CRYSTALLINE ENZYMES

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PREFACE TO THE SECOND EDITION

WHEN the Jesup Lectures were delivered in 1938 ten enzymes had been isolated, crystallized, and found to be proteins. Nevertheless, the protein nature of these compounds was still a very controversial subject. Those workers who had themselves isolated enzyme proteins were convinced that the crystalline proteins obtained actually represented the enzymes in pure form, but most other workers were somewhat skeptical. At the present time about thirty-five enzymes have been obtained as crystalline proteins and the identity of the enzyme and the crystalline protein has been widely accepted. The chemical structure responsible for the activity and the question as to whether or not prosthetic groups are necessary is, however, still under discussion.

The protein nature of some viruses at least has also been pretty well demonstrated. As a result three hitherto apparently independent problems, the formation of proteins, of enzymes, and of viruses, which are of the first importance for the comprehension of the mechanics of living matter, have been reduced to a single problem—the mechanism of the synthesis of proteins in general.

The subject matter has been restricted, as in the first edition, to work carried out at the Laboratory of General Physiology of The Rockefeller Institute for Medical Research at Princeton, New Jersey, or to closely related subjects.

General reviews may be found in Sumner and Somers, *Chemistry and Methods of Enzymes*, in Nord and Weidenhagen's *Handbuch der Enzymologie*, and in the various volumes of *Ergebnisse der Enzymforschung* and *Advances in Enzymology*.

The authors are indebted to Miss Jocelyn Farr for valuable assistance in the preparation of this manuscript.

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Laboratories of The Rockefeller Institute for Medical Research
Princeton, New Jersey
November, 1946

PREFACE TO THE FIRST EDITION

THE present monograph is based on the Jesup Lectures given at Columbia University in the spring of 1938. It contains the results of a series of investigations on the isolation and chemistry of bacteriophage and the proteolytic enzymes carried out in the writer's laboratory. Bacteriophage has not been crystallized and may not be an enzyme, but the results of the experiments with bacteriophage are essentially similar to those obtained with the enzymes and are, therefore, included in the present volume.

The problems presented formidable technical difficulties, owing partly to the unstable nature of the enzymes and partly to the large quantities of material which it is necessary to handle. The results are in large part due to the ability and perseverance of my collaborators, Dr. M. Kunitz, Dr. M. L. Anson, Dr. Roger M. Herriott, and Dr. A. P. Krueger. The work has entailed also a very great amount of analytical and preparative procedure for which acknowledgment is due to faithful technical assistants, especially to the late Mr. Nicholas Wuest, to Miss Margaret MacDonald, and to Miss Elizabeth Shears, who has also been most helpful in the preparation of the manuscript.

I am indebted to the *Journal of General Physiology* for permission to reproduce the figures and photographs used.

References to the older literature will be found in the original papers.

JOHN H. NORTHROP

Laboratories of The Rockefeller Institute for Medical Research
Princeton, New Jersey
September, 1938

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CRYSTALLINE ENZYMES

I: GENERAL CHEMISTRY OF ENZYMES

ONE of the most striking characteristics of living things is the rapidity and precision with which the chemical changes necessary for their existence are carried on. The process of digestion is a familiar example. Proteins are split in the stomach into much smaller compounds, and this process is continued in the small intestine. The final products are precisely those needed for the nutrition of the animal and are formed from proteins with little or no evolution of heat or expenditure of energy. The process cannot be duplicated without the aid of enzymes, since chemical hydrolysis of proteins yields different products and in any case can be accomplished only by violent treatment and the expenditure of considerable energy. Similar examples of the efficiency of the reactions which take place in the animal could be multiplied indefinitely. It is now known that these specific accelerating effects which living cells exert on the reactions occurring within them and in their vicinity are due to the presence of minute amounts of some substances formed by the living cell which have come to be known as enzymes. Without enzymes life could not exist, and yet enzymes themselves are not living.

For many years this characteristic ability of living matter to direct these reactions was regarded as a vital activity entirely outside the realm of experimental science. Evidence gradually accumulated, however, to show that the living cell was not necessary for some, at least, of these characteristic reactions; and one case after another was found in which the reaction could be made to take place without the living cell.

Thus Spallanzani (1783) was able to cause the digestion of meat to take place outside the animal by allowing gastric juice to act on the meat *in vitro*. This experiment was one of the first examples of the separation of a "vital activity" from the living organism. Similar observations were soon made by Fabroni, Kirchhoff, Payen and Persoz, Schwann, Kühne, Buchner, and others. Nevertheless, enzymes

were classified for a long time as "organized" and "unorganized," depending upon whether or not the reaction in question could be made to take place *in vitro*. The discussion revolved principally around the question of yeast fermentation and was the cause of the celebrated controversy between Pasteur and Liebig. Pasteur found that fermentation occurred only when yeast cells were present and hence that Liebig's idea of "spontaneous" fermentation was incorrect.

In 1897, however, Buchner was able to cause fermentation by an extract of yeast, without the presence of actual yeast cells. This experiment broke down the old distinction between "organized" and "unorganized" ferments and it has since been considered possible to cause any of the reactions which occur in cells, to take place without the cell, provided the necessary enzyme can be extracted.

Buchner's experiments also showed that Liebig was correct in saying that yeast cells were not necessary for fermentation. He was mistaken, however, in assuming that the enzyme which causes the fermentation could produce itself without the yeast cell.

It had been suspected long before Buchner that the process of gastric digestion was due to the presence of some specific substance. Schwann (1836) definitely assumed the existence of such a substance and named it "pepsin." The existence of trypsin in the intestine had also been suspected early in the nineteenth century, but was not definitely assumed to exist until the time of Corvisart (1857–1858) and of Kühne (1867), who gave it its present name. A large number of other enzymes were then discovered by means of their characteristic reactions. It was assumed that since these reactions occur enzymes must exist to cause them, but there was no direct proof of the actual existence of enzymes, and, in fact, their existence as ordinary chemical compounds was frequently questioned. The problem is analogous to that of the causative agent of an infectious disease. A causative agent is assumed to exist because the disease occurs, but the assumption cannot be proved until the etiological factor is actually isolated.

During the time enzymatic processes were being discovered the chemists found that many purely chemical reactions are accelerated by small amounts of substances which apparently are not changed by the reaction. Berzelius (1825) pointed out that the properties of these substances are strikingly similar to those of the active agents

found in living cells. He named the general phenomenon "catalysis" and considered what are now called enzymes to be a special class of catalysts.

The name "enzyme" was proposed by Kühne (1867) for these organic catalysts. In the last fifty years enzymes and the enzymatic reactions have been studied intensively by chemists and physiologists. The chemists have been interested primarily in the mechanism of the reactions, and the physiologists in the nature of the reactions, and both chemists and physiologists have spent a great deal of time trying to isolate the enzymes. Rapid progress was made in the study of the mechanism of the reactions caused by enzymes, and many of the anomalies characteristic of enzymes were explained.

THEORY OF CATALYSIS

The fundamental law of catalysis was formulated by Ostwald, who defined catalysts as substances which affect the rate but do not affect the equilibrium state of a reaction.[1]

Theoretical proof of this statement was furnished by Van't Hoff who showed by thermodynamic reasoning that a substance which is not changed during the course of a reaction cannot affect the equilibrium condition (cf. Taylor 1925, page 318).[2]

This peculiarity of catalytic reactions also furnishes the most useful method of determining experimentally whether a reaction is catalytic or not. If the substance under investigation acts as a catalyst (enzyme) the addition of increasing concentrations of the substance will not change either the nature or concentration of the final products formed, but only the time required for the reaction to take place. If, on the other hand, the substance takes part in the reaction, the nature or

[1] Bredig (1902) modified the definition slightly in order to make it conform more closely to the experimental facts. He defined catalysts as substances which do not appear in the reaction products in simple stoichiometric relationship.

[2] This conclusion is strictly true only when the catalyst is present in low concentration and does not combine with any of the components of the reaction. If the catalyst is present in high concentration it may affect the activity of the components in the same way as would any indifferent substance and so change the equilibrium (Taylor 1925). If the catalyst combines with one or more components the equilibrium will also be changed (Euler 1925, page 305). Enzymes, in general, are present in such low concentration that effects on the activity are negligible. Many enzymes combine with the products of reaction, however, and in such cases this combination may affect the equilibrium point, although the principal result is to slow down the rate of reaction so that experimentally it may be very difficult to reach final equilibrium.

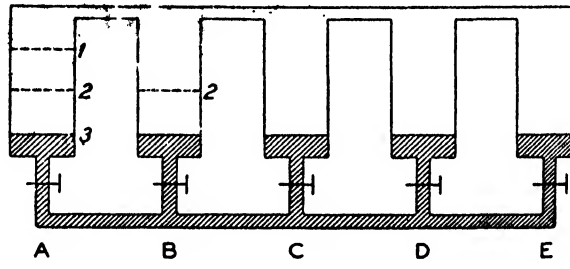
quantity of the products, as well as their rate of formation, will change as the quantity of substance added is increased.

Since the catalyst does not change the equilibrium it follows that if any one catalyst causes the reaction to proceed from A to B then any other catalyst which affects the reaction must also cause the reaction to proceed in the same direction and to the same extent. The assumption of "hydrolyzing" and "synthetic" catalysts for the same reaction under the same conditions is therefore in contradiction to theory (cf. Borsook 1935).

It is true, however, that under some conditions the addition of a catalyst can greatly increase the concentration of one or more reaction products and it is for this reason that enzymes are so important in cellular reactions and catalysts so useful in industry. Assume the presence of a substance, A, which decomposes "spontaneously," i.e., with the liberation of energy, to form a series of substances, B,C,D,E— and that at equilibrium all substances are present in equal concentration. Since they are all in equilibrium with A they are also in equilibrium with each other. If A is then allowed to decompose spontaneously the system at the end of the reaction will contain equal concentrations of A,B,C,D, and E, and no catalyst can cause any further change to occur. Now suppose that A alone is present and a catalyst is added which accelerates the reaction $A \rightleftharpoons B$ but not the other reactions. Addition of this catalyst will result in a rapid formation of B, until [A] = [B] with the result that a large amount of B and very little C,D, or E is formed. The reaction is not at equilibrium and if allowed to proceed by itself will end at the same state as though the catalyst had not been added. In the meantime, however, large amounts of B have been formed due to the catalyst and could be removed. Similarly, if the concentration of B is greater than that of A at the beginning, addition of the same catalyst will now cause A to be formed until [A] = [B] and very little C,D, or E will be formed. The reaction does not take place in the direction $B \rightarrow A$ simply because of the addition of the catalyst but because the energy changes require the reaction to go in this direction and the catalyst merely accelerates the rate. In this case, also, the high concentration of A is only temporary since if left to itself the same final equilibrium conditions, A=B=C=D=E, will be reached.

The condition may be roughly exemplified by the arrangement

shown in the diagram below. The five tubes are connected at the top by open tubing and at the bottom are all connected to tube A by means of tubes which can be closed. If tube A is now filled with water to position 1, the water will distribute itself until it is at the same level in all tubes, since this is the equilibrium condition. If the connecting tubes at the bottom are closed at the beginning the water will distribute itself slowly through the vapor phase. This corresponds to the "spontaneous" reaction rate. If all the bottom tubes are opened the same final condition will be reached much more rapidly. This corresponds to what will happen if a catalyst is added which accelerates all the reactions. If, however, only the tube connecting A and B is opened the level of B will rise to position 2 and no measurable change will occur in the levels of C,D, and E during this time.



The preceding example resembles more or less the hydrolysis of proteins; hydrolysis with acid leads to complete hydrolysis and results in the liberation of the amino acids and, hence, any other catalyst must also lead only to amino acids *at equilibrium* under the same conditions. Pepsin or trypsin, however, increase the rate of hydrolysis of some protein linkages much more than others, so that for all practical purposes the reaction stops before any amino acids are formed, although it is still far from equilibrium.

ENZYMES AS THEORETICAL CATALYSTS

The agreement between enzyme reaction and catalytic theory has been much more fully confirmed in connection with carbohydrates and esters than with proteins.

The first experimental synthesis by an enzyme was reported by Croft Hill, who found that a disaccharide which he thought was mal-

tose was formed by the action of maltase on concentrated glucose solutions. The substance, however, was eventually identified by Emmerling as iso-maltose which is formed, under the same conditions, by acid catalysis. Iso-maltose is not hydrolyzed by maltase and this fact led Armstrong to suggest that enzymes synthesize substances which they do not hydrolyze. This assumption is contrary to the law of catalysis since it predicts that the direction of the reaction, and, hence, the equilibrium condition, vary with the catalyst. It was found later that the "maltase" preparation used by Croft Hill contained emulsin which does hydrolyze iso-maltose and, hence, also synthesized it. This is one of many examples of the confusion caused by the use of impure enzyme preparations.

Bourquelot studied the simpler case of glucoside hydrolysis and synthesis and found in several cases that enzymes caused the reaction to proceed towards the equilibrium point from either side. This furnished experimental proof of the statement that enzymes do not change the equilibrium point.

Kastle and Loevenhart showed that the hydrolysis of esters by lipase also agrees with theory.

Pottevin found that pancreatic lipase synthesizes esters from oleic acid and methyl alcohol and that the equilibrium condition was independent of the concentration of enzyme. (For review of the older literature see Euler 1925, page 295, and Bayliss 1925.)

Borsook and Schott have found (1931) from a study of oxidation potentials that the enzyme, fumarase, which catalyzes the reaction, succinate=fumarate, also acts as a theoretical catalyst.

The synthesis of glycogen described by Cori and Cori (1939) is another example since in this case the reaction proceeds in either direction, depending upon conditions. The synthetic reaction is remarkable in that a trace of the end product, glycogen, is required to start it. The enzyme, phosphorylase, however, is also necessary. The reaction as a whole, therefore, appears to be an example of autocatalysis superimposed on an enzyme reaction.

The experimental results with esters and glucosides in the presence of enzymes are, therefore, in good agreement with theory. In the case of the proteolytic enzymes, however, the results are more complicated and there is some doubt as to the occurrence of protein synthesis in the presence of proteinases (cf. Chapter XIII).

MEASUREMENT OF CATALYTIC EFFICIENCY

According to the accepted definition, the efficiency of a catalyst must be expressed as the ratio of the velocity of the reaction in the presence of unit concentration of catalyst to that of the uncatalyzed reaction, and not as the difference between the two rates as is usually done. Thus, if a reaction proceeds at the rate of 1 mol per second without the catalyst and at 10 mols per second with unit concentration of catalyst, the actual increase due to the catalyst is 9 mols per second but the relative increase is 10 times. If a second reaction is proceeding at the rate of 1/1,000 mols per second without the catalyst and 1 mol per second with the catalyst, the actual increase is about 1 mol per second but the relative increase in rate is 1,000. The second catalyst is, therefore, much more efficient than the first if the ratio of the rates with and without catalyst is compared, but less efficient if the difference in rates is taken as a measure of efficiency. It appears to the writers that the ratio is the only rational measure of the efficiency of the catalyst, but unfortunately experimental data are usually lacking for the calculation of this figure.

CHARACTERISTICS OF ENZYME REACTIONS

Enzyme reactions in general are distinguished from ordinary catalytic reactions in that they are more specific, they have a temperature optimum, there is an optimum acidity (pH) for the reaction, and the effect of changing substrate or enzyme concentration is not always that predicted by simple chemical theory.

The specificity of the reaction is usually emphasized in any discussion of enzymes and has even been considered a qualitative distinction between enzymes and other catalysts. It is clear, however, that this is not the case. The specificity of enzyme reactions differs quantitatively but not qualitatively from that of other catalytic reactions and there is no reason to believe that a special theory is necessary to account for the facts.

For example, the hydrolysis of dipeptides with acid or enzymes was found by Levene, Simms, and Pfaltz (1924) to depend, in either case, on the dissociation constants of the peptides. The interesting enzyme models made and studied by Langenbeck (1935) also show marked specificity.

Steinhardt's and Fugitt's recent results (Steinhardt and Fugitt 1942) furnish another striking example of the same fact. It has been stated frequently that enzyme hydrolysis differs qualitatively from acid hydrolysis in that the rate of acid hydrolysis is approximately the same for different acids of the same strength, whereas the rate of enzyme hydrolysis is specific for the particular enzyme used.

Steinhardt and Fugitt have found, however, that hydrolysis of wool protein and of egg albumin are about one hundred times as fast in the presence of long-chain fatty acids, such as cetyl sulfonic, as in the presence of HCl. Cetyl sulfonic acid, therefore, is an efficient "artificial" pepsin, since the addition of the substance to an acid solution of a protein increases the rate of hydrolysis enormously.

The theory of catalysis, although still incomplete, is rapidly taking form and when completed will apply as well to enzymes as to other catalytic reactions (Schwab 1937).

HOMOGENEOUS OR HETEROGENEOUS CATALYSIS

Enzyme reactions have been considered by many workers to be examples of heterogeneous catalysis, and such models as those of Bredig (1902) show many of the characteristics of enzymes. On the other hand, Arrhenius (1908) showed long ago that equally good analogies could be found in homogeneous reactions. In the absence of definite knowledge of the chemical nature of enzymes themselves it was impossible to decide the question. The experiments on the solubility of the crystalline enzymes described in subsequent chapters show that a solution of these enzymes consists of one phase only. These enzyme reactions are therefore a case of homogeneous catalysis.

EFFECT OF TEMPERATURE

As the temperature is increased above 0°C., the velocity of enzyme reactions, as of reactions in general, increases. As the temperature increases still more, the velocity of enzyme reactions apparently decreases so that they appear to have an optimum temperature which is usually about 30–40°C. Tammann (1889, 1895) showed that this apparent optimum was merely the result of the fact that the enzymes are inactivated with increasing speed as the temperature is raised while the velocity of the catalytic reaction increases regularly with temperature just as any other reaction. The temperature coefficient

of inactivation of the enzyme is greater than that for the speed of the reaction. As a result, the amount of enzyme present at any time decreases rapidly as the temperature rises so that the longer the time interval used in the measurement, the lower the "optimum" temperature is found to be.

pH OPTIMUM

It was early recognized that enzyme reactions were very sensitive to acidity. Pepsin, for instance, acts only in acid solution, while trypsin acts on most proteins only in neutral or slightly alkaline solution. The early experiments were complicated and difficult to interpret since it appeared that the optimum acid concentration varied with each acid. The problem was greatly simplified by Sørensen (1909), who showed that the deciding factor was the hydrogen ion concentration. If the rate of reaction is plotted against the hydrogen ion concentration, the divergent effects supposed to be due to the different acids disappear and reactions taking place at the same hydrogen ion concentration are found to proceed at the same rate irrespective of the acid used to adjust the reaction. A further step in the explanation of this phenomenon was made by Michaelis (1914), who found that the curves expressing the rate of hydrolysis as a function of the pH could be accurately calculated on the assumption that the enzyme was a weak acid or base and that the activity was due either to the ion or the undissociated molecule, depending on the nature of the enzyme.

It was found (Northrop 1922a), on the other hand, that the pH optimum for the digestion of proteins by pepsin or trypsin varied with different proteins and could be predicted fairly well from the titration curve of the proteins if it were assumed that pepsin reacted with the positive protein ion and trypsin with the negative protein ion (Figure 1). It was later shown by Willstätter, Grassmann, and Ambros (1926a,b) that a similar assumption would account for the effect of pH on the activity of papain and other enzymes of that group if it were assumed that the enzyme attacked the undissociated protein molecule.

This explanation accounts for the increase in the rate of trypsin digestion as alkali is added to an isoelectric solution of the protein, since more ionized protein is formed. It does not, however, account for the fact that the reaction rate decreases again in the presence of

an excess of alkali. A study of purified trypsin solutions at various pH showed (Kunitz and Northrop 1934) that the enzyme exists in an active and inactive form which are in equilibrium with each other and that the equilibrium is shifted toward the inactive form as the pH becomes more alkaline than 8.0. The effect of pH on the amount of active enzyme and on the rate of digestion of casein is shown in Figure

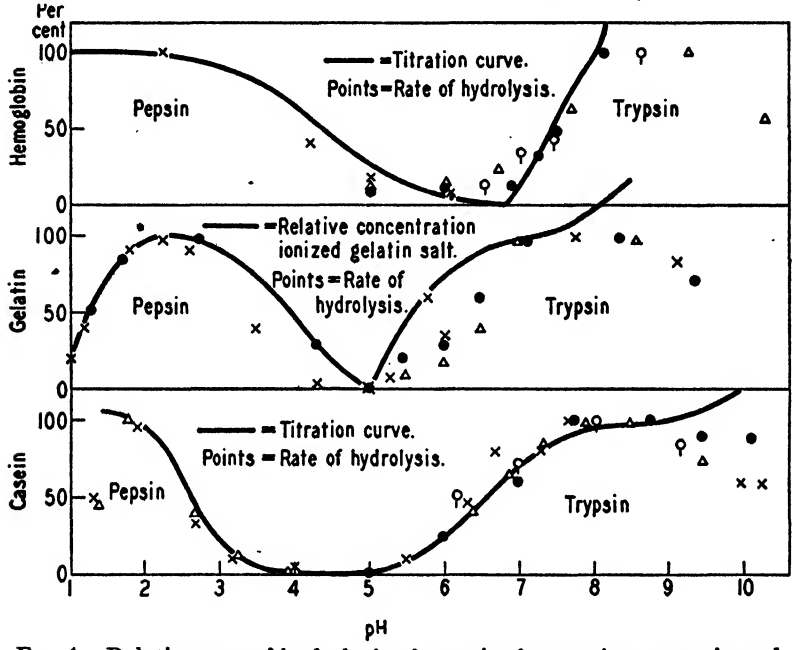


FIG. 1. Relative rate of hydrolysis of proteins by pepsin or trypsin and percent of protein present as salt, at various pH.

2. The decrease in reaction rate in strong alkali is therefore due to the formation of inactive enzyme as Michaelis assumed. The effect of the pH on the ionization of the protein, together with its effect on the equilibrium between active and inactive enzyme, therefore, furnishes a complete picture of the pH activity curve for trypsin. In acid solution the enzyme is present in the active form, but the substrate protein is present in the form of positive ions which are not attacked by the enzyme. As the alkalinity increases, the percentage of protein present as negative ions increases, and therefore the rate of digestion increases. As the alkalinity is increased still further, the

percentage of trypsin present in the active form begins to decrease. Since the rate of digestion is proportional to the product of the concentration of the negative protein ions times the concentration of active trypsin, there is a point at which this value is a maximum, and the position of this maximum will depend upon the particular protein used.

ANOMALIES OF ENZYME KINETICS

The theoretical equation for a simple catalytic reaction states that the amount of substrate decomposed is proportional at any time to

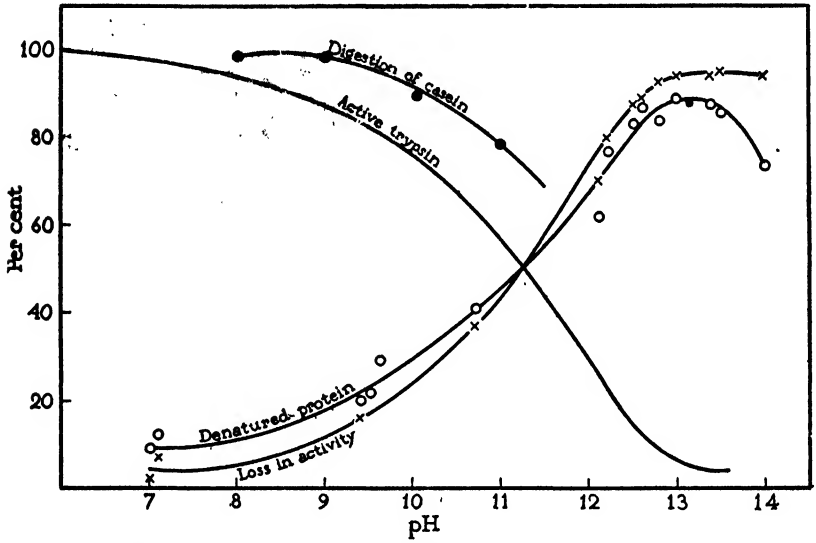


FIG. 2. Effect of pH on the rate of digestion of casein by trypsin and on the relative amount of active trypsin present in solution.

the enzyme concentration and to the substrate concentration, i.e., $-dS/dt = KES$. The percentage substrate decomposed per unit time, therefore, should be proportional to the enzyme concentration and independent of the substrate concentration. Actually, however, it is often found that the percentage substrate decomposed in unit time increases less rapidly than the enzyme concentration. In high substrate concentrations a constant quantity of substrate may be decomposed in solutions of various concentrations instead of a constant percent as the simple theory demands. The time course of the reac-

tion also diverges from the theoretical prediction, and it is frequently found that the amount of substrate decomposed is proportional to the square root of the time. This square root relationship is known as Schütz's rule.

