owing to its glutathione content, pineapple juice prevents darkening of cut fruit. Because of the complexity of the various oxidizing systems and the large number of substances which interact, a single formula cannot be presented for the natural darkening of fruit tissues.

Oxidation is, of course, the basic cause of browning. This can be more or less eliminated by destroying the oxidases by heat, by storing

TABLE LII

THE BROWNING OF PEACH JUICE

	Observations Taken in 30 Minutes		
Variety	Juice only	With Guaiacol (oxidase)	With Guaiacol and H_2O_2 (peroxidase)
Sunbeam	O	O	++++
Oriole	+	+	+++
Rochester	++	++	++++
Valiant	+	+	++++
Elberta	+	+	++++
St. John	+	+ (+)	++++
South Haven	+	+	++++
Massasoit	+	+	++++
Golden Jubilee	+	+	++++
Arp	+	+	+++
Foster	+ (+)	+ (+)	++++
Viceroy	+	+	++++
Early Charlotte	++	++	++++
Livingston	++	++	++++
Vedette	++	++	++++
Ingold	++	++	++++
Eclipse	+	+	++++
Veteran	+ (+)	++	++++
Fitzgerald	+	+	++++
National	+	+	++++
Ideal	+	+	+++
Muir	+	+	++++
Armstrong	+	+	++++
Orange Cling	++	++	++++
Niagara	++	++	++++
Early Crawford	++	++	++++

O = no change; + = some darkening; + + = light brown; + + + = brown; + + + + = dark brown.

at a low temperature, by reduction of *p*H, by halides, and by sugar syrup. Complete elimination, however, is possible only if sulfur dioxide and other reducing substances are used. For an extensive bibliography see the excellent paper by Joslyn (67).

Non-enzymic or autocatalytic oxidation may be effected by intermediary enzymic oxidation products, loss of vitamin C, absorption of oxygen, and formation of peroxide followed by polymerization and the interaction of these products. Metallic salts and traces of metals play an important part in autocatalytic oxidation.

A Successful Attempt at Eliminating the Browning of Yellow Peaches

Sunbeam, a yellow peach originated by the New Jersey Experiment Station, showed only very slight darkening. "Slices and juice of Sunbeam peaches were still of the original color when practically all other varieties showed definite darkening." Table LII shows the

TABLE LIII

TANNIN CONTENT AND CATECHOL REACTION OF PEACHES

Variety	Average Percentage of Tannin in the Fresh Peach	Catechol Reaction In Juice	Catechol Reaction In 50 Per Cent Alcohol Extract
Sunbeam	0.005	O	O
Sunbeam*	0.009	O	O
Golden Jubilee	0.026	++	++
Massasoit	0.034	++	++
Eclipse	0.053	++	+++
Niagara	0.059	+++	+++
Fitzgerald	0.062		+++
St. John	0.067	+++	+++
Foster	0.069	+++	+++
South Haven	0.116	+++	+++
Early Crawford	0.127	+++	+++
Elberta	0.132	+++	+++†
Arp	.....	++	
Oriole	.....	+++	+++
Rochester	.....	+++	+++
Ingold	.....	+++	
Orange Cling	.....	+++	+++
Armstrong	.....	+++	+++

* Later picking.

† Very strong reaction.

O = no change; + = some darkening; + + = light brown; + + + = brown; + + + + = dark brown.

browning of the juice of this peach, as compared to that of other peaches, with guaiacol as a substrate for phenol oxidase and hydrogen peroxide as a substrate for peroxidase. In the first column the color changes as observed on the original juices are recorded. Sunbeam peaches did not darken. Arp, one of Sunbeam's parents, was somewhat better than other varieties. Definite darkening occurred, however, in 30 minutes. The other parent, not recorded in Table LII, showed discoloration. The phenol oxidase test could not be observed, owing to the darkening of all the juices without the substrate (guaiacol). All showed the peroxidase reaction. Kertesz (68) found that the fact that slices of Sunbeam peaches did not darken when left in air was not due to lack of oxidases. As may be seen in Table LIII, failure of these peaches to discolor is due to the absence of catechol tannins in this variety of peach.

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CHAPTER XXVII

PECTIN-DECOMPOSING ENZYMES AND THEIR USE IN THE FRUIT JUICE, WINE, AND JELLY INDUSTRIES

Pectins are parts of the cell walls of plants. They are polymerization compounds of galacturonic acid of an unknown constitution. The pectic compounds of the middle lamella are believed to be the cementing link between cells. Softening of fruits during ripening is probably caused by enzymic breakdown of the cementing pectins (1, 2). Pectins are soluble carbohydrates of colloidal nature. They are formed from an insoluble compound, called protopectin, by boiling in water or dilute acids or by the action of enzymes. Lemons (albedo), oranges (albedo), apples, sugar beets, flax stalks, strawberries and raspberries, and many other fruits are good sources of pectin.

The pectins, or the pectic substances as they are sometimes called, are very important industrially. They are used in the setting of jams and jellies, and as emulsifying agents in the manufacture of oil emulsions and other foods. The pectic substances and the pectin-decomposing enzymes have been extensively studied in connection with the preparation of textile fibers, the clarification of fermented and unfermented juices, and the stabilization of " clouds " in tomato and citrus juices.

The Agricultural and Food Chemistry Division of the American Chemical Society proposed the following nomenclature for the pectic substances (3):

Pectic Substances. " Pectic substances " is a group designation for those complex, colloidal carbohydrate derivatives which occur in or are prepared from plants and contain a large proportion of anhydrogalacturonic acid units which are thought to exist in a chainlike combination. The carboxyl groups of polygalacturonic acids may be partly esterified by methyl groups and partly or completely neutralized by one or more bases.

Protopectin. The term " protopectin " is applied to the water-insoluble parent pectic substance which occurs in plants and which upon restricted hydrolysis yields pectin or pectinic acids.

Pectinic Acids. The term " pectinic acids " is used for colloidal polygalacturonic acids containing more than a negligible proportion of methyl ester groups. Pectinic acids, under suitable conditions, are capable of forming gels with sugar and acid or, if suitably low in methoxyl content, with certain metallic ions. The salts of pectinic acids are either normal or acid pectinates.

Pectin. The general term "pectin" (or pectins) designates those water-soluble pectinic acids of varying methyl ester content and degree of neutralization which are capable of forming gels with sugar and acid under suitable conditions.

Pectic Acids. The term "pectic acids" is applied to pectic substances mostly composed of colloidal polygalacturonic acids and essentially free from methyl ester groups. The salts of pectic acids are either normal or acid pectates.

Many bacteria, molds, and higher plants contain active pectin-destroying enzymes. For excellent reviews see references 2 and 4. Three pectic enzyme systems are now known: *protopectinase, pectinase* (*polygalacturonase, pectolase*), *and pectase* (*pectin-methoxylase*).

1. Protopectinase. Protopectinase is the enzyme that softens plant tissue by hydrolyzing the middle lamella of plants (protopectin). The exact nature of this reaction is not known. Various pathogenic microorganisms, fungi, and bacteria-infecting plants contain this enzyme.

Good sources of protopectinase are *B. carotovorus* (5), *B. mesentericus* (6), *Botrytis cinerea* (7), *Rhizopus* (8), *Sclerotinia cinerea* (the plum-rotting organism), and *Fusarium chromiophthoron* (9). The softening of fruits such as apples, pears, peaches (10, 11), and of some vegetables such as tomatoes is caused by protopectinase, which is present in these plants. Ehrlich (12) prepared a highly active protopectinase by extracting the mycelium of *Penicillium* with water and precipitating the enzyme with alcohol. This preparation dissolved up to 60 per cent of the sugar-beet tissue in 24 hours.

2. Pectinase (Pectolase, Polygalacturonase). This enzyme splits polygalacturonic acid into monogalacturonic acid by opening glycosidic linkages. It is mostly present in fungi and bacteria and is frequently accompanied by pectase (pectin-methoxylase) (2).

Although barley and barley malt do not contain pectin, pectinase may be prepared from the barley malt (13). Pectinase is present in *Sclerotinia cineria* (14) and in a great variety of bacteria (15), especially those microorganisms thriving on fruits (16). Various fungi such as *Rhizopus tritici* (17), *Sclerotinia cinerea, Botrytis cinerea* (18), *Penicillium ehrlichii* (19), and other penicillia (20) contain pectinase. Menon (21) described the pectinases of the parasite molds *B. cinerea, Monilia fructizena, Pythium de baryanum, Phytophthora erythroseptica, Fusarium fructizenum,* and *Glocosporium fructizenum.*

Jansen and MacDonnel (22) have reported on the rate of glycosidic hydrolysis of pectin and enzyme- and alkali-prepared pectic acids by the commercial enzyme mixture "Pectinol 100 D." This paper contains many interesting observations. However, the preparation of specific enzymes from molds offers no difficulties, and results

obtained with enzymes of known origin and definite purity are of greater value to the enzymologist.

The optimum *p*H of mold pectinase is at *p*H 3.0 to 3.5, and it is influenced, as in other enzymes, by the composition of the reaction mixture, source of the enzyme, purity of the enzyme, etc.

3. Pectase (Pectin-Methoxylase, Pectinesterase). Pectase, the enzyme that splits off methoxy groups (methyl alcohol) from pectin and converts soluble pectin, in the presence of calcium salts, into a gel, was discovered by Fremy in 1840. There are some indications that pectase is a non-specific esterase identical with plant esterases (lipases).

Preparation and Properties of Pectase. The press juice of fresh-cut alfalfa converts pectin into a solid gel within a few seconds. Mehlitz (23) obtained 1682 grams of crude press juice from 5000 grams of alfalfa. He further purified this juice by preserving it with chloroform, placing it in a dark place, and allowing it to settle for 22 hours. Then the crude juice was filtered and the pectase was precipitated by the addition of 2 volumes of 90 per cent alcohol. The precipitate was collected and dissolved in 400 cc. of distilled water by allowing it to remain in the water for 15 hours with occasional shaking. Then the pectase was separated from the insoluble residue by filtration. The enzyme was precipitated again with 90 per cent alcohol. The precipitate was dried over calcium chloride in a vacuum desiccator. The dry enzyme powder kept well for 4 months, whereas its 10 per cent solution lost most of its activity in 3 to 4 days. A calcium concentration of 0.15 per cent was found to be the optimum amount for gel formation, and the *p*H optimum of the alfalfa pectase was at 4.8 to 5.0 at a calcium pectate concentration of 0.7 per cent.

Paul and Grandseigne (24) prepared active pectase by extracting sprouting legumes with water. The extract was mixed with a colloid, such as starch or tragacanth, and was precipitated with acetone. The resulting gel was dried. Tzerevitinov and Rozanova (25) examined a series of plants and grasses and found that potato plants and Swedish clover were rich sources for pectase.

It was found that this enzyme removes the methoxyl group from pectin and that gel formation is a secondary reaction influenced mainly by the state of the substrate. For this reason Kertesz (26) differentiates between the " methoxylase " reaction and the " pectase " (gelation) reaction. According to Kertesz, gel formation depends on the composition of the mixture and is influenced by *p*H changes brought about by the liberated carboxyl groups. Acid formation may increase the *p*H of the medium to such an extent that gel formation is greatly

delayed. Kertesz proposes the method applied by Knaffl-Lenz (27) for lipase for the titration of the carboxyl groups of the galacturonic acids set free during the reaction. By this method alkali is added at short intervals, the *p*H being kept constant (*p*H 6.2) during the course of the reaction. This cannot be accomplished with buffers. The pectin methoxylase shows increased activity with increasing *p*H. In an alkaline solution, however, pectin is demethoxylated, so that enzyme activity can be determined on the acid side of the *p*H scale only.

Table LIV shows the pectin methoxylase content of some plants

TABLE LIV

ENZYMATIC PECTIN DEMETHOXYLATION BY VARIOUS MATERIALS

Material	Applied in Determination (cc.)	Dry Matter (per cent)	Pectin Methoxylase Units	
			Per Cc.	Per Gram
Filtered juice of Windsor sweet cherries	5.0	11.14	0.182	1.6
	10.0		0.189	1.7
Juice of shipped Florida tomatoes	1.5	4.25	2.39	56
	2.0		2.42	57
	2.5		2.57	60
	3.0		2.48	58
				58 (Average)
Juice of ripe hothouse tomatoes, Forcing Wonder	1.0	3.59	5.02	140
	2.0		4.90	137
10 per cent extract of dried tobacco powder	2.0	3.86	0.44	11.4
	3.0		0.42	10.9
Press juice of leaves of hothouse-grown (Kentucky) green White Burley tobacco	1.0	3.82	1.69	44
	2.0		1.66	43
Press juice from alfalfa	1	6.99	1.08	15.5
	2		1.17	16.7
	3		1.13	16.1
	4		1.04	14.9
				15.8 (Average)

Steapsin "Difco" (6 years old) 5.6 units per gram per cc.

and Table LV shows that of some commercial enzyme mixtures as reported by Kertesz (26).

It had been known for some time that pectin methoxylase is an esterase similar in action to certain lipases and perhaps identical with plant lipases. Experiments supporting this view had been reported by Kertesz, who showed that castor-bean lipase and pancreatic lipase have both pectin methoxylase and pectase activity. Owing to the non-

specificity of the enzyme, Oppenheimer (28) suggests that it should not be called pectin methoxylase. Indeed, this enzyme is a typical esterase, liberating methanol or other alcohols from its esters (29). Thornberry (30) determined the pectase activity of several microorganisms by the

TABLE LV

DETERMINATION OF PECTIN METHOXYLASE IN MIXTURES OF PECTIC ENYZMES (PECTINASES)

Material	Applied in Determination (cc.)	Dry Matter (per cent)	Pectin Methoxylase Units	
			Per Cc.	Per Gram
Malt extract (10 per cent, fresh)	5	2.58	0.033	1.3
	10		0.034	1.3
Commercial pectinase, No. 29AP (made of *Monilia* sp.)	2	2.00	0.73	37
	4		0.65	33
	6		0.71	36
	8		0.68	34
				35 (Average)
Commercial pectinase, No. 46AP (made of *Aspergillus* sp.)	6	2.00	0.30	15
	8		0.38	19

rate of hydrolysis of the ester linkage of the monocalcium salt of monoethyltartaric acid.

Kertesz and Loconti (31) have reported that precipitation of pectic compounds by the tomato enzyme, in the presence of calcium, may take place very rapidly and that only 40 per cent or less of all the methoxyl groups available had to be removed. Owing to the rapidly acting enzyme, most commercial tomato juices contain pectin which is partially demethoxylated and precipitated.

McColloch and coworkers (32) have described a method by which they obtained dry pectase preparations from tomatoes. These preparations were 100-fold more active than the most active dry ones prepared by other investigators. The main phases of one procedure were to adjust the comminuted tomatoes to *p*H 8, freeze out the water, and remove the ice from the comminuted tomato slurry. The enzyme was precipitated from the supernatant liquid by dialysis against distilled water. In a second procedure, the pectase was obtained by washing the pulp with hydrochloric acid after the serum had been removed, and extracting the pulp with 10 per cent sodium chloride followed by dialysis. The precipitated enzyme was redissolved in saline solution and assayed.

Lineweaver and Ballou (33) found that *alfalfa pectase,* prepared

according to Mehlitz, is, at *p*H 5.7, about 30 times as active in the presence of 0.2 *M* monovalent cations or 0.02 *M* divalent cations as in the absence of cations, but at *p*H 8.5 these concentrations of cations have no effect on the activity. Pectase is only slightly inhibited by sodium pectate at *p*H 8.5. It is considerably inhibited at *p*H 5.7 in the absence but not in the presence of cations. The enzyme-substrate dissociation constant at *p*H 5.7 is the same (0.04 per cent) at 0.025 and 0.2 *M* sodium ion. These investigators observed that pectase is adsorbed on Celite near *p*H 5 and may be eluted with the aid of dilute salt solutions.

Weber and Deuel (34) observed that, when three unidentified molds were cultivated on a medium containing 3.3 per cent malt extract and 0.7 per cent pectin, there was no definite ratio between the pectinase content of the mycelium and the medium (liquid). In one case, the dried mycelium contained twice as much pectinase as the liquid; in the second case, liquid was about 5 times more active, whereas the third mold produced approximately equal amounts of the enzyme in the mycelium and liquid respectively.