Arrhenius (1915) pointed out that this effect could be predicted if it were assumed that the product of the reaction formed an inactive compound with the enzyme in accordance with the mass action law

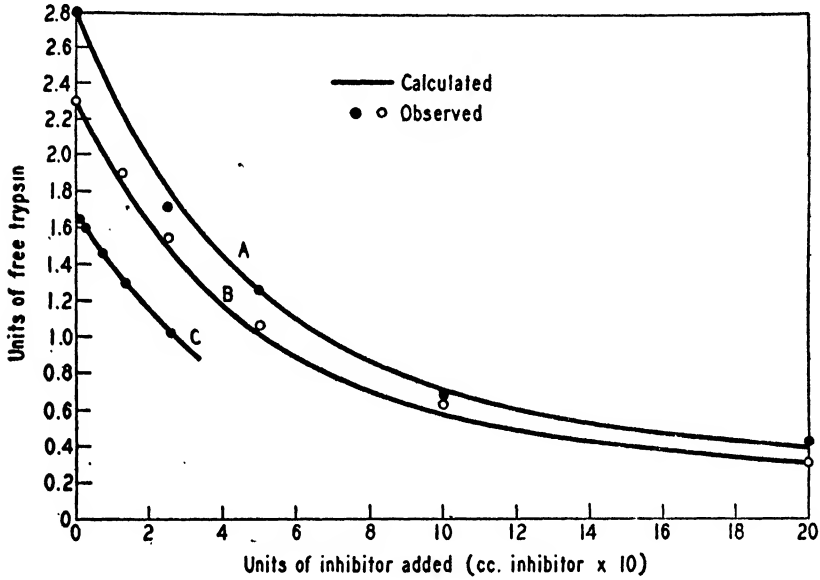


FIG. 3. Effect of adding increasing amounts of inhibitor to trypsin solutions of different strengths. The solid curves are the calculated values, and the points the observed units of active trypsin present.

for a dissociating complex. Northrop (1920, 1922b) found that such an inhibiting substance was actually formed during the digestion of proteins by pepsin or trypsin and also that the reaction between the substance and the enzyme agreed with the mass action theory. The effect of adding increasing amounts of this inhibitor to trypsin solutions is shown in Figure 3. The presence of such a complex between enzyme and inhibitor in crude enzyme solutions also accounts for the abnormal effect of changing the enzyme concentration which occasionally is observed. An example of this effect is shown in Figure 4. These results are experimental evidence for the correctness of

Arrhenius's explanation of this anomaly. The presence of such complexes also accounts for anomalies connected with the rate of inactivation of crude enzyme solutions. There remains, however, the anomalous effect of changing the substrate concentration.

It is a general assumption in chemical kinetics that the formation of an intermediate addition compound is the first step in chemical

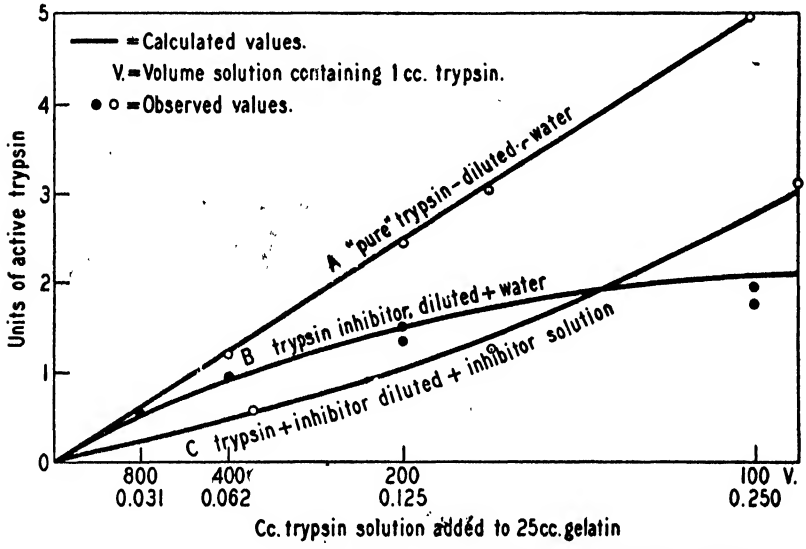


FIG. 4. The influence of the presence of inhibitor on the concentration-activity curve of trypsin. Curve A, "pure" trypsin diluted with water. Curve B, mixture of trypsin and inhibitor diluted with water. The ratio of trypsin to inhibitor is therefore constant. Curve C, mixture of trypsin and inhibitor diluted with a solution of inhibitor of the same concentration as was present in the trypsin solution. The concentration of inhibitor is therefore constant in this experiment.

reactions. Henri (1902, 1905) and others pointed out that the existence of such a compound would account for the fact that the amount of substrate decomposed approaches a constant value as the substrate value is increased instead of increasing in proportion to the substrate. Michaelis and Menten (1913) stated this assumption quantitatively and found that the prediction agreed very well with the experiment. Van Slyke (1914, 1942) derived a similar equation which agrees well with the kinetics of the urea-urease reaction. The theory assumes that the substrate combines with the enzyme to form a com-

pound which exists for a considerable length of time in solution. As the substrate concentration is increased more enzyme substrate compound is formed, until finally all the enzyme is combined with the substrate and therefore the addition of more substrate has no effect on the reaction. The calculation involves the use of several constants, so that agreement between observed and calculated figures is not entirely convincing and there are, in addition, some results which are difficult to explain on this basis. It was found (Northrop 1922c, 1932a), for instance, that the digestion of casein and of gelatin proceeded at the same rate whether present in separate solutions or in the same solution (Figure 5). The kinetics of either reaction can be explained by supposing that the enzyme is saturated with one protein, say casein, but the rate of reaction of the mixture cannot be explained on this basis, since if the enzyme is already saturated with casein it could not act on the gelatin. It becomes necessary, then, from the point of view of the addition compound theory to assume either that there are two enzymes or two different active groups, which amounts to practically the same thing.

Euler, Josephson, and Myrbäck (1924) pointed out that the results of the calculation would be just the same if it were assumed that the enzyme substrate compound did not decompose, but that the rate of reaction was proportional to the uncombined enzyme times the uncombined substrate. This mechanism would be more analogous to acid hydrolysis, since in this case it is possible to show that the rate of hydrolysis is proportional to the hydrogen ion, i.e., the free acid rather than to the total acid of the solution.

Rothen's recent results (1946) are of interest in this connection. Rothen finds that pepsin and trypsin can act on a film of protein covered by a film of a plastic, "Formvar." Further, thin films of protein require thinner films of Formvar to protect them than do thick protein films. This result shows that diffusion through the protecting film can hardly be assumed to occur. If enzyme action can occur across a film it is evident that no intermediate substrate-enzyme complex can be formed.

Medwedew (1937) has shown that the kinetics of invertase agree with the modern theory of chemical reaction velocity which predicts that the rate of reaction is proportional to the number of effective collisions between reacting molecules. Butler (page 144) has found

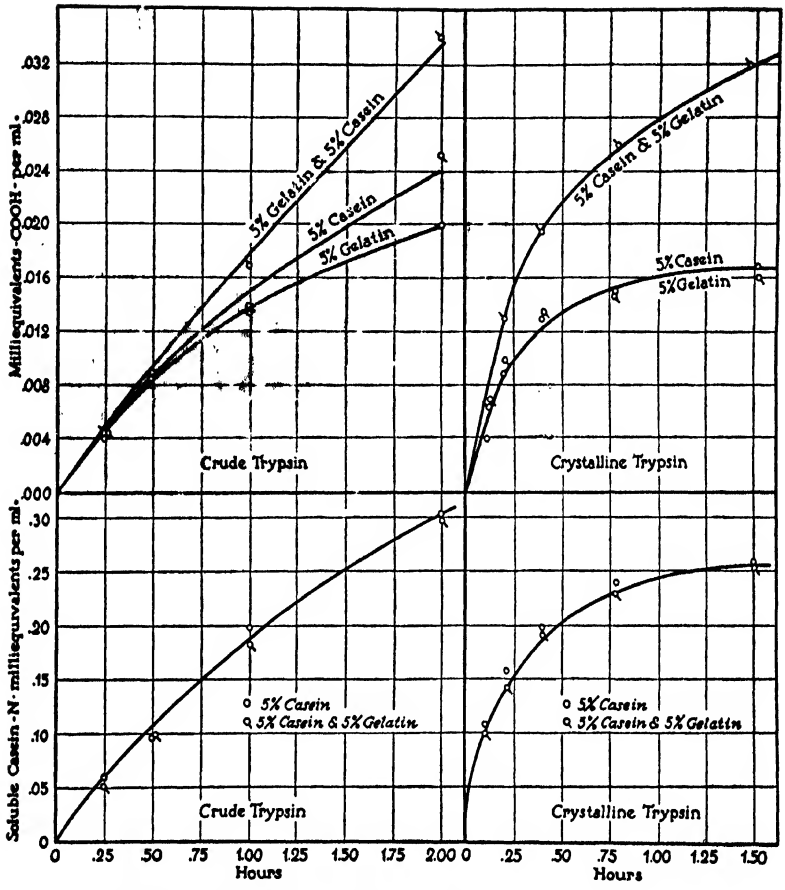


FIG. 5. Digestion of mixtures of casein and gelatin with crude and crystalline trypsin. Upper figures show increase in formol titration of mixture and of two proteins separately, and lower figures show digestion of casein when present alone and in presence of gelatin.

that the same theory may be applied to the kinetics of trypsin reactions. If this theory turns out to be of general application, the kinetics of enzyme reactions will at last be explainable in terms of chemical theory.

THE RESISTANCE OF LIVING ORGANISMS TO DIGESTION BY PROTEOLYTIC ENZYMES

The fact that living organisms are not digested by pepsin or trypsin, whereas dead organisms are rapidly digested, has attracted the attention of biologists and physiologists for many years. The early work was centered chiefly about the defense of the digestive tract, but it was soon recognized that all living matter was resistant to these enzymes. The problem is evidently closely connected with the resistance of the living cell to autolysis by the enzymes already in the cell. It differs from the latter in that by using concentrated solutions of the proteolytic enzymes the digestion of dead cells may be made very rapid in comparison to autolysis. There is much more striking difference, therefore, in the behavior of dead and living cells when exposed to trypsin or pepsin than in the autolysis of dead and living cells.

Fermi (1910) was able to show in a series of careful and ingenious researches that Claude Bernard's theory of a protective mucus or epithelium, Pavy's theory of the alkalinity of the tissues, Gaglio's theory of the absorption of the enzymes, and Wieland's anti-enzyme theory were all insufficient to account fully for the results. There is no doubt that the alkalinity of the blood and the presence of anti-enzymes have an effect on the process, but, as Fermi points out, they are insufficient to account for the complete absence of digestion of living cells. Fermi concluded that the configuration of the protein molecule in the living cell was different from that after the death of the cell, and that the "living molecule" could not be attacked by the enzyme. This theory is not very different from Hunter's "living principle," except that the attribute of "living" is applied specifically to the protein molecule. Fermi considered that the assumption of a mechanism which prevented the enzyme from entering the living cell was untenable, since there are innumerable types of cell membranes, and it is not reasonable to suppose that they are all impermeable to the enzyme while the cell is living, and permeable after the death of the cell. It is evident, however, that if there were such a mechanism it

would account for the failure of the enzymes to destroy the cell. The work of Osterhout (1922) and others has shown that the permeability of the cell membrane is one of the most characteristic attributes of the living cell and that this permeability changes in a remarkable way when the cell is dead or injured, so that it is not at all unlikely that the permeability to enzymes is also greatly different in the living and the dead cell. Experiments were undertaken (Northrop 1926a), therefore, to determine whether or not pepsin and trypsin actually did enter the living as well as the dead cell. It was found in every case that as long as the cell was alive, no detectable quantity of enzyme was taken up; whereas when the cell died, the enzyme was rapidly removed from solution and concentrated in the cell.

Effect of trypsin on living and dead organisms.—The results of several experiments in which living and dead organisms were exposed to the action of a powerful dialyzed trypsin solution are shown in Table 1. They confirm the results of Fermi and other workers in showing that the living cell is not attacked, whereas cells which have been killed by heat or mechanical injury are rapidly digested. The results were more striking than the figures show, since in some cases the dead organism was almost completely disintegrated although the formol titration increase was small. It may be added that normal animals lived just as long in the active enzyme solution as in the inactivated enzyme. The experiment also shows that the slow digestion due to autolysis is negligible compared to that due to the trypsin.

The digestion of the organisms killed by heat could be readily accounted for by assuming a change in the chemical nature of the proteins or by the destruction of the anti-enzyme. These objections, however, do not apply to the digestion of the organism when killed simply by mechanical injury. It is difficult to imagine that this would cause a change in the chemical nature of the protein, unless Fermi's "living molecule" is assumed. The anti-enzyme is present and in the case of earthworms its action is marked, yet, owing to the large excess of enzyme, the tissue is digested.

Permeability of dead and living tissue to pepsin and trypsin.—In order to see whether the enzyme can penetrate into the organisms the experiments were repeated, with the modification that the organisms were placed in a small volume of the enzyme solution. This is necessary, since otherwise the change in concentration of the surrounding

enzyme solution, on removal of a small quantity of trypsin, would be too small to measure. The concentration of the enzyme in the surrounding solution was then determined at intervals. Since the volume of the enzyme solution is known, the total amount of enzyme removed can be calculated. In every case the dead organism removed large quantities of the enzyme from solution, while the living organism removed little or none (Table 2). Pepsin and trypsin, therefore,

TABLE 1

EFFECT OF TRYPSIN ON LIVING AND DEAD ORGANISMS

Organisms added to concentrated dialyzed trypsin and left at 20°C. for 24 hours; increase in formol titration and visible digestion determined

	APPEARANCE AND INCREASE OF FORMOL TITRATION, 0.1 N NaOH PER CC. SOLUTION AFTER 24 HOURS					
	Living Organisms		*Killed by Cutting*		*Killed by Boiling 10 Min.*	
	Active Trypsin	Inactive Trypsin	Active Trypsin	Inactive Trypsin	Active Trypsin	Inactive Trypsin
Earthworm*	0.8, alive	0.9, alive	3.50, nearly all digested	1.0	3.50	—
Euglena	0, "				Cells dissolved	
Yeast	0, "				0.75, cells dissolved	
Fundulus	0.1, "	0.1, "	0.3, partially digested	—	0.8, partially digested	0.10
Meal worm†	0.1, "	0, "	2.0	0.8	0.4	—
Goldfish‡	0, "	0, "	0.20	—	0.35	—

* Lumbricus terrestris. † Tenebrio molitor. ‡ Caracius auratus.

cannot enter the living tissue. It might be objected that even though it cannot enter, the enzyme could attack the surface of the cell. There is good reason to suppose that the surface of the cell is not protein in nature, but, even though hydrolysis did take place at the surface, it would be so slow compared to the rate when the enzyme was distributed throughout the cell that it could not be detected experimentally.

It may be shown that the enzyme is really taken up in an active form and is not merely inactivated or destroyed. Pieces of earthworms which have been killed by mechanical injury or heat are placed

in strong trypsin solution for one hour. The trypsin is rapidly removed from solution, but in this time no noticeable digestion occurs. The tissue is then removed from the enzyme solution, washed with water, and placed in a small volume of water. After 24 hours at 20°C. the tissue is almost completely digested, while control pieces treated in the same way with inactivated trypsin are unchanged.

If the lack of hydrolysis of living cells were really due to the protective action of the membrane, it should be possible to show that

TABLE 2

REMOVAL OF TRYPSIN FROM SOLUTION BY LIVING AND DEAD ORGANISMS

Material washed with dilute trypsin solution and placed in an equal volume of dilute trypsin at 0°C. for 24 hours; supernatant solution tested for trypsin

	TOTAL UNITS TRYPSIN REMOVED FROM SOLUTION				
	Living	*Killed by Cutting*	*Killed by Boiling*	*Killed by HCl*	*Killed by 50 Percent Alcohol*
B. coli	0		4.0		
Earthworm	1.0	10.0	14.0		
Euglena	0		6.0		
Meal worm*	4.0	6.0	7.0		
Goldfish	0		4.0		
	PEPSIN				
Earthworm	0	6.0	8.0	10.0	4.0

* Worms injured, there is some motion, but they do not recover on removal from solution.

digestion occurs if the enzyme is actually injected into the cell. Attempts were made to carry out this experiment, but the results were not entirely convincing owing to the difficulty of performing the operation without injury to the cells.

Digestion of living cells by papain (Robbins and Lamson 1934; Berger and Asenjo 1940).—The proteolytic enzyme, papain, does kill and dissolve living ascaris. It would be of interest to determine whether or not this enzyme were taken up by the living cell. If it is not taken up by the living cell, but still causes death, then the explanation found for the behavior of pepsin and trypsin evidently is not of general ap-

plication. Sang (1938) has concentrated an inhibitor from *ascaris* which inhibits trypsin and pepsin but not papain. These results indicate that resistance of these worms to pepsin and trypsin and their digestion by papain is due to properties of this inhibitor. On the other hand, live tadpoles or arbacia eggs are not digested by papain or ficin any more than by trypsin (Northrop 1947a).

CHEMICAL NATURE OF ENZYMES

Most of the early workers considered enzymes to be proteins, but no pure preparations were obtained and the evidence was almost entirely indirect. It was known, for instance, that the temperature coefficient for the rate of inactivation was extremely high and agreed with that for the denaturation of proteins. No other reaction is known to have such a high temperature coefficient.

The work of Willstätter (1928) and his collaborators resulted in the preparation of much more highly purified and active preparations than had ever been obtained before. These preparations, however, were obtained only in very dilute solution. These solutions, although highly active, gave none of the tests for proteins or carbohydrates, and it was concluded that the enzymes were not proteins or carbohydrates. Since the activity of the pure enzymes was unknown, it is evidently not possible to draw conclusions from negative tests. The crystalline enzymes which have been prepared are proteins, but their activity may easily be detected in solutions which are too dilute to give positive protein tests. The bacteriophage is the most striking example of this fact. This substance may be detected (Northrop 1938) in a solution containing about 10^{-15} gm. per cc., i.e., one part of the active preparation in one thousand million million parts of water. Such a solution, of course, gives completely negative chemical tests, not only for proteins and carbohydrates but for any other chemical compound. If these negative tests were to be taken as significant, it would be necessary to conclude that bacteriophage did not exist at all as a chemical substance. Willstätter's work has been of great value, nevertheless, since it has shown that enzymes are many times more active than had previously been supposed and has also resulted in the discovery of a large number of new enzymes.

In the last twenty years a number of enzymes have been isolated and crystallized and have been found to be proteins (see Table 3).

TABLE 3

LIST OF CRYSTALLINE ENZYMES*

Enzyme	*Reference*
Urease	Sumner (1926)
Pepsin	Northrop (1930)
Trypsin	Northrop and Kunitz (1931)
Chymo-trypsin	Kunitz and Northrop (1933)
Yellow enzyme	Theorell (1934)
Carboxypeptidase	Anson (1935)
Alcohol dehydrogenase	Negelein and Wulff (1937)
Catalase (beef liver)	Sumner and Dounce (1937)
Lysozyme	Abraham and Robinson (1937), Alderton, Ward, and Fevold (1945)
Papain	Balls, Lineweaver, and Thompson (1937), Balls and Lineweaver (1939)
Ficin	Walti (1937)
Hemocuprein and hepatocuprein	Mann and Keilin (1938)
Lecithinase	Slotta and Fraenkel-Conrat (1938)
Tyrosinase	Dalton and Nelson (1938)
Ascorbic acid oxidase	Tadokoro and Takasugi (1939)
Glyceric aldehyde-diphosphate dehydrogenase	Warburg and Christian (1939)
Pepsin (salmon)	Norris and Elam (1939)
Ribonuclease	Kunitz (1939a)
Lactic dehydrogenase	Straub (1940)
Myogen (aldolase)	Baranovskii (1940)
Catalase (beef erythrocytes)	Laskowski and Sumner (1941); Dounce (1942)
Chymopapain	Jansen and Balls (1941)
Enolase	Warburg and Christian (1941)
Fumarase	Laki and Laki (1941)
Urine and serum protective enzymes	Mall and Bersin (1941)
Carbonic anhydrase	Scott (1942)
Peroxidase	Theorell (1942)

TABLE 3 (Continued)

Enzyme	*Reference*
Phosphate-transmitting fermentation enzyme	Bücher (1942)
Phosphorylase (animal)	Green, Cori, and Cori (1942)
Asclepain	Carpenter and Lovelace (1943)
Rennin	Berridge (1943), Hankinson (1943)
Tumors, fermentation enzyme from	Kubowitz and Ott (1943)
Zymohexase	Warburg and Christian (1943)
Lactoperoxidase	Theorell and Paul (1944)
Phosphomonoesterase.	Nguyen-Van-Thoai, Roche, and Sartori (1944)
D-glyceraldehyde 3-phosphate dehydrogenase	Cori, Slein, and Cori (1945)
Hexokinase	Kunitz and McDonald (1946a)
β-amylase	Balls, Thompson, and Walden (1946)
α-amylase	Meyer, Fischer, and Bernfeld (1947a and b)

* The identity of the crystalline protein and the enzyme has not been fully established in the case of some of the recent preparations included in this list.

Since these proteins exhibit peculiar properties, they must have peculiar chemical structure, and since the chemical properties of molecules are not additive, the entire structure of the molecule must be known before the properties of the molecule are completely understood. It is usually true, however, that certain chemical properties depend markedly on specific groups or atoms in the molecule and, to a much less extent, upon other groups or atoms. These groups which markedly influence the activity are frequently referred to as "active groups," but it must be realized that the term is relative and that theoretically a change in any part of the molecular structure would be expected to influence the properties of the molecule. In the case of the yellow respiratory ferment, Warburg has shown that the activity depends on the presence of a characteristic prosthetic group, and his work, together with that of Theorell (1934) and of Kuhn (1937), has determined the structure of the group so that the most important part of the structural chemistry of this enzyme is now known.

The prosthetic group and the protein of acetaldehyde reductase have also been isolated and crystallized. The chemistry of these "dissociating proteid" enzymes has been reviewed by Warburg (1938).

The proteolytic enzymes may also have prosthetic groups but there is, as yet, no evidence for their presence, so that the activity appears to depend on some peculiar arrangement of the amino acids, as is probably the case in insulin.

The amino acid content of several of the enzymes analyzed by Professor Brand is shown in Table 4. The results show that all but 1 or 2 percent of the nitrogen of pepsin, ribonuclease, and chymo-trypsinogen is accounted for by the amino acid content. If prosthetic groups are present therefore they do not contain nitrogen or they are a very small part of the molecules. The table also shows that no characteristic number of any one amino acid occurs in all the enzymes.

Attempts to isolate fragments of the molecule possessing some peculiar structure have failed since the activity is lost as soon as the molecule is ruptured and there is no means, at present, once the enzymatic activity is lost, of recognizing the fragments which possess the "active group."

It has been possible, however, to prepare several proteins closely related to the active enzymes, but which are themselves inactive, and a study of the difference between these inactive proteins and the active enzymes has thrown some light on the structure responsible for the activity as well as upon the method of formation of the active enzyme in the animal body.

The results in general indicate that comparatively slight changes in an inactive protein may result in the formation of an active enzyme and that under certain conditions this reaction is autocatalytic, i.e., some enzymes possess the power to form themselves from inert proteins.

GENERAL METHODS OF ISOLATION

Experience unfortunately has shown that no one method can be relied upon to lead to the isolation of an enzyme. The most that can be said is that they are best handled by the technique of protein chemistry and that large quantities of material must be used so that weighable solid precipitates are available and not simply dilute solutions.

In the work reported here this has been the guiding principle, and

TABLE 4

AMINO ACID COMPOSITION*
(Brand)†

Crystalline Enzyme	*Pepsin*‡	*Ribonuclease*§	*Chymotrypsinogen*§	*α Chymotrypsin*§	*β Chymotrypsin*§	*γ Chymotrypsin*§
Percent						
Nitrogen	14.6	16.5	16.18	16.06	16.24	16.00
Sulfur	0.94**	2.84	1.48	1.57	1.56	1.59
Phosphorus	0.09					
Amino Nitrogen	0.162	1.30	0.97	1.22	1.31	1.34
	gm Amino Acid per 100 gm of Protein					
Constituents						
Glycine	6.4	1.3	5.3			
Alanine						
Valine	7.1	7.3	10.1			
Leucine	10.4	0	10.4	9.1	9.4	8.5
Isoleucine	10.8	3.1	5.7			
Proline	5.0	3.6	5.9			
Phenylalanine	6.4	3.6	3.6			
Cysteine	0.5	0.60	1.29	1.22	1.29	1.27
Half-Cystine	1.64	6.51	3.30	3.66	3.51	3.59
Methionine	1.7	4.43	1.22	1.25	1.29	1.28
Tryptophane	2.36	0	5.57	5.81	6.40	6.27
Arginine	1.0	5.16	2.83			
Histidine	0.9	4.22	1.23	1.26	1.22	1.26
Lysine	0.9	10.4	8.0			
Aspartic Acid	16.0	14.2	11.3			
Glutamic Acid	11.9	13.0	9.0			
Amide Ammonia	1.6	2.49	1.86	1.76	1.76	1.71
Serine	12.2	12.0	11.4			
Threonine	9.6	9.00	11.4	11.2	10.6	10.7
Tyrosine	8.5	7.93	2.96	2.83	2.87	3.09
Hydroxyproline	0	0	0			
Total	115.15	108.84	112.36	38.09	38.34	37.63
Total, Calculated as Amino Acid Residues	97.47	92.17	94.98	33.33	33.58	32.99
Total, Amino Acid N as % of Total N	99.2	98.7	97.7	35.1	34.9	34.6

* The methods of analysis were described by Brand, Saidel, Goldwater, Kassell, and Ryan (1945). All analyses are reported for ash, sulfate, and moisture-free proteins.

† The authors are grateful to Dr. E. Brand, Department of Biochemistry, College of Physicians and Surgeons, Columbia University, New York, N. Y., for permitting the inclusion of these analyses before the appearance of his publications.

‡ The pepsin was purified and crystallized as described by Herriott, Desreux, and

several grams or, better, several hundred grams, of each step in the process of purification have been at hand before proceeding to the next step. The same general procedure that is found to be successful in the isolation of the proteolytic enzymes has led to the purification of the bacteriophage and, in Stanley's hands, to the isolation and crystallization of tobacco mosaic virus protein (Stanley 1936).

Northrop (1940b), Table VI, Steps 1–11. A dialyzed solution of this crystalline preparation of constant solubility was cooled to 5°C.; titrated to pH 9; back to pH 7; warmed to 50°C.; and then, after acidifying to pH 3, the precipitated protein was washed and dried while frozen. The filtrate and washings contained not more than 3 percent of the total nitrogen present before alkali denaturation.

§ These materials were prepared as described in the original papers by Kunitz and Northrop and discussed in other sections of this book. The preparations were 3–5 times recrystallized and salts were removed by dialysis.

** The sulfur content reported earlier by Northrop (1930) is in agreement with the present values. Those reported in the pepsinogen paper (Herriott 1938a) were determined by a commercial analyst and are incorrect.

II: PEPSIN

(Northrop 1930)

ISOLATION AND CRYSTALLIZATION

A NUMBER of methods have been proposed for the purification of pepsin, such as precipitation with safranin (Marston 1923), etc., fractionation by various adsorbents, and precipitation by dialysis from acid solution (Pekelharing 1896). Brücke reported the preparation of a "protein-free" pepsin by adsorption on copper phosphate. This method has been investigated by Kraut and Tria (1937), who found that the enzyme obtained in this way was different from the crystalline pepsin described below and represented only a small fraction of the total proteolytic activity present in gastric juice.

These and several other methods were tried and more or less active preparations were obtained. The results with Pekelharing's method seemed the most encouraging, however, since the loss of activity was less and there was some indication that a constant activity was reached. This result had been reported by Pekelharing and also by Fenger, Andrew, and Ralston (1928), using a similar method. It was found, however, that the dialysis could be dispensed with and the process made more rapid and efficient by solution with alkali and subsequent precipitation with acid, after a preliminary precipitation with half-saturated magnesium sulfate or ammonium sulfate. The amorphous material so obtained contains about half the activity present in the original material and is three to six times as active, as measured by the liquefaction of gelatin, and about five times as active, as measured by the digestion of casein or by the rennet action on milk. This material appeared to be a protein, as previous workers had found, and was reasonably stable. Efforts were therefore made to isolate this protein in crystalline form.

Isolation of the crystalline enzyme.—It was noticed that the precipitate which formed in the dialyzing sac when the procedure of Pekelharing was followed appeared in more or less granular form and

FIG. 6. Pepsin crystals. × 90.

filtered rather easily as though it were on the verge of crystallization. This precipitate dissolved in very dilute acid on warming the suspension, and it was eventually found that it could be induced to crystallize by warming to 45°C., filtering, and allowing the filtrate to cool slowly. The crystals so obtained were regular hexahedra and showed a tendency to grow in clusters, especially when appearing from more acid solutions. On one occasion a few crystals with truncated pyramids were obtained. They had the same activity and optical activity as the usual form. The crystals showed positive double refraction and were optically active in solution. They possessed proteolytic activity, when dissolved, equivalent to five times that of the U. S. P. 1 : 10,000 pepsin, as measured by hydrolysis of casein, and two and one-half times, as measured by the liquefaction of gelatin.

Improved method for the preparation of the crystals.—The isolation of the crystals in bulk by the above method was difficult owing to the dialysis. It was found that this could be avoided and the purification carried out as outlined above for the amorphous preparations, except that the acid precipitate was dissolved at 45°C. in concentrated solution. On inoculation this solution set to a solid paste of crystals (Figure 6). Table 5 is an outline of the method as finally developed.

Isolation of crystalline pepsin from bovine gastric juice (Northrop 1933a).—The possibility exists that the enzyme present in commercial pepsins was formed from some more complicated compound during the process of extraction and does not represent the enzyme as secreted normally in the gastric juice. In order to determine whether the activity of the gastric juice is due to the same protein, experiments were undertaken to isolate the enzyme from gastric juice.

Preliminary determinations of the activity of swine gastric juice showed that if the activity were due to the crystalline enzyme isolated from the gastric mucosa, the quantity of this protein in the gastric juice was extremely small (about 10 mg. per liter) and hence a very large quantity of gastric juice would be necessary before any attempt at isolation could be made. It was found to be impossible to obtain sufficient quantities of swine gastric juice, but bovine gastric juice may be collected in quantity as described by Williams and Vander Veer (1932).

Fractionation with various salt solutions was tried and it was found

that the activity could be concentrated in the protein fraction. This fraction, however, contained a large amount of a mucilaginous substance which rendered the solutions extremely difficult to work with, since they could be centrifuged or filtered only with the greatest difficulty. This difficulty has been encountered by other workers, and it

TABLE 5

ISOLATION AND CRYSTALLIZATION OF SWINE PEPSIN FROM COMMERCIAL PEPSIN PREPARATIONS

	ACTIVITY PER MG. PROTEIN NITROGEN		
	Gelatin Viscosity	*Rennet*	*Hemoglobin*
30 percent solution of commercial pepsin, precipitated at pH 3.0 with half-saturated magnesium sulfate	7.5	14×10^4	0.04
Filtered. Precipitate dissolved in water with dilute alkali	15.0	0.25×10^4	0.10
Precipitated with sulfuric acid at pH 3.0, filtered			
Precipitate dissolved in 2–3 volumes water at 45°C., acid added until near precipitation point, cooled slowly, filtered			
Precipitate, pepsin crystals	15.0	0.7×10^4	0.20

was found by Fenger, Andrew, and Ralston that the active material could be freed from this mucilaginous impurity by precipitating in the cold with 75 percent acetone. This process entails a loss of nearly half the total activity, but no more satisfactory method could be found. A method of fractionation was eventually worked out which consisted essentially in preliminary precipitation with saturated ammonium sulfate followed by solution in 60 percent acetone and precipitation with 75 percent acetone (Table 6). This process yielded a white amorphous precipitate free from most of the mucilaginous impurity and possessing about half of the total original activity. It was further purified by repeated precipitation with one-half saturated magnesium sulfate and was finally crystallized from warm water.

The activity per mg. of nitrogen, as determined by the hemoglobin method, increases from 0.0025, which is the value found in the original gastric juice, to about 0.19, which is the characteristic value al-

ready found for the crystalline protein isolated from swine. The yield of crystalline material is very poor and represents only a few percent of the total original activity. Of this loss, about one half occurs during the precipitation with acetone and the remainder during the repeated precipitation with magnesium sulfate. Actually about 100 mg. of crystalline material was obtained from 15 liters of

TABLE 6

PREPARATION OF CRYSTALLINE PEPSIN FROM BOVINE GASTRIC JUICE

	ACTIVITY PER MG. PROTEIN NITROGEN (*Hemoglobin Method*)
Contents of fourth pouch filtered	0.0023
Saturate with ammonium sulfate, filter, and dissolve precipitate in dilute hydrochloric acid	
Cool to −10°C., precipitate plus 60 percent acetone. Centrifuge and supernatant precipitated plus 75 percent acetone	
Precipitate dissolved in hydrochloric acid and precipitate plus half-saturated magnesium sulfate	0.12
Repeat magnesium sulfate precipitation 3–5 times	0.18
Dissolve precipitate in small volume sodium acetate and crystallize as described for preparation from commercial preparations	0.18

gastric juice. The crystals are small hexagonal bipyramids and are indistinguishable by inspection from the crystals obtained previously from the gastric mucosa of swine. They may be distinguished from swine pepsin by solubility experiments.

Differentiation of bovine and swine pepsin by means of solubility.—The crystalline pepsin isolated from bovine gastric juice is indistinguishable from swine pepsin in crystalline form or by the rate of digestion, as measured on a number of substrates, nor can it be distinguished from swine pepsin by precipitin tests. The solubility is the same as that of swine pepsin, but the protein differs from swine pepsin, since the solubilities of swine and bovine pepsin are independent; i.e., a solution saturated to swine pepsin is not saturated to bovine pepsin. The results of an experiment of this kind are shown in Table 7.

Crystalline salmon pepsin (Norris and Elam 1940).—Salmon pepsin was obtained as needle-shaped crystals. The preparation had constant

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Crystalline salmon pepsin (Norris and Elam 1940).—Salmon pepsin was obtained as needle-shaped crystals. The preparation had constant

solubility. The temperature optimum for the digestion of proteins is lower than that for swine pepsin and the specific activity slightly higher.

TABLE 7

SOLUBILITY OF PEPSIN FROM SWINE MUCOSA AND FROM BOVINE GASTRIC JUICE IN {0.01 pH 4.65 ACETATE / 0.50 SAT. MAGNESIUM SULFATE} AT 26°C.

Specific gravity saturated magnesium sulfate $\frac{22°}{H_2O\ 22°} = 1.294$

	No.	SWINE PEPSIN		BOVINE PEPSIN		SWINE + BOVINE	
		N/*ml.*	$[P.U.]^{Hb.}_{ml.}$	N/*ml.*	$[P.U.]^{Hb.}_{ml.}$	N/*ml.*	$[P.U.]^{Hb.}_{ml.}$
		mg.		mg.		mg.	
About 1 gm. amorphous pepsin stirred with 10 ml. solvent, centrifuge and filter, repeat 4 times, filtrate	1	0.80	0.17	0.69	0.12		
5 ml. 0.02 pH 4.65 acetate added to precipitate, clear solution							
5 ml. sat. magnesium sulfate added, precipitate centrifuge and filter supernatant	2	0.84					
5 ml. supernatant No. 2, stir with precipitate No. 1 (swine pepsin). Filtrate with 5 ml. supernatant No. 2 from swine pepsin, added to precipitate No. 1 from bovine pepsin, stir, centrifuge and filter, supernatant		0.85				1.60	0.32
Calculated if solubilities additive						1.49	0.29

The specificity of the enzyme differs from that of swine pepsin as tested with synthetic substrates (Fruton and Bergmann 1940).

Shark pepsin (Sprissler 1942).—Highly purified preparations of pepsinogen and pepsin have been prepared from shark mucosa by

Sprissler. The reactions, in general, are very similar to those of the corresponding compounds from swine.

The milk-clotting property, although weak, is still present even in this species which certainly does not normally have access to milk.

Evidence that the crystalline pepsin represents the enzyme present in gastric juice.—It was mentioned in discussing the method of isolation of the enzyme from gastric juice that a mucilaginous substance from which the active protein can be separated only with great difficulty is present in quite large amount in the original gastric juice. The possibility is suggested, therefore, that the enzyme is present in the gastric juice in some form of combination with this mucilaginous material. The methods used in the isolation would not be expected to split any chemical compound but might be considered to separate an adsorption complex. If such an adsorption complex existed in the gastric juice, it would be expected that the size of the active particles (or molecules) in the juice would be considerably larger than that of the molecules of the purified enzyme. The diffusion coefficient of the active substance in the gastric juice should therefore be smaller than that of the purified enzyme. The measurements gave a value for the diffusion coefficient in N/1 pH 4.5 acetate buffer at 6°C. of 0.038 ± 0.002 cm.^{2}/day, which agrees, within the experimental error, with that found for the crystalline swine pepsin (cf. page 54). These measurements are experimental evidence that the active molecule present in the gastric juice is the same, or very nearly the same, size as that in a solution of the purified crystalline protein and hence that the active molecule in the gastric juice does not exist in the form of a large complex.

PRESENCE OF A GELATIN LIQUEFYING ENZYME IN CRUDE PEPSIN PREPARATIONS (Northrop 1931a)

In the course of a study of the fractionation of crude pepsin preparations it was found that amorphous precipitates could be obtained which were extremely active with respect to liquefaction of gelatin. In the study of the properties of the crystalline protein isolated from crude pepsin preparations it was also noted occasionally that a small amount of amorphous material was obtained which had this strong gelatin liquefying power. When these preparations were crystallized the abnormal activity disappeared, and the crystalline material had

the same activity as the usual crystalline pepsin. A careful study of this material showed that the activity was due to a distinct enzyme, "gelatinase," which is extremely active with respect to the liquefaction of gelatin. This gelatinase is present in very small quantities in the original material and is completely removed from the crystalline pepsin only with considerable difficulty. The conclusion that the abnormal activity is due to this gelatinase depends upon the fact that the abnormally active material cannot be obtained from crystalline pepsin which has been crystallized four or five times. In addition, it was found that the activity of the crystalline pepsin could be destroyed in slightly alkaline solution, while the gelatin-liquefying power of the amorphous preparations was only slightly reduced. This accounts for the occasional abnormal results which were obtained in the study of the alkali inactivation of crystalline pepsin.

A method was worked out for the purification of the gelatinase and a small amount of protein material was obtained which was about 450 times as active as the crystalline pepsin, as measured by the liquefaction of gelatin, or about 1,000 times as active as the crude pepsin preparation. The activity of this material could not be increased by any method tried and solubility measurements gave an indication that it might be a pure protein. Owing to lack of material, however, no conclusive results were obtained as to the final purity of the preparation.

A study has been made of the activity of this material in affecting the hydrolysis of gelatin, casein, egg albumin, and edestin, as measured by the change of viscosity, increase in formol titration, and production of non-protein nitrogen. The activities of the crude pepsin and crystalline pepsin have also been determined in the same way. The gelatinase is considerably less active than the crystalline pepsin in all respects except the hydrolysis of gelatin. It is three or four times as active as crystalline pepsin in the hydrolysis of gelatin, as measured by the formol titration, and 450 times as active as measured by the change of viscosity. It is possible that the activity on proteins other than gelatin is due to small amounts of the crystalline pepsin present in the gelatinase preparations. On the other hand, crystalline preparations which have apparently been entirely freed from the gelatinase still possess the power of digesting gelatin.

TESTS OF PURITY

The experimental evidence in favor of the protein nature of enzymes has accumulated to such an extent that this view is now widely accepted. The question as to whether the enzyme preparations are pure proteins is, however, much less certain. The problem is complicated by the difficulty of defining a pure protein. If homogeneity in the ultracentrifuge or electrophoresis cell is considered as a sufficient test of purity then many proteins, including several enzymes, have been obtained in pure form. If constant solubility be considered as the criterion of purity then no protein, with the possible exception of trypsin (Kunitz 1938b) and chymo-trypsinogen (Butler 1940; Kunitz and Northrop 1938a) has been prepared in pure form. The earlier results with pepsin may be taken as an example. Thus this protein is homogeneous in the ultracentrifuge (Philpot 1935) and in the electrophoresis cell (Tiselius, Henschen, and Svensson 1938; Herriott, Desreux, and Northrop 1940a). The earlier preparations of Northrop (1930) showed constant solubility in certain solvents but not in others. Steinhardt was unable to obtain samples which had constant solubility in dilute acid. In addition, Northrop (1933b, page 173) found that some samples of crystalline pepsin contained nearly 50 percent inert protein and that all samples contain more or less of another enzyme especially active on gelatin (Northrop 1931a). Herriott (1938a, page 518) found that pepsin prepared from different sources varied in specific activity. Holter (1931) and Dyckerhoff and Tewes (1933) also found slight variations in activity. Desreux and Herriott (1939) have shown that these variable results are probably due to the fact that crude pepsin preparations may contain more than one active component which were not separable by the original method of purification and which differ in solubility. It became necessary, therefore, to develop a method of separating and isolating one or more of these proteins.

Desreux and Herriott (1939) showed that there were probably at least two active proteins present, a more soluble one which has an activity of about 0.33 $[P.U.]_{P.N.}^{Hb}$ and a less soluble one having an activity of about 0.2 $[P.U.]_{P.N.}^{Hb}$ or less.

The more active and more soluble component has been isolated by repeated extraction with 0.6 saturated magnesium sulfate at pH 5.0

and crystallized. It has constant activity and constant solubility in several solvents. It appears to be at least as pure as any other known protein, with the possible exception of chymo-trypsinogen.

A more insoluble fraction has been prepared by repeated precipitation with 0.45 saturated magnesium sulfate at pH 5.0 which has about two thirds the activity of the soluble fraction as measured by hemoglobin, but the same or slightly higher activity as measured by clotting of milk, digestion of casein, or casein or gelatin viscosity changes. This preparation does not have constant solubility and is undoubtedly still a solid solution of two or more proteins. When mixed with the pure soluble fraction variable solubility curves are obtained which resemble those of the original crude pepsin preparation.

A third fraction has been obtained by dialysis in salt-free solution at pH 4.0 which resembles this insoluble fraction (Desreux, unpublished experiments).

A further fraction having a specific activity of about 0.6 $[P.U.]_{P.N.}^{Hb}$ as measured by hemoglobin digestion has been obtained in small amounts from certain samples of pepsinogen. This fraction is extremely unstable and does not appear in commercial preparations.

ISOLATION OF CRYSTALLINE PEPSIN OF CONSTANT ACTIVITY AND CONSTANT SOLUBILITY IN CERTAIN SOLVENTS
(Herriott, Desreux, and Northrop 1940b)

Desreux and Herriott (1939) have shown that the solubility curves for crude pepsin preparations demonstrate the presence of at least two proteins. These preliminary experiments indicated that the proteins were present as distinct solid phases; i.e., the crude preparation is a mixture. The present experiments show conclusively, however, that this is not the case but that the proteins are present as one solid phase of varying composition; i.e., a solid solution. Application of Raoult's law to such systems (Northrop and Kunitz 1930) predicts that the residue left when nearly all the material has dissolved will contain more of the insoluble components than the original sample, while the fraction dissolved when only a small amount of solid is dissolved will contain more of the soluble components. The solubility curve, therefore, not only indicates the composition of the original material but also furnishes a method of separation of the components. This method is much more delicate than the usual method of fractionation by which

the salt concentration is varied, instead of the protein concentration.

The fractionation was found to be more complete the more alkaline the solution and, as predicted, the smaller the fraction dissolved. It was eventually found that a nearly complete separation could be obtained by extracting the crude protein with a large volume of a solution of 0.6 or 0.65 saturated magnesium sulfate in 0.2 M pH 5.0 acetate. The pH is that of the maximum stability of pepsin. The method has been found satisfactory and reproducible with three preparations of

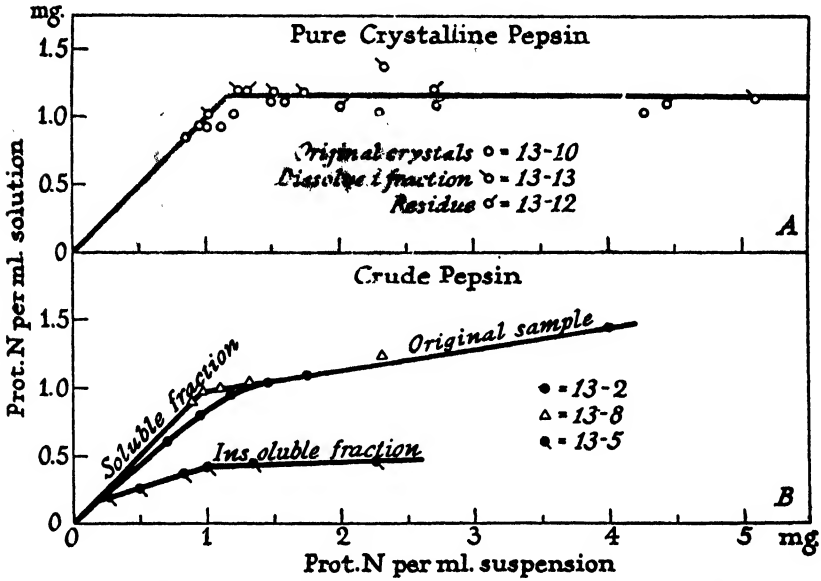


FIG. 7. Solubility curves of pepsin at various stages in the purification.

pepsin from pepsinogen and with eight different preparations of Cudahy U.S.P. 1 : 10,000 soluble pepsin. One preparation from pepsinogen which had been stored at −13°C. for two years did not give a complete separation by this method and some of the commercial preparations required more repeated extraction than others. The solubility curves of the various fractions, in the amorphous form in [0.5 saturated magnesium sulfate, 0.05 M pH 4.6 acetate] are shown in Figure 7.

It will be seen that the solubility of the extracted protein is more nearly constant than that of the original preparation. The residue after

extraction is less soluble and also inhomogeneous (Figure 7B). The soluble fraction after crystallization gives the theoretical curve for a protein containing only one component. Further extraction of this preparation causes no significant change in the solubility curves. The curves for the original sample, the portion dissolved, and the residue are the same (Figure 7A).

The solubility of the fraction extracted increases with each step in the purification while the solubility of the residue decreases. This result confirms the conclusion that the original preparation is a solid solution rather than a mixture since in the latter case the saturated solution in the presence of excess solid must contain all the components and repetition of the solubility determination on this fraction must therefore yield the same value as that obtained from the original preparation.

The solubility of the crude material is intermediate between that of the more soluble and the more insoluble components as would be predicted for a solid solution. The curve for the insoluble fraction also agrees with that expected for solid solutions rather than mixtures. In the case of mixtures the solid phase which first appears must be a pure or nearly pure component but the curves show that this fraction gives again a smooth rounded curve, characteristic of a solid solution.

The solubility curves of these fractions in the amorphous form are shown in Figure 8. The preparations obtained from the commercial preparation have the same activity and solubility as those from pepsinogen. The solubility (in the amorphous state) is constant in [0.5 saturated magnesium sulfate, 0.05 M pH 4.6 acetate] and also in [0.6 saturated magnesium sulfate, 0.2 M pH 5.0 acetate].

The absolute value for the solubility agrees with that previously reported (Northrop 1930). Slight variations in this figure are due to slight variations in temperature, salt concentration, or non-protein nitrogen. The primary interest is in comparison of the different values of the solubility in the same experiment and for this reason no special precaution was taken to have the composition of the solvent or the temperature *exactly* the same for different experiments.

The solubility is constant and independent of the amount of solid present. The preparation, therefore, is a pure protein, or a mixture of two proteins present in proportion to their solubilities or a solid solution of two or more proteins having very nearly the same solubility

(Northrop and Kunitz 1930; Kunitz and Northrop 1938a; Butler 1940; Herriott 1942).

It will be noted that crystallization does not change the solubility curves markedly and in the case of pepsin from pepsinogen improves them. The fact that the solubility is constant in two different solvents and is not changed by recrystallization renders it very unlikely that the preparations consist of a mixture of proteins, present in proportion to their solubility, or solid solutions of proteins having nearly the same solubility. This possibility, however, cannot be completely excluded but may be rendered more improbable by repeating the measurements in several other solvents. From what is known of solubility in general and that of proteins in particular it is unlikely that two or more proteins would have even approximately the same solubility in a variety of solvents at different pH's.

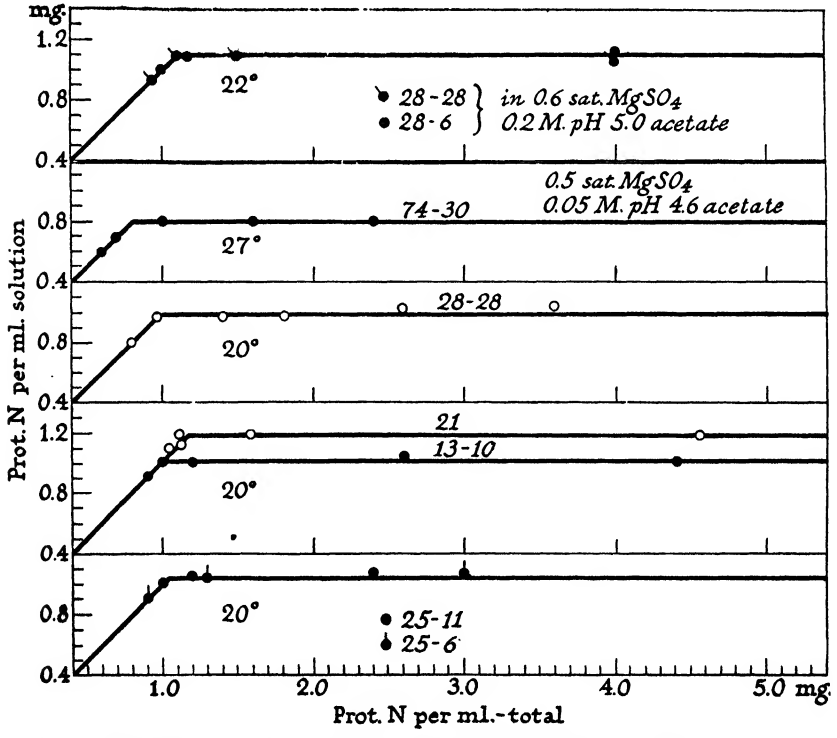


FIG. 8. Solubility curves of different samples of soluble pepsin in the amorphous form in magnesium sulfate solution.

In order to extend the solubility measurements to other solvents and to obtain values for the solubility of the crystalline protein, the measurements were repeated with the crystalline protein in several different solvents.