Enzymic Production of Pectinates for the Use of Industrial Gels. By partial removal of methoxyl groups from pectin, the resulting pectinic acid forms insoluble precipitates, or gels, with calcium or other metals. Acids, alkalis, or enzymes may be employed as the hydrolyzing agents. Viscosity tests or titration with sodium hydroxide may be used as a measure of desirability of a given product.

In a patent, assigned to the Secretary of Agriculture of the United States, Willaman and associates (35) described the preparation of a pectinic acid of predetermined methoxyl content and desired viscosity. In this process, tomato pectase is used for the partial removal of the methoxyl groups from any commercial pectin. The preferred pectin concentration is from 0.3 to 1 per cent. The *p*H is 6.00 and the temperature between 40° and 45° C. The reaction period is 1 to 2 hours. The *p*H of the mixture is kept constant by the continuous addition of sodium hydroxide. The reaction is terminated by the addition of a strong acid in order to bring the *p*H to 4.00 and the temperature to about 80° C. The mixture is cooled below 40° C., and the pectinic acid is precipitated with alcohol, filtered, and dried. This patent contains details for production and control. See also the articles by Mottern and Hills (36).

Baker and Goodwin (37) described a method in which acid is employed for the partial removal of the methoxyl groups.

Enzyme Activities and Composition of "Pectinol A." Fish and Dustman (38) studied the composition and activities of "Pectinols," which are industrial enzymic fruit juice clarifiers. Pectinol A con-

tains about 91.5 per cent sugar, mainly dextrose and levulose. These sugars can be extracted with 80 per cent ethyl alcohol, an active sugar-free enzyme mixture being left. By means of optical rotation and iodine-reduction methods, it is shown that the sugar-free Pectinol A completely splits 10 times its weight of pectin to galacturonic acid in 24 hours at 39° C. The *p*H of the reaction mixture changed from 3.6 to 2.8. The pectase activities and optimum *p*H's of various pectinols have been determined. Sugar-free Pectinol A also hydrolyzes sucrose, starch, and maltose.

ENZYMIC CLARIFICATION OF FRUIT JUICES

The manufacture of fruit juices has grown enormously. In 1939 about 35 million cases of fruit and vegetable juices were processed, as compared to 2 million cases in 1931, which was the third season of production. This increase in consumption is due to the development of better processing methods.

The object of the fruit-juice manufacturers is to make a clear, sterile, stable beverage which retains the food values and the flavor of fresh fruit. The use of sterile equipment makes possible the manufacture of uncooked apple juice which is kept under pressure of carbon dioxide. According to Charley (39), pasteurization of fruit juices should be carried out at a temperature which does not change the flavor, by passing the juice through a heated tube containing two metal surfaces between which an electric current is passed. Keeping quality may be improved by adding sugar, concentrating the juices, withholding feeding elements for yeast, and maintaining vacuum storage.

It appears that all countries make use of pectin-decomposing enzymes as an aid in clarifying fruit juices. Indeed all authors agree that some clarification process must be applied in order to make fruit juices filterable, and most of them use enzyme clarifiers. Others, however, use tannin and gelatin or a filter aid, such as diatomaceous earth.

In 1922 Gusmer (40) obtained a patent for the clarification of fruit juices with mold diastase. In 1933 a patent was granted to Mehiltz (41) for the use of bacterial filters (Seitz, E. K.) in connection with enzymatically clarified fruit juices. He recommended the use of enzyme mixtures in general, such as *Aspergillus oryzae* and *A. wentii*, and of malt for the clarification of fruit juices. At the same time Willaman and Kertesz (42, 43) patented the use of the pectinases of *A. niger, A. flavus, Penicillium glaucum, A. oryzae, A. fumigatus, A. parasitians, A. tamarii, Rhizopus tritici, R. nigricans, A. wentii,* and those of malt for fruit-juice clarification.

Bohne (44) prepared mixtures for clarifying fruit juices, wines, and beer from a fungus (*Aspergillus*) which yielded pecteolytic, diastatic, and proteolytic enzymes. The fungus was cultivated in the presence of an albumin insoluble in cold water, e.g., gelatin or casein, until the albumin was converted into compounds of lower molecular weight which are soluble in cold water. The mixture was then worked up in the usual way into a dry powder. This preparation may be used with 0.5 to 2 per cent active carbon to prevent the introduction of an unpleasant odor or taste to liquids (45).

Horovitz-Vlasova and Rodionova (46) reported that the usual practice of combining pectin removal with fermentation in the manufacture of fruit juices is not necessary. Pectin can be destroyed without fermentation by employing a pectase-producing acido-yeast such as *Saccharomyces oxycocci* (from cranberries). Some strains of *S. oxycocci* also ferment sugars in an acid medium. By means of the proper yeasts the pectin and sugar fermentation can be carried out separately or together. Thus fruit or berry juices can be freed from pectin with or without the removal of sugar. In making expressed cranberrry juice, conservation of sugar has a commercial advantage.

PECTIN-DECOMPOSING ENZYMES IN APPLE JUICE MAKING

At Michigan State College (47) the following method is employed. The apples are ground and the juice is expressed during the afternoon. The clarifying enzyme is added to the cider in the late afternoon. Then the cider is placed in cold storage and filtered the following afternoon or about 16 hours after pressing. Twenty-five ounces of Pectinol per 100 gallons of cider gives good results under these conditions. Table LVI shows the amounts of Pectinol (an enzyme product of Rohm

TABLE LVI

OUNCES OF PECTINOL REQUIRED PER 100 GALLONS OF CIDER UNDER DIFFERENT CONDITIONS OF TIME AND TEMPERATURE

Temperature (° C.)	5 hours	15 hours	30 hours	48 hours
40	..	30	15	10
60	54	18	9	6
100	14	5	..	..

and Haas) that should be used per 100 gallons of cider for different temperatures and different periods of time as suggested by Fabian and Marshall (47). Figure 51 shows the effect of the clarifying enzyme on apple cider.

According to Fabian and Marshall (47), cider clarified by Pectinol and filtered shows little, if any, loss of color and a negligible loss of flavor. Marshall (48), however, encountered some difficulty with Pectinol. Apple juice that had been treated with this product and

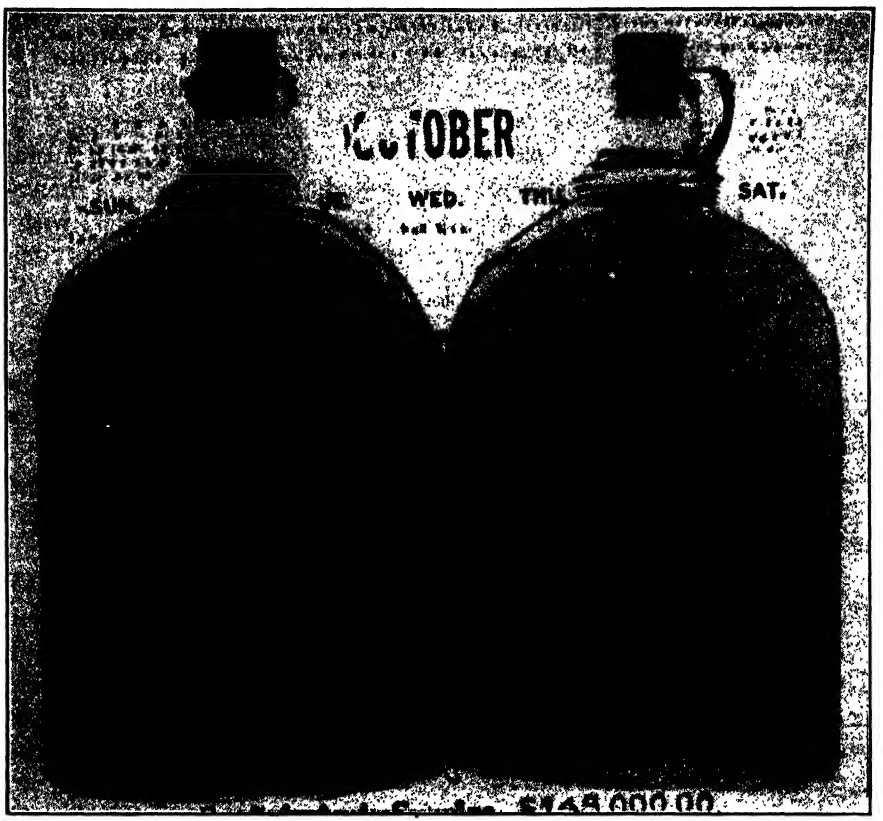

FIG. 51. The cider in the jug at the left was strained twice through two thicknesses of cheesecloth. That in the jug at the right was subjected to enzymic clarification followed by filtration. The unfiltered product is opaque whereas the filtered one is clear enough so that the figures on the calendar behind it are clearly visible.

preserved by filtration by the use of germ-proofing filters formed quite a heavy precipitate. He suggests that after enzyme clarification the apple juice either be flash-pasteurized or subjected to the ordinary pasteurization in bottles. Apple juice intended for cold sterilization or germ-proofing filtration should not be clarified with Pectinol. Figure 52 shows equipment used by the Michigan Experiment Station in making experimental packs of apple juices (49).

According to Hilton (50), tannin, gelatin, or pectic enzyme products

are employed in Canada for the clarification of apple juice. The pectic enzyme treatment was found to be the simplest method. Hilton found that stored apple juice that had been treated with "Pectinol A (soluble)," or Filtragol, "often throws a light sediment after a few months, in bottle or can."

A very ingenious application of the enzymic clarification procedure was recommended by Widmer (51) in 1933. In his method the pectin-

FIG. 52. Some of the equipment used in making experimental packs of apple juice at the Michigan Experiment Station (47). *A* is flash sterilizer with juice intake at 1; steam intake is through 2 to casing 3, which encloses the flattened and coiled tubing. Bottles may be filled by the siphon fillers, 4, which are connected by rubber tubing to the coil of flattened tubing. Cans are filled with rubber tube 5. *B* is an automatic closing machine. When the closed cans leave this machine, they invert onto the belt of *C*, which delivers them to the cooler at *D*. This can cooler consists of a belt moving 100 feet per minute, which causes the 20-ounce cans to rotate approximately 100 r.p.m. while being moved forward slowly under a spray of cold water.

ase is added to the fruit mashes before pressing. This effects a loosening of the complex colloids and decomposition of the interfering protopectins, and results in a higher yield of fruit juices.

PECTIN-DECOMPOSING ENZYMES IN WINE MAKING

It is very important to the maker of wines that the wine should be clear and sparkling as soon as possible after fermentation.

Mehlitz and Scheuer (52) reported that the cloudiness of fruit juices and musts is due mainly to pectic compounds. These investigators studied this problem in great detail and concluded that added pectic enzymes completely decomposed the pectin into methyl alcohol and

galacturonic acid. Hickenbotham and Williams (53), confirming Widmer's work, found that the must of white grapes was most readily cleared when the clarifying enzyme was added before fermentation. Addition of the enzyme to the crushed grapes resulted in a higher yield of juice and greater ease in pressing. Small amounts of sulfur dioxide, employed to prevent fermentation during settling, did not affect the pectic enzymes. This was confirmed by Besone and Cruess (54) in commercial practice; e.g., the natural enzymes of grapes clarify by acting on the pectic substances, often yielding brilliant, clear supernatant fluid. Some samples of unheated grape juices of the same type do not clear satisfactorily, however.

Experiments Showing the Action of Natural Pectic Enzymes (52). Five hundred cubic centimeters of Muscat of Alexandria and Thompson seedless juices is heated to 81.5° C. and cooled. Similar volumes of juice are not heated. To both are added 175 p.p.m. of SO_2 as $K_2S_2O_5$ (containing 57 per cent SO_2), and the samples are allowed to settle for 2 days. The unheated samples clot and settle almost clear, whereas the heated samples remain turbid.

In a similar set of experiments the juice of Semillon grapes was employed. Both heated and unheated samples failed to clear, however, nor did all samples of Muscat juice clear satisfactorily. This shows the great variations in the concentration of the natural enzymes in the grape juices.

Experiments Showing the Action of Added Pectic Enzymes. Samples of 400 cc. of the press juice of Muscat grapes are prepared as follows:

1. Use 175 p.p.m. sulfur dioxide, no enzyme. Allow to stand 24 hours. Add pure yeast and ferment. Rack. Store. Note velocity of clarification.

2. Add sulfur dioxide; add yeast immediately. Continue as in 1 after fermentation.

3. Heat to 79° C. Cool. Add yeast. Continue as in 1.

4. Control. Do not add SO_2. Do not let settle. Add yeast immediately.

5. Heat to 79° C. Cool. Add yeast.

6. Add SO_2. Add Pectinol, 1:1000. Allow to settle 24 hours. Rack. Add yeast.

7. Heat to 79° C. Cool. Add SO_2 and pectinase.

Results. Number 3 was turbid and cloudy; 1 was moderately clear; 6 and 7 were very clear. After fermentation, racking, and 2 weeks' storage, 1 was clearer than 2, 3, 4, and 5. Numbers 3 and 5 were much

less clear than 7. Thus the samples to which pectinase was added cleared best whereas the unboiled sample containing the natural enzyme produced only partial clarification.

Experiments with Excessive Natural Pectin. Crushed Concord grapes (a red variety) are washed and boiled for 2 minutes to extract pectin and the blue-red color. After cooling to room temperature the juice is filtered through a small piece of cotton. To 10 cc. of the juice 10 milligrams of a pectinase preparation is added. Another 10-cc. portion is kept as a control. The pectinase-containing sample will form a heavy dark blue precipitate within 4 or 5 hours whereas the control remains turbid. It should be noted, however, that the rate of clarification of the juice differs markedly with the variety.

Semi-Industrial-Scale Experiment. Besone and Cruess have treated 50 gallons of Folle Blanche juice with 1 gram of Pectinol W per liter and with 150 p.p.m. of sulfur dioxide. The juice was clear in 24 hours and was racked; it resulted in 7 gallons of precipitate which, on continued settling, yielded 2 gallons of heavy precipitate. The clarified juice was fermented with pure yeast. Another lot was fermented with pure yeast without the addition of Pectinol. The wine made with the aid of Pectinol was clear within 2 days after cessation of fermentation. The wine that was not treated with Pectinol remained hazy 4 months after fermentation was complete. Besone and Cruess state that the difference was very striking, but that they have no results on wines stored for 1 year or longer.

The best temperature for the enzymic clarification of wines is at 15° to 21° C.

Few studies are available concerning the other enzymes of wine. Wine contains oxidase, invertase, and catalase. It does not contain amylase and carboxylase.

Fatty acid esters are produced by enzymic action during fermentation. These are the chief contributors to the flavor of wines. Esters of polybasic acids are produced by non-enzymic action; these, however, are not flavor contributors. The *p*H of wine is a directive factor in the esterification process. The theoretical limit of esterification is not attained even in wines 50 years old (55).

PECTIC ENZYMES IN CITRUS FRUITS

Citrus juices contain pectic enzymes which produce clear undesirable juices. Joslyn and Sedky (56) investigated the conditions required for the heat inactivation of the precipitate-forming pectic enzymes in

citrus juices. They found that heating orange juice or grapefruit juice at *p*H 4 for 1 minute at 80° C. destroys the pectic enzymes. Lemon juice requires only a temperature of 70° C. under the same conditions. At *p*H 2.5 the pectic enzymes of orange juice and grapefruit juice are inactivated at 75° C. and those of lemon juice at 70° C.

MacDonnel, Jansen, and Lineweaver (57) studied the pectase content of citrus fruit tissues and found that this enzyme is bound to the solid particles, very little being present in the edible portion of the orange or the press juice of the flavedo. Optimum extraction of the enzyme was obtained at *p*H 8 and a sodium chloride concentration of 0.25 *M*. Similar to the alfalfa esterase, the orange enzyme is considerably activated by salts. Orange esterase shows optimum response at *p*H 7.5 and a concentration of 0.02 to 0.005 *M* divalent cations. For monovalent cations, a concentration of 0.12 *M* was required for highest activity and inhibition did not take place until a concentration of 0.4 *M* was reached. Salts also affected the extraction, adsorption, elution, and stability of the pectase.