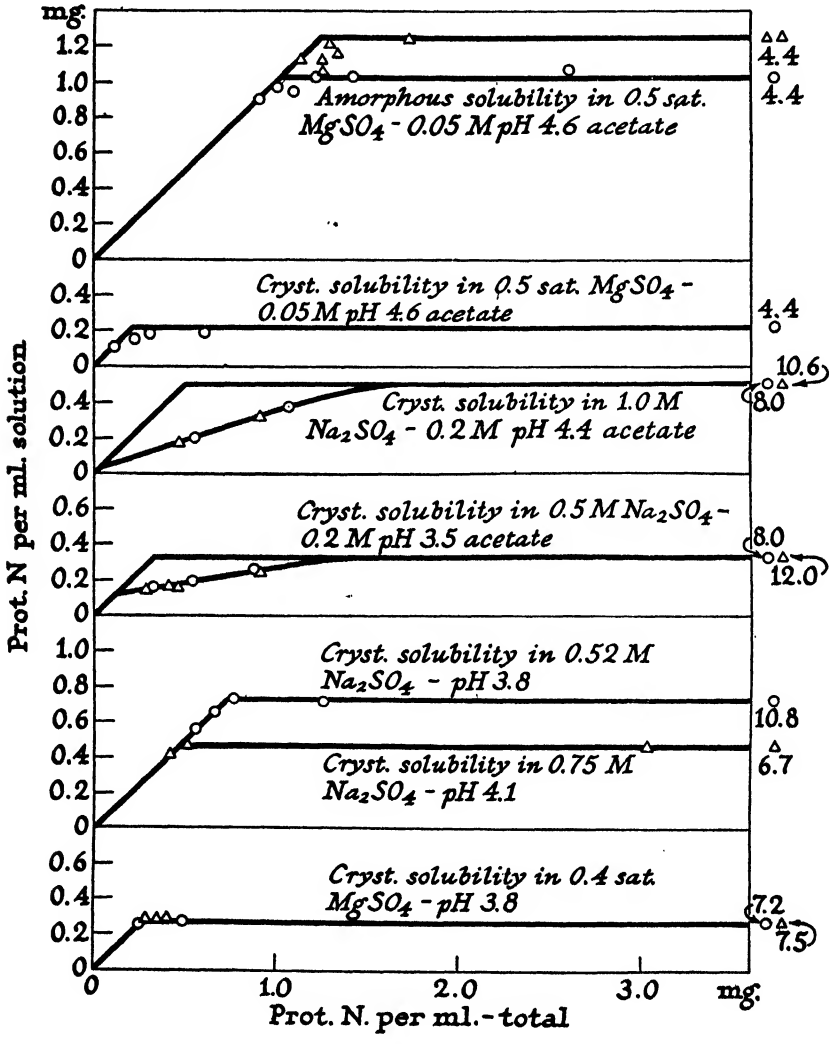


FIG. 9. Solubility of crystalline pepsin, sample No. 13–10 circles and No. 21 triangles, in various solvents at 20°C.

The results of the solubility measurements on various preparations of the crystalline protein are shown in Figures 9–12.

The curves show that the solubility is constant and independent of the amount of solid phase present in the following solvents: amorphous form in [0.5 saturated magnesium sulfate, 0.05 M pH 4.6 acetate] or

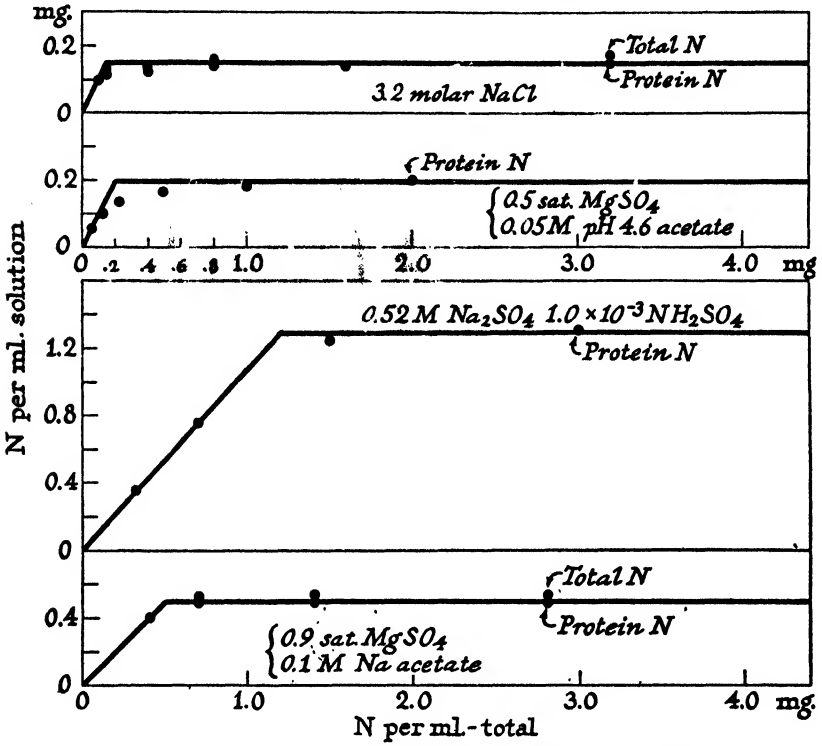


FIG. 10. Solubility of crystalline preparation No. 75–8 in various solvents at 20°C.

[0.6 saturated magnesium sulfate, 0.2 M pH 5.0 acetate]; crystalline form in 0.75 M sodium sulfate pH 4.1; 0.52 M sodium sulfate pH 3.9; [0.9 saturated magnesium sulfate pH 6.0, 0.1 M sodium acetate]; 3.2 M sodium chloride pH 3.8; or 0.4 saturated magnesium sulfate pH 3.8.

The solubility of both the amorphous and crystalline forms in [0.5 saturated magnesium sulfate, 0.05 M pH 4.6 acetate] and of the crystalline form in 0.52 M sodium sulfate agrees closely with that previously determined (Northrop 1930).

The original and the present preparations both gave constant solubility in the amorphous form in [0.5 saturated magnesium sulfate, 0.05 M pH 4.6 acetate] and in the crystalline form in sodium sulfate. The solubility curves of the crystals in [0.5 saturated magnesium sulfate, 0.05 M pH 4.6 acetate] are not constant but are rounded in the dilute suspensions. This abnormality is discussed below.

The earlier experiments were made with pepsin which had been crystallized directly from a Parkê, Davis preparation without previous

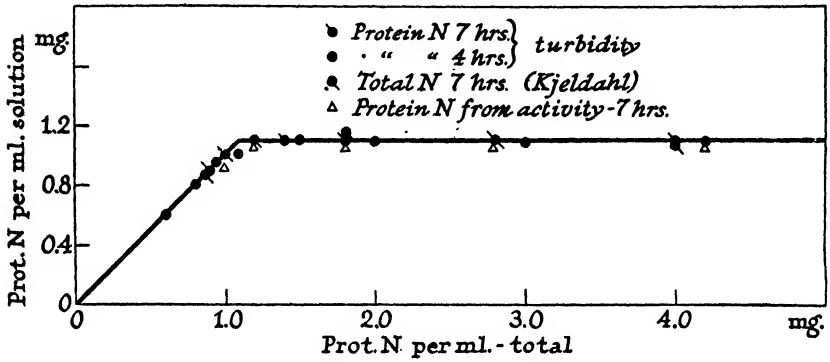


FIG. 11. Solubility of crystalline preparation No. 22–29 in 0.52 M sodium sulfate, 2.5×10^{-3} sulfuric acid at 20°C.

fractionation of the protein. Pepsin prepared in this way from commercial preparations now available does not give constant solubility in any solvent tried. The original preparation was repeated many times but only one lot of commercial pepsin was used. Evidently this particular lot of crude pepsin was more homogeneous than usual.

In [0.2 M pH 4.4 acetate, 1.0 M sodium sulfate] or [0.2 M pH 3.5 acetate, 0.5 M sodium sulfate] or [0.5 saturated magnesium sulfate, 0.05 M pH 4.6 acetate], however, the points do not fall on the theoretical curve for a system of one solid phase of one component but are below this curve in the dilute suspensions (Figure 9). The same result was obtained in the original solubility experiments. All these solvents contain acetate buffer, while the corresponding solvents without the acetate buffer give theoretical curves.

There are two possible explanations for this discrepancy:

1. The variation in non-protein nitrogen affects the solubility of the

protein in the presence of acetate buffer but does not do so in the other solvents. This explanation predicts that the addition of non-protein nitrogen will affect the solubility of the protein in the presence of acetate but will not do so in the absence of acetate buffer. As will be seen below this is the case.

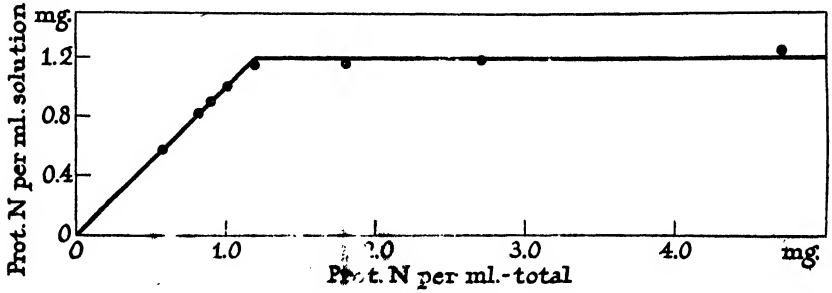


FIG. 12. Solubility of crystalline preparation No. 25–11 in 0.52 M sodium sulfate, 2.5×10^{-3} sulfuric acid at 20°C. Protein nitrogen determined by turbidity.

2. More than one protein is present. The solubilities of these proteins are nearly the same in the absence of acetate but are different in the presence of acetate. This explanation predicts that the protein can be further separated by fractionation in the solvents containing acetate. The results show that this cannot be done.

Effect of non-protein nitrogen on the solubility of pepsin in [0.5 saturated magnesium sulfate, 0.05 M pH 6.4 acetate] and in 0.52 M sodium sulfate, pH 3.9.—The substances grouped together as non-protein nitrogen may be quite different in different solvents. All that is known about them is that they are formed by decomposition of the protein and that they do not precipitate from hot 2½ percent trichloracetic acid. In order to test their effect on the solubility of the protein therefore it is necessary to have a solution prepared as nearly as possible under the actual conditions of the solubility experiment. The solution containing the non-protein nitrogen was prepared therefore from a saturated solution of the protein in the solvent by heating the solution as rapidly as possible and filtering off the denatured protein. There is no change in the pH or non-protein nitrogen content of the magnesium sulfate-acetate solvent upon removal of the protein in this way. In sodium sulfate the non-protein nitrogen increases and the pH becomes slightly more alkaline upon heating. This difference is due to the fact

that pepsin is more active in the less concentrated sodium sulfate than it is in the magnesium sulfate so that more non-protein nitrogen is formed from the denatured protein before inactivation is complete.

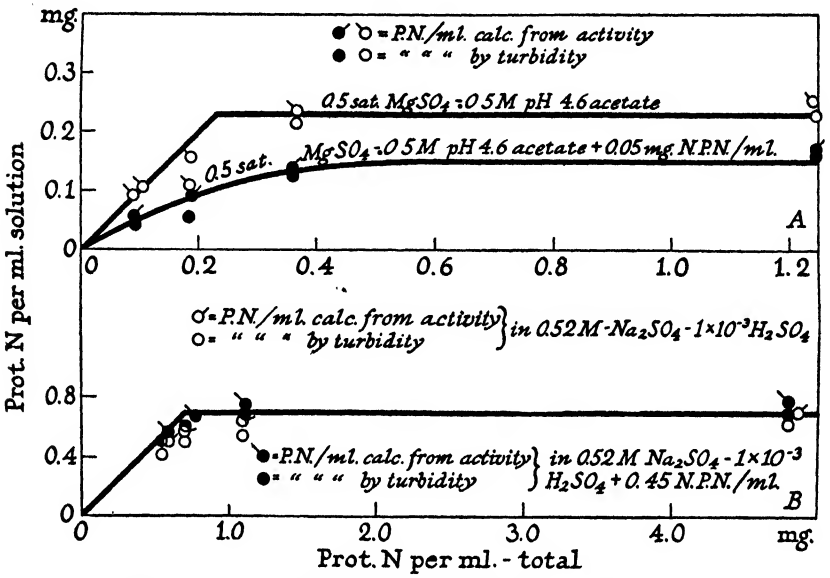


FIG. 13. Effect of the addition of non-protein nitrogen on the solubility of crystalline pepsin in magnesium sulfate or sodium sulfate.

The effect of the solvents on the solubility of the protein is shown in Figure 13. The solubility in the magnesium sulfate-acetate solvent is decreased markedly by the presence of the non-protein nitrogen and the resultant curve deviates more from the theoretical than does the curve in which pure solvent was used. It must be remembered that the suspension in the pure solvent also contains the non-protein nitrogen which is formed during the solubility experiment. It is evident that this effect of non-protein nitrogen on the solubility is quite sufficient to account for the deviation of the results from the theoretical curve in this solvent. It is quite possible that this effect of the non-protein nitrogen is due to the formation of a solid solution of the non-protein with the protein as Steinhardt has suggested (1938, 1939). Experimental test of this mechanism is difficult since the amount of non-protein nitrogen in the solid is too small to determine accurately. Expressed as mol fraction, however, it may be very large owing to the

great difference in molecular weight between the protein and non-protein components.

In sodium sulfate the non-protein nitrogen has less effect on the solubility of the protein and what effect it does have is in the opposite direction, that is, the protein is slightly more soluble in the presence of increased amounts of non-protein nitrogen. In this case the non-protein nitrogen added was four times that formed during the solubility experiment. Evidently the amount of non-protein nitrogen formed during the experiment would not affect the solubility to a measurable extent. The non-protein nitrogen may therefore be considered an inert component in this solvent.

Results of fractionation in [0.5 saturated magnesium sulfate, pH 4.6 acetate].—The preceding section shows that the effect of non-protein nitrogen on the solubility of the protein is sufficient to account for the divergence of the results from the curve for a pure protein. This result, however, does not exclude the possibility that more than one protein is present. If two or more proteins are present, however, then their relative concentrations must be different in those solid phases having different solubilities and it should, therefore, be possible to separate them at least partially by re-extracting the protein with the solvent. The extract obtained when only a small percent of the solid is dissolved should contain more of the soluble component while the residue left after most of the protein is dissolved should be high in the insoluble component. It may be recalled that such a separation does occur with crude preparations which give solid solution curves in all solvents (Figure 7). Preparations giving good curves in other solvents but solid solution curves in [0.5 saturated magnesium sulfate, 0.05 M pH 4.6 acetate] cannot be separated by extraction with this solvent. The protein extracted gives curves differing slightly if at all from the residue left after nearly complete solution. Both curves are indistinguishable from that of the original preparation (Figure 14). The figure shows that preparation 74–11 gave a solid solution curve when tested in the amorphous form. Preparation 74–30 was prepared from 74–11 by extraction in the solvent used for solubility measurements. This preparation (74–30) has constant solubility in the amorphous form. This preparation, however, gives a slightly rounded curve in the crystalline form in [0.5 saturated magnesium sulfate, 0.05 M pH 4.6 acetate]. Upon further extraction the same curve is obtained either from

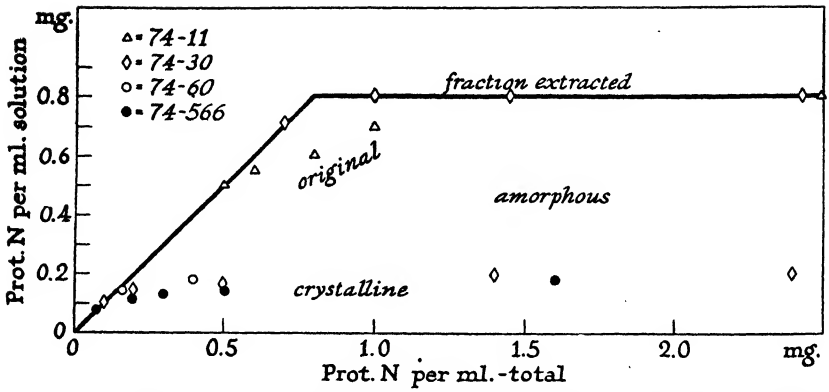


FIG. 14. Effect of fractionation with [0.5 saturated magnesium sulfate, 0.05 M pH 4.6 acetate] on the solubility of pepsin preparations. Solubility determined in [0.5 saturated magnesium sulfate, 0.05 M pH 4.6 acetate.] 74–11, original crystals; 74–30, 50 percent of 74–11 extracted with the solvent, precipitated, and crystallized; 74–60, 10 percent of 74–30 extracted in the solvent, precipitated, and crystallized; 74–566, residue left after repeated extraction of 74–30 in the solvent, equivalent to 5 percent of 74–30.

the residue or from the protein extracted (lower curves in Figure 14).

It may be concluded, therefore, that the preparation contains only one protein component and that the rounded curves obtained in [0.5 saturated magnesium sulfate, 0.05 M pH 4.6 acetate] are due to variation in the non-protein nitrogen.

PREPARATION OF HIGHLY ACTIVE PEPSIN FROM PEPSINOGEN

In connection with some solubility experiments on pepsinogen (Herriott 1938a, page 514) it was found that a small amount of protein was obtained which differed in certain properties from the main bulk of the preparation. Thus, when pepsinogen was fractionated a few percent of the protein was recovered which had a much lower solubility and 2½ times higher "potential specific hemoglobin activity" than the starting material. This fraction behaved in other respects like ordinary pepsinogen, i.e., upon acidification it rapidly changed into pepsin and about 15 percent of its nitrogen was split off during activation and part of this nitrogen is a pepsin inhibitor (Herriott 1938a, page 535). Although highly active on hemoglobin this fraction had the same specific activity as the bulk of the materials as measured with other substrates such as gelatin and milk.

A highly active preparation of pepsin could also be obtained by fractionation of pepsin prepared from ordinary pepsinogen. This highly active pepsin is much less stable than ordinary pepsin, particularly near pH 2.0. Nearly 50 percent of the highly active pepsin was destroyed in 15 hours at 25°C. as compared to only 5–10 percent of the ordinary crystalline pepsin. It is estimated that the highly active pepsin is present as not more than 3–4 percent of the original pepsin protein.

Specificity of the active fraction.—If this active fraction represented a distinct enzyme, comparable to the gelatinase (Northrop 1931a) it would be expected to hydrolyze part of the protein molecule not attacked by the other fraction. This is not the case. Addition of the highly active fraction to casein which has been previously hydrolyzed by the less active fraction causes no further increase in amino groups. Addition of the less active fraction to casein previously hydrolyzed by the active fraction likewise causes no increase in hydrolysis. Both fractions, therefore, attack casein at the same points.

It is interesting to note that the method of preparing the highly active pepsin from pepsinogen and the low active 0.1 $[P.U.]^{Hb}_{mg.P.N.}$ pepsin from commercial products (Desreux, unpublished results) is practically identical; i.e., dialysis at pH 4.0. Borgstrom and Koch (1943) have prepared very active pepsin by absorption on coagulated egg white.

Discussion.—The experiments reported show that pepsin prepared as originally described from commercial preparations or from pepsinogen contains two or more proteins. These proteins are indistinguishable by ultracentrifuge or electrophoresis but may be distinguished by solubility measurements. It is probable that more than one of these proteins is active but it does not necessarily follow that more than one pepsin occurs in any one animal.

It is quite possible that the insoluble fraction is a decomposition product of the more soluble fraction. In fact, heating a pure preparation of the soluble fraction results in a preparation which has a solubility curve very similar to the original crude pepsin.

The possibility that the protein originally came from different animals must also be considered. It is already known that pepsin from cattle is different from swine pepsin (Northrop 1933a) and it is quite possible that the solubility method is sufficiently sensitive to distin-

guish between proteins from different varieties of animals or between animals of different ages as well as between proteins from animals of different species (Landsteiner and Heidelberger 1923). All the preparations used in this work represent extracts from a number of different individuals of different ages, varieties, and physiological condition. Attempts to prepare the enzyme from one individual were unsuccessful owing to the small amount present. It appears quite possible, therefore, that the enzyme may be homogeneous in any one animal but differs slightly in different varieties or age groups.

Wyman, Rafferty, and Ingalls (1944) have recently found that hemoglobin from young animals is different from that of old animals so that there is now experimental evidence for the fact that different proteins may be found in different individuals.

ELECTROPHORESIS EXPERIMENTS (Herriott, Desreux, and Northrop 1940a)

The experiments described in the preceding section show that pure pepsin of constant solubility may be obtained from some crude samples by fractionation with salt solutions. Other samples, however, could not be separated completely by this method.

Ågren and Hammarsten (1937a) and Tiselius, Henschen, and Svensson (1938) have found that electrophoresis of pepsin solutions yields a substance of higher activity than the original solutions as calculated on a total nitrogen basis. According to Tiselius, *et al.*, this is a protein, shows a sharp boundary, and migrates as a homogeneous substance. It seemed possible, therefore, that the electrophoresis method might furnish a means of separating the various protein components of a pepsin solution. A number of electrophoresis experiments were therefore carried out with various pepsin preparations. The results of these experiments are summarized in Table 8.

The results show that there is no separation of the protein components from each other but that they migrate at the same rate and show a homogeneous boundary. This accounts for the fact, noted by Tiselius, that the specific activity of the migrating component is different in different preparations. More or less of the non-protein nitrogen is left behind at pH 4.4 and the migrating protein therefore has a higher activity on a total nitrogen basis than the original solution, as Tiselius and his collaborators and Ågren and Hammarsten stated.

There is, however, no increase in activity on a protein nitrogen basis.

The "glycerine standard" pepsin of Table 8 is the same solution as that used by Tiselius. It is a twice-crystallized preparation from Cudahy pepsin. The solubility diagram of this preparation is shown in Figure 15. The diagram shows that more than one protein is present. The migrating boundary, however, is homogeneous (Figure 19) as Tiselius states but this migrating material still contains more than one protein component.

TABLE 8

ELECTROPHORESIS OF VARIOUS PEPSIN SOLUTIONS

Exp. No.	Pepsin Preparation	Cell Used	pH	Distance of Migration	Moving Boundary	Stationary Boundary	Percent N.P.N.		$[P.U.]^{Hb}_{Total\ N}$		$[P.U.]^{Hb}_{Protein\ N}$	
							Orig.	*Anode*	*Orig.*	*Anode*	*Orig.*	*Anode*
				cm.								
1*	Glycerine standard dialyzed	Tiselius	4.6	2	Sharp	—	3	3	0.29	0.29	0.29	0.31
2*	Same as No. 1	"	4.43	8	"	—	4	3.3	0.30	0.30	0.30	0.305
3	Same as No. 1	U tube	4.6	8	"	Faint	5	5.0	0.28	0.28	0.30	0.30
4	Glycerine standard dialyzed and partly inactivated	"	4.6	2	Diffuse	"	18	13.0	0.25	0.29	0.29	0.31
5	Same as No. 4	"	4.6	4	"	"	30	20.0	0.19	0.26	0.28	0.33
6	Same as No. 4	"	4.6	10	"	"	16	13.0	0.25	0.27	0.31	0.33
7	Crystallized from Cudahy, dialyzed	Theorell	4.1		—	"	12	1.5	0.26	0.29	0.29	0.29
8	Same as No. 7	U tube	4.6	6	Sharp	"	10	5.0	0.26	0.30	0.29	0.31
9	Same as No. 7	"	4.6	9	"	"	10	5.0	0.26	0.28	0.29	0.28
10	Same as No. 7	"	4.6	8	"	"	15	3.0	0.25	0.30	0.29	0.31
11	Same as No. 7	M cell	4.6	3	"	"	15	4.0	0.26	0.29	0.29	0.30
12	Crystalline Parke, Davis	U tube	4.6	9	"	—	5	1.5	0.23	0.24	0.24	0.24
13†	Pure "A" pepsin	Tiselius	4.6	2	"	—	2	2.0	0.33	0.33	0.33	0.33
14	Mixture: 0.44 activity pepsin + 0.28 activity pepsin	U tube	5.2	5	"	—	10	10.0	0.31	0.32	0.34	0.36

* The electrophoresis in these experiments was carried out by Dr. Alexandre Rothen, Laboratory of Physical Chemistry at the Rockefeller Institute.

† The electrophoresis was carried out by Dr. Lewis G. Longsworth, Laboratory of Physical Chemistry at the Rockefeller Institute.

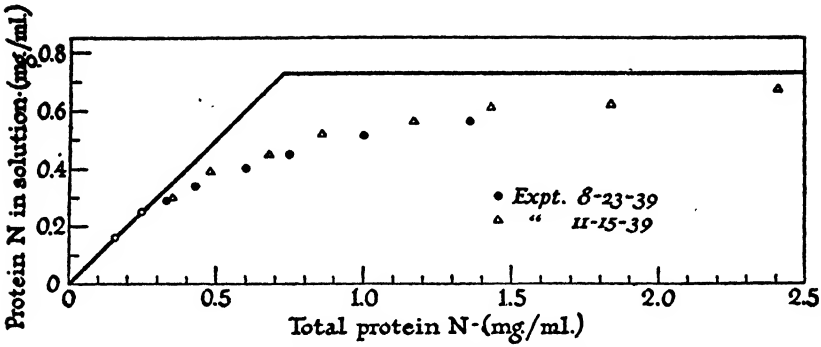
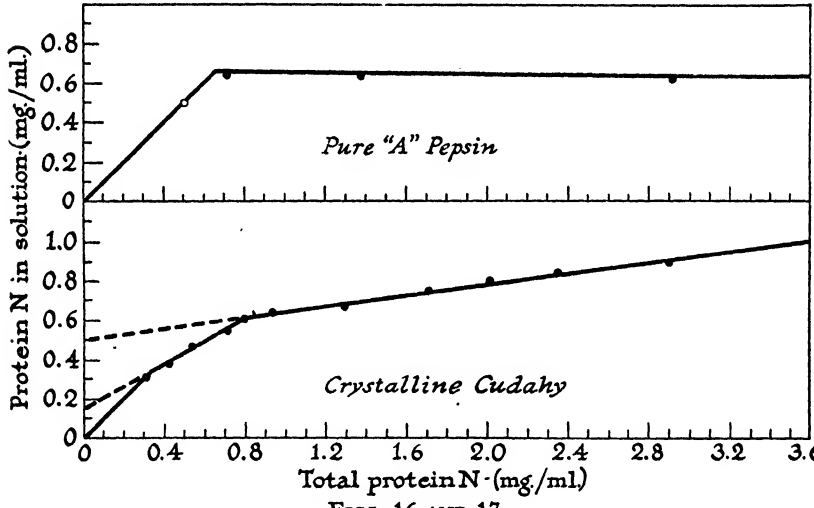


FIG. 15. Solubility diagram of "standard glycerine" pepsin in half-saturated magnesium sulfate 0.05 M pH 4.6 acetate at 22°C.

The solubility diagram of another sample of crystalline pepsin prepared from Cudahy pepsin is shown in Figure 17 and that of pure "A" pepsin in Figure 16. The electrophoresis of a sample of pure "A" pepsin is reported in experiment 13 of Table 8 and Figure 18, and that



FIGS. 16 AND 17.

16. Solubility diagram of pure "A" pepsin prepared from specially active preparation of Cudahy pepsin. Conditions the same as in Figs. 15 and 17.

17. Solubility diagram of crystalline pepsin prepared from Cudahy pepsin in half-saturated magnesium sulfate 0.05 M pH 4.6 acetate at 22°C.

of the Cudahy sample in experiments 7–11 of Table 8. The solubility diagrams show that the crystalline Cudahy pepsin contains several proteins while the "A" pepsin contains only one. The electrophoresis shows only one boundary in both samples.

The electrophoresis of a mixture of highly active pepsin (specific

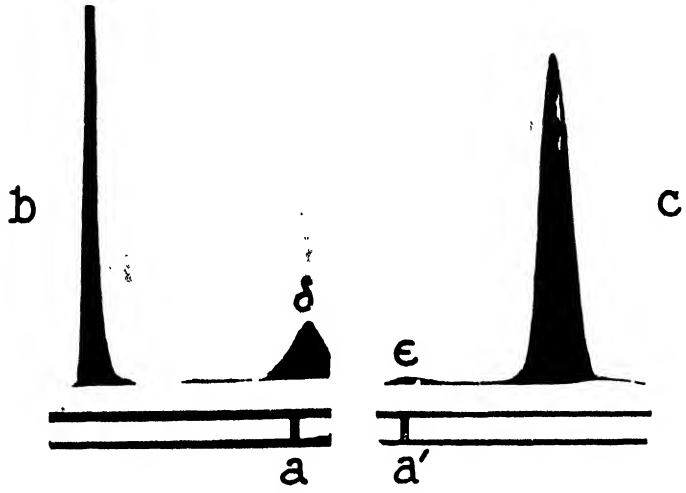


FIG. 18. Electrophoretic patterns at 0°C. of pure "A" pepsin in an 0.014 N sodium acetate buffer at pH 4.46. *aa′*, positions in the cell to which the boundaries were shifted before the potential was applied. *b*, *c*, patterns of the rising and descending boundaries, respectively, after 4000 sec. at 6.9 volts/cm. The rising boundary was still too sharp to be recorded completely. The δ and ϵ boundaries are concentration gradients remaining near the initial boundary positions and *do not* represent electrically inert material. The mobility computed from the displacement of the descending boundary is $u = -7.5_3 \times 10^{-5}$ cm.²/volt/cm.

activity 0.44 P.U.) and a fraction of low activity (0.28) is shown in experiment 14, Table 8. There is again no separation. The activity of the migrating mixture is the same as that of the original mixture.

Electrophoresis at pH 3.5.—In the preceding experiments at pH 4.1–5.2 the protein migrates to the anode and the non-protein nitrogen remains stationary. At pH 3.4, however, some of the non-protein nitrogen migrates to the cathode and the protein to the anode. This confirms the results of Ågren and Hammarsten, Table 9. There is, however, no change in the activity of the protein and the change in

total activity observed is due merely to the separation of protein and non-protein compounds.

Isoelectric point of pepsin.—Michaelis and Davidsohn (1910) studied the migration of pepsin in an electrophoresis cell. The enzyme moved to the anode at pH 3.6 to 5.0, to both anode and cathode at pH 3 to 3.4, and to the cathode at pH 2.2 to 1.5. Ringer (1915) found that most

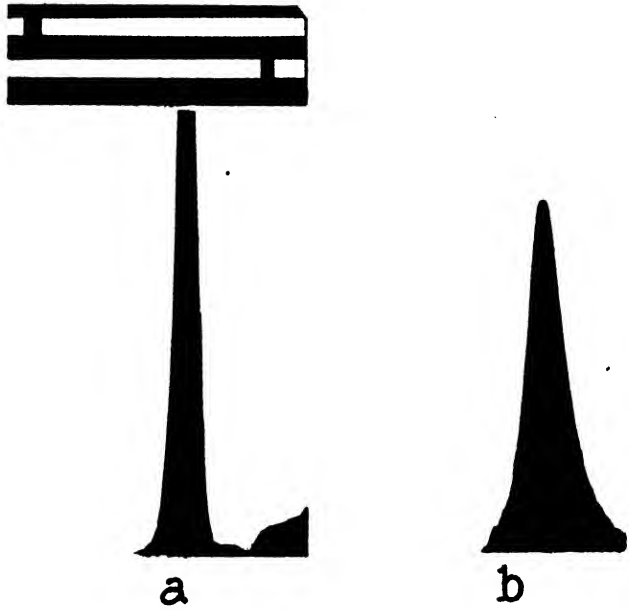


FIG. 19. Longsworth pattern of dialyzed "standard glycerine" twice crystallized Cudahy 12–7–33 pepsin at pH 4.43, and 3.3 volt/cm. (*a*) Rising anode boundary after 6900 seconds; 1.28 cm. migration; mobility = -5.6×10^{-5} cm.^{2}/volt/sec. (*b*) Rising anode boundary after 57,300 seconds; 9.0 cm. migration; mobility = -4.7×10^{-5} cm.^{2}/volt/sec.

samples of pepsin, as obtained from gastric juice, migrated always to the anode, even in strongly acid solution. The addition of protein split products, however, caused the enzyme to migrate to the cathode on the acid side of about pH 3.0. Ringer concluded that the enzyme was an acid. This result was confirmed by Northrop (1925) from a study of the distribution of pepsin between egg albumin particles and the surrounding solution. The distribution was found to be the same as that

of the chloride ion indicating that pepsin was a negatively charged monovalent acid, at least as far acid as pH 1.0.

Tiselius, Henschen, and Svensson (1938) have recently obtained the same result by electrophoresis measurements. Cataphoresis measurements on collodion particles coated with crystalline pepsin, however, showed an isoelectric point at about pH 2.7 and a minimum solubility was observed near this point (Northrop 1930, page 769). This result has now been found to be due to the effect of decomposition products on the measurement as originally described by Ringer. Thus, solutions of pure "A" pepsin, when freed from non-protein nitrogen, are negatively charged even in N/10 hydrochloric acid. After a few hours

TABLE 9

ANALYSIS OF PEPSIN FRACTIONS OBTAINED BY ELECTOPHORESIS

Exp. No.	Pepsin Preparation	Cell Used	pH	Distance of Migration	Boundaries		Percent N.P.N.			$[P.U.]^{Hb}_{Total\ N}$			$[P.U.]^{Hb}_{Protein\ N}$		
					Anode	*Cathode*	*Orig.*	*Anode*	*Cathode*	*Orig.*	*Anode*	*Cathode*	*Orig.*	*Anode*	*Cathode*
				cm.											
15	Crystalline Cudahy in N/10 pH 3.4 citrate	M	3.4	3	Sharp	Diffuse	10	6	70	0.27	0.30	0.10	0.30	0.31	0.30

at 30°C., however, during which time about 5 percent non-protein nitrogen appeared in the solution, the particles became positively charged at pH 1.5.

The fact that pepsin contains at least two primary amino groups but does not become positively charged even in 0.1 M acid indicates the presence of a very strong acid group. An indication of such a group is also found in the titration curves but cannot be determined definitely in this way. The amino acid content (Table 4) does not indicate the presence of any unusual organic acid group. Pepsin contains one atom of phosphorus (Northrop 1930) and this acid group may be that of phosphoric acid.

RELATION OF THE ACTIVITY TO THE PROTEIN

The results described in the preceding section show that the crystalline enzyme preparations behave like a pure substance and indicate therefore that the proteolytic activity is really the property of the protein. This relationship between the protein and the proteolytic activity may be further tested in a number of ways by studying the effect of chemical changes in the protein upon the activity of the preparation.

Measurement of the rate of migration of the protein as determined by chemical analysis and of the enzyme as determined by activity measurements also indicates the identity of the enzyme and the protein. Such measurements may be made in the electrophoresis cell as described above or by diffusion.

Rate of diffusion as measured by activity or protein-nitrogen.—It may be shown experimentally that the rate of diffusion of the active material is the same as that of the protein by determining the rate of diffusion by activity or by protein determinations. The results of such an experiment give a diffusion coefficient at 8° of 0.0369 ± 0.0005 $cm.^2$/day from rate of diffusion of the active substance and 0.0367 $cm.^2$/day for the rate of diffusion of the protein. This may be considered experimental proof that the active substance and the protein diffuse at the same rate. This diffusion coefficient corresponds to a molecular volume of 80,000, which is larger than that found by osmotic pressure measurements or by the ultracentrifuge. This discrepancy means that the molecule is either hydrated or non-spherical (Anson and Northrop 1937).

Inactivation by alkali.—It is known that pepsin is very rapidly inactivated by weak alkali, and Michaelis found that the rate of inactivation was proportional to about the fourth power of the hydroxyl ion concentration between pH 6.0–8.0. Wasteneys and Borsook considered that there were two reactions in this range, one of which was instantaneous, but Steinhardt (1937) has recently shown with purified pepsin solution that the rate of activation is inversely proportional to the fifth power of the hydrogen ion concentration in this range. If a pepsin solution is titrated to a series of different pH between pH 5.6 and 8.0 and then made acid, it will be found that as the pH is increased, an increasing percentage of the activity has been

lost and that at the same time denatured protein has been formed. If such solutions are then analyzed for denatured protein and activity it is found that the percentage loss in activity is just equal to the percent of the protein which has been denatured (Figure 20). The rate of formation of denatured protein is therefore exactly equal to the rate of loss of activity.

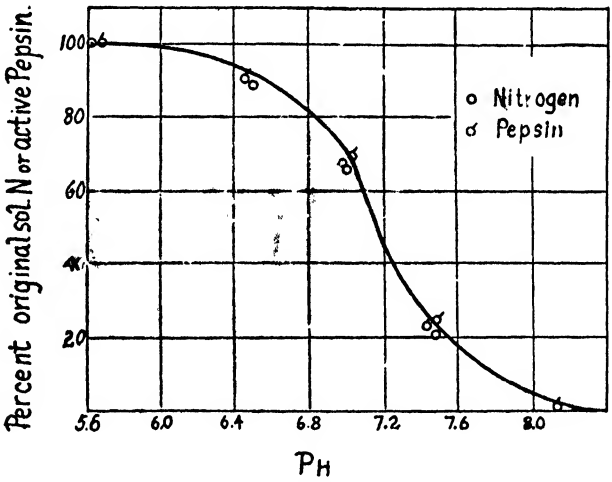


FIG. 20. Percentage inactivation and percentage denaturation of pepsin at various pH at 20°C.

Heat of denaturation (Conn, Gregg, Kistiakowsky, and Roberts 1941).—The titration curves of native and denatured pepsin show that between pH 4.5–9.5 the same total equivalent of alkali is combined, but the curves differ markedly near pH 6.0. It is in this region that the rate of inactivation of the enzyme increases rapidly with increasing alkalinity. The heat of denaturation was found to be 85 Kg. cal. per mol.

Preparation of active pepsin from denatured pepsin (Northrop 1931b). —It was found by Pavlov and Parastschuck that pepsin solutions which had been inactivated by alkali recovered some of their activity when allowed to stand in nearly neutral solution. This procedure is identical with that which Anson and Mirsky found to result in the reversal in the denaturation of proteins. It was found that solutions of purified pepsin which had been completely denatured and inacti-

vated by adjusting to pH 10.5 recovered some of their activity when allowed to stand at pH 5.4. The percentage recovered in this way is small but increases as the solution is diluted. Crystalline pepsin was isolated from such reactivated solutions and found to be identical with the original crystalline protein. The reappearance of activity under these conditions therefore is accompanied by the reappearance of the native protein, so that again changes in activity are paralleled by chemical changes in the protein.

Inactivation in acid solution (Northrop 1932b).—In strongly acid solution pepsin gradually loses activity, but under these conditions there is a loss in total protein and no denatured protein appears. It is probable that denatured protein is formed as an intermediate step in the reaction but is hydrolyzed so rapidly that its presence cannot be detected, since in very strong acid (more than 2 M) denatured protein does appear and there is no subsequent hydrolysis.

A pepsin solution was allowed to inactivate slowly at 50°C. and pH 1.8 and was analyzed at various stages for protein nitrogen and activity. The results in Table 10 show that the activity per milligram protein remains constant throughout the experiment and therefore that destruction of the protein molecule results in practically complete loss of activity. If the split products of the protein have any activity, it must be less than one percent of the activity of the original protein molecule.

TABLE 10

HYDROLYSIS OF PEPSIN AT 50°C. AND PH 1.8

SAMPLE	PROTEIN NITROGEN PER MILLILITER			[P.U.]$_{mg.\ P.N.}$ (SPECIFIC ACTIVITY OF PROTEIN N)										
	Alk.	*2.5 CCl_3COOH*	*Ph. T*	*Gd. V⁻*	*Cas. V⁻*	*Ed. V⁻*	*Cas. V⁺*	*Rent.*	*Cas. S*	*Ed. S*	*Cas. F*	*Ed. F*	*Gd. F*	*Hb*
1	1.90	1.80	1.90	13	1000	880	460	73×10^4	0.47	0.42	0.16	0.08	0.002	0.18
3	0.62	0.60	0.90	13.7	1050	870	350	69×10^4	0.42	0.39	0.17	0.08		0.17
5	0.20	0.21	0.50	13.0	1020	880	350	71×10^4	0.46	0.42	0.17	0.085	0.0025	0.18
6	0.06	0.06	0.45	18.0	980	890	420	71×10^5	0.38	0.46				0.18

FIG. 21. Pepsin crystals from 15 percent alcohol. × 225.

"Alcohol soluble" pepsin.—Albers, Schneider, and Pohl (1943b) have described the preparation from old autolyzed gastric mucosa extracts of an active fraction which is soluble in 65 percent alcohol. The material does not precipitate with sulfosalicylic acid and is considered by the authors to be "non-protein" in nature and to be different from the crystalline pepsin protein described in the preceding pages. The experiments of Albers, Schneider, and Pohl were repeated with the result that the "non-protein" alcohol soluble substance was found to

TABLE 11

PREPARATION OF "ALCOHOL SOLUBLE" PEPSIN FROM GASTRIC MUCOSA
(Albers, Pohl, and Schneider 1943a)

	No.	Pepsin Units by Hemoglobin	
		Total	*Per Mg. Protein N*
Fundus from 20 swine stomachs ground up and added to 8 liters 0.5 percent HCl. Stirred and stood at 37°C. for 30 hours. Filtered and concentrated to 2.6 liters at 30°C. in vacuum. Titrated to pH 2.6 with HCl.	1	150	.26
Stood at 0°C. for 3 months.		150	.30
450 ml. Solution No. 1 filtered. *Filtrate*	2	27	.35
Adjusted to pH 3.2 (methyl orange) with 10 M ammonia. 300 ml. saturated $MgSO_4$ added slowly. Stood at 0°C. for 30 hours. Filtered. *Filtrate*	3	2	.2
Precipitate (20 ml.) stirred with 140 ml. 70 percent alcohol, 25°C. for 1 hour. Filtered. *Filtrate*	4	10	.32
Precipitate		.1	.1
140 ml. Filtrate No. 4 plus 100 ml. ethyl alcohol, stood at 0°C. for 24 hours. Filtered. *Filtrate*	5	3	.1
Precipitate (about 3 gm.) dissolved in 3 ml. H_2O.		6.5	.25
Titrated to pH 3.5, stood at 25°C. Few needles and some amorphous.			

be identical with the usual crystalline pepsin protein. No indication of any non-protein active material could be found. Crystalline pepsin, itself, is quite soluble in 70 percent alcohol and may be readily crystallized from 30 percent alcohol (Northrop 1946). In fact the method is a convenient one for the preparation of crystalline pepsin from crude pepsin preparations. The protein crystallizes as needles from the solvent. These needle crystals, when recrystallized from water, appear

as the usual hexagonal pyramids. Crystalline pepsin does not precipitate with cold sulfosalicylic acid but both crystalline pepsin, prepared in the usual way, and that prepared from 60 percent alcohol solution precipitate with hot sulfosalicylic acid.

Albers, Schneider, and Pohl (1942) also describe an active proteolytic enzyme "pepsidin," which is soluble in boiling 20 percent acetic acid and in butyl alcohol. The enzyme is said to be activated by hydrogen peroxide. These experiments were repeated several times with negative results. No proteolytic activity could be detected in such a preparation, either before or after treatment with hydrogen peroxide.

Inactivation of pepsin with ultraviolet light and beta and gamma rays from radium (Northrop 1934).—Pepsin solutions exposed to ultraviolet light or radium are inactivated with a corresponding loss in native protein. The results of an experiment with ultraviolet light are shown in Table 12. The decrease in activity is again proportional to

TABLE 12

CHANGE IN ACTIVITY AND PROTEIN NITROGEN IN PEPSIN SOLUTIONS OF VARIOUS pH EXPOSED TO ULTRAVIOLET LIGHT

pH	0			1.7			3.0			4.65		
BUFFER	1.0 N HYDROCHLORIC ACID			N/50 HYDROCHLORIC ACID			N/65 ACETIC ACID			N/50 4.65 ACETATE		
Time	$[P.U.]^{Hb}_{ml.}$	*P.N./ml.*	$[P.U.]_{mg.\ P.N.}$	$[P.U.]^{Hb}_{ml.}$	*P.N./ml.*	$[P.U.]_{mg.\ P.N.}$	$[P.U.]^{Hb}_{ml.}$	*P.N./ml.*	$[P.U.]_{mg.\ P.N.}$	$[P.U.]^{Hb}_{ml.}$	*P.N./ml.*	$[P.U.]_{mg.\ P.N.}$
hrs.		mg.			mg.			mg.			mg.	
0	0.019	0.098	0.20	0.020	0.098	0.21	0.020	0.098	0.22	0.020	0.098	0.21
2.55	0.014	0.076	0.18	0.015	0.070	0.215	0.017	0.080	0.21	0.017	0.084	0.20
5.00	0.0092	0.058	0.16	0.0095	0.050	0.19	0.0118	0.070	0.17	0.013	0.078	0.17
8.75	0.0054	0.030	0.18	0.0050	0.018	0.28	0.0077	0.058	0.13	0.0086	0.063	0.14

the loss of the protein, although at pH 4.65 there is some indication that a small amount of denatured protein accumulates in the solution in the latter stages of the reaction. Since energy must be absorbed in order to inactivate the pepsin molecules, it should be possible to pre-

dict the rate of inactivation of pepsin solutions by various wave lengths from the absorption spectrum of such solutions. Proteins have a very high absorption coefficient for ultraviolet light so that there is no doubt that the absorption spectrum of pepsin solutions is due to the protein itself.

The inactivation, on the other hand, is evidently a property of the active molecule so that, as Kubowitz and Haas (1933) have pointed out, agreement between the calculated and observed efficiency of

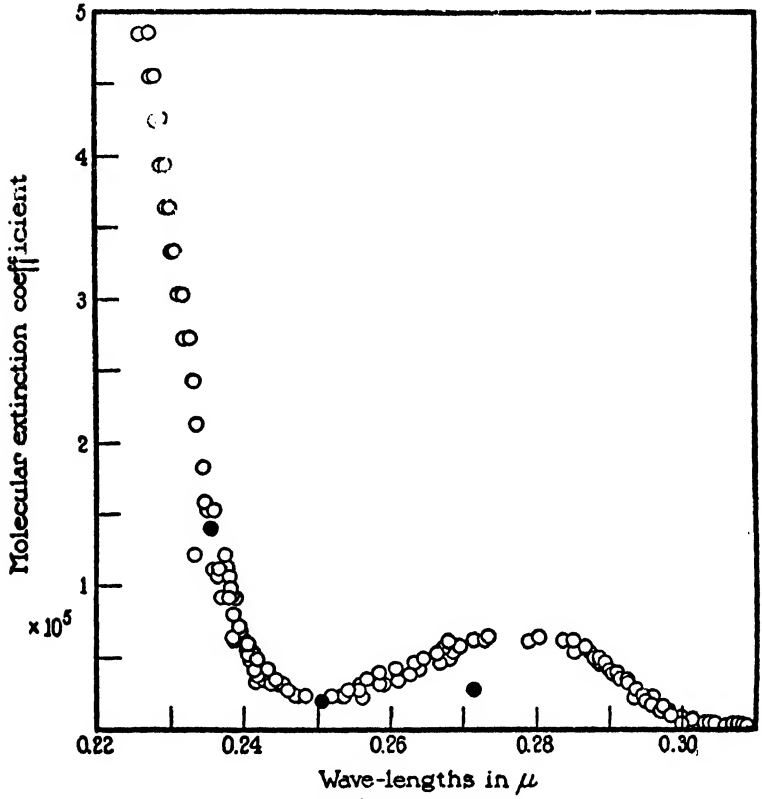


FIG. 22. Comparison of the destruction spectrum of crystalline pepsin with its absorption spectrum. Clear circles represent points obtained with a pure crystalline pepsin preparation (0.2376 mg. per ml.) in N/100 HCl, pH 2.54, on the normal absorption curve. Solid circles represent the reciprocals of the energy/100 required to inactive 50 percent of the pepsin (0.1412 mg. pepsin per ml.) in M/100 HCl, pH 2.06, plotted against the point wave length.

inactivation by various wave lengths would be very strong evidence that the activity was a property of the protein molecule. The possibility exists, however, that a different amount of energy is necessary to inactivate at different wave lengths and it is also possible for energy to be absorbed without causing inactivation. The relation between the absorption spectrum of pepsin solutions and the inactivating efficiency of various wave lengths has been investigated by Gates (1934a,b). The result of the experiments is shown in Figure 22. The figure shows that the inactivating efficiency of the various wave lengths agrees with that determined by the absorption spectrum, at 2,357Å and at 2,500Å, but is less than that calculated from the absorption spectrum at 2,700Å. This means either that more energy is required to inactivate at this wave length than at the shorter wave length or else that the solution contains some material which absorbs more highly at 2,700Å. It is known that the products of the reaction absorb more highly than does pepsin, and it is possible that the increased absorption noted at 2,700Å is due to these products. The results in general are very similar to those obtained by Kubowitz and Haas for the destruction spectrum of urease.

Absorption of pepsin by crystalline proteins (Northrop 1933b).—Pepsin, like other enzymes, is removed more or less completely from solution by various insoluble substances. Dauwe showed that insoluble proteins were particularly efficient in this respect. These results were confirmed by Abderhalden and co-workers. The writer found that the quantity of pepsin removed by insoluble proteins depended largely upon the pH of the solution and that under certain conditions the ratio of the enzyme in the precipitate to that in the solution was the same as the chloride ion ratio. This result suggested that the pepsin was a negative ion and was distributed like any other ion in accordance with the Donnan equilibrium. In the acid range, however, between pH 2.0 and 5.0 the results were anomalous from this point of view, since much more pepsin was absorbed than would be expected from the Donnan equilibrium.

It was found by Dyckerhoff and Tewes and by Waldschmidt-Leitz and Kofrányi that crystalline proteins such as edestin or melon globulin also possess the property of absorbing pepsin from pepsin solutions, and Waldschmidt-Leitz considered that the crystalline foreign protein removes the active group from the pepsin protein. If this explanation

were correct a convenient means would be at hand to separate the active group of pepsin from the protein-pepsin molecule, since the foreign protein (edestin or melon globulin) is rapidly and completely digested by pepsin and since there is little or no loss in activity during peptic digestion. It would only be necessary, therefore, to allow the complex of foreign protein and pepsin to digest until all the protein had been destroyed and the active pepsin must then be found in solution free from protein. When this experiment is performed, however, it is found that there is left in the digested edestin solution an amount of pepsin protein just equivalent to the peptic activity present and equivalent to the loss in pepsin protein from the original pepsin solution. The absorption of pepsin by crystalline foreign protein, therefore, consists in the absorption of the pepsin protein, as such, and does not separate the pepsin protein into an inert protein and an active pepsin group.

The absorption of pepsin by edestin shows a sharp maximum at about pH 4.0, and pepsin may be removed completely from dilute solutions by stirring with a suspension of edestin crystals at this pH. The pepsin protein may be recovered from the "edestin-pepsin" complex by allowing the "edestin-pepsin" to autolyze, or by simply extracting (Table 13) the edestin-pepsin with N/4 sulfuric acid at 0°C. The recovered pepsin may be identified by its tyrosine-tryptophane content, which is twice that of edestin, and by its content of basic nitrogen, which is about one quarter that of edestin. It may be readily recrystallized and obtained in the characteristic crystalline form and with the characteristic specific activity of the original crystalline pepsin. If a suspension of edestin crystals at pH 4.0 is added to increasingly concentrated solutions of either crystalline or crude pepsin surprisingly large amounts of pepsin are taken up by the edestin crystals and preparations may be obtained which contain nearly 50 percent pepsin and are, therefore, one half as active as crystalline pepsin itself and much more active than commercial pepsin preparations. The general form and appearance of the edestin crystals is not markedly changed, but if the suspension of edestin crystals in the pepsin solution is allowed to stand for several hours at room temperature, the edestin gradually dissolves and the pepsin content of the remaining precipitate increases. On longer standing the precipitate becomes less and less in bulk and finally dissolves completely, so that

TABLE 13

EXTRACTION OF PEPSIN FROM EDESTIN-PEPSIN

	Total N/ml.	*Pepsin N/ml.*	$[P.U.]^{Hb}_{ml.}$	$[P.U.]^{Hb}_{mg.\ N}$	$[P.U.]^{Hb}_{mg.\ Pepsin\ N}$	*pH*
0.5 gm. pepsin-edestin + 10 ml. N/4 sulfuric acid	10	0.30	0.05	0.005	0.17	0.8
Stir at 0°C. for 20 min.						
Centrifuge—supernatant	1.3	0.23	0.038	0.029	0.165	
Precipitate stirred + 10 10 ml. N/10 sulfuric acid						
Centrifuge—supernatant	1.3	0.02	0.003	0.0025	0.15	
Precipitate stirred + 10 ml. N/10 sulfuric acid						
Centrifuge—supernatant	1.4	0.02	0.0024	0.0017	0.12	
Precipitate stirred + 10 ml. N/10 sulfuric acid						
Centrifuge—supernatant	1.2	0.02	0.0020	0.0016	0.10	
Precipitate + 10 ml. water—suspension edestin crystals	4 5	0.02	0.001	0.0002	0.05	
Total $[P.U.]^{Hb}$						
in washings			0.454			
in precipitate			0.010			
			0.464			
Total original $[P.U.]^{Hb}$			0.50			

the final result is a solution of digested edestin containing the original quantity of pepsin.