OTHER FRUIT JUICES

Tzerevitinov found that a press juice of leaves of clover, potato, or alfalfa containing pectase was effective in clarifying fruit and berry juices. A 0.1 per cent pectase press juice completely clarified, precipitating pectic acid within 6 days, whereas without added pectase 6 months was necessary. The optimum temperature of the pectase was at 55° to 60° C. The optimum *p*H varied with the kind of juice, less acid being required for clarifying cider and more acid (*p*H 3.3) for raspberry juice (58, 59).

Charley (60) obtained satisfactory results with Filtragol, an enzyme which decomposes pectin at the rate of 0.2 per cent, using 2 grams per 1000 cc. Pectin was completely removed in 30 hours from strawberry juice, the slowest of a number of juices to respond to the treatment. The juice was clarified by means of a Sharples supercentrifuge. Filtration was greatly aided by increasing the acidity from 0.4 to 1.1 per cent by the addition of citric acid.

Joslyn and Sedky (61) studied the rate of decomposition of natural pectins of various fruits by the enzymes occurring within these fruits: apples, cucumbers, oranges, lemons, and grapefruit. The decomposition of pectin in the fruit extracts was followed by their clarification.

For the technology of fruit juices the excellent book by Tressler, Joslyn, and Marsh (62) is recommended.

THE USE OF ENZYMES IN THE MANUFACTURE OF FRUIT JELLIES

Pectins, in the presence of definite concentrations of sugar and acid, form fruit jellies and jams. Table LVII shows qualities of various

TABLE LVII

Fruits Rich in Pectin and Acid*	Fruits Containing Less Pectin and Acid†	Fruits Rich in Pectin, but Low in Acid‡	Fruits Containing Acid but Low in Pectin§	Fruits Low in Pectin and Acid‖
Apples, sour and crab	Apples, ripe	Apples, kinds low in acid	Apricots, sour	Apricots, ripe
Blackberries, sour	Blackberries, ripe	Bananas, unripe	Cherries, sweet varieties	Elderberries
Cranberries	Cherries, sour varieties	Cherries, sour	Peaches, sour	Peaches, ripe
Currants	Fejoias	Figs, unripe	Pineapples	Pomegranates
Gooseberries	Grapes, California	Grapefruit, peel	Rhubarb	Prunes
Grapes, eastern	Loquats	Guavas, ripe	Strawberries	Raspberries
Guavas, sour	Plums	Oranges, peel		Strawberries
Kumquats		Pears		Over-ripe fruits
Loganberries		Pie melon		
Lemons		Quince, ripe		
Oranges, sour				
Plums, sour				

* Make jelly easily.
† Make jelly if care is exercised.
‡ Require addition of acid or acid fruits.
§ Require addition of pectin or fruit rich in pectin.
‖ Require addition of pectin and acid.

fruits for jelly manufacture on the basis of pectin and acid content (63).

It is interesting to note that Baker (64), of the Delaware Agricultural Experiment Station, discovered that edible non-sugar pectinate jellies may be prepared from partially demethoxylated pectins. Small amounts of calcium salts are required for the formation of these jellies. Lemon and grapefruit juice have been jellied without added sugar. Other fruit juices, such as strawberry, raspberry, and cherry, when prepared by the new method with a small amount of added sugar had an excellent appearance and flavor. This method, according to Baker, requires further research before the products can be placed on the market.

In order to obtain clear, sparkling, and appealing jellies, diastatic treatment of the fruit juices appears to be necessary. Fruit for jelly making must be used at the stage between "greenness" and "ripe-

ness." At this stage the fruits contain large quantities of starch which becomes intermixed with the pectin and other constituents of the fruit juices during pressing. Although in ripe fruits most of the starch becomes hydrolyzed, there is also considerable breakdown of pectin during ripening.

By using diastatic enzymes the colloidal starch suspension causing muddy turbidities may be readily removed. Subsequent filtration results in a clear product.

The Diastatic Treatment. McIntire (65) recommends the following method for the enzymic treatment of fruit juices used for jelly making. The fruits are thoroughly cooked and then pressed the usual way. When the temperature has cooled to 29° to 38° C. the enzyme is added. This temperature, however, is most suitable for a *p*H at or about 4.0. If the *p*H is as low as 3.0 the temperature should not be more than 29° C. McIntire employed 1 part of diastase to 6000 parts of apple, quince, or crabapple juice (calculated by weight), and for pectin liquors about 1 part of diastase to 12,000–15,000 parts of pectin liquor. The starch content of these materials must also be considered, however.

The hydrolysis of the starch is very rapid; it requires only 30 minutes. The end point is controlled by observing the changing of the original iodine-blue color to the definite brown or reddish brown, indicating that the dextrin-maltose stage has been reached. Now the juice is centrifuged or filtered and heated to 71° C. to destroy the enzyme. The juice is then concentrated and sugar is added in the usual way.

The Iodine Reagent. McIntire suggests the use of the following iodine reagent. Eighteen grams of potassium iodide is dissolved in 100 cc. of distilled water; 12.692 grams of iodine is dissolved in the potassium iodide solution. This mixture is made up to 1000 cc. with distilled water. Forty cubic centimeters of this solution is diluted to 100 cc. with distilled water and used as the reagent in the starch hydrolysis. It is of course best to determine the action of the enzyme on a small quantity of fruit juice at first.

The Effect of Commercial Enzymes on Pectin. Baker and Kneeland (66) studied the effect of commercial diastatic enzymes on a pectin solution. They claim that the diastases tested by them contain some pectinase. The pectinase has a detrimental effect on the viscosity and grade of the pectin solution. In Fig. 53 the effect of the commercial diastases on the viscosity of the pectin solution between *p*H 3.3 and 5.0 is shown as found by Baker and Kneeland. In a *p*H range of 3.3 to 5.0 at 40° C., hydrolysis is least at *p*H 3.3. A more acid *p*H than the

usual *p*H of 5.0 is recommended for the hydrolysis of starch in pectin solutions with the aid of diastases. For details concerning fruit jelly manufacture see reference 67.

Debittering Citrus Pectin. Hall (68) has prepared a new glucosidase from the leaves of the tree *citrus decumana*. This enzyme

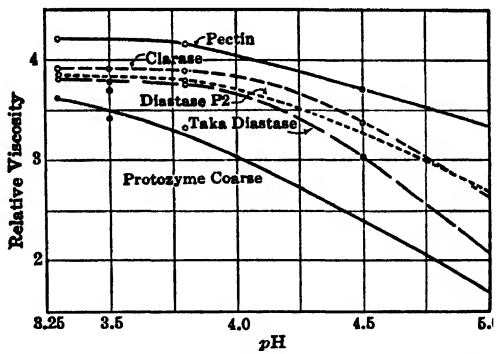

FIG. 53. Viscosity of pectin after 30-minute activity of diastatic preparations at various *p*H values.

hydrolyzes the bitter principle, *naringin*, of citrus fruits, thus preventing bitterness of citrus products. The use of this enzyme may be of interest to the citrus-pectin industry, where bitterness must be eliminated before a marketable product can be obtained.

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CHAPTER XXVIII

VITAMIN-DESTROYING ENZYMES

LIPOXIDASE

In 1928, Bohm and Hass found that seeds of legumes, such as soybean and navy bean, contain an enzyme that has the ability to oxidize carotene and unsaturated fats. They obtained patents for bleaching flour by this method. This enzymic reaction is very striking. The deep reddish brown carotene becomes decolorized within a few minutes when shaken with a suspension of ground soybeans. A vitamin-A-destroying enzyme has been identified in alfalfa (1). Wilbur and collaborators (2) found that soybeans added to the ration of dairy cows destroyed a considerable amount of the vitamin A of milk. Sherman and Salmon (3) reported that excessive weathering of soybeans, resulting from letting plants remain in the field 2 to 3 weeks after the seeds matured, caused 50 per cent destruction of the carotene. This enzyme is also present in Labiatae (4) and in animal tissues (5).

It has been shown (6, 7) that carotene oxidase, as previously described, does not exist, and that carotene oxidation is brought about indirectly by an unsaturated fat oxidase or lipoxidase. The unsaturated fats are oxidized by the unsaturated fat oxidase, and the oxidation products in turn oxidize the carotenoids. Commercial carotene products contain vegetable oil (unsaturated fat) as the solvent. Crystalline β-carotene dissolved in mineral oil is not oxidized by the oxidase unless a drop of olein is added to the mixture. It will be seen that this enzyme system is quite complicated.

Purification and Mode of Action. Balls, Axelrod, and Kies (8) have purified the soybean lipoxidase. They found that crude preparations are accompanied by a heat-stable factor, which increases the catalytic action of lipoxidase upon unsaturated fats. The purified enzyme was almost inactive. It became highly active when extracts of various sources were added. This activator was found to be of peptide nature. Theorell and coworkers (9) have also purified soybean lipoxidase. They have shown that lipoxidase action of a soybean extract depends on the interaction of two different proteins, the " activation enzyme " and the lipoxidase. The pure lipoxidase was completely inactive on a

colloidal solution of sodium linolate unless this substrate had first been treated with the activation enzyme. Theorell's lipoxidase system acts as well in the presence as in the absence of oxygen. Thus, no oxidation and peroxide formation of the linolic acid can occur. The Swedish investigator obtained lipoxidase by a lengthy method, which cannot be described here, in pure state. Lipoxidase is a globulin. Cosby and Sumner (10) reported that they have concentrated soybean lipoxidase 60-fold, and that their enzyme preparation does not depend on an activator.

Holman and Burr (11) have shown that impure lipoxidase converts linoleic, linolenic, and arachidonic acids into conjugated, unsaturated carbonyl compounds, which display a strong absorption in the ultraviolet region. The exact mechanism of this oxidation, however, is not yet known. Lipoxidase has been extensively reviewed by Süllmann (12).

THIAMINASE

Green and associates (13) found that foxes became severely ill when they were kept on a diet containing raw carp. Spitzer and coworkers (14) have shown that a typical thiamin avitaminosis could be produced in chicks when 25 per cent of raw whole carp was incorporated into their diet, which was otherwise adequate in thiamin, thus confirming the work of Green and collaborators. Spitzer and coworkers further found, by *in vitro* experiments, that carp tissues contain an enzymelike substance that destroys thiamin, and that boiling eliminates the destructive action. Deutsch and Hasler (15) examined 31 species of fresh-water fish in the Great Lakes region and found that the thiaminase is present in about half of them.

Mode of Action. Krampitz and Wooley (16) have shown that in this reaction thiamin is split into 4-methyl-5-hydroxyethylthiazole and 2-methyl-4-amino-5-hydroxymethylpyrimidine. Sealock and Livermore (17) have verified this observation by demonstrating that, if bicarbonate buffer is present in the digest, carbon dioxide is liberated. Thus they have proved that the over-all reaction is the hydrolytic cleavage of a carbon to quarternary nitrogen linkage, with the addition of the hydroxyl group to the methylene substituent in the pyrimidine and the change of the quaternary nitrogen to a tertiary nitrogen. In this reaction hydrogen is produced, since the hydroxyl group of the water molecule is involved in the formation of the alcohol:

$$R'CH_2N^+R_3 + H_2O \longrightarrow R'CH_2OH + NR_3 + H^+$$

Thus, thiaminase is a hydrolytic enzyme.

Sealock and Goodland (18) reported that cupric, zinc, and ferric ions markedly decreased the activity of thiaminase. However, cyanide, fluoride, iodoacetate, sulfhydryl, and sulfite were less effective. Compounds resembling thiamin in one or more ways, such as *o*-aminobenzyl and *β*-aminoethyl-4-methylthiazolium chloride, were strongly inhibitory. Beloff and Stern (19) found that cocarboxylase is appreciably inhibited by carp spleen thiaminase at *p*H 6.5, but not at *p*H 7.8.

Purification. Agner (20) obtained thiaminase in a semi-pure state. The solution of this preparation was yellow, and its behavior was that of an albumin with an isoelectric point of about *p*H 5.0. There was a parallelism between the color and activity. In the cataphoretic field, they moved together; after inactivation with hydrogen peroxide or by dialysis, the enzyme can be reactivated by small amounts of glutathione. Agner believes that when thiaminase acts on thiamin it reduces the vitamin.

ENZYMIC DESTRUCTION OF ASCORBIC ACID

Ascorbic acid (vitamin C) may be oxidized by ascorbic acid oxidase, phenolases, peroxidases (21), by the cytochrome system (22), and by heavy metals. In order to prevent the formation of biologically inactive oxidation products, Kertesz, Dearborn, and Mack (23) recommended the introduction to industry of the appropriate blanching time for vegetables. The enzymes may be destroyed by a short boiling period.

The juice of legumes and other plants contains glutathione and other substances that reduce dehydroascorbic acid. These substances protect ascorbic acid from oxidation. The reduction of dehydroascorbic acid by these substances is catalyzed by an enzyme. After dehydroascorbic acid is reduced, an enzyme catalyzes the reduction of the oxidized glutathione and probably the reduction of other substances oxidized by dehydroascorbic acid. These substances interfere with the estimation of ascorbic acid by the 2,6-dichlorophenolindophenol (24). See also the section on ascorbic acid oxidase.

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CHAPTER XXIX

ENZYMES IN TEXTILE, PAPER, AND RELATED INDUSTRIES

SIZING AND DESIZING WITH THE AID OF ENZYMES

All woven fabrics contain starch and, often, other sizings, such as glues, gums, and inorganic salts, the sizings being applied to warp threads before they are woven into fabrics. The purpose of sizing is to strengthen the yarn by increasing its tensile strength and its resistance to friction during the spinning process. To increase its smoothness the yarn is also treated with some oily substance, such as wax, oil, paraffins, or binder. Sometimes it is necessary to give the fabric an extra heavy weight; for this purpose a rather large amount of mineral salts is applied. The loading is held by the starch. If the fabrics are to be bleached, printed, or dyed, the sizings must first be removed. Otherwise the warp threads will not adsorb the dye, whereas the filling or crossing threads will. Starch sizing interferes with reducing agents employed during certain printing operations, etc. The use of diastases has proved most profitable for manufacturers of both textiles and enzymes. Digestion of the starch loosens the sizing, which can serve no useful purpose at this stage. Oily sizes are removed by scouring with ammonia and soap, etc.

Desizing can also be accomplished with alkalis or acids. These chemicals, however, also attack the cellulose fibers. The use of enzymes saves time and labor. Enzyme action is more effective and more easily controlled than hydrolysis by acids and alkalis.

In the United States the following enzyme-desizing products are marketed: Degomma (Röhm and Haas), Diastafor (Standard Brands), Exsize (Pabst), Polidase (Schwarz Laboratories), Polyzyme (Takamine Laboratory), Rapidase (Wallerstein Co.), Serizyme (Wallerstein Co.), and Diazyme (Wolf & Co.). The sources of these enzymes are the pancreas, malt, bacteria, and molds.

Sizing

In the preparation of starch sizings it is extremely important that enzyme action should not go beyond the liquefying stage. Starch of low viscosity, but still capable of giving a blue color with iodine, is used in sizing.

Desizing

Desizing of starch must be carried out in an extensive manner. The starch must be broken down to products that are readily removed from the fabrics by water. The removability will depend on the nature of the enzyme, the temperature, and the nature of the sizing. Sometimes it may be necessary to allow the enzyme to act overnight.

Bennett (1) suggests the following method for desizing textiles. Two pounds of malt concentrate (Stachexo) per 100 gallons of water is heated to 60° C. The fabric is placed in this liquid for about 30 minutes. This treatment hydrolyzes the starch, and only a washing is needed to complete the operation. If the sizing was heavy the amount of enzyme used must be about doubled, and the time must be increased to 1 hour.

The progressive desizing action of the diastases can be readily followed by testing the textiles with an iodine solution. The iodine, however, must be washed out again, as even traces of it are very poisonous to diastases. The present author found the following iodine solution very suitable for testing desizing:

Stock Iodine Solution (2). This consists of 11 grams of iodine (C.P.) and 22 grams of potassium iodide (C.P.) made up to 500 cc. with distilled water. From this stock solution a dilute (working) solution should be made up fresh every day.