The rate of autolysis can be increased by dissolving the edestin-pepsin precipitate in hydrochloric acid. The edestin protein is then very rapidly destroyed and there is left the pepsin protein (Figure 23). There is no change in activity during this process so that the autolysis of "edestin-pepsin" differs strikingly from the autolysis of pepsin itself, since in the latter case the destruction of the protein is paralleled by a corresponding loss in activity, while in the case of "edestin-pepsin" the edestin is destroyed without any corresponding loss in

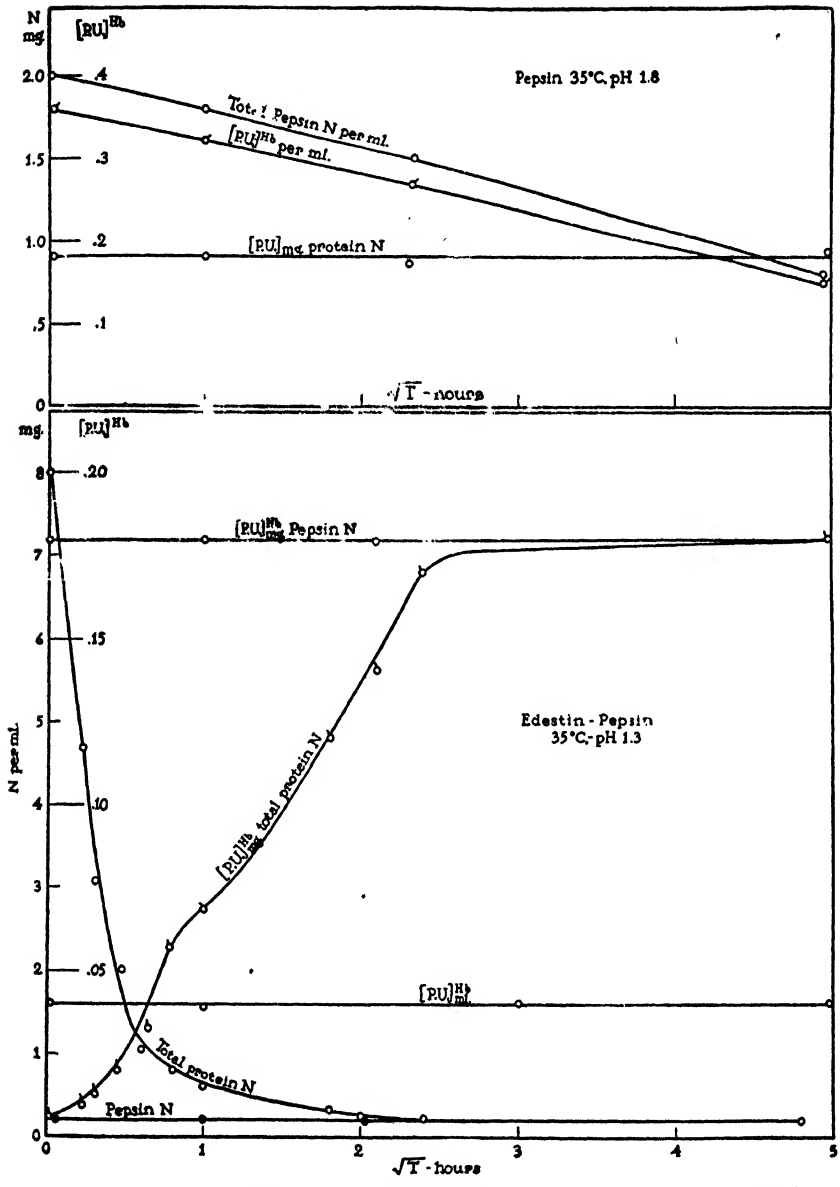


FIG. 23. Autolysis of pepsin (upper part) and of edestin-pepsin (lower part) in acid solution at 35°C.

activity. There is, therefore, no reason to consider the "edestin-pepsin" complex as having any activity of its own aside from that due to the content of pepsin protein.

If supersaturated solutions of autolyzed edestin-pepsin or autolyzed solutions of pepsin alone are allowed to stand, the pepsin precipitates out in the form of spheroids, as Dyckerhoff and Tewes have shown. It is characteristic of proteins to appear in this spheroidal form when conditions are not quite right for crystallization or when they have not been sufficiently purified. The pepsin spheroids consist largely of pepsin but contain from 10 to 30 percent non-protein nitrogen. They may be purified by solution and precipitation with acid or magnesium sulfate and the pepsin may then be obtained in the usual crystalline form.

The edestin-pepsin complex may also be formed by mixing cold solutions of edestin with solutions of pepsin. A precipitate forms which varies in composition and quantity with the pH of the solution. The maximum quantity and the maximum activity again are found at about pH 4.0. If the relative concentrations of pepsin and edestin are varied at pH 4.0, the quantity of pepsin in the precipitate is a maximum when equal concentrations (by weight) of pepsin and edestin are mixed. Under these conditions the precipitate contains nearly 75 percent pepsin and is about three quarters as active as crystalline pepsin itself.

Since pepsin and edestin both have an equivalent weight[1] of about 1,000, the precipitate having maximum activity corresponds approximately to three equivalents of pepsin to one of edestin. Since the molecular weight of pepsin is only one sixth that of edestin, this corresponds approximately to eighteen molecules of pepsin to one of edestin.

Similar experiments may be performed with the globulin from melon seed (*Cucumis*), as Waldschmidt-Leitz and Kofrányi have found, and also with gelatin. In both cases the pepsin protein removed from the pepsin solution and taken up by the solid protein corresponds to the loss of activity of the solution.

[1] The equivalent weight of the protein is the number of grams which will combine with a gram molecule of alkali.

EFFECT OF ACETYLATION AND IODINATION ON THE ACTIVITY OF PEPSIN
(Herriott and Northrop 1934)

The experiments described in the previous sections have shown that crystalline pepsin has the general chemical structure of a protein. Since it exhibits peculiar and powerful catalytic properties, there must be present in the molecule some characteristic structure not present in ordinary proteins. No evidence for a prosthetic group has been found, and hydrolysis or denaturation of the protein results in complete loss of activity so that identification of the "active structure" by isolation of fragments of the molecule has not been successful.

Indirect information as to the nature of the active group may be obtained, however, by altering the structure of the original pepsin molecule with reagents known to react with certain chemical groups and determining the effect of this change in structure upon the activity. By analogy with other compounds, such as acids or dyes, it would be expected that any change in the molecule would affect the activity, but that the extent of the effect would depend upon a number of factors such as the nature of the reagents, the reacting groups of the molecule, and their relation to the active part of the molecule. Pepsin is known to have such free groups as carboxyl (COOH), amino (NH_2), and phenolic hydroxyl (OH). The effect on the activity resulting from the modification of these groups should furnish information as to their relation to the molecular structure responsible for the activity.

If the modification of these groups in native pepsin resulted in a complete loss in activity, it would be probable that the active group, or one closely associated with the active group, had been altered by the treatment. If the modification of these groups failed to affect the activity, it would follow that the modified groups are not closely related to the active part of the enzyme. Experiments were designed to determine what effect acetylation of the various groups of pepsin would have on the activity.

In these experiments pepsin was acetylated by treatment with ketene, and it was found that the activity decreased as the acetylation proceeded. In the study of the course of the acetylation reaction it became apparent that the time rate of change in the specific activity of the enzyme during acetylation depends upon the pH of the

solution. Acetylation of pepsin at pH 5.5 causes a greater drop in specific activity per acetyl group introduced than acetylation at pH 4.5. If the pH of the pepsin solution is kept constant, the specific activity of the reaction mixture when plotted against the increase in number of acetyl groups per molecule of protein drops off logarithmically as acetyl groups are introduced. There is no sharp break in the curve; the activity drops gradually and is 10 percent or less of the original activity after the introduction of more than twenty acetyl groups per molecule of enzyme.

A preparation of acetylated pepsin has been obtained in crystalline form which has 60 percent of the activity of the original pepsin, as measured by several methods. This material contains one or less primary amino groups per molecule of protein instead of the three or four originally present in pepsin. It also possesses from six to eleven acetyl groups per molecule, the number depending upon the conditions during the acetylation. From the acetyl determinations of several different preparations and the solubility experiments it appears that there may be several acetyl products possessing approximately 60 percent of the original pepsin activity and which may form solid solutions with one another.

Another crystalline acetylated pepsin preparation was obtained by subjecting the above acetyl pepsin to normal sulfuric acid. The specific activity of this acid solution increased to the value of pure pepsin and the acetyl content dropped from nine to four acetyl groups per molecule. There was no measurable increase in the primary amino nitrogen, and it is probable that the remaining acetyl groups are on the primary amino groups. It was also possible to prepare this same, or a closely related material, starting with pepsin. By careful acetylation an acetyl product was obtained which had lost none of its activity but which had lost all of its primary amino groups. This material had three acetyl groups per molecule, the equivalent of the amino groups lost.

Still another acetylated product was prepared by acetylation for from thirty-five to forty hours at pH 5.5 in a dialyzing bag. This material was only 10–15 percent as active as pepsin and had on the average twenty acetyl groups per molecule. On further acetylation the decrease in activity was very slow. It was much less soluble than

the other preparations and crystallized with difficulty and in very small crystals.

The different acetyl preparations could be separated from one another by fractiona. precipitation from concentrated magnesium sulfate solution. They had constant composition and activity after repeated recrystallization, but the solubility curves indicate that they are solid solutions of similar proteins (Figure 24). Under the micro-

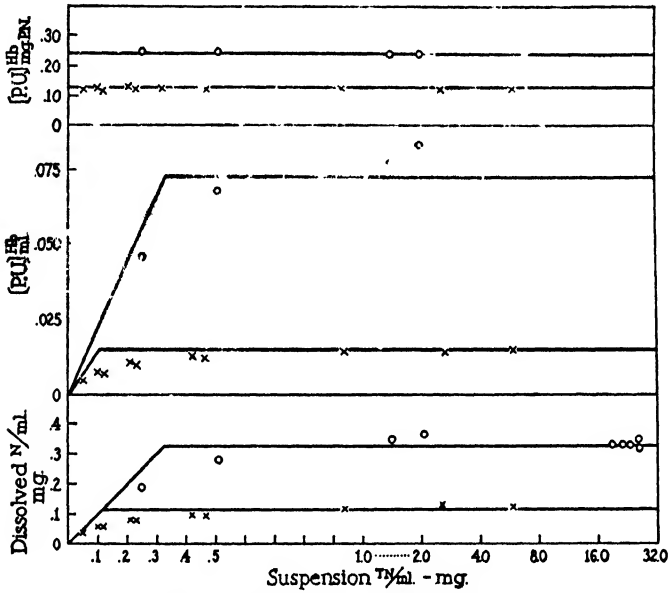


FIG. 24. Solubility of amorphous "100 percent active" acetyl pepsin and of amorphous "60 percent active" acetyl pepsin in 0.05 M pH 4.65 acetate buffer and 0.5 saturated magnesium sulfate.

scope the crystals of all of the acetyl preparations appeared alike and indistinguishable from those of pepsin, as may be seen in Figure 25.

The results of these acetylation experiments show that acetylation of primary amino groups does not change the specific activity of pepsin by more than 10 percent, but that addition of acetyl groups in some other places causes a marked decrease in activity.

Similar results were obtained by Hollander (1943) who studied the effect of acetylation of pepsin on the rate of hydrolysis of carboben-

zoxy-l-glutamyl-l-tyrosine. Acetylation of the OH group decreased the rate of hydrolysis, while acetylation of the amino group was without effect.

Tracy and Ross (1942a) have found that malonyl groups may be added to pepsin without change in specificity.

The addition of carboxyl groups to the substrate protein increases the rate of hydrolysis.

The primary amino groups of proteins in general are probably those of lysine, and since acetylating these groups has little or no effect on

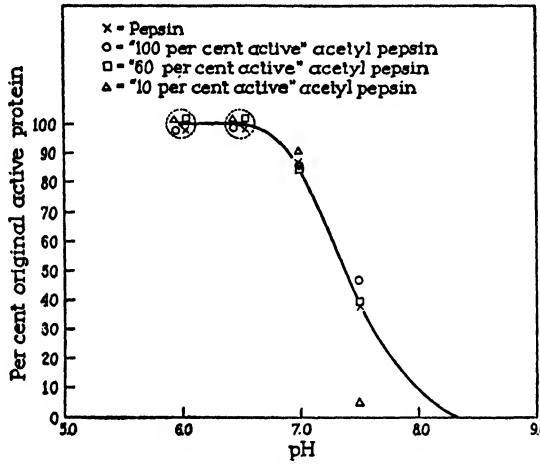


FIG. 26. Percent inactivation of pepsin and three different acetyl derivatives of pepsin in phosphate buffer at various pH and 23°C.

the activity it is probable that the lysine groups are not part of, or are not intimately associated with, the molecular structure responsible for the activity. Further acetylation, however, causes a marked decrease in activity, which would indicate that certain groups of the protein more closely related to the active part of the molecule have been acetylated. Ketene reacts with NH, OH, or SH groups. It will be shown below that those groups which decrease the activity are attached to the phenolic OH group of tyrosine.

The fact that alkali inactivation of acetyl-pepsin is the same as that of pepsin (Figure 26) renders it unlikely that the amino groups are involved in this reaction, since they are not free in acetyl pepsin.

The titration curves of the acetyl preparations examined were not greatly different from the same properties of the parent substance, pepsin.

Position of the acetyl groups which cause a loss in activity.—The experiments just described show that acetylation of the primary amino groups of the pepsin molecule has no effect upon the activity, while further addition of acetyl groups causes a decrease in activity. Evidently these latter groups are closely related to the activity and their identification would be the first definite connection between chemical structure and activity.

It is probable (Herriott 1935) that those acetyl groups in the 60 percent active acetyl pepsin which are responsible for the decrease in specific enzymatic activity are attached to the phenolic hydroxyl groups of some of the tyrosine components of the protein. In the 60 percent active preparation there are three acetyl groups which are not in the 100 percent active acetyl pepsin. These three additional acetyl groups are hydrolyzed by molar acid or by alkali at pH 10.0, whereas the acetyl groups on the amino groups are not hydrolyzed under the same conditions. These easily hydrolyzed acetyl groups have been designated as "pH 10.0 labile" acetyl groups. There are three less tyrosine phenol groups in the 60 percent active acetyl pepsin, as measured colorimetrically with the Folin phenol reagent under conditions which will not hydrolyze an acetylated phenol.

When the 60 percent active material is changed back into 100 percent active acetyl pepsin by the acid treatment, there is an accompanying loss of the pH 10.0 labile acetyl groups and the number of tyrosine phenol groups returns to that of the original pepsin.

The conclusion that the acetyl groups are attached to the phenolic hydroxyl group of tyrosine is further indicated by the fact that the rate of acetylation of glycyl-tyrosine by ketene and the effect of pH on this reaction are similar to the corresponding values for the acetylation of pepsin.

Inactivation of pepsin by iodine and the isolation of diiodo-tyrosine from iodinated pepsin (Herriott 1937).—The experiments on the acetylation of pepsin point to a close relationship between the tyrosine of pepsin and the proteolytic activity of the protein molecule. In the hope of obtaining direct and decisive evidence concerning the role played by tyrosine in pepsin it was decided to study the action of

iodine on pepsin. Iodine is reported to react with the benzenoid part of tyrosine in proteins, and since this type of iodine linkage is known to be relatively stable, it seemed likely that an iodine-tyrosine compound could be isolated from iodinated pepsin.

Under certain conditions solutions of native pepsin readily absorb iodine, and the proteolytic activity decreases gradually. Pepsin is practically inactive when the number of iodine atoms per molecule of pepsin is 35–40. There is no appreciable oxidation of pepsin or of glycyltyrosine by iodine under the conditions used for iodination. The rates of iodination of pepsin and of glycyl tyrosine are affected by a variation of pH in a like manner. The effect of pH is nearly identical with that already noted for acetylation of these two materials.

Completely iodinated pepsin was hydrolyzed, and the products of hydrolysis containing iodine were fractionated. A solution containing 82 percent of the total iodine was obtained from the iodinated pepsin. The analyses of this solution were similar to those of a solution of diiodo-tyrosine. Also present in this solution was a dark material which prevented crystallization of the diiodo-tyrosine and which could be removed only with difficulty and at the expense of the yield of crystalline diiodo-tyrosine. The yield of crystalline diiodo-tyrosine finally obtained represented 53 percent of the original iodine.

Philpot and Small (1939), using much less iodine, found that there was no decrease in the Folin phenol-color value of pepsin as the enzyme activity decreased. Since diiodo-tyrosine yields only half as much color per mol as does tyrosine they expected a decrease in color as the tyrosine in pepsin was iodinated. They concluded that a reaction of iodine with some structure other than tyrosine was responsible for the decrease in enzyme activity.

Herriott (1941a) treated pepsin with dilute iodine until only 2 iodines per mol pepsin had been introduced. The activity decreased only 15 to 20 percent. Monoiodo-tyrosine was isolated from this iodinated pepsin. Monoiodo-tyrosine has nearly the same molar color value as tyrosine. Hence, Philpot's and Small's failure to observe a decrease in color after iodination is explained. Treatment with small amounts of iodine results in the formation of monoiodo-tyrosine, while larger quantities of iodine form diiodo-tyrosine.

Recently (1944a) Harington and Pitt Rivers have synthesized optically active l monoiodo-tyrosine and inactive d-l monoiodo-

(a) (b)

(c)

FIG. 25. (a) Crystalline pepsin, × 90; (b) crystalline "100 percent active" acetyl pepsin, × 34; (c) crystalline "60 percent active" acetyl pepsin, × 28.

tyrosine. Since the properties of these synthetics did not correspond quantitatively with those reported by Herriott (1941a), they concluded that the nature of the material isolated from iodinated pepsin is unknown. The points of disagreement are: optical rotation, "water of crystallization," as determined from elementary analyses, and a color reaction.

Herriott (1947) has synthesized d-l monoiodo-tyrosine and compared it with the product isolated from iodinated pepsin. Melting (decomposition) points, mixed solubility, cross distribution experiments with butyl alcohol and water, ultraviolet absorption spectrum and its variation with pH, all indicate that the material isolated from iodinated pepsin is monoiodo-tyrosine. Variations in the elementary analyses prevented their use as a basis of comparison. Only one determination of the optical rotation of the product from pepsin could be made. It was lower than that previously reported but the difference is hardly more than the experimental error. The results of the tests of physical properties are strong evidence and far outweigh the less reliable elementary analyses. It appears that the product isolated from pepsin *is* monoiodo-tyrosine, but whether it is optically active or racemic is still uncertain.

The close relation between the tyrosine and the activity of the enzyme has been confirmed by the work of Philpot and Small (1938). These workers have found that nitrous acid reacts with the pepsin tyrosine to form a diazo compound which has about half the activity of the original pepsin.

Sizer (1946) has found that tyrosinase attacks the tyrosine group in pepsin, without affecting the activity of the enzyme. The action of the tyrosinase, however, took place at pH 7.3 and 37°C. at which pepsin is instantly and practically completely denatured and inactivated (cf. page 54). The effect of tyrosinase on active pepsin cannot be determined under these conditions.

Sizer's experiments have been repeated by Edman (1947), using highly purified preparations of chymo-trypsin, trypsin, and pepsin. No reaction occurred when tyrosinase was added to pure trypsin or chymo-trypsin. When trypsin or chymo-trypsin preparations containing non-protein impurities were used, the tyrosine in the non-protein impurity was attacked by the tyrosinase. Pepsin is completely inactivated at the pH of the tyrosinase reaction so that denatured pepsin

only is present. No significant reaction occurred when tyrosinase was added to this pepsin solution, unless non-protein compounds were present.

It appears that the reactions observed by Sizer were due to the presence of non-protein impurities in the enzyme samples used, and were not due to a reaction of the tyrosinase with tyrosine in the proteins, themselves.

GENERAL PROPERTIES OF CRYSTALLINE PEPSIN

Specificity.—The specificity of the crystalline enzyme, i.e., the relative rate with which it hydrolyzes various proteins, does not differ significantly from the specificity of gastric juice (cf. Table 14) (Northrop 1933a). The enzyme causes a rapid decrease in the viscosity of gelatin, casein, and edestin solutions. Casein solutions in the presence of large concentrations of enzyme show a rapid decrease in viscosity followed later by an increase. The milk clotting power is about the same in bovine gastric juice and in the crystalline enzyme. It seems probable, therefore, that the proteolytic enzyme, pepsin, also clots milk, as stated by Tauber and Kleiner (1933), and that in the adult animal there is no special milk clotting enzyme. In the young animal there is evidently a distinct rennet. It may be noted that pepsin from chickens or sharks

TABLE 14

PROPERTIES OF CRYSTALLINE PEPSIN FROM BOVINE GASTRIC JUICE AND FROM SWINE STOMACH

PEPSIN	PROTEOLYTIC ACTIVITY PER MG. NITROGEN										
	Gel. V⁻	*Cas. V⁻*	*Ed. V⁻*	*Cas. V+*	*Rennet*	*Cas. S.*	*Ed. S.*	*Cas. F.*	*Ed. F.*	*Gel. F.*	*Hb*
Bovine gastic juice	10.6	1300	640	430	170,000	0.27	0.20	0.16	0.072	0.0008	0.180
Swine stomach Standard pepsin Solution 5/9/32	11.0	1400	625	500	168,000	0.30	0.22	0.11	0.075	0.0007	0.183

also clots milk, although this property, in the case of the shark at any rate, does not appear to be of any practical value. The ratio of rennet to pepsin activity differs somewhat in different animals, and pepsin prepared from some commercial preparations shows a small varia-

tion in this ratio. This may be due to the presence of material from young animals or from a different species of animal in the different commercial preparations. The rennet activity of pepsin prepared from pepsinogen is constant and reproducible. Crude commercial preparations contain, in addition to pepsin, an enzyme which digests gelatin much more rapidly than does pepsin and also another enzyme which may be purified by the method of Brücke (Kraut and Tria 1937). These enzymes represent a relatively negligible part of the total proteolytic activity, and it seems possible that they are derived from the cells of the gastric mucosa.

The question as to the existence of a special milk-clotting enzyme, "rennet," which has been a subject of controversy for many years, has finally been settled by Berridge (1945), who has isolated and crystallized the enzyme.

Commercial rennet preparations, which are made from calf stomachs, were used as the starting point for these experiments. The enzyme was purified by fractional precipitation with sodium chloride, absorption and elution from alumina. It was crystallized from ammonium sulfate and magnesium sulfate. The crystals have almost a true constant solubility and are evidently quite pure. The pH optimum for the digestion of hemoglobin with rennet is pH 4.0, whereas the pH optimum for crystalline pepsin is pH 1.8.

Pepsin hydrolyzes carbobenzoxy-l-glutamyl-l-tyrosine (Fruton and Bergmann 1938).

The pH optimum varies with the substrate. Free carboxyl groups increase the rate of hydrolysis. Free amino groups decrease the rate (Fruton, Bergmann, and Anslow 1939).

Harington and Pitt Rivers (1944b), however, found that cysteyl tyrosine and tyrosyl-cysteine and the corresponding cystine compounds are hydrolyzed by pepsin. These peptides contain both free amino and free carboxyl groups and should not hydrolyze rapidly, on the basis of Bergmann's results. The reduced forms of these peptides are split by pepsin more rapidly than the odixized.

The rate of hydrolysis of these synthetic substrates is extremely slow, however, compared to the rate of hydrolysis of proteins. For instance, crystalline pepsin hydrolyzes edestin at the rate of about 15 percent per minute per .16 milligrams of pepsin nitrogen per milliliter (Northrop 1932d, page 52). Carbobenzoxy-l-glutamyl-l-tyrosine

TABLE 15

PRELIMINARY DATA ON THE AMINO ACID COMPOSITION OF PEPSIN (Brand)*

PREPARATION OF PEPSIN†

Analysis	*Percent Protein*		*Molecular Weight*
Total N	14.6		
Total S‡	0.94		34,000
Total P	0.09		34,400
Free alpha amino N§	0.081		34,600
Constituent	*Gm. Amino Acid Residue per 100 Gm. Protein*	*Molecular Weight of Pepsin Calculated from Residues per Mol*	*Residues Mols per Mol Protein***
Glycine	4.9		29
Alanine			
Valine	6.0		21
Leucine	9.0		27
Isoleucine	9.3		28
Proline	4.2		15
Phenylalanine	5.7		13
Cysteine	0.4		2
Half-Cystine	1.4		4
Methionine	1.5	35,100	4
Tryptophane	2.15	34,600	4
Arginine	0.9	34,800	2
Histidine	0.8	34,500	2
Lysine	0.8	32,500	2
Aspartic acid			
Glutamic acid	10.4		28
Amide NH_3			
Asparagine	10.7		32
Free asp. acid	3.0		9
Serine	10.1		40
Threonine	8.2		28
Tyrosine	7.7		16
"Terminal H_2O"††	0.10		
H_3PO_4§§	0.22		
Total	97.47	Average: 34,300 ± 500	306

* The authors are grateful to Professor E. Brand of the College of Physicians and Surgeons, Columbia University School of Medicine, New York, N. Y., for the privilege of including this preliminary report of his analyses.