Dilute Iodine Solution. This requires 2 cc. of stock iodine solution and 20 grams of potassium iodide made up to 500 cc. with distilled water.

Desizing is complete if no more color is produced when a piece of the material is treated with a few drops of the iodine solution. Sometimes, however, it is impossible to remove the last trace of the substance producing iodine color. The bulk, however, is always removed.

FINISHING

By employing diastases for starch hydrolysis the use of the more expensive dextrin can be eliminated. Bennett uses a finishing mixture of 50 gallons of water, 50 pounds of cornstarch (other starches are just as suitable), and ½ pound of malt concentrate at a temperature of 60° C. He allows 30 to 45 minutes for the reaction, after which he destroys the enzymes by boiling the mixture for a few minutes. In an alternative method Bennett suggests that the starch and water be brought to 60° C. Then the enzyme preparation is added and the mixture is stirred for 30 minutes and boiled for a few minutes. If

desired, the starch and water may be boiled in a steam-jacketed pan for a few minutes. This will burst the starch granules and will allow a more rapid enzyme action after adjustment of the starch solution with cold water to 60° C.

Concentrated finishing solutions naturally require more starch and more diastase. Solutions up to 30 per cent concentration, which are extremely viscous without previous diastatic treatment, may readily be employed provided jacketed pans and revolving agitators are used.

Epsom Salts Finishes. These finishes are difficult to prepare without enzyme-treated starch. By using enzymatically hydrolyzed starch, however, epsom salt finishes of any concentration may easily be prepared (Bennett).

Oily Finishes for Rayon Fabrics. Any " oily " finish may be added to the enzymatically hydrolyzed starch. This will result in soft, full-bodied fabrics.

Desizing after Calico Printing. Diastatic desizing agents are also very useful in after-processing printed fabrics or in processing preliminary to printing. The soaping and washing are aided without affecting shades and colors, and some materials do not have to be soaped at all (3).

Scott (4) has described methods for testing the desizing efficiency of enzymes. Nopitsch (5) reported that no evidence of cellulose destruction could be detected by various commercial diastatic enzymes.

THE USE OF ENZYMES IN SILK DEGUMMING

Owing to a considerable saving in the quantity of soap required in this process, proteolytic enzymes are often employed in the " degumming " of silk. Silk fibers are bound together by a small amount of a protein (gum) called sericin which is readily digested by proteolytic enzymes. The main constituent of silk, the protein fibroin, however, is very resistant to proteolytic enzymes.

Enzymic treatment of silk is much milder than continued exposure to hot, concentrated soap solutions. Degumming imparts a light color and fine texture to the silk.

THE USE OF ENZYMES IN LAUNDERING AND DRY CLEANING

Enzymes in Laundering. Röhm (6) found that fabrics can be cleaned much more easily and at low temperatures when pretreated with fat- and protein-digesting enzymes. A concentration of 2 grams

of pancreatin per 100 liters of water gave satisfactory results. The textiles were not injured.

Röhm also recommended the addition of pancreatin to the bath and wash water. Here the role of the enzyme is to remove fat and protein excretions which are deposited within the pores and serve only to clog them up. For this purpose, the use of 0.5 to 1 gram of pancreatin per 100 liters of water is suggested.

Balantine and Sons (7) mixed soap with diastase to improve its cleansing action.

Altenburg (8) recommends the use of ricinus lipase as a fat remover in laundry work. For this purpose he prepared a stable lipase preparation by mixing the enzyme obtained by usual methods with salts such as sodium or potassium sulfate or sodium borate. The enzyme salt mixture was dried at low temperature and defatted with ether, chloroform, or gasoline.

Enzymes in Dry Cleaning. Ausband (9) points out the importance of enzymes in dry-cleaning plants. He stated that by the use of enzymes it is possible to remove stains that cannot be removed by any other method. Glue, gelatin, and starchy sizings can be readily and completely removed from material before redyeing. According to Ausband, the enzyme process " has contributed immeasurably to the growth of the redyeing industry." The author suggests that, although most commercial enzymes contain a buffer yielding a *p*H close to the optimum of the enzyme, a colorimetric *p*H meter should be available to the dyer and the water should be nearly neutral. Traces of spotting aids should be rinsed from the fabrics as buffers cannot completely take care of excessive acids and alkalis. Indeed, many substances, especially certain reducing and oxidizing agents, inhibit the activities of certain enzymes considerably.

Gale (10) expressed similar views. He stained one sample of spun-rayon fabric with egg albumin, and another with a mixture of chocolate, malt, egg albumin, and ice cream. The stained samples were studied under different conditions. The digestion time was 30 minutes, and the temperature of the enzyme solution was 48° C. The enzyme concentrations were 0.5 per cent and 1 per cent, respectively. The nature of the enzyme used is not given in the paper. Gale concludes that the cleaner must take the following precautions when handling albumin-spotted spun rayons:

1. Avoid overheating or prolonged drying, or local superheating of the spotted garment.

2. Use a warm solution of a good enzyme, and extend the digestion time to 2 hours if necessary.

ENZYMES IN PAPER MAKING

Diehm (11) suggests that the enzymic conversion of starch for paper surfacing should be carried out by mixing the starch with cold water, adding the enzyme, and heating to about 70° C., holding that temperature until the enzyme has converted the starch, and then destroying the enzyme by heating to 85° C. or higher. It is best to keep the solution close to the optimum *p*H of the enzyme used. Conversion should require from 15 to 30 minutes. The advantage of enzymic conversion is that, with one grade of starch, the paper mill may produce solutions of any desired fluidity and concentration. Usually, solutions from 2 to 18 per cent are employed. The cost is lower than for chemically treated starches.

Diastases containing *proteases* have been extensively used in the production of adhesives, and several enzyme products are on the market for this purpose. The role of the enzyme preparation in this connection is to produce the most desired viscosity for the adhesive. The viscosity of the starch solution decreases with increasing quantities of enzyme.

According to coating mill specialists the enzyme-converted starch products are as efficient as any starch product employed for binding pigments.

Invertase may also be used in paper making. Invert sugar is frequently used as a plasticizing agent. The invert sugar is made by enzymic or acid hydrolysis of cane sugar. The plasticizing function is a property of levulose. Hydrolysis by invertase, if employed by the paper maker himself, should result in considerable saving (12).

Enzymes have been used only in recent years by the paper industry. Extensive studies are not available.

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CHAPTER XXX

THE USE OF ENZYMES IN THE MANUFACTURE OF LEATHER

GENERAL CONSIDERATIONS

The various operations which precede bating with enzymes are the preservation of the fresh hides and skins, trimming, soaking, liming, fleshing, dehairing, and scudding. Those following bating are pickling, tanning, and finishing. Some or all of these phases of leather manufacture, however, may vary from tannery to tannery. Details concerning the various phases of leather manufacture have been admirably described by Wilson (1). During certain of these processes the hides and skins must be protected against the destructive action of microorganisms. The bacteria carried with the raw hides must be destroyed, and new growth must be prevented. The fresh hides and skins used in leather manufacture are preserved (cured) by salting, by drying, or by a combination of these two methods, by pickling, and, of course, by the use of disinfecting agents. Apparently one of the most successfully applied disinfectants in this connection is chlorine gas, and a special chlorinator has been developed for the treatment of tannery soak waters (Wallace & Tiernan Co., Newark, N. J.).

The fibrous part which consists of collagen is the protein that is made into leather. Solutions of lime have been used for hundreds of years as dehairing agents. Lime adversely affects the skin only slightly. The composition of a solution of lime liquor is readily controlled. At 21° C. a saturated solution ($N/20$) has a pH of only 12.5. Dehairing with lime is a slow process, lasting 1 to 4 weeks. However, it was found years ago that the addition of certain sulfides to the lime liquor hastens the reaction considerably. Sulfides loosen S—S linkages, changing them to SH linkages, and the insoluble protein is thus changed to a soluble one. When properly controlled the collagen is not adversely affected by this reaction. Besides sodium sulfide and hydrosulfide, arsenic sulfide, dimethylamine, and other compounds have also found use as " sharpeners " of lime in dehairing. It is important to be acquainted with the composition of the agent which was employed for dehairing before using an enzyme-containing bate. Inadequate washing of the dehaired skins and hides may result in inhibition of enzyme action by toxic chemicals adsorbed on or combined with the skin.

DEHAIRING WITH ENZYMES

Various improvements have been suggested for the use of enzymes for the dehairing of hides and skins. Neugebauer (2) treats hides and skins simultaneously or successively with an alkali-metal sulfite, using a proteolytic enzyme in conjunction with a heavy metal salt which is said to act as an accelerator. One part of copper sulfate per 10,000 parts of solution, according to the author, has a considerable effect.

The following are examples of Neugebauer's method:

1. Soaked calf skins or skins of other animals are treated for 20 hours at about 20° C. in a solution of 10 kilograms of sodium sulfite, 10 kilograms of sodium bicarbonate, 0.3 kilogram of copper sulfate, and 4 kilograms of peptone per 1000 liters of water. For prevention of putrefaction, the use of 0.1 per cent of an active disinfectant such as U. S. patent 1,919,293, known by the trade name Preventol fl. I, is suggested. The skins are rinsed for a short time and then left for about 20 hours at 20° C. in a bath of the following composition: 0.7 kilogram of a commercial enzyme preparation from *Bacillus mesentericus*, 10 kilograms of sodium bicarbonate, 1 kilogram of Preventol fl. I, and 1000 liters of water. The skins are then freed from hair. Instead of peptone, 10 kilograms of 15 per cent aqueous ammonia or a suitable amino acid, an amine, a salt of an oxyacid, or thiourea may be employed. If desired the thiourea may be omitted. About 100 kilograms of skins may be treated with these liquors.

2. Soaked calf skins are treated for 20 hours at room temperature with a solution of 10 kilograms of sodium sulfite, 10 kilograms of sodium bicarbonate, 3 kilograms of sodium nitrilotriacetate, and 2 kilograms of copper sulfate in 1000 liters of water to which is added about 0.1 per cent of Preventol fl. I. After the sulfite bath the skins are rinsed for a short time and then immersed at 22° C. in an enzyme bath of the following composition: 0.1 kilogram of pancreatin, 10 kilograms of sodium bicarbonate, 1 kilogram of Preventol fl. I, and 1000 liters of water. The skins remain in this bath for about 20 hours. Instead of the pancreatin another proteolytic enzyme may be used, for example, an enzyme obtained with the aid of *Bacillus mesentericus*, *Aspergillus oryzae*, or *A. flavus*.

DELIMING WITH MINERAL ACIDS PRELIMINARY TO BATING

After dehairing, washing, and scudding, deliming with mineral acids (mostly hydrochloric acid is used) is important but not practiced by all tanneries.

Grasser (3) studied the velocity of the deliming of limed pelt. He found that, of a series of acids and mixtures of inorganic salts and acids, sulfuric acid showed the quickest deliming action.

Stiasny (4) considers the following factors most important in deliming and bating: Weak buffer-forming acids such as lactic, formic, butyric, and boric acids, as well as acid salts (bisulfite), acid anhydrides (lactic anhydride), ammonium salts, ammonium chloride, sulfate, or lactate, and sugarlike materials may be used for deliming. Concentrated salt solutions, however, have a destructive action on the hides. Ammonium sulfate is better for deliming than ammonium chloride, which forms calcium chloride which has a stronger peptizing action than calcium sulfate. The presence of ammonium salts, however, is not necessary for the bating of neutral stock. A buffer such as sodium carbonate should be used to furnish a slightly alkaline *p*H (8.0) if trypsin is used as the bate.

According to Wilson (1), the *p*H of the solution during acid deliming should be left at 5.0 and never be allowed to go below 4.7. Equilibrium should be reached from 30 to 90 minutes after introduction of the skins (calf) to the solution. This varies according to the lime content of the stock. After the deliming process the skins are ready for bating. Wilson recommends the *p*H control of the solution after the addition of the enzyme bate. The solution is to be kept at the optimum *p*H of the enzyme — a very important suggestion indeed, since there is a tendency to a shift to the alkaline side of the *p*H scale during bating, especially if the acid-deliming step has been omitted. It should be noted in this connection that commercial bates contain ammonium salts which are powerful buffering agents.

BATING WITH ENZYMES

The process of bating, which used to be a secret process, is probably hundreds of years old. The purpose of bating is the preparation of the dehaired, plumpy alkaline skins and hides for tanning. To achieve this the early tanners kept the dehaired skins in a warm suspension of the dungs of dogs or birds. Now, however, only " artificial " bating products are employed. The yearly world consumption is probably 1000 carloads.

Wood (5) was the first to show that the bating action of the unpleasant dungs was caused by the enzymes which they contained. He demonstrated that the dungs contained pepsin, trypsin, amylase, and lipase. In 1898 Wood (6) prepared trypsin from dog dung by precipitating an aqueous extract with alcohol. To this enzyme he

added ammonium sulfate and found that it acted as an excellent bate. As a result of Wood's important investigations the first commercial bate, called Erodin, was prepared; cultures of *Bacillus erodiens* were used as the source of enzyme material (7). The bacterial cultures were adsorbed on wood meal and mixed with ammonium chloride. In 1907 Röhm (8) patented the application of a mixture of a pancreatic extract and ammonium salts as a bating agent. This product bore the trade name Oropon. Priority claims concerning the application of enzymes to bating were later disputed (9, 10).

The Mechanism of Bating

The main function of artificial bates is to remove degradation products of hair, glands, epidermis, certain coagulable tissue proteins, and reticular tissue, which were not removed in previous operations. This can be accomplished only by enzyme-containing bates. The *p*H of the bating solution should be as close as possible to the optimum *p*H of the enzyme contained in the bate. The time and amount of enzyme required vary with the nature of the skins and hides. Skins and hides appear quite different after pickling and tanning if the bating process has been omitted (11). The unbated material has a thick, spongy, and irregular grain surface, distinctly noticeable on the finished leather.

Bates containing ammonium salts, reacting with the calcium hydroxide attached to the collagen, liberate ammonia and thus always result in an alkaline (*p*H 8.3 to 8.5) solution: $Ca(OH)_2 + 2NH_4Cl = CaCl_2 + 2NH_4OH$. For proper bating it is best, therefore, to follow the suggestion of a number of prominent leather chemists and delime the stock with acidified water.

Although bacteria played an important role in the earlier methods of bating, now their interaction during bating should be eliminated. The effect of the enzymes on collagen fibers is negligible unless bating is prolonged much longer than is necessary.

The removal of the epidermal degradation products by the bate enzyme cannot easily be followed up by microscopical study. These proteins are precipitated when treated with dilute acids. Unhairing, scudding, and washing operations do not remove all these proteins, and the unremoved ones remain adsorbed on the dermostatic layer to form jellylike solutions.

Bating as Carried out on Various Stocks

Skins as used in the United States differ greatly. Most goat skins, for instance, are imported and are in the dry state. The tanner must first experiment to determine the best conditions for each kind of material in question. Dry stock requires a more extensive procedure than soft material, and goat skins require a more drastic action than calf skins. Heavy hides, such as steer and cow hides, which are used for the production of sole or belting leather, are sometimes bated with enzymes but are more often partly delimed by chemical treatment. According to Wilson, however, the bated finished products are of higher quality.

Calf skins used for shoe uppers or other purposes are always bated with enzyme products. Bating plays a very important role in the manufacture of fine glove leather from lamb skins.

It is not very difficult to apply a certain bate for a particular skin. Although some indications of the progress of bating may be obtained at the end of the bating experiment, final conclusions can be drawn only after examination of the quality of the finished leather.

In the following are a few examples of bating procedures as given by Wilson (1).

Deliming and Bating Calf Skin

A 500-pound pack of unhaired, scudded, washed, and cheeked calf skin is placed in a 2500-gallon paddle vat filled with 1900 gallons of fresh water at 26° C. A solution of dilute sulfuric acid is made up by adding 1 pound of concentrated sulfuric acid per gallon of water. This acid is added while the paddle is running. The *p*H is measured every 5 minutes. More acid is added whenever the *p*H is above 5.0; great care is taken not to allow the *p*H to become more acid than 4.7. The adjustment of the *p*H is continued until two successive readings of 5.0, at intervals of 5 minutes, are obtained. The average amount of acid required is ascertained on the first few packs; after this the process becomes simplified. The amount of acid varies from 10 to 50 pounds. The time varies from 30 to 90 minutes. The paddle is run continuously.

After the deliming, the skins are transferred to a 2500-gallon paddle vat for bating. The bate is prepared by leaving in the vat about 500 gallons of the liquid from the previous lot, and by adding to this 1400 gallons of fresh water and 40 pounds of Oropon C.S. The stock is placed in the liquid and the temperature is brought to 33° C. The paddle is allowed to run for 90 minutes, after which the skins are

removed. Wilson found that the finished product was best when the *p*H was kept at 7.4 by the addition of some sulfuric acid.

After this operation the stock must be pickled or tanned, whichever is required.

Bating Goat Skin

A pack of 1600 pounds (dry weight) of goat skin which have been limed, unhaired, and washed is delimed by the same method as that described above for calf skin. Fifty pounds of Oropon A and a bating time of 3 hours are employed for soft skins, and 100 pounds of Oropon A and 5 hours for the hardest skins.

After the skins are removed from the bate they are scudded and washed with running water at 21° C. in a drum for 30 minutes, and then pickled or tanned.

Bating Sheep Skin

The dewooled and limewater-treated stock is placed in a paddle vat for bating. Five thousand pounds of limed weight stock is placed in a paddle vat of 2500-gallon capacity containing 1900 gallons of water at 40° C. The paddle is run continuously. Seventy-five pounds of Oropon A is added, and the temperature is raised to 38° C. Here, too, Wilson suggested first the deliming of the stock with sulfuric acid, as in the case of calf skin, and the maintenance of the *p*H close to 7.4. The time required may be 90 minutes or more. After bating the stock is washed in a drum for 2 minutes with running water at 21° C. and is taken to be pickled or tanned.

Bating Hog Skin

Hog skin is seldom bated. Wilson claims to have obtained good results by applying the same method as for calf skin and by doubling the Oropon concentration.

Bating Steer Hides and Other Stock

As a rule, steer hides for sole are not bated. Methods have been devised for this purpose, however. The resulting finished products are said to have a finer grain and to be better tanned. Kangaroo skins and other types of dry skins may be bated with good results by methods described above, only slight modifications being necessary (12).

It is important to note that optimum conditions for bating vary with

the stock, and for best results the tanner should make adjustments according to the nature of the stock.

In the United States the following companies produce enzyme bates for the leather industry: American Cyanamide Company, American Extract Company, Jaques Wolf and Company, L. H. Lincoln Company, Röhm and Haas Company, Schwarz Laboratories, Inc., The Martin Dennis Co., and Wallerstein Laboratories.

Enzyme Sources for Bates

Since the early researches (13) numerous articles and patents dealing with improvements in the preparation of bate enzymes have appeared. These are, of course, all based on the classical work of Wood and of Röhm. Some of the most important work will be discussed in the following pages. The action of all these preparations is based on the proteolytic and lipolytic enzymes they contain.

Bacterial Enzyme Bates

The first commercial bate was prepared from cultures of *Bacillus erodiens* in 1896 (7); its trade name was Erodin. The cultures were adsorbed on wood meal and mixed with ammonium chloride. As has been stated above, this product was developed as a result of the basic finding of Wood, who had shown that bacterial enzymes and ammonium sulfate may be used as a bate with excellent results.

In 1896 Popp and Becker (7) prepared a bate by using the bacteria of dog and bird feces for the inoculation of media. They had previously shown that the bacteria of such cultures, after maximum growth has been obtained, may be destroyed by short boiling of the media, and that the sterile liquid may be used as a bate. This method was later improved by Wood (14).

Tröger (15) applied to bating bacterial preparations obtained by steeping decayed fish in nutrient solutions. Wallerstein (16) prepared an enzymic liquor by growing bacteria of the *mesentericus* or *subtilis* group under aerobic conditions. This product was employed for the simultaneous dehairing and bating of hides. The cultures were treated with antiseptics and sterilizing substances such as pine oil and formaldehyde. The enzyme-containing solutions were employed at *p*H 8 to 9. Others used cultures of *Bacillus megatherium* and *Bacillus vulgatus* which were cultivated in media containing keratin (17). Birch-Hirschfeld and collaborators (18) prepared a proteolytically active bate by isolating *Bacillus sphaericus* from a sweated hide and

inoculating it into split leather hide. After 6 days the hide was washed with acetone, dried, and powdered.

Bates Containing Pancreatic Trypsin

In 1907 Röhm (8) obtained a German patent, and in 1908 an American patent (19), for the application of pancreatic extract and ammonium salts in bating. This product was given the trade name Oropon.

The use of Oropon and other enzyme bates during the early part of this century has completely eliminated the old-fashioned methods of bating.

According to Eitner (20), the Oropon bates of Röhm and Haas contain pancreatic extract absorbed on straw meal which has been dried and reground. The preparation contains trypsin and ammonium chloride. It is made by varying these components to suit the work with various hides. Payrache and Bailly (21) used a mixture of pancreatin and intestinal enzymes as a bate.

Kubelka and Nemec (22) found that the artificial bates contain, in addition to the enzymes, 60 to 80 per cent ammonium sulfate and 12 to 20 per cent wood meal. The role of the wood meal is to transfer the adsorbed enzyme to the skin during bating. The skin adsorbs the enzyme from the wood meal.

According to Wilson bating should be carried out, with trypsin-containing bates, at the optimum *p*H of trypsin. It should be noted, however, that crude trypsin, or pancreatin, is a mixture of a group of proteolytic enzymes which appears to have an optimum *p*H of 8.3, whereas pure crystalline trypsin is a single enzyme with an optimum *p*H of 7.4. To obtain the optimum *p*H, sulfuric acid must be added to the bating liquor every 2 or 3 minutes during the entire period of bating. Wilson (23) suggests the use of sodium metaphosphate, sodium pyrophosphate, or a mixture of the two, or acid pyrophosphate, as a buffer when sulfuric acid is used for the adjustment of the *p*H of the bate.

Mold-Enzyme Bates

Krall (24) cultivated *Aspergillus oryzae* on rice, elastin, and keratin. To this culture bating salts were added, and the mixture was used as a combination dehairing-bating agent. Gerngross (25) cultivated *A. oryzae* on bran. To the aqueous extract of the bran mycelium he added ammonium sulfate and employed it as a bate. LePetit (26) used cultures of *A. oryzae, Penicillium,* and *Mucor* for dehairing hides.

Papain Bates

The use of papain as a bate has been suggested by a number of authors (27, 28). Pickard (29) proposed a process for "soaking back" hides and skins which have been sun-dried, and for dried skins of animals which have died instead of being slaughtered. The hardened skins are much more difficult to soak back, and trypsin is often used for this purpose. According to Pickard's method, papain or bromelin may be used instead of trypsin.

Example. The bate consists of papain, 1 kilogram; soda ash, 4 kilograms; ammonium chloride, 1 kilogram; dextrin, 13.5 kilograms; wood flour, 0.5 kilogram. The 20 kilograms of the mixture is dissolved in 4000 liters of water at 40° C. Two kilograms of sodium sulfate is added per 4000 liters of water. One thousand kilograms of skin may be treated with this mixture.

The skins are tumbled or drummed in this liquor for an hour or two. If they are a resistant type they may be "broken on beam" to facilitate the penetration of the enzyme. They are then returned to the liquor and drummed or left lying for 2 to 4 hours at 40° C. As a result the skins are soaked back in a short time. They are better prepared for subsequent processing, and their quality approaches more closely that of the slaughtered animals.

Owing to the high price of papain this improvement is used as a supplement to trypsin in a concentration of not over 0.25 per cent of the bating solution. Bromelin, fig, banana, or mulberry proteases may also be used as a supplement (30).

Example. Five hundred kilograms of goat skins are limed, dehaired, washed, and drained. They may be partially delimed by lactic acid or other deliming agents, and then paddled or drummed at 40° C. with 1250 liters of water containing approximately 3¾ kilograms of the pancreatic enzyme bate known as Pancreol 5A, 20 grams of refined papain, and 75 grams of fused sodium sulfide (unless sufficient sulfide has already been used in dehairing). The bating is completed after 4 hours at a temperature of not less than 35° C. This improved method yields leathers in which the growth marks are reduced; the "spread" is improved; there is increased "fullness" as well as finer grain. Also the skins are more receptive of the tan and take the dye more evenly. The authors recommend that the concentration not exceed 0.25 per cent of vegetable enzyme in water.

Other Bates

Cathepsin. Geigy (31) employed as a bate aqueous extracts of various animal organs containing cathepsin, to which reducing substances were added to serve as activators.

Pepsin. Pepsin cannot be employed for dehairing or bating since it attacks the collagen (32).

Lipase. Burns (33) recommended for bating purposes a brei of castor beans because of their lipase content. He used the following mixture: 199.5 pounds of water, 55.33 pounds of castor bean brei, 2.5 pounds of sodium sulforicinate, and 12.5 pounds of manganous sulfate. The antiseptic employed was sodium fluoride.

In the medium of dilute hydrochloric acid or dilute organic acids (formic, lactic, or acetic acid) Altenburg (34) used lipase-containing plant materials, such as castor beans and sunflower seeds, in conjunction with fat emulsifiers, such as sodium phosphate.

Keeping Quality of Bates. Kubelka and Nemec (35) analyzed samples of commercial bates which had been tested 1, 5, or 10 years previously. Only products containing 60 to 80 per cent ammonium sulfate and about 20 per cent wood meal were selected. During the first year there was 25 to 40 per cent loss in enzyme activity. After 1 year, however, the activity of the samples remained constant. The Kubelka-Wagner (36) modification of the Fuld-Gross casein method was employed in these tests.

Method for the Estimation of Proteolytic Activity of Bates

The principle of this method (Lohlein-Volhard) (37) is as follows: Casein which has been dissolved in hydrochloric acid and precipitated with sodium sulfate releases a certain amount of hydrochloric acid which may be estimated by titration with alkali. On digestion of the casein there is a corresponding increase in the liberated hydrochloric acid. The increase of liberated hydrochloric acid is proportionate to the trypsin content of the bate, or to that of another proteolytic enzyme.

Solutions. 1. Alkaline-casein solution: 40 grams of Hammerstein casein dissolved in dilute sodium hydroxide and adjusted to *p*H 8.40 and diluted to 1 liter.

2. *N*/5 hydrochloric acid.

3. 10 per cent sodium sulfate.

4. 0.1 per cent α-naphtholphthalein in approximately 50 per cent alcohol.

5. *N*/10 sodium hydroxide.

Procedure. Into a 50-cc. flask 0.1 gram of bate is weighed. To this is added 10 cc. of water; the solution is kept at 37° C. for 15 minutes. Then 5 cc. of the alkaline-casein solution is added and the mixture is permitted to digest for 1 hour. At the end of 1 hour 5 cc. of $N/5$ hydrochloric acid is added to stop enzyme action. Then 10 cc. of sodium sulfate solution is added to precipitate undigested casein. After several minutes of cooling the mixture is filtered. To 10 cc. of the filtrate, 1 drop of indicator is added, and the solution is titrated until the first definite color change. By also titrating two blanks, the bound and unbound hydrochloric acid may be determined.

The Two Blanks. (*a*) To 5 cc. of casein solution 10 cc. of water and 10 cc. of sodium sulfate are added, and the freed hydrochloric acid is estimated in the filtrate as in the original bate-containing digest. (*b*) In the second blank only the bate is titrated without the casein. By this titration that portion of the sodium hydroxide which corresponds to the ammonium salt content of the bate is obtained. Both blank values are subtracted from the first titration. The bating value is expressed as cubic centimeters of $N/10$ sodium hydroxide equivalent to the digested casein and calculated per gram of the bate. When this method is used the optimum *p*H of Oropon is at *p*H 8.3 (38).

Other methods for testing bates are the Fuld-Gross (36) method and the formol titration method.

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CHAPTER XXXI

ENZYMES IN OTHER INDUSTRIES

ENZYMES IN TOBACCO MAKING

Tobacco making may be divided into two main phases: drying and fermenting. Drying, both natural and artificial, includes the so-called hunger period and the dehydration period. The hunger period is of short duration; it brings about the destruction of the cells. During both the hunger period and the dehydration period the important enzymic decomposition of carbohydrates, proteins, pectins, and chlorophyll takes place. A brown pigment forms as the result of the enzymic oxidation of the glucoside rutin. Bacterial action has no influence on these autolytic reactions (1). Proper fermentation is necessary to obtain the desired aroma of the finished product.

Proteolysis. Incompletely autolyzed tobacco proteins release unpleasant odors on burning, whereas the products of protein hydrolysis do not. Proteolysis is considerable during the drying period (2).

Nicotine Destruction. Fodor and Reifenberg (3) have shown that, during fermentation, enzymic nicotine destruction takes place and the maceration juice of tobacco could also split nicotine.

According to Barta (4) there is a relationship between the catalase content of tobacco and the loss of nicotine during fermentation. Tobacco with high catalase content shows a decrease in the nicotine content after fermentation. The enzyme catalase itself does not act on pure nicotine. Thus the catalase effect must be an indirect one. Peroxidase and oxidase concentrations are of no importance in this respect.

Bodnar and Barta (5) studied the mechanism of nicotine breakdown during fermentation and concluded that pyridine, ammonia, and methylamine are not formed during the process. They suggest that nicotine is destroyed by certain oxidizing enzymes.

Pigmentation. Neuberg and Kobel (6) found that the slightly soluble quercitin-rhamnoglucoside (rutin) is changed by a tobacco enzyme (a glucosidase) into a soluble brown pigment. The brown color is intensified by the addition of hydrogen peroxide in concentration of 0.003 per cent. In the presence of hydrogen peroxide, the almost insoluble rutin dissolves very rapidly.

The Effect of Drying on the Tobacco Enzymes. Neuberg and Kobel (7) showed that in the currently used method of preparing roof-

dried tobacco the enzymes are not destroyed. In this process the water is expelled by gradually heating the tobacco leaves to a maximum temperature of 100° C. and then gradually cooling them. The procedure requires 60 minutes. The enzymes amylase, invertase, phosphatase, pectase, glycolase, and ketonaldehydemutase were studied.

The Effect of Sugars on Tobacco. Glucose and fructose, which are products of tobacco fermentation, as well as added glucose, increase the quality of the finished product (8). In common with other plants, fresh green tobacco leaves have also the ability to change 6-carbon sugars (hexosediphosphate) to 3-carbon sugars (9).

Artificial Fermentation. Sweating tobacco leaves inoculated with cultures of *Debaryomyces nicotiana* and of *Micrococcus* sp. improved the aroma of the product. The nicotine content of the cigars obtained from the leaves inoculated with the organisms was 5.12 to 5.38 per cent, whereas the untreated control leaves had a nicotine content of 6.25 to 6.38 per cent (10). Street (11) noted that the addition of yeast had a beneficial effect on tobacco fermentation.

The Tobacco Virus is Not a Living Thing. It is interesting to note that Ryshkow and Sukhov (12) found that the virus of tobacco mosaic showed negative results when tested for the following enzymes: oxidase, peroxidase, catalase, protease, asparaginase, urease, amylase, chlorophyllase, and phosphatase. This property of the virus is in contrast to the usual characteristics of living things. The findings of the Russian investigators, however, do not rule out the enzymic nature of the virus.

ENZYMES IN THE PREPARATION OF DRUGS

Desirable Enzyme Actions. The enzymes continue to act during the harvesting and drying of the drug-containing plants and, in some instances, also during preparation and storage. Enzyme action may be either beneficial or detrimental to the final quality of the drug. The liberation of methylsalicylate in the leaves of *Gaultheria procumbens,* of eugenol in *Geum urbanum,* the mustard oils, and the odor of certain flowers (13) become evident only after enzyme action has taken place. The fresh fruit of *Vanilla planifolia* and *pompona* is almost odorless. It contains glucovanillyl alcohol which is acted upon by oxidases, which convert it to glucovanillin. Glucovanillin is hydrolyzed to glucose and vanillin by a glucosidase (14, 15). In the preparation of these products, especially some perfumes, enzyme action is indispensable, and natural or artificial heat is employed to accelerate enzyme activity.