† The pepsin used in these analyses was prepared by Herriott as described by Herriott, Desreux, and Northrop (1940b), Table VI, Steps 1–11. A dialyzed solution of this crystalline preparation of constant solubility was cooled to 5°C.; titrated to pH 9; back to pH 7; warmed to 50°C.; and then, after acidifying to pH 3, the precipitated protein was washed and dried while frozen. The filtrate and washings contained not more than 3 percent of the total nitrogen present before alkali denaturation.

‡ The sulfur content reported earlier by Northrop (1930) is in agreement with the present values. Those reported in the pepsinogen paper (Herriott 1938a) were determined by a commercial analyst and are probably incorrect.

(Fruton and Bergmann 1938) is only 40 percent hydrolyzed in 1,440 minutes in the presence of 1 milligram of pepsin per milliliter. The rate of hydrolysis of edestin is, therefore, at least 100 times as fast as that of the synthetic substrate.

Holter (1931) has found that solutions of crystalline pepsin after being treated with aluminum hydroxide show slightly different properties when the relative rates of formation of amino nitrogen and of non-protein nitrogen are compared. Similar differences are found in comparing various gelatinase fractions and it is possible that Holter's experiments resulted in a partial separation of pepsin and gelatinase.

Molecular weight.—The molecular weight from osmotic pressure in M/1 acetate pH 4.6 is 35,000 ± 1,000; from diffusion coefficient, 70,000; from sedimentation data 37,000 (Philpot and Eriksson-Quensel 1933); from one atom phosphorus per mol 34,000 (see Table 15).

Amino acid composition of pepsin.—The results of amino acid analyses of pepsin are shown in Tables 4 and 15. The relative quantities of the various amino acids are quite different from those of any other protein. The free amino nitrogen and the basic nitrogen is low. The tyrosine, the hydroxyamino acids, and the dicarboxylic acids are high. The molecular weights calculated from the methionine, tryptophane, arginine, histidine, and lysine, phosphorus, sulfur, and free α amino nitrogen are remarkably concordant and agree with those found by osmotic pressure or ultracentrifuge determinations.

The arrangement of amino acids in pepsin.—Pepsin contains one phosphorus atom which is apparently an integral part of the protein. Such a system is ideal for the study of the arrangement of amino acids in a well-defined enzyme (or protein) and of the differences in specificity of the various purified proteases when acting on naturally occurring proteins and peptides.

With the phosphorus as a common component, peptides isolated from enzymatic digests of pepsin (as substrate) will probably be different, depending on the protease used as catalyst. After determining the arrangement of amino acids in each peptide it should be possible to picture the more or less complete arrangement of amino acids in the vicinity of the phosphorus, and thus to make a start on the determination of the structure of this enzyme.

Pepsin contains only two histidine and two arginine residues, either

of which amino acids may be readily determined. Any procedure which will separate the peptides containing one of these amino acids will, of course, give a new focus for the study of the structure of a different segment.

Preliminary treatment of the native pepsin protein with reagents which react with the "surface" or "free" groups (see page 65), followed by digestion and isolation of the phosphorus peptide, will bring out the relationship of these surface groups to the other structures or arrangements of amino acids.

The phosphorus containing peptide from autolyzed pepsin, i.e., a peptic digest of pepsin, has been isolated and a study of its structure is under way. A preliminary examination reveals that it contains about six amino acids and that tyrosine, tryptophane, and cystine are not constituents of the peptide.

Ultraviolet spectra.—The ultraviolet spectrum of pepsin, as usually determined, has already been discussed and resembles that usually obtained for proteins. If the determination is made with a continuous light source and at low temperature (Lavin and Northrop, 1935) the curve is seen to be composed of bands which resemble and may be identified with those of tryptophane, tyrosine, and phenylalanine. No peculiar absorption either in the ultraviolet or visible has been observed, so that if pepsin does contain a prosthetic group it seems quite certain that the prosthetic group does not have a strong absorption spectrum.

X-ray patterns.—Bernal and Crowfoot (1934) have found the X-ray pattern of pepsin is that of a typical protein crystal. The dimensions of the unit cell are found to be A = 67Å, C = 154Å.

III: PEPSINOGEN

ISOLATION (Herriott 1938a)

LANGLEY found that gastric mucosa contained a substance which was not changed by alkali as is pepsin but which was transformed into pepsin when the solution was acidified. He considered it to be an inactive precursor of pepsin called "pro-pepsin" or "pepsinogen." This result has frequently been confirmed, but, as Sørensen has pointed out, the question of the activity of pepsinogen is complicated by the fact that pepsin itself is active only in acid solution, and it is therefore difficult to determine whether pepsinogen is active or not. It can easily be shown that pepsinogen does not clot milk, but there is a further possibility that the milk clotting is due entirely to a separate enzyme. In view of the results described under pepsin, this seems unlikely, and, in addition, it may be shown that the activity of pepsinogen, measured by the change in viscosity of gelatin solutions at pH 4.5, is negligibly small. Preliminary experiments by Holter and Northrop resulted in the preparation of a protein fraction which, after activation, had about half the activity per milligram nitrogen as does pepsin itself. An improved method has recently been worked out by Herriott, and the pepsinogen has been obtained in crystalline form.

TESTS OF PURITY

Constant composition.—The protein is obtained by this method in the form of needle-shaped crystals (Figure 27). They have constant optical rotation and constant potential activity (i.e., activity after transformation into pepsin) through at least three crystallizations (Table 16).

Solubility experiments.—The determination of solubility in the presence of increasing amounts of solid phase furnishes the most dependable criterion of the purity of protein preparations. The use of this method was discussed in relation to the solubility of pepsin. The

TABLE 16

COMPOSITION AND ACTIVITY OF VARIOUS PEPSINOGEN PREPARATIONS

MATERIALS	C	H	N DUMAS	N KJELDAHL	P	Cl	ASH
	Per-cent	*Per-cent*	*Per-cent*	*Per-cent*	*Per-cent*	*Per-cent*	*Per-cent*
Uncrystallized pepsinogen	52.80	6.88	15.92	15.1	0.09 0.084	0.00	1.72
Uncrystallized pepsinogen			14.40	13.8	0.084		
3 × crystallized pepsinogen			14.45	14.3			
3 × crystallized (Na) pepsinogen			14.00	14.00			
3 × crystallized No. 9 pepsinogen			13.59	13.4			

MATERIALS	METHODS*			
	Hemoglobin $[P.U.]^{Hb}_{mg.\ P.N.}$	*Rennet*† $[P.U.]^{Ren.}_{mg.\ P.N.}$	*Gel. viscosity* $[P.U.]^{Gel.\ V.}_{mg.\ P.N.}$	*Edestin N.P.N.* $[P.U.]^{Ed.\ Sol.}_{mg.\ P.N.}$
Specific potential activities of pepsinogen preparations†				
3 × crystallized pepsinogen	0.20	275	17	0.42
Average of 10 preparations of pepsinogen	0.22	250–300		
3 copper treatment but uncrystallized pepsinogen	0.21	270	17	0.46

* With the exception of the rennet method the methods were carried out as described by Northrop (1932d).

† It will be noted that the specific activity by hemoglobin units of these preparations is higher than those reported for the earlier pepsin preparations. The determinations were made at different times and with different hemoglobin solutions. The apparent increase in activity is probably due in part to differences in the hemoglobin method. All recent preparations of pepsin show consistently 0.22–0.26 hemoglobin units per mg. protein nitrogen.

results of an experiment of this kind with pepsinogen are shown in Figure 28. The curve is very close to that of a pure substance in equilibrium with its saturated solution, except for a narrow range in which a very small amount of precipitate is present. This is the most difficult part of the curve to obtain experimentally and it is not possible from the present results to decide whether the discrepancy is due to experimental difficulties or whether there is a small amount of second component present. The activity measurements follow very closely

FIG. 27. Pepsinogen crystals. × 340.

the concentration as determined by protein nitrogen. This correspondence indicates the identity of the active molecule and the protein.

GENERAL PROPERTIES

Reversible heat inactivation.—Neutral, salt-free solutions of pepsinogen may be heated to boiling and then cooled without loss of potential activity. If salt is added to the hot solution, however, it is found that the protein is entirely denatured in the hot solution, but, in the absence of salt, reverts to the native condition again when cooled. The amount of native protein present in the solution at various temperatures may be found by adding salt, which precipitates the denatured protein, and by analyzing the solution for pepsinogen. Such experiments show that the protein is *practically completely in the native* form at or below 50°C. and practically completely dena-

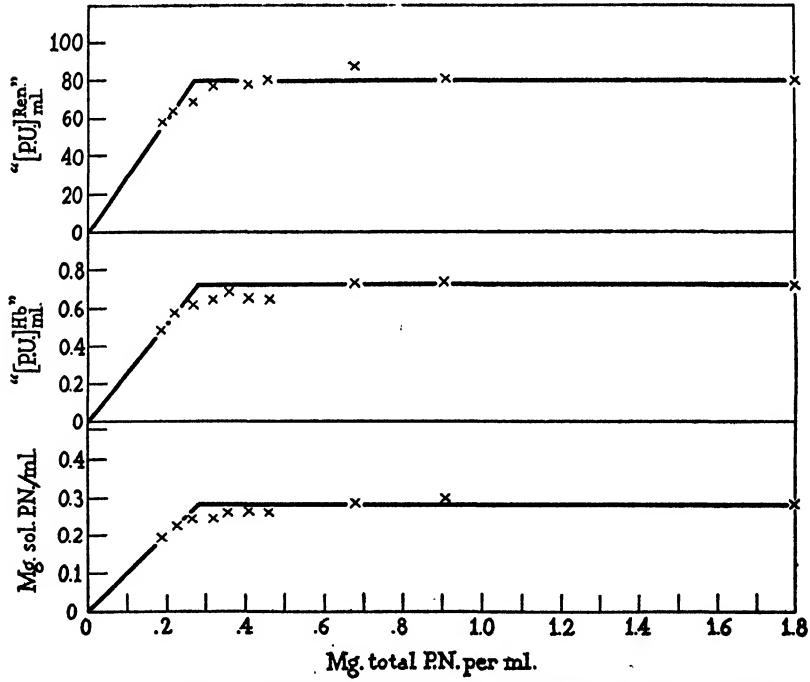


FIG. 28. Solubility of pepsinogen in 0.55 saturated ammonium sulfate—M/10 pH 6.8 phosphate at 21°C.

tured at 70°C., indicating a very high heat of reaction. The fact that the reaction is an equilibrium one shows that Van't Hoff's relation between the equilibrium constant and heat of reaction is involved. The results are very similar to those obtained with trypsin, to be discussed later.

Reversible alkali inactivation.—The protein is denatured by strong alkali, i.e., pH greater than 9.0, but this reaction is also reversible, at least for some time. These two reactions of pepsinogen are entirely different from those of pepsin under the same conditions, since pepsin in similar concentrations is instantly and almost irreversibly denatured by temperatures of over 70°C. or by alkali.

If the logarithm of the ratio of native to denatured pepsinogen at 25°C. is plotted against pH, a straight line with a slope of 2 is obtained. This agrees with the assumption that the equilibrium between native and denatured pepsinogen is expressed by the following reaction:

Native pepsinogen + $2OH^- \rightleftarrows$ Denatured pepsinogen $(OH^-)_2$ or

$$\log \frac{[\text{Pepsinogen}]}{[\text{Pepsinogen } (OH^-)_2]} = -2 \log [OH^-] + \log K.$$

Properties of pepsin and pepsinogen.—A summary of the elementary composition and other properties of pepsinogen and pepsin are shown in Table 17. The only significant difference in composition is the figure for amino nitrogen, which is much higher for pepsinogen than for pepsin. The isoelectric point of pepsinogen is more alkaline than that of pepsin. The molecular weight appears to be slightly higher, although the difference is hardly outside the experimental error.

The tyrosine and tryptophane content of pepsinogen is the same when measured at pH 11.0–12.0, but is less than pepsin at pH 8.0. This difference at pH 8.0 indicates, as a rule, a difference in the phenolic tyrosine groups, but in the present case the result is complicated by the fact that pepsin is denatured at pH 8.0 while pepsinogen is not. There is also a difference in specific optical rotation, which is less for pepsinogen.

Titration curves.—The titration curves of pepsin and pepsinogen are shown in Figure 29. Pepsinogen is converted to pepsin so rapidly below pH 5.0 that this part of the curve is of no significance and it is therefore impossible to fix the isoelectric point of pepsinogen on its titration curve. Likewise, in the case of pepsin from pH 6.0 or 7.0 on

toward the alkaline side, pepsin is present in the denatured form, while pepsinogen is present in the native form. The curves show that pepsin combines with more alkali between pH 6.0–9.0 in the region where it is denatured, whereas pepsinogen combines with more alkali between pH 9.0–12.0 in the region where it is denatured. The total groups combining with alkali over the whole range between pH 6.0–12.0 is the same in both proteins. Steinhardt (1937) has suggested that alkali inactivation of pepsin is the result of the ionization of a

TABLE 17

COMPOSITION AND PROPERTIES OF PEPSINOGEN AND PEPSIN

	Pepsinogen	*Pepsin*
C percent dry weight	52.8	51.7
H " " "	6.88	6.86
N " " "	14.40	14.7
P " " "	0.09	0.09
S " " "		0.94
Amino nitrogen as percent total nitrogen	4.0	1.4
Isoelectric point—pH	(2.5) denatured 3.7 cataphoresis 3.6–4.3 minimum precipitation	None
Molecular weight, osmotic pressure	42,000 ± 3,000	38,000 ± 3,000
Tyrosine plus tryptophane (percent dry weight as tyrosine)		
pH 8.0	3.2	5.0
pH 11.0–12.0	8.7	8.6
Optical rotation $[\alpha]_D^{25}$ per gm. dry weight	−61°	−71°

series of groups, and the titration curves obtained here agree with this mechanism.

Ultraviolet absorption spectra.—Direct inspection of the plates of ultraviolet absorption spectra of pepsin and pepsinogen show a very slight difference. One of the tyrosine absorption bands in pepsinogen appears to be slightly different from that in pepsin.

Immunological relation of pepsin and pepsinogen (Seastone and Herriott 1937).—The immunological reactions of pepsin are complicated by the fact that the protein is undoubtedly denatured almost immediately upon injection into the blood stream, and the antibodies

obtained, therefore, probably correspond to the denatured and not to the native protein. For this reason it is necessary to carry out precipitin reactions with the denatured form of the enzyme. It is known that denatured proteins are not as specific immunologically as native proteins, and this fact may explain some of the non-specific reactions observed. The results show that pepsin from swine, cattle, or guinea pig reacted with serum from a rabbit immunized with swine pepsin,

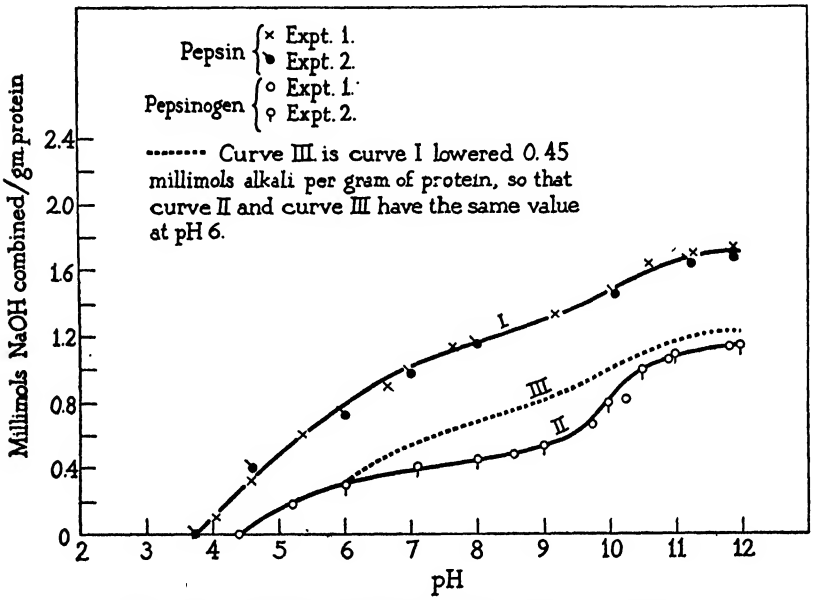


FIG. 29. Titration curves of pepsin and pepsinogen.

but pepsin from rabbit, chicken, and shark did not. There is evidently a species specificity in pepsin but it is not so highly specific as that found for the serum proteins. This broadening of the range of specificity may be due to the fact that it was necessary to use denatured protein. Pepsin anti-sera reacted with both pepsin and pepsinogen but did not react with the serum proteins from the same species of animals as the pepsin. The pepsin, therefore, is a foreign protein just as is the lens protein in the sense that it is immunologically distinct from the serum proteins. The cross reaction between pepsin and pepsinogen may again be due to the use of denatured antigen, since anti-pepsinogen sera do not react with pepsin. In this case the antigen is a

native protein. Pepsinogen anti-sera do not react with the serum proteins of the same species. Anti-sera made with serum proteins do not react with either pepsin or pepsinogen from the same species.

The results, in general, show that the enzyme and its precursor have two distinct specificities. They are immunologically distinct from each other and from the normal serum proteins and also from pepsin of other species. Thus, they show a species specificity and, in addition, a functional specificity analogous to those of the lens proteins.

FORMATION OF PEPSIN FROM PEPSINOGEN
(Herriott 1938b)

If pepsinogen solutions are made more acid than about pH 6.0, the pepsinogen is transformed into pepsin by an autocatalytic reaction. The results of an experiment at pH 4.6 are given in Figure 30. The hemoglobin activity $[P.U.]^{Hb}$ determinations represent the amount of pepsin found by difference from the amount of pepsinogen at the beginning and that present at any time. The gelatin activity, on the other hand, is a direct measure of the pepsin present. It may be seen that both methods give concordant values. Under these conditions determination of pepsin by the rennet method also gives values which agree with the two just mentioned. The figure also shows that the experimental determinations agree quite well with the theoretical curve calculated from the simple autocatalytic equation

$$\frac{dA}{dt} = KA(A_\infty - A).$$

A is the activity at the time, t, and A_∞ is the final activity. The rate of reaction depends upon the acidity, salt concentration, and pepsinogen and pepsin concentration. The results of varying these conditions may be briefly summarized as follows:

1. The addition of pepsin or increasing the concentration of pepsinogen increases the rate of reaction under all conditions. The reaction is therefore essentially autocatalytic throughout.
2. The reaction is catalyzed slowly by hydrogen ions.
3. The reaction has a maximum rate near pH 2.
4. Increasing salt concentration increases the rate of reaction on the alkaline side of pH 2.0 but decreases it on the acid side of pH 2.0.

5. In solution more acid than pH 4.5 or in the presence of high concentration of salt the pepsin appears in solution in combination with an inhibiting substance. This compound dissociates slowly in solutions more acid than pH 5.4 into free active pepsin and the inhibitor. On long standing with pepsin the inhibitor is destroyed.

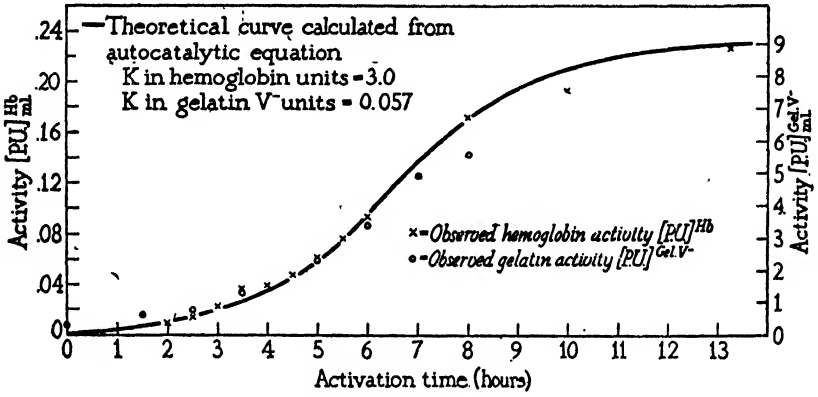


FIG. 30. Conversion of 1.0 mg. pepsinogen nitrogen per ml. into pepsin at pH 4.6 and 25°C.

The entire reaction, therefore, takes place as follows:

$$\text{Pepsinogen} \xrightarrow[\text{acid}]{\text{(Pepsin)}} \text{Pepsin-Inhibitor Compound} \underset{\text{pH} > 5.4}{\overset{\text{pH} < 5.4}{\rightleftharpoons}} \text{Pepsin} + \text{Inhibitor} \downarrow \text{X}$$

The presence of the inhibitor causes the kinetics of the reaction to differ in some instances from that predicted by the simple autocatalytic equation.

The inhibiting substance may be separated from pepsin. It is soluble and stable in boiling trichloracetic acid, insoluble in 0.8 saturated ammonium sulfate, and dialyzes slowly through collodion (cf. page 88).

Chemical changes accompanying the formation of pepsin from pepsinogen.—The fact that the reaction is autocatalytic shows that it is caused by the pepsin itself. With ordinary proteins the action of pepsin results in the hydrolysis of the protein into a number of smaller

molecules so that the substrate protein is completely destroyed. In the present reaction this is not the case. There is only a slight decrease in the total protein and the final result is the disappearance of the protein, pepsinogen, and the appearance of a new protein, pepsin. At the same time about 15 percent of the nitrogen appears in non-protein form. If the pepsinogen is really a single chemical individual, it follows that this non-protein nitrogen must be split off from the pepsinogen molecule during the course of the reaction.

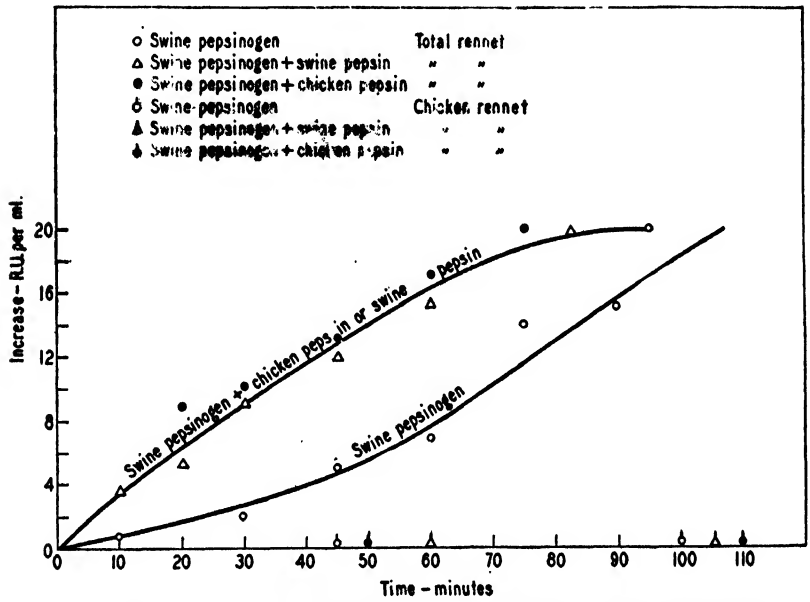


FIG. 31. Effect of the addition of chicken pepsin on the formation of pepsin from swine pepsinogen.

It could be assumed that the pepsinogen contains a small amount of an inert protein in spite of the results of the tests for purity and that the non-protein nitrogen results in hydrolysis of this inert protein. It may be shown, however, that this explanation is unlikely. In the first place, when activation is carried on at different pH, the amount of non-protein nitrogen formed is the same and reaches its maximum value in all cases at the same time as the pepsin activity reaches its maximum value, showing that the formation of the non-

protein nitrogen is affected in the same way and to the same extent by changes in the pH as is the formation of pepsin from pepsinogen. The possibility that the non-protein nitrogen comes from a secondary reaction between pepsin and pepsinogen may be ruled out by adding pepsin to the activation mixture. If the pepsinogen were digested by pepsin this experiment would result in an increase in non-protein nitrogen. As a matter of fact, the amount of non-protein nitrogen formed under these conditions is the same as that formed without the addition of excess pepsin.

Transformation of swine pepsinogen into swine pepsin by chicken pepsin (Herriott, Bartz, and Northrop 1938).—The fact that the formation of pepsin from pepsinogen is an autocatalytic reaction raises the question as to what form of pepsin will be obtained if pepsinogen of one species is activated by pepsin from another. It has been found that chicken pepsin differs strikingly from swine pepsin in that it is very slowly inactivated by standing in solution at pH 8.0–9.0, whereas swine pepsin is very rapidly inactivated under these conditions. It is therefore possible to distinguish between swine and chicken pepsin. If swine pepsin or chicken pepsin is added to a solution of chicken pepsinogen, the rate[1] of formation of pepsin from the pepsinogen is accelerated but in either case chicken pepsin is formed no matter whether the reaction is accelerated by chicken pepsin or swine pepsin. Similarly, if swine pepsinogen is activated by swine pepsin or chicken pepsin, only swine pepsin is formed (Figure 31). The species specificity of the enzyme is therefore present in the precursor

[1] The velocity constant, however, is smaller when pepsin is added than in the control solution with pepsinogen alone; not the same, as stated in the original paper (Herriott, Bartz, and Northrop 1938). This discrepancy was called to the writer's attention by Dr. C. D. Coryell. It is due to the fact that the total (final) pepsin concentration in the experiments to which pepsin was added is twice that of the solution of pepsinogen alone, and the velocity constant, therefore, cannot be compared to the value for the more dilute solution since this reaction, like most enzyme reactions, does not agree with the simple kinetic theory in regard to changes in concentration (Herriott, 1938b). The autocatalytic reaction equation predicts that doubling the total concentration will double the *rate* of reaction and therefore give the same value for the velocity constant. Actually, increasing the total concentration has less effect on the reaction *rate* than is predicted by theory (Herriott 1938b) and therefore gives a lower velocity constant as the concentration increases.