Wiechowski (16) claims that he obtained a higher active ergot preparation by the application of yeast fermentation.

Undesirable Enzyme Actions. Rapid heating or rapid removal of moisture is, of course, the simplest method for preventing destruction by enzymes. A radical *p*H change or the use of enzyme inhibitors may also be applied. Since enzymic catalysis requires water, maceration of the plant with lactose which absorbs the water may also be employed (16).

By the procedure of Wiechowski the enzymes are salted out from the plant extracts with ammonium sulfate and the drug is extracted with a water-insoluble organic solvent. Stoll (17) successfully applied this method to the study of the digitalis glucosides. Water-free organic solvents such as absolute alcohol are also being used for the preparation of enzyme-free extracts of glucosides, ethereal oils, and perfumes.

Products obtained from slowly dried squill are physiologically inactive. From the heated plant, however, active preparations may readily be obtained (18). The heart glucoside zymarin of *Strophanthus komba* is changed by enzyme action to an inactive isomer alloglycoside (allozymarine) (19). To destroy the enzymes it is necessary to heat the plant for 30 minutes at 80° to 100° C.

The characteristic odors of many plants such as radishes, cress, scurvy grass, and garlic are produced by the action of glucosidases on odorless glucosides. Most plants that contain glucosides also contain glucosidases. To prevent hydrolysis the enzymes must be inactivated.

The latex of *Papaver somniferum* contains oxidases that have a destructive action on opium (20). The enzymes are also present in some commercial extracts, and they continue to act during storage, resulting in the destruction of considerable quantities of the alkaloids. Heat should be used in the preparation of the extracts in order to destroy the enzymes.

Many undesired color formations (*Cinchona, Mentha piperita,* etc.) may be prevented by heating the plant in water for 60 minutes at 80° C. before processing.

THE USE OF LIPASE IN THE FAT INDUSTRY

Connstein, Hoyer, Wartenberg, and others (21) have shown that castor beans, owing to their high lipase content, may be used for the industrial hydrolysis of fats into glycerol and fatty acids.

Procedure (21). Peeled castor beans are ground up with an equal weight of a 1 per cent chloralhydrate solution. The mixture is kept at 15° to 40° C. for a few days. This effects a hydrolysis of the natural

fats as shown by a considerable increase in the acidity of the mixture. The acid formed must be occasionally neutralized. A slightly acid *p*H is necessary, however, for the action of the ricinus lipase. It is best to adjust the mixture at the beginning of the reaction and continue to do so until all the fat is hydrolyzed.

The addition of 0.15 to 0.20 per cent of manganous sulfate acts as an accelerator of lipolysis, and an excess is not harmful. The selection of the castor beans is of major importance. The peeled beans must not be brown or defective, since such beans contain little lipase. A quantitative lipase test must be applied before a purchase for industrial production is made.

THE USE OF CATALASE IN VARIOUS INDUSTRIES

Vita-Zyme Laboratories, Inc., offer a concentrated catalase solution for the use in connection with the bleaching of furs, textiles, woods, fruit, and feathers, with hydrogen peroxide. Their product is also recommended to be used in decomposing hydrogen peroxide employed in the manufacture of floating soaps, foam rubber, and cake baking, and for destroying hydrogen peroxide when used as a preservative in the preparation of gelatin, milk for cheese, and drinking water.

THE ROLE OF ENZYMES IN SEWAGE DISPOSAL

Microbes and the enzymes secreted by them play an important role in the purification and disposal of domestic and industrial sewage. According to Buswell (22) all organic wastes are submissive to biological treatment. Chamberlin (23) reported that the amounts of lipase, amylase, and pepsin present in sewage are closely related to properly seeded mixtures from the standpoint of gasification. Rennin and trypsin occur in concentrations not related to the gas curve. The addition of enzymes, with the exception of lipase, to properly seeded mixtures does not affect the gas curve. Lipase was the only enzyme that brought about an appreciable and favorable change in the physical condition of properly seeded or fresh solid digestion mixtures. Chamberlin concludes that it may not be possible to effect a more favorable action of the other enzymes even if optimum conditions could be maintained and that in any case it would not be feasible or economical.

Rudolfs (24) studied the effect of adding trypsin, lipase, and other enzymes to fresh solids and fine screening and to activated sludge in order to find out their effect on liquefaction, drainability, and gas and odor formation. Optimum conditions for each enzyme were maintained. Only trypsin and lipase gave promising results.

The experiments of Wooldrige and Standfast (25) showed that the most important factor in bio-oxidation of sewage is a series of catalytic oxidation-reduction reactions brought about by the enzymes of living and dead cells.

Buswell (26) showed that a fuel gas can be produced by the anaerobic fermentation of the solids precipitating out of domestic wastes. The quantity of gas made available apparently justifies its collection for use. The amount of gas so produced, however, is too small to supply the needs of a community. An average of 150 analyses of the gas showed that it consisted of 64 per cent methane, 28 per cent carbon dioxide, 3.4 per cent hydrogen, and 4.3 per cent nitrogen. It had a heat value of 640 B.t.u. per cubic foot.

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CHAPTER XXXII

MICROBIOLOGICAL METHODS FOR THE ESTIMATION OF VITAMINS AND AMINO ACIDS

Many vitamins necessary for animal life are also required for the metabolism of the lower organisms. This principle has been successfully applied to the assaying of vitamins by employing certain yeasts and bacteria. These microorganisms are exceedingly sensitive to minute quantities of the vitamins. The results are accurate, inexpensive, and much more rapid than the animal assays. Thus, for the assay of thiamin the increase in carbon dioxide formation effected by using a certain type of yeast as the test organism is the basis of the test. For six B vitamins the titration of the acid formed by *Lactobacillus casei* is employed as the basis of the test. Similar methods are applied by using other organisms for the estimation of other vitamins and of amino acids.

DETERMINATION OF THIAMIN BY THE YEAST FERMENTATION METHOD

This method was developed in the Fleischmann Laboratories by Schultz, Atkins, and Frey (1). It is one of the best microbiological methods for the estimation of thiamin. The principle of the procedure is that the addition of thiamin to certain commercial bakers' yeasts which are poor in this vitamin show an increased fermentation rate. Under suitable conditions this method is highly specific. The only interfering substance is 2-methyl-5-ethoxymethyl-6-aminopyrimidine. The corresponding 5-hydromethyl compound is a product of hydrolysis of thiamin. It is also active in this reaction. This type of substance does not occur in nature to any extent except in urine and may be eliminated by sulfite treatment.

Procedure. Two preparations are made of each sample to be tested; one is treated with sulfite and one remains untreated, and their action on fermentation is determined. The difference, expressed in terms of pure thiamin chloride, is taken as the basis for determining the thiamin content of the unknown.

Preparation of the Unknown. Water-soluble samples are diluted to a desired concentration. Solid samples must be finely divided and dispersed in water to allow the measurement of an exact aliquot. The

solution or suspension is adjusted to a slightly acid reaction to Congo red paper with sulfuric acid and heated at 100° C. for 20 minutes. For this an Arnold steam sterilizer may be used. After cooling, the solution is made up to volume and an aliquot is used for sulfite treatment (see below). The remaining portion is neutralized with sodium hydroxide, litmus paper being used for the indicator. The neutralized portion is taken for testing total fermentation activity (before sulfite treatment).

Sulfite Treatment. In a 50-cc. Pyrex Erlenmeyer flask are placed a portion (not over 20 cc.) of the prepared sample, 0.7 cc. of *N* sulfuric acid, and 5 cc. of water containing 0.2 gram $Na_2SO_3 \cdot 7H_2O$. The sulfite solution must be freshly prepared. The *p*H must be adjusted to 5.2 to 5.6, using dilute sulfuric acid and sodium hydroxide and brom cresol purple as the indicator. The flask is covered with a 30-cc. beaker, heated at 100° C. for 30 minutes, and then cooled. Excess sulfite is decomposed with an excess of 3 per cent hydrogen peroxide. The end point is tested by using as an outside indicator a drop of a mixture composed of 1 drop of 5 per cent potassium iodide, 1 drop of 1 per cent soluble starch, and 1 drop of 50 per cent sulfuric acid. A drop of sulfite-containing solution added to a drop of this mixture, which has a pink color, discharges it. When all the sulfite is oxidized, the pink color remains and becomes deep violet when an excess of peroxide is added. When the excess sulfite is decomposed, the solution is neutralized with sodium hydroxide, using neutral litmus paper as the indicator. The solution is made up to volume, and an aliquot is employed for the estimation of residual fermentation activity.

Reagents

Solution A. In 1 liter of water are dissolved 180 grams of ammonium dihydrogen phosphate, 72 grams of diammonium hydrogen phosphate, and 0.2 gram of nicotinic acid. This solution is sterilized by heating at 100° C. (Arnold sterilizer) for 30 minutes, or it may be handled in the same manner as solution B.

Solution B. In 1 liter of water are dissolved 200.0 grams of dextrose (anhydrous), 7.0 grams of magnesium sulfate ($MgSO_4 \cdot 7H_2O$), 2.2 grams of potassium dihydrogen phosphate, 1.7 grams of potassium chloride, 0.5 gram of calcium chloride ($CaCl_2 \cdot 2H_2O$), 0.01 gram of ferric chloride ($FeCl_3 \cdot 6H_2O$), and 0.01 gram of manganese sulfate ($MnSO_4 \cdot 4H_2O$). Solution B is distributed in 500-cc. Erlenmeyer flasks, cotton-plugged, and sterilized at 100° C. on three successive days. Thereafter the flasks are capped with foil or paper and stored at room

temperature. After a flask has been opened, it is kept in a refrigerator at 4° C.

Thiamin Standard Solution. Crystalline thiamin chloride is dried over phosphorus pentoxide for 24 hours. A weighed amount is dis-

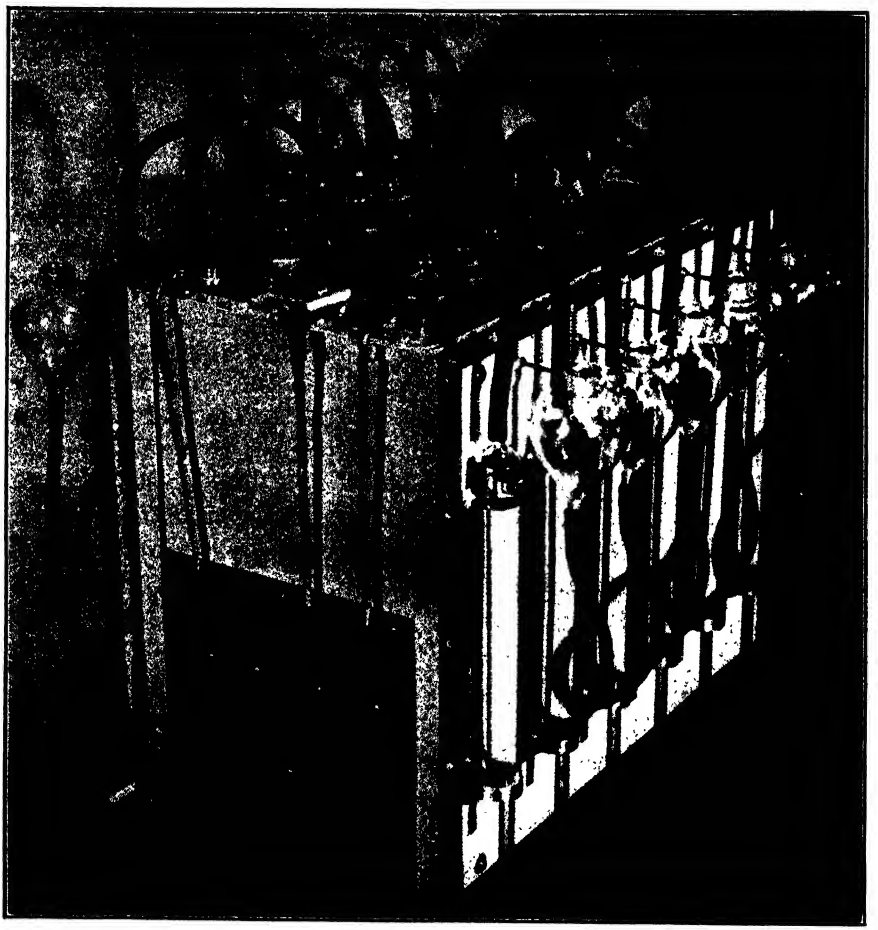

FIG. 54. Fermentometer.

solved in distilled water to make a solution containing 0.1 mg. of thiamin chloride per cubic centimeter. A subdilution is made to contain 1.0 microgram per cubic centimeter. This solution is distributed in 200-cc. flasks (cotton-plugged), sterilized at 100° C. for 20 minutes on two successive days, and then treated like solution B.

Yeast Suspension. Fleischmann's bakers' yeast (10.0 grams) or its equivalent is weighed into a small beaker. The small foil-wrapped

cakes of yeast or the high-vitamin-B_1 bakers' yeast should not be used. Distilled water is added to the yeast until a heavy cream has been produced and then enough more to make the volume of the suspension 200 cc. This suspension is prepared just prior to each determination.

The Fermentometer. The fermentometer is shown in Fig. 54. The drive is a Boston ratiomotor producing 96 r.p.m. The temperature is controlled (at 30° ± 0.2° C.) by a Fenwal thermoregulator, controlling a 200-watt heater. An auxiliary 500-watt heater for rapidly raising the temperature is manually controlled. The gasometers are of 260-cc. capacity with graduations at each 2 cc. The gasometer fluid is a 10 per cent calcium chloride colored blue with a small amount of cupric chloride.

A Typical Assay. Into each of six reaction bottles 2.5 cc. of solution A and 7.5 cc. of solution B were placed, followed by the addition of the thiamin-containing solutions; i.e., to No. 1, 0.8 cc. of a 1 per cent Hi-B_1 bakers' yeast suspension; to No. 2, 4.0 cc. of sulfite-treated yeast plus 1.0 cc. of a solution containing 1.0 microgram of the thiamin; to No. 3, 5 cc. of a wheat-germ suspension; to No. 4, 20 cc. of sulfite-treated wheat-germ suspension plus 1 microgram of thiamin; to No. 5, 1 microgram of thiamin; to No 6, 2 micrograms of thiamin. Distilled water was added to make a volume of 40 cc. Then the time was noted, and 10 cc. of a yeast suspension containing 0.5 g. of moist compressed yeast was rapidly added to each bottle.

The reaction bottles were placed in the fermentometers and connected to the gasometers. Shaking was started and after 2 to 3 minutes the initial reading of the gas volumes was made. After 3 hours the final reading was taken. The difference represents the gas evolved. Table LVIII shows how the fermentation activity of each test is determined. The evaluation of the results, in terms of thiamin content of the unknown, is given in Table LIX.

Josephson and Harris (2) modified this procedure by reducing it to a micro scale, using the Warburg-Summerson equipment. Caster and collaborators (3) published a critical review concerning the yeast fermentation method, pointing out some of its inaccuracies and suggesting improvements. According to these authors errors occur when this procedure is employed for thiamin estimation in materials having a high sulfite blank. Many foods usually have low blanks, but most urines have high blanks. It is suggested that position differences may be eliminated by increasing the speed of shaking.

Schopfer's Method for the Estimation of Thiamin (4). In this method the mold *Phycomyces blackesleeanus* is the test organism. The medium contains 10 per cent glucose, 1 per cent asparagine, 0.5 per cent

TABLE LVIII

TYPICAL ASSAY RUN WITH SIX GASOMETERS

(Analysis of two unknowns)

Bottle No.	Addition to Control Reaction Mixture		Gas evolved in 3 Hours	Fermentation Activity per Test
	mg.		cc.	μg. of thiamin
1	8	Yeast (bakers' Hi-B_1)*	192	1.75
2	40	Yeast (bakers' Hi-B_1)* SO_2-treated, plus 1 μg. of thiamin†	180	1.32
3	50	Wheat germ‡	186	1.54
4	200	Wheat germ,‡ SO_2-treated,§ plus 1 μg. of thiamin	182	1.39
	μg.			
5	1	Thiamin	171	1.00
6	2	Thiamin	199	2.00

* 0.8 cc. of 1 per cent suspension of Hi-B_1 bakers' yeast heated at 100° C. flowing steam, for 20 minutes at reaction blue to Congo red, cooled, and neutralized.

† 4.0 cc. = 40 mg. of sulfite-treated Hi-B_1 bakers' yeast. One gram of yeast suspended in 20 cc. of H_2O, 0.2 gram of $Na_2SO_3 \cdot 7H_2O$ added, reaction adjusted to *p*H 5.2, and then heated at 100° C. for 20 minutes. Cooled, excess sulfite destroyed, neutralized, and made to 100-cc. volume.

‡ 5 cc. of 1 per cent suspension treated as in *.

§ 20 cc. = 200 mg. of sulfite-treated wheat germ treated as in †.

TABLE LIX

EVALUATION OF RESULTS OF TEST DESCRIBED IN TABLE LVIII

	Hi-B_1 Bakers' Yeast	Wheat Germ
	micrograms per gram	
Total fermentation activity	219	30.8
Residual fermentation activity after sulfite treatment	8	1.95
True thiamin content	211	28.85

magnesium sulfate, and 1.5 per cent KH_2PO_4. The organism does not grow in this medium unless thiamin is added. After the addition of as little as 0.0005 gamma of thiamin per cubic centimeter growth is appreciable, and, within certain limits, growth is directly proportional to the thiamin concentration.

Mattoso and Chaves (5) suggest that the test be carried out at *p*H 6, which is optimum for the growth of the organism, and that potassium hydroxide be employed for the adjustment of the medium. Schopfer (6) published a critical review concerning the application of the *Phycomyces* test for thiamin. The article contains 78 references.

ASSAY METHOD FOR SIX B VITAMINS

Landy and Dicken (7) published the following microbiological assay method which permits the estimation of pantothenic acid, riboflavin, nicotinic acid, pyridoxine, folic acid, and biotin by a single acidimetric method, using only one test organism, *Lactobacillus casei*. Casein hydrolysate is the only chemically undefined constituent of the medium employed in the test. Omission of any of the required vitamins results in absence of growth and acid production.

Preparation of Sample (8). A 1-gram sample of finely divided tissue is rinsed into a test tube with 8 cc. of acetate buffer containing approximately 1 per cent solids and having a *p*H of 4.5 to 4.7. To this are added 20 milligrams of taka-diastase suspension in 1 cc. of cold water and 1 cc. of papain (" caroid ") suspension. The papain suspension is made by mixing equal quantities of papain and glycerin and dispersing the paste in water. It should be diluted to contain 20 milligrams of papain per cubic centimeter of solution. To 10 cc. of enzyme-tissue suspension is added 0.5 cc. of benzene. Then the sample is corked and incubated for 24 hours at 37° C. The enzymic treatment is necessary in order to liberate tissue-bound vitamins. Blanks, for the vitamin content of the enzyme products, must be run.

After enzyme treatment the sample is heated in flowing steam for 30 minutes to destroy the enzymes and to remove the benzene. The sample is filtered through a very thin layer of Filter Cel on a Hirsch filter, and the residue is washed with a volume of water equal to about twice that of the filtrate. The combined filtrates and washings are diluted, usually to a concentration of 25 milligrams per cubic centimeter on the basis of the weights of the fresh tissue. The extracts are placed in tubes or flasks stoppered with cotton plugs, steamed 5 to 10 minutes, and stored in a Dry Ice refrigerator until tested.

In order to prevent decomposition of riboflavin, the samples should not be exposed to light. Aqueous solutions, materials in a homogeneous state, do not have to be extensively ground or mixed. Cereals give higher results of nicotinic acid when extracted at an alkaline *p*H.

Rabinowitz and Snell (9) recommended an extraction procedure for the microbiological determination of vitamin B_6. Hydrolysis of pyridoxal phosphate requires a concentration of hydrochloric acid not over 0.055 *N* for best results.

Medium. Table LX shows the composition of the basal medium. All constituents must be of the highest purity. For best results the medium should be prepared just before use. Stock solutions of tryptophan, cystine hydrochloride, salt solutions A and B, and purine bases

TABLE LX

COMPLETE MEDIUM FOR *Lactobacillus casei*

Casein hydrolysate	0.5 gram (dry weight)
Sodium acetate ($NaC_2H_3O_2 \cdot 3H_2O$)	0.6 gram
Glucose	1.0 gram
Asparagine	25 mg.
Tryptophan	10 mg.
Cystine	10 mg.
Salt solution A	0.5 cc.
Salt solution B	0.5 cc.
Guanine hydrochloride	500 μg.
Adenine sulfate	500 μg.
Xanthine	500 μg.
Uracil	500 μg.
Thiamin hydrochloride	10 μg.
Biotin (free acid)	0.5 μg.
Folic acid concentrate (50 per cent folic acid)*	1.0 μg.
Riboflavin	20 μg.
Calcium pantothenate	20 μg.
Nicotinic acid	20 μg.
Pyridoxine hydrochloride	40 μg.
Distilled water to	100 cc.

*p*H adjusted to 6.8

SALT SOLUTION A		SALT SOLUTION B		
K_2HPO_4	5 grams	$MgSO_4 \cdot 7H_2O$	10	grams
KH_2PO_4	5 grams	NaCl	0.5	gram
Water	50 cc.	$FeSO_4 \cdot 7H_2O$	0.5	gram
		$MnSO_4 \cdot 2H_2O$	0.337	gram
		Water	250	cc.

* Obtainable from Lederle Laboratories.

may be kept on hand and may be preserved with toluene. The purine and pyrimidine bases may be dissolved in the casein hydrolysate. After the addition of riboflavin the medium should not be exposed to light.

Preparation of Casein Solution. Most vitamin-free caseins contain traces of vitamins too large to be negligible for microbiological assays. The casein must be reprecipitated several times in order to remove traces of the interfering B vitamins.

To 1 liter of distilled water at 50° C. 100 grams of casein are added with stirring. The mixture is stirred for 30 minutes, 3 grams of sodium bicarbonate (dissolved in a convenient volume of water) are added, and the stirring is continued until the casein is dissolved. The *p*H is adjusted to 4.6 with 10 per cent hydrochloric acid and the casein precipitate is collected by filtration. The precipitation is repeated several

times, until satisfactory blanks can be obtained in a culture medium with the resulting hydrolysate.

One hundred grams (on basis of dry weight) of the reprecipitated casein are hydrolyzed with 500 cc. of 25 per cent sulfuric acid for 10 hours at 15 pounds pressure. The sulfuric acid is neutralized with barium hydroxide and the barium sulfate is removed by filtration.

The casein hydrolysate solution, containing 10 per cent total solids, is adjusted to *p*H 3 and treated with 1 gram of Nuchar (Industrial Chemical Sales Co., New York) per 10 grams of casein. After 1 hour of stirring the mixture is filtered through Filter Cel. This resulting casein hydrolysate is preserved under toluene or it may be sterilized by autoclavin. A casein hydrolysate for microbiological assay procedures is obtainable from S. M. A. Corp., Chagrin Falls, Ohio.

Assay Procedure. For the estimation of any of the six B vitamins, a basal medium of twice the concentration given in Table LVIII is prepared, omitting the vitamin for which the assay is to be made. The samples are placed in culture tubes and diluted with water to make a total volume of 5 cc. in each tube. Five cubic centimeters of the two-fold concentrated medium is added. In a similar manner, dilutions of the vitamin used as a standard are made up. The test tubes are plugged with cotton and sterilized at 15 pounds' pressure for 15 minutes. The tubes are allowed to cool and are inoculated with a suspension of *L. casei* (A.T.C.C. 7469) and are incubated at 37.5° C. for 72 hours. After incubation, the degree of growth is determined by titrating the acid produced with 0.1 *N* sodium hydroxide. Brom thymol blue may be used as an indicator, or a *p*H meter may be employed. Values obtained from the dilutions of the vitamin standard are used to construct a standard curve. From the curve the vitamin content of any dilution of sample may be calculated. See Fig. 55.

Preparation of Inoculum. Stock cultures of *L. casei* are kept in yeast dextrose agar stabs. A 24-hour culture of *L. casei* is 10 cc. of yeast dextrose broth is centrifuged. The bacteria are washed in 10 cc. of 0.85 per cent sodium chloride solution. The washed residue is resuspended in 4 cc. of saline, and 1 cc. of the resulting suspension is added to 85 cc. of saline. One-tenth of a cubic centimeter of the diluted suspension per tube serves as the inoculum.

It should be noted that for all the six vitamins growth and acid formations are, within a limited range, proportional to the concentration of the vitamin which is being tested. The extreme lower and upper parts of the standard curve do not give dependable values. Only that part of the curve which is linear, or nearly so, may be employed for the

calculation of assay values. The large dilutions which are necessary eliminate the effect of non-specific growth-stimulatory substances.

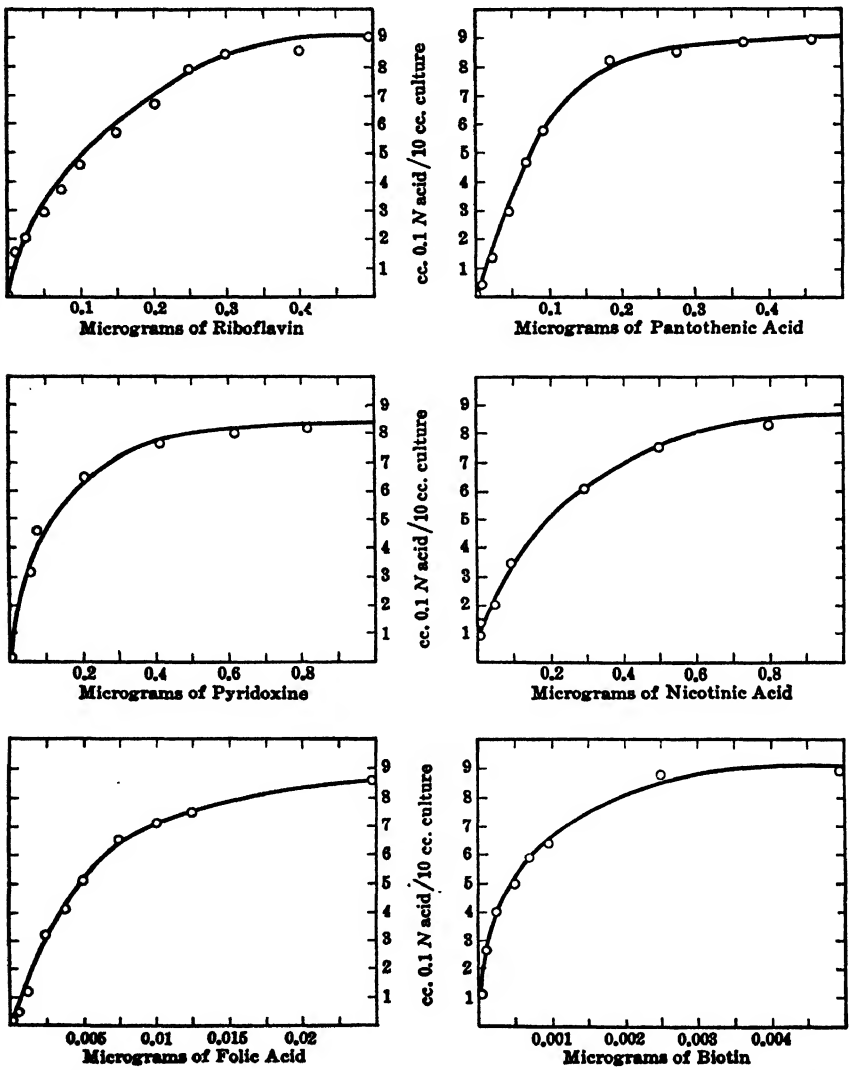

FIG. 55. Standard assay curves for six B vitamins using *Lactobacillus casei* and a medium in which casein hydrolysate is the only chemically undefined constituent.

Landy and Dicken found that the absence of pantothenic acid, riboflavin, folic acid, and pyridoxine produced perfect blanks (no growth or acid production). In the nicotinic acid and biotin blanks, however, a small amount of growth took place, equivalent to about 1.0 cc. of 0.10 *N*

acid. These slight blanks do not interfere with the accuracy of the procedure and are an indication that there remained this small quantity of nicotinic acid and biotin in the constituents of the medium.

The Microbiological Method for *p*-Aminobenzoic Acid. Landy and Dicken (10) described a microbiological method for the estimation of *p*-aminobenzoic acid using *Acetobacter suboxydans* as the test organism. See also reference 11.

MICROBIOLOGICAL METHODS FOR THE DETERMINATION OF AMINO ACIDS

Knowledge of the growth-factor requirements of various lactic acid bacteria, especially *Lactobacillus casei, L. arabinosus* 17–5, and *Streptococcus faecalis* R, has led to the development of useful microbiological methods for the quantitative determination of most of the B vitamins. Since lactic acid bacteria also require most of the amino acids for growth, it has been possible to develop satisfactory microbiological methods for the ten essential amino acids, namely, lysine, histidine, arginine, leucine, isolcucine, valine, methionine, threonine, tryptophane, and phenylalanine (12), and also for glutamic acid (13), aspartic acid (14, 15), and tyrosine (16, 17).

These methods are based on the use of an organism which requires the particular amino acid in question for normal growth and which will respond in a regular fashion to given amounts of the amino acid. The growth response may be measured by determining the increases in turbidity of the culture tubes with graded amounts of amino acid, or more commonly by titrating the lactic acid produced in the tubes with alkali. The number of cubic centimeters of alkali necessary to neutralize cultures grown with known amounts of the pure amino acid are plotted against the amounts of amino acid to give the " standard curve." The cubic centimeters of alkali necessary to neutralize the cultures grown with given amounts of the preparation under assay are then read from the standard curve directly in terms of the pure amino acid. From such data, the exact amino acid content of the unknown is calculated.

These methods can be applied to the assay of foods, feeds, and other natural materials, as well as to purified proteins and amino acid mixtures. Because of their specificity, accuracy, sensitivity, and ability to yield many replicate results in a short time, these microbiological methods are coming into widespread use. As little as 1 to 2 grams of material is sufficient for the assay of all the amino acids mentioned above, and one individual can assay a half dozen substances in about

two weeks. The techniques and equipment required are relatively simple, although elementary knowledge of bacteriological technique is essential in maintaining purity of stock cultures and preventing contamination of assays.

In order to standardize and, therefore, simplify microbiological amino acid methods, a single uniform method was developed for the ten essential amino acids and tyrosine (12, 16, 17). Nine of the amino acids are assayed with *Streptococcus faecalis* R and phenylalanine, and tyrosine with *Lactobacillus delbrückii* LD5. The details of this method are given below.

Inoculum. Stab stock cultures of the organisms *Streptococcus faecalis* R and *Lactobacillus delbrückii** are carried in a medium of the following composition: 1 gram of glucose, 0.5 gram of Bacto-peptone, 0.6 gram of anhydrous sodium acetate, Salts A and B in half

TABLE LXI

BASAL MEDIUM*

dl-Leucine	100 mg.	Sodium acetate (anhydrous)	3 grams
dl-Isoleucine	100 mg.	Adenine	5 mg.
dl-Valine	100 mg.	Guanine	5 mg.
l(−)-Cystine	100 mg.	Uracil	5 mg.
dl-Methionine	100 mg.	Pantothenic acid	100
dl-Tryptophan	200 mg.	Riboflavin	100
l(−)-Tyrosine	100 mg.	Thiamin HCl	100
dl-Phenylalanine	100 mg.	Nicotinic acid	100
dl-Glutamic acid	100 mg.	Pyridoxamine†	200
dl-Threonine	100 mg.	*p*-Aminobenzoic acid	20
dl-Alanine	100 mg.	Biotin	0.1
dl-Aspartic acid	100 mg.	Folic acid‡	1.0
l(+)-Lysine	50 mg.	Salts A	
l(+)-Arginine	100 mg.	K_2HPO_4	250 mg.
l(+)-Histidine	100 mg.	KH_2PO_4	250 mg.
dl-Serine	100 mg.	Salts B	
l(−)-Proline	100 mg.	$MgSO_4 \cdot 7H_2O$	100 mg.
l(−)-Hydroxyproline	100 mg.	NaCl	5 mg.
dl-Norleucine	100 mg.	$FeSO_4 \cdot 7H_2O$	5 mg.
Glycine	100 mg.	$MnSO_4 \cdot 4H_2O$	5 mg.
Glucose	5 grams	Adjust to *p*H 6.8	
		Add distilled H_2O to 250 cc.	

* The amino acid being assayed is omitted from the medium.
† Sold by Merck & Co.
‡ Obtainable from Lederle Laboratories.

* These organisms can be obtained from the American Type Culture Collection, Georgetown University School of Medicine, Washington, D. C., where *S. faecalis* is listed as No. 9790 and *L. delbrückii* as 9595.

the concentration given in Table LXI, and 1.5 gram of agar per 100 cc. of medium at *p*H 6.8. The cultures are stored in a refrigerator and subcultured each month. Inoculum for the assay is prepared by transferring a small amount of growth from a stab culture to a centrifuge tube containing 8 cc. of the same medium but without agar. After incubation for 16 to 24 hours at 37°, the cells of the liquid culture are centrifuged, washed with water, and suspended in 100 cc. of water. One drop (about 0.05 cc.) of this cell suspension is used to inoculate each tube in the assay.

Assay Medium. The composition of the assay medium is shown in Table LXI. The amount indicated is sufficient for 50 assay tubes, since each tube receives 5 cc. of medium. In preparing medium for assays, allowance should be made for 20 tubes for the standard and 10 tubes for each unknown. The amino acid being determined is omitted from the medium. Stock solutions of most of the amino acids are prepared in distilled water in a concentration of 10 milligrams per cubic centimeter. Only 5 milligrams of leucine is added per cubic centimeter to avoid crystallization on standing. Glutamic and aspartic acids are prepared in a concentration of 20 milligrams per cubic centimeter. Tyrosine is dissolved in 1 *N* sodium hydroxide and then diluted with water so that a solution of 10 milligrams per cubic centimeter of 0.1 *N* sodium hydroxide is obtained. Cystine is dissolved in 2 *N* hydrochloric acid and diluted with water to a final concentration of 5 milligrams per cubic centimeter of 0.2 *N* hydrochloric acid. Tryptophan is dissolved with 0.2 *N* hydrochloric acid to give a concentration of 20 milligrams per cubic centimeter. All the other amino acid solutions are adjusted to *p*H 7. It has been found convenient to prepare 500-cc. quantities of each amino acid at one time and to store them in brown glass-stoppered bottles under a thin layer of toluene in the refrigerator. They can be kept in this manner for several months without noticeable deterioration. The remainder of the ingredients of the medium, with the exception of glucose and sodium acetate, also are prepared as solutions, so that they can be pipetted conveniently. Salts A is made by dissolving 50 grams each of K_2HPO_4 and KH_2PO_4 in 500 cc. of distilled water. Salts B is made by dissolving, in order, 20 grams of $MgSO_4 \cdot 7H_2O$, 1 gram of NaCl, 1 gram of $FeSO_4 \cdot 7H_2O$, and 1 gram of $MnSO_4 \cdot 4H_2O$ in 500 cc. of distilled water. One cubic centimeter of concentrated hydrochloric acid is added to Salts B to prevent precipitation of the salts on standing. A stock solution containing 1 milligram per cubic centimeter of adenine, guanine, and uracil is prepared by dissolving 870 milligrams of adenine sulfate, 620 milligrams of guanine hydrochloride, and 500

milligrams of uracil in 200 cc. of water containing 10 cc. of concentrated hydrochloric acid, and adjusting the volume to 500 cc.

The vitamin solutions are prepared individually as follows: riboflavin, 25 gammas per cubic centimeter of 0.02 *N* acetic acid; calcium pantothenate, 27.2 gammas; nicotinic acid, 100 gammas; thiamin hydrochloride, 100 gammas; and pyridoxamine dihydrochloride, 100 gammas per cubic centimeter of water; *p*-aminobenzoic acid, 10 gammas per cubic centimeter of water; biotin (free acid), 0.02 gamma per cubic centimeter of water prepared from an initial solution of the solid in 20 per cent ethanol and folic acid, 1.0 gamma per cubic centimeter of water. All the above solutions are stored in brown bottles under toluene in the refrigerator. The vitamin solutions should be renewed once a month. The remaining solutions can be kept almost indefinitely.

Preparation of Samples for Assay. Fresh, moist substances, such as vegetables or meats, are sliced and dried sufficiently at 100° to permit grinding into a homogeneous mass. It is not necessary to free the samples of fatty materials or any other constituent prior to hydrolysis or assay. One gram of dried material is placed in a glass ampule of 20-cc. capacity, along with 10 cc. of 10 per cent hydrochloric acid. The ampule is sealed in a flame and autoclaved for 10 hours at 15 pounds' steam pressure. After being cooled, the ampule is carefully broken open and the hydrolysate washed into a small beaker with approximately 25 cc. of water. It is then adjusted to *p*H 6.8 with 5 *N* sodium hydroxide, filtered through paper if an appreciable amount of insoluble material is present, and brought to a volume of 50 cc. with water. It may be stored under toluene in the refrigerator. The 1-gram quantity should be sufficient for determining nine of the essential amino acids. Tryptophan and tyrosine require a separate alkaline hydrolysis. With purified proteins and other materials available in only small quantities, the amount hydrolyzed may be reduced considerably, the degree depending upon the amino acid content of the sample. This can be calculated, approximately, from the sensitivity of each assay as indicated by the standard curves. A further reduction is possible by decreasing, proportionately, the scale of the assay from 10 cc. volume per assay tube to 5 cc. or 2.5 cc.

For the determination of tryptophan and tyrosine, 0.5 gram of sample is hydrolyzed and prepared as described above, except that 5 *N* sodium hydroxide is used in place of hydrochloric acid. Frequently, a copious precipitate appears on neutralization of the alkaline digest. This may contain considerable amounts of silica dissolved from the glass. The precipitate is best removed by centrifugation, since filtration through paper may be extremely slow. It is washed

twice with water, which is then combined with the remainder of the precipitate-free hydrolysate.

Activity of Optical Isomers and Preparation of Standards. With the exception of histidine, of which only the *l* isomer was available,

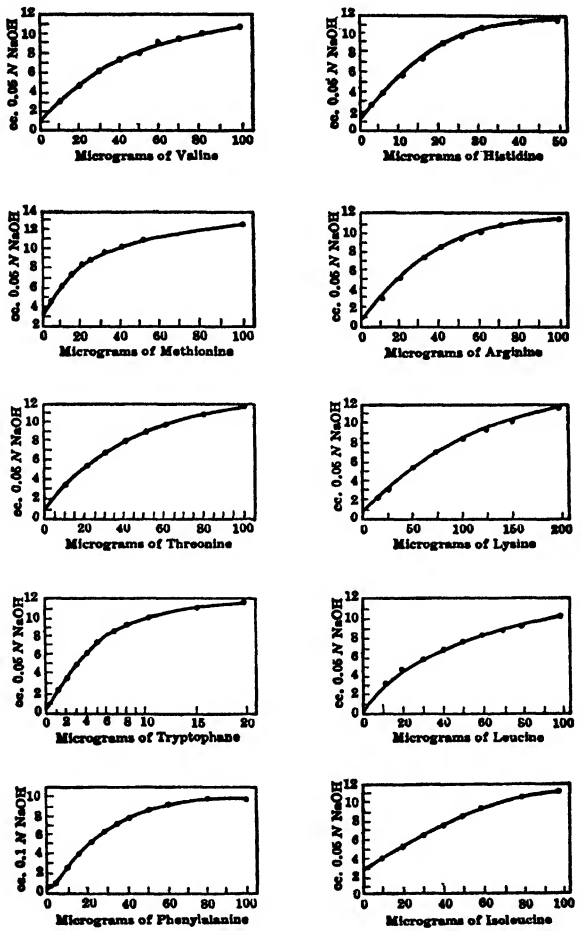

FIG. 56. Typical standard curves. The quantities of each amino acid are those of the *l* or naturally occurring isomer.

the synthetic *dl* racemate of each amino acid is exactly one-half as active as the *l* isomer, indicating that the *d* or unnatural isomer is inactive. Identical standard curves are obtained with the *l* and *dl* forms when twice as much of the *dl* as of the *l* isomer is used. Therefore, either form can be used as the standard. It is important, obviously, that amino acids of known purity be employed. It is convenient

to prepare solutions of the amino acids, to be used as standards, in a concentration of 100 gammas per cubic centimeter of the *l* isomer. These may be stored under toluene in the refrigerator. Dilutions are made from these primary solutions so that the quantities needed for construction of the standard curves (Fig. 56), which are in terms of the *l* isomer, can be pipetted conveniently. These secondary dilutions, also, may be preserved. The dilutions must be made so that the maximum volume of standard added to the assay tube does not exceed 5 cc.

Assay Procedure. The assay of a protein for threonine is taken as an illustration of the basic procedure, which is applicable, of course, to the assay of the remaining ten amino acids. Five-cubic-centimeter quantities of the basal medium shown in Table LXI, free of threonine, are placed in lipless test tubes (180 by 22 mm.) supported in wire racks. A standard curve is prepared by adding, in duplicate, amounts ranging from 0 to 5 cc. of the standard threonine solution, equivalent to 0 to 100 gammas of the *l* isomer (see Fig. 56 for intermediate values) to separate tubes of medium. To another set of tubes containing 5 cc. of the threonine-free medium are added in duplicate 1.0-, 1.5-, 2.0-, 2.5-, and 3.0-cc. quantities of the appropriate dilution of the protein hydrolysate being assayed. The total volume in all tubes is brought to 10 cc. with water. The tubes are plugged with cotton and sterilized by autoclaving at 15 pounds' pressure for only 13 minutes. On autoclaving, the medium becomes light brown; dark brown discoloration indicates excessive sterilization and interferes with the subsequent colorimetric titrations. Each tube is inoculated, aseptically, with 1 drop of *S. faecalis* suspension prepared as described above. The racks of inoculated tubes are incubated for 40 to 48 hours at 37° to permit development of the assay organism to the maximum degree permitted by the available threonine. Small variations in time or temperature do not affect the assay significantly. After incubation, the lactic acid formed by *S. faecalis* from the glucose in the medium is titrated directly in each tube with approximately 0.05 *N* sodium hydroxide, using brom thymol blue as indicator. The cubic centimeters of sodium hydroxide required to neutralize the tubes of the standard are plotted against micrograms of threonine to give the reference curve shown in Fig. 56. Similar standard curves are prepared for each assay. Dilutions of the protein hydrolysate under test are chosen to give titration values which fall on the sharply ascending portion of the standard curve, and the threonine content is read from the standard threonine curve. The final value is an average of the figures obtained at the various levels, which agree closely. Phenylalanine and tyrosine determinations with *L. delbrückii*

are made in the same way as described above, except that the incubation period is 64 to 72 hours and the titrations are made with approximately 0.1 *N* sodium hydroxide.

A convenient method for recording and calculating results is shown in Table LXII. One gram of liver was hydrolyzed with acid, neutral-

TABLE LXII

ASSAY OF BEEF LIVER FOR THREONINE

Diluted Sample Added to Assay Tube	NaOH Required to Neutralize Assay Tube after Incubation	Amino Acid Equivalent of Titration Figure as Read from Standard Curve	Amino Acid Content Calculated for 1 cc. Diluted Sample at Each Assay Level
cc.	cc.	γ	γ
0.5	3.6	10	20
1.0	5.1	19	19
1.5	6.8	31	21
2.0	7.85	40	20
3.0	9.6	62	21
Average			20.2

$$20.2 \times \overbrace{12.5 \times 100}^{\text{dilution}} = 25.25 \text{ milligrams of threonine per gram, or } 2.53 \text{ per cent.}$$

ized, filtered, and adjusted to 100 cc. A further 1:12.5 dilution was made prior to assay. Alkaline hydrolysis, used to liberate tryptophan and tyrosine from proteins, results in complete racemization of these amino acids. Since the *d* form of tryptophan is inactive for *S. faecalis,* and the *d* form of tyrosine is inactive for *L. delbrückii,* assay values must be multiplied by a factor of 2 to arrive at the final correct tryptophan and tyrosine contents of the protein.

Discussion. The wide variety of materials that can be satisfactorily assayed for amino acid content by the microbiological method is shown in Table LXIII. The method fulfills, for each of the amino acids, the following criteria of reliability: (*a*) Essentially the same amino acid values are obtained for a particular protein, irrespective of the amount of sample assayed, thus indicating that the method is stable to non-specific stimulatory or inhibitory substances which may be introduced with samples. (*b*) The results are readily reproducible, so that the same values are obtained for a given protein in repeated assays with fresh hydrolysates, different batches of medium, and different operators. (*c*) Recoveries of known amounts of amino acids added to proteins before hydrolysis are quantitative generally within ±2 per cent. (*d*) Compounds related chemically or physiologically to the amino acids, other than a few very closely related exceptions, are

TABLE LXIII

Amino Acid Content of Natural Products

Substance	Nitrogen % of Dry Weight	Per Cent, Calculated to 16 Per Cent Nitrogen, on Dry Basis									
		Histidine	Arginine	Lysine	Leucine	Isoleucine	Valine	Methionine	Threonine	Tryptophan	Phenylalanine
Rye, seed	1.95	1.72	4.3	4.2	6.2	4.0	5.0	1.26	3.0	1.31	5.6
Wheat, seed	2.22	2.0	4.2	2.9	6.8	3.6	4.5	1.20	2.5	1.37	5.1
Flour, patent	2.28	1.54	3.1	2.2	7.5	3.7	4.2	0.96	2.5	0.98	5.6
Soybean flour, defatted	9.32	2.3	7.1	5.4	7.4	4.5	4.6	0.84	3.9	1.20	5.3
Linseed meal	6.94	1.50	8.4	3.3	5.3	4.2	5.1	0.81	3.0	1.46	5.2
Alfalfa meal	2.90	1.21	3.1	4.9	6.6	3.6	4.4	0.15	3.3	1.44	4.1
Carrots	1.30	0.74	0.68	1.14	4.8	2.9	3.4	0.56	2.7	0.24	2.8
Peas	4.76	1.21	8.9	5.0	6.4	4.1	4.0	0.43	3.9	0.71	4.8
Yeast, brewers'	9.14	2.1	4.5	6.4	7.1	4.2	5.4	1.37	5.1	1.05	4.4
Whole milk	4.34	2.4	3.6	8.7	9.9	5.2	6.6	2.1	4.0	1.32	5.3
Blood meal	14.96	5.63	4.2	8.8	12.2	1.13	7.7	1.11	4.1	1.28	7.3
Tankage	10.75	2.4	5.9	7.2	7.7	2.7	5.4	1.28	3.0	0.83	4.2
Liver, beef	12.98	1.87	3.4	6.1	8.3	4.0	5.7	2.0	3.8	1.38	5.3
Potatoes, peeled*	Per Cent of Dry Weight										
		0.10	0.37	0.33	0.56	0.29	0.46	0.09	0.37	0.13	0.43

*Results are not calculated to 16 per cent nitrogen because of difficulty in obtaining concordant nitrogen values.

inactive. (*e*) In general, the microbiological values for purified and impure proteins are in reasonably good agreement with those obtained by the more recent improved chemical methods.

Satisfactory microbiological methods are not yet available for the determination of glycine, alanine, serine, proline, hydroxyproline, and cystine in natural materials.

Enzymic Methods for Analysis of Amino Acids. A series of methods, though not often used, have been developed for the enzymic analysis of amino acids in protein hydrolysates. Thus, arginase has been applied for arginine determination; decarboxylases for lysine, ornithine, tyrosine, arginine, glutamic acid, and histidine; and succinic dehydrogenase for glutamic acid, the last being first converted to succinic acid with chloramine T (18).

Determination of Protein Value with *Tetrahymena geleii* H. Dunn and Rocklan (19) described a method for the determination of biological values of proteins with the aid of the ciliated protozoan *Tetrahymena geleii* H. This small animal utilizes unhydrolyzed proteins, and its amino acid requirements approximate those of certain mammals.

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SUBJECT INDEX