If the reaction rate of various mixtures of pepsinogen and pepsin, all of the same total concentration, are compared it is found that all give the same velocity constant, showing that the reaction, at constant concentrations, agrees with the autocatalytic equation and that pepsin itself is the catalyst.

and is not modified by the transformation of the precursor into the active enzyme. As described above, however, the enzyme and its precursor are immunologically distinct proteins, so that a new specificity is conferred upon the protein when it is formed from the precursor without, however, affecting its species specificity.

IV: PEPSIN INHIBITOR

(Herriott 1941b)

THE experiments reported in the preceding chapter show that a substance which inhibits pepsin is formed during the transformation of pepsinogen to pepsin. The present chapter describes the isolation, crystallization, and properties of this substance.

Preparation of the inhibitor consists of activation of the pepsinogen at pH 1.0–2.0 for a very short time followed by rapid alkali inactivation of the pepsin. Precipitation of the denatured pepsin is brought about with trichloracetic acid. The inhibitor remains dissolved in the trichloracetic acid filtrate. The total nitrogen in the trichloracetic acid filtrate is about 15 percent of the original pepsinogen nitrogen; half of this non-protein nitrogen is inhibitor nitrogen. Separation of the inhibitor from the inert polypeptides has been accomplished by repeated fractional precipitation first with tungstic acid at pH 1.0–2.0 and second by magnesium sulfate in the presence of trichloracetate ion at about pH 3.0. When fractionation has brought the specific inhibiting activity, i.e. the inhibiting activity per milligram nitrogen $[I.U.]_{mg.N}$ to above 60 percent of the value of the pure inhibitor, the preparation may be crystallized. Half saturated ammonium sulfate, pH 5.0, room temperature, and 3.0–5.0 mg. of inhibitor nitrogen per ml. are the conditions required for crystallization. The material first precipitates as clear spheroids which on standing form rosettes of fine needles as may be seen in Figure 32.

Fractional recrystallization and solubility experiments indicate the presence of not more than 20–25 percent impurity in the material of highest specific activity.

The inhibitor is destroyed by pepsin between pH 2.0–5.0, with a rate maximum near pH 4.0.

The reversible combination of pepsin with the inhibitor follows quantitatively the simple mass law equation derived for a similar reaction.

FIG. 32. Crystalline pepsin inhibitor. × 1590.

The proteolytic action of pepsin as well as the milk clotting action is inhibited at pH 5.7. Dissociation of the pepsin inhibitor complex prevents tests at a more acid pH. There was no demonstrable effect of the inhibitor on crystalline trypsin as measured by the digestion of hemoglobin at pH 7.0–8.0, nor the milk clotting action of crystalline chymo-trypsin or commercial rennet at pH 5.7. The crystalline trypsin inhibitor (Kunitz and Northrop 1936) had no effect on the milk clotting action of pepsin. This indicates a high degree of specificity among the inhibitors and is additional proof that the enzyme, rennet, is different from pepsin. An interesting result was obtained when pepsin from different species was tested with swine pepsin inhibitor. Bovine pepsin was inhibited to the same degree as swine pepsin but chicken pepsin was not inhibited at all. On the other hand, crude inhibitor solution prepared from chicken pepsinogen inhibited both swine and bovine pepsin but had no effect on the chicken pepsin (see Table 18).

Certain chemical and physical properties have been determined, such as the isoelectric point, optical rotation, elementary analysis, amino nitrogen, and rate of diffusion. A few amino acids making up the inhibitor have been roughly estimated as well as the number of peptide linkages. The indications are that the inhibitor has basic groups exposed since it is precipitated by many reagents used to precipitate basic substances, namely, tungstic, phosphotungstic, flavianic, picric, and picrolonic acids. The main basic amino acid is probably arginine. The molecular weight, as determined by diffusion and combining equivalence with pepsin, lies somewhere between 4,000 and 10,000.

TESTS OF PURITY

Fractional recrystallization.—An inhibitor preparation was fractionally recrystallized to see if there was any drift in properties indicating the presence of impurities. No significant change in properties was found.

Solubility curve.—The solubility curve shown in Figure 33 indicates that the preparation having a specific inhibitory value of 0.9 probably contains not more than 25 percent impurity.

INACTIVATION OF INHIBITOR BY PEPSIN

It was soon found that whereas pepsin free inhibitor solutions are stable for long periods of time at acidities varying from pH 1.0–10.0, in the presence of pepsin the inhibitor is rapidly inactivated with a pH maximum near pH 3.5 (Figure 34). There is an increase in amino nitrogen, as measured by Van Slyke's gasometric method, amounting to approximately 8 percent of the total nitrogen or about 5 amino groups per molecule, assuming a molecular weight of 5,000 for the inhibitor.

These experiments are strong evidence that the inactivation of the inhibitor is in fact an hydrolysis catalyzed by pepsin. In this connection it might be pointed out that the pH maximum at pH 3.5–4.0 shown in Figure 34 is close to that found by Fruton, Bergmann, and Anslow for the hydrolysis of their synthetic substrates by pepsin (1939).

APPLICATION OF THE MASS LAW TO THE COMBINATION REACTION OF PEPSIN AND INHIBITOR

Some evidence has been obtained to indicate that the combination of pepsin with the inhibitor is a simple reversible dissociation as illustrated in equation I

$$\text{Pepsin} + \text{inhibitor} \rightleftharpoons \text{inhibitor pepsin compound} \qquad (I)$$

This type reaction should follow the mass law which in its simplest form is equation II

$$\frac{[\text{Pepsin}] \times [\text{inhibitor}]}{[\text{Pepsin-inhibitor compound}]} = \text{constant} \qquad (II)$$

where the values within brackets are concentrations. In using the simplest form it is assumed that one molecule of pepsin reacts with one molecule of inhibitor.

Equation II cannot be used as such but may be rearranged (Northrop 1925–26) so that it will contain terms that are easily measurable. The equation used in the present work is the same as that used by Northrop with a few minor changes in symbols and is equation III,

$$P_f = \pm \sqrt{\left(\frac{I_t - P_t + K}{2}\right)^2 + KP_t} - \frac{I_t - P_t + K}{2} \qquad (III)$$

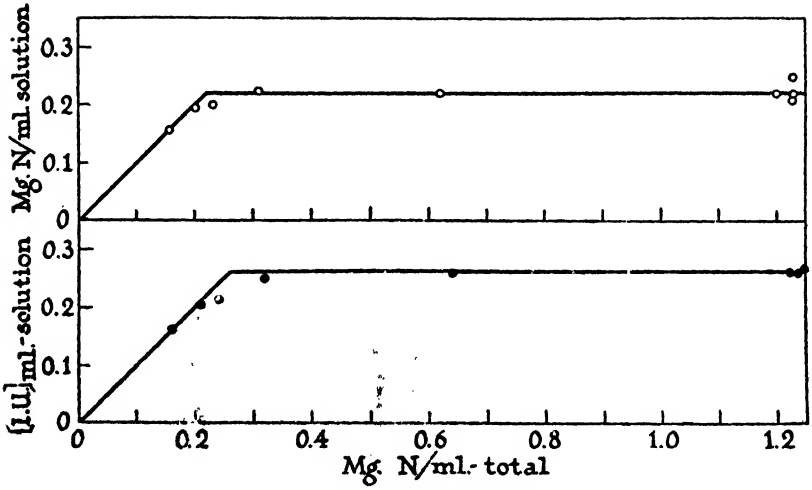


FIG. 33. Solubility curve of amorphous inhibitor in a magnesium sulfate-trichloracetate solution at 23°C.

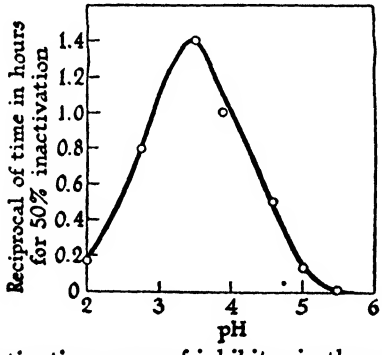


FIG. 34. pH-inactivation curve of inhibitor in the presence of pepsin.

where P_f = free pepsin; P_t = total pepsin; I_t = total inhibitor expressed in terms of pepsin units; and K = the constant for the equation.

In order to use this equation, the total inhibitor concentration used must be constant and the total pepsin concentration varied. The free

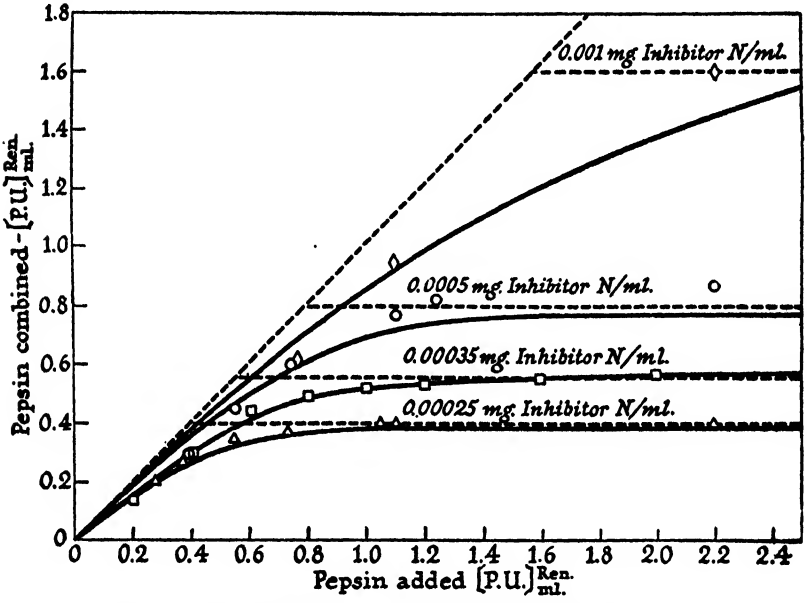


FIG. 35. Effect of increasing amounts of pepsin and inhibitor on the amount of pepsin bound by the inhibitor at pH 5.7. The points are the determined values. The solid lines are the theoretical curves calculated from the mass law as indicated in the text. The broken lines indicate the course if the reaction were stoichiometric.

or uncombined pepsin is determined and the combined pepsin obtained by the difference between the total and free pepsin. It is also necessary to make certain assumptions which, as nearly as could be tested, are valid, namely, that the pepsin inhibitor compound has no activity and that on addition of the compound into the Klim solution there is no appreciable dissociation due to dilution before the Klim is clotted.

When the amount of total pepsin was plotted against the combined pepsin a smooth curve was obtained, as seen in Figure 35, which approached a limiting value depending on the amount of inhibitor used.

Since the above equation (III) calls for the inhibitor in terms of pepsin units, this limiting value or maximum value of pepsin to combine with the indicated amount of inhibitor can be substituted for the inhibitor concentration. In other words, the total inhibitor is expressed as that amount of pepsin with which the inhibitor will combine when there is a large excess of pepsin.

In the present instance the pepsin equivalent value for the inhibitor was obtained only from curve I; i.e., 0.00025 mg. inhibitor nitrogen was equivalent to 0.4 pepsin rennet units. The pepsin equivalent values for the inhibitor in curves II, III, and IV were calculated from curve I. This was possible since the amount of inhibitor nitrogen used in these curves was predetermined.

The solid lines are the calculated curves obtained by calculating back with the equation using an average value of the constant K.

It may be seen in Figure 35 that the experimental points show a reasonable approach to the calculated curves.

If one takes the figures obtained from the above experiment, namely that 0.00025 mg. inhibitor nitrogen is equivalent to 0.4 rennet units of pepsin or approximately 0.0012 mg. pepsin nitrogen, one may then calculate the molecular weight of the inhibitor. Such a calculation has been performed and it indicates a molecular weight of about 6,000.

TABLE 18

REACTION OF INHIBITORS FROM DIFFERENT SPECIES WITH DIFFERENT PEPSINS

Source of Inhibitor	*Enzyme*	*Substrate*	*pH*	*Inhibiting Action*
Swine pepsinogen	Swine pepsin	Klim (milk clotting action)	5.7	+
" "	" "	Denatured pepsin	5.7	+
" "	" "	" hemoglobin	5.7	+
" "	Bovine "	Klim (milk clotting action)	5.7	+
" "	Chicken "	" " " "	5.7	−
Chicken "	Swine "	" " " "	5.7	+
" "	Bovine "	" " " "	5.7	+
" "	Chicken "	" " " "	5.7	−
Bovine trypsin inhibitor	Swine "	" " " "	5.7	−
Swine pepsinogen	Bovine chymo-trypsin	" " " "	5.7	−
" "	" rennet	" " " "	5.7	−
" "	" trypsin	Denatured hemoglobin	7.6	−

CHEMICAL AND PHYSICAL PROPERTIES

Some of the chemical and physical properties of the purified inhibitor $[I.U.]_{mg.N} = 0.95$, have been collected together in Table 19.

In Table 19 *B* are a few amino acid analyses along with certain other analyses and certain values deduced from them. For instance, assuming the molecular weight to be 5,000 there are then 57 atoms of nitrogen per molecule of inhibitor. In the intact inhibitor there are 8

TABLE 19

CHEMICAL AND PHYSICAL PROPERTIES OF PEPSIN INHIBITOR

A

Property	*Method of Analysis*	*Percent of Moisture Free Material*
C		48.07
H		8.07
N	Dumas	16.65
N	Kjeldahl	16.7
Ash		0.5
$[\alpha]_D^{pH\ 7}$ gm.		−104°
Diffusion constant at 10°C.	Northrop and Anson	0.09 cm.²/day
I. E. P.	Cataphoresis of collodion particles	pH 3.7
Molecular weight	Diffusion	8,000
Molecular weight	Combining equivalent with pepsin	6,000

B

		Percent of Total Nitrogen	*No./ Molecule**
Primary amino N	Van Slyke gasometric	18	8–9
Amide N	Alkaline distillation after acid hydrolysis	6.5	3
Non-amino N	Difference between total N and NH_2N after acid hydrolysis	23	19
Peptide linkages	Difference between total NH_2-N after hydrolysis and original NH_2-N plus amide N		27
Arginine	Sakaguchi	31	8
Tryptophane	May and Rose	0	0
Tyrosine	Folin phenol	0.4	0.1

* Assuming a molecular weight of 5,000.

free amino nitrogens while after acid hydrolysis there are 38. It follows therefore that there has been an increase of 30 amino groups but 3–4 of this increase of amino groups was found to be the amide nitrogen which on acid hydrolysis yields ammonia. Therefore there are a possible 26 peptide linkages. There must also be some 19 non-amino nitrogens. The arginine content of 31 percent represents about 7–8 molecules of arginine per inhibitor. Since 3 of the 4 nitrogens in the arginine molecule are non-amino all of the non-amino nitrogen can be explained by the arginine content. The tyrosine content determined by the Folin phenol reagent was so low that it must be an impurity. The tryptophane test of May and Rose is negative.

It seems very likely that the inhibitor has a number of strongly basic groups exposed or it is precipitated from dilute solution practically quantitatively by tungstic, phosphotungstic, flavianic, picric, and picrolonic acids, all of which are supposed to precipitate basic substances. The relatively high content of arginine would tend to substantiate this.

V: CHYMO-TRYPSINOGEN AND CHYMO-TRYPSIN

IT HAS been known since the time of Corvisart and Kühne that pancreatic juice possesses the property of digesting proteins. Kühne assumed that this property was due to the presence in the juice of an "unorganized ferment" or enzyme which he called trypsin.

Subsequent work by Fischer and Abderhalden, Cohnheim, Bayliss, Vernon, Schaffer and Terroine, Willstätter and Waldschmidt-Leitz, and their collaborators has added greatly to our knowledge of the enzymatic properties of pancreatic juice, and it is now known that several proteinases and several peptidases are present. The name "trypsin" has been retained to designate the most important proteinase (Northrop and Kunitz 1932a).

Kühne and Heidenhain showed that extracts of fresh pancreas or freshly secreted pancreatic juice have no proteolytic activity. The preparations become active when mixed with the contents of the small intestine, as found by Schepowalnikow, or when the pancreas is allowed to stand in slightly acid solution. The mechanism of this activation has been the subject of controversy for many years. Pavlov, Bayliss, Zunz, Wohlgemuth, Vernon, Delezenne, and others found the activation reaction to be catalytic and considered the activator present in the intestine (enterokinase) to be an enzyme. Hamburger, Hekma, Dastre, and Stassano and Waldschmidt-Leitz found the reaction to be stoichiometric and considered that the enterokinase formed an addition compound with the inactive zymogen. Vernon found that activation could be caused by trypsin as well as by enterokinase, but this was denied by Bayliss and Starling. The contradictory nature of the numerous experimental results indicates that there is more than one proteolytic enzyme in pancreatic extracts and more than one method of activation. Vernon showed that the activity as determined by the clotting of milk, could be partially separated

from the proteolytic activity, as determined by protein hydrolysis, and concluded that there were at least two enzymes. He also showed that one of these was more stable than the other and that activation was caused by the less stable one.

In contrast to the marked advances in knowledge of the properties of the enzymes of the pancreatic juice, as shown by their catalytic effect in various reactions, little or no knowledge was obtained until recently as to the chemical nature of these enzymes. The early workers, Kühne, Mays, Hammarsten, and Michaelis, considered them to be associated with the nucleic acid fraction, but Levene showed that they were not nucleic acids themselves, since hydrolysis of the nucleic acids did not destroy the activity.

The methods used in the present work were those known to be favorable for the isolation of proteins, i.e., concentrated solutions in concentrated neutral salt and low temperatures. These methods have led to the isolation of three crystalline enzymes—trypsin, chymotrypsin, and carboxypeptidase—and to the isolation and crystallization of the inactive precursor of trypsin (trypsinogen) and of chymo-trypsin (chymo-trypsinogen). A compound which has a very powerful inhibiting effect on trypsin has also been isolated and crystallized. It is not a protein but is composed of amino acids and has a molecular weight of about 5,000.

Trypsin and chymo-trypsin are the enzymes principally responsible for the proteinase activity of pancreatic juice. Neither alone digests protein very far, but the two together cause hydrolysis to proceed to the polypeptide stage. Trypsin accelerates the clotting of normal or hemophilic blood but under ordinary conditions does not clot milk. Chymo-trypsin, on the other hand, clots milk but not blood. It probably represents the pancreatic rennin of Vernon. Trypsin appears to be identical in its specificity with Waldschmidt-Leitz's "proteinase."

Bergmann and Fruton (1937) described the existence of a third proteolytic enzyme in pancreatic extracts which they called "heterotrypsin." Further experiments, however, showed that "heterotrypsin" was a partially purified preparation of crystalline trypsin (Hofmann and Bergmann 1939). There is no evidence, therefore, for the existence of "heterotrypsin."

PREPARATION OF CHYMO-TRYPSINOGEN, CHYMO-TRYPSIN, TRYPSINOGEN, AND TRYPSIN

Kunitz & Northrop (1935, 1936)

An outline of the methods of preparation of chymo-trypsinogen (Figure 36) chymo-trypsin (Figure 37), trypsinogen, and trypsin is shown in Table 20. The success of the method depends largely on the sharp fractionation obtained in the first extraction with 0.25 N sulfuric acid. Most of the mucin and inert proteins are denatured and rendered insoluble by acid of this strength and are thus removed in the first operation.

PURITY OF CHYMO-TRYPSINOGEN

Chymo-trypsinogen is a stable protein and furnishes excellent material for a rigorous test of the application of the phase rule to protein solutions (cf. Appendix). The following experiments were undertaken in order to establish whether or not a protein could be prepared which satisfied strictly the criteria for a single phase of one component as defined by the phase rule. The results (Butler 1940) show that this protein does behave as a single component.

The data for the solubility curve previously reported (Kunitz and Northrop 1938b) were obtained by means of turbidity measurements. This procedure is open to the objection that non-protein impurities may be overlooked.

In the experiments described below the total N content of the solutions was used as a measure of the protein concentration since this is the most accurate method known (Butler 1940, page 195).

Under these circumstances ammonium sulfate solutions cannot be used as the solvent and magnesium sulfate was substituted. The first experiments showed that when recrystallized seven or eight times from magnesium sulfate, this material gave quite good solubility curves, the solubility being practically independent of the amount of solid phase from the first appearance of turbidity, but the actual solubility varied somewhat from one preparation to another, and the residue left after dissolving part of the substance had an appreciably different solubility. This suggested that the material might be a solid solution and in an attempt to effect a separation a series of fractional crystallizations was carried out on twice-crystallized material.

FIG. 36. Chymo-trypsinogen crystals. × 260.

FIG. 37. Chymo-trypsin crystals. × 120.

TABLE 20

ISOLATION OF CHYMO-TRYPSIN AND TRYPSIN FROM BEEF PANCREAS

Remove pancreas from cattle within 1 hour after slaughter, immerse in cold N/4 H_2SO_4, drain off acid, mince, suspend for 24 hours in 2 volumes N/4 H_2SO_4 at 5°C. Strain through gauze.

Acid extract of pancreas 7 liters

Add solid $(NH_4)_2SO_4$ to 0.4 saturation.

Filter

Filtrate

Add solid $(NH_4)_2SO_4$ to 0.7 saturation. 2 days at 5°C

Filter

Precipitate
100 grams

Dissolve in H_2O. Refractionate between 0.4 and 0.7 sat. $(NH_4)_2SO_4$.

Filter

Precipitate from 0.7 sat. $(NH_4)_2SO_4$

90 grams

Dissolve in 0.25 sat. $(NH_4)_2SO_4$. Adjust to pH 5.0, 2 days at 25°C.

Filter

Crystals of chymo-trypsinogen 25 grams	Filtrate
Recrystallize in 0.25 sat. $(NH_4)_2SO_4$ pH 5.0.	Adjust to pH 4.0, precipitate in 0.7 sat. $(NH_4)_2SO_4$ and refractionate between 0.4 and 0.7 sat. $(NH_4)_2SO_4$.
Filter	Filter
8 × Recrystallized chymo-trypsinogen	Precipitate from 0.7 sat. $(NH_4)_2SO_4$ 40 grams
Dissolve in N/50 H_2SO_4. Adjust to pH 7.6. Add trace of trypsin, 2 days at 5°C.	Wash with sat. $MgSO_4$. Dissolve in 0.4 M borate pH 9.0, cool to 5°C. Bring to 0.5 sat. $MgSO_4$, 3 days at 5°C.
Solution of chymo-trypsin	Filter
Adjust to pH 4.0. Salt out in 0.7 sat. $(NH_4)_2SO_4$.	Filtrate (See Table 35)
Filter	Crystals of trypsinogen 10 grams
Amorphous precipitate of chymo-trypsin 5 grams	Wash with 0.5 sat. $MgSO_4$. Dissolve in N/50 H_2SO_4. Bring to 0.5 sat. $MgSO_4$. Add 0.4 M borate pH 9.0, 1 day at 5°C.
Dissolve in N/100 H_2SO_4, 24 hours at 25°C.	Filter
Filter	Crystals of trypsin 6 grams
Chymo-trypsin crystals	

In this first series a solution of the protein was prepared at pH 4.0 and sufficient magnesium sulfate was added to cause the crystallization of about one third of the total protein. The addition of more magnesium sulfate gave a further crop of crystals and the remainder was precipitated from a nearly saturated solution of the salt. This process was repeated on the fractions five times, the "less soluble" precipitate from one fraction being systematically united with the "more soluble" precipitate from the next. In this way three fractions were obtained, *A*, *B*, and *C*; *A* being obtained from the first precipitates and *B* and *C* from the middle and last fractions. The three fractions were crystallized twice from a pH 4.0, 0.4 saturated magnesium sulfate solution and the solubility curves were determined with this solvent. The curves obtained are shown in Figure 38. Both *B* and *C* gave very good solubility curves, the solubility being practically constant from the first appearance of turbidity, but the solubility of *C* is slightly greater than that of *B*. The curve of *A* has a distinct break and the solubility continues to rise after the first appearance of turbidity at *P* to the region of *Q*, indicating that this material is complex.

The whole fractionation was repeated with new material in a somewhat simplified form in the course of which the middle fraction (B) was divided between the two end fractions. In this way two fractions *A'*, *C'* were finally obtained. It was found that the solubility curve of *A'* was identical with that of *A* and *C'* with the previous fraction *C*.

Since *B* and *C'* were obtained by adding more magnesium sulfate to a saturated solution of protein in approximately the same solvent as that in which the solubility was determined, it was possible that these fractions might contain two substances present in the same proportion as their solubility in this solvent. Solubility curves of *C'* were therefore determined in two other solvents of pH 5.0 and 8.0, with suitable concentrations of magnesium sulfate (Figure 38, *d* and *e*). In both cases a good solubility curve was obtained and since it is very unlikely that the ratio of the solubilities of two substances would be identical at three different pH's, this may be taken as strong evidence that *C* and *C'* are a single substance.

That a true equilibrium was established in these experiments was shown by the following facts.

1. The solubility of the crystals in a given solvent was independent of the pH of the solution in which the crystals were formed. Figure 39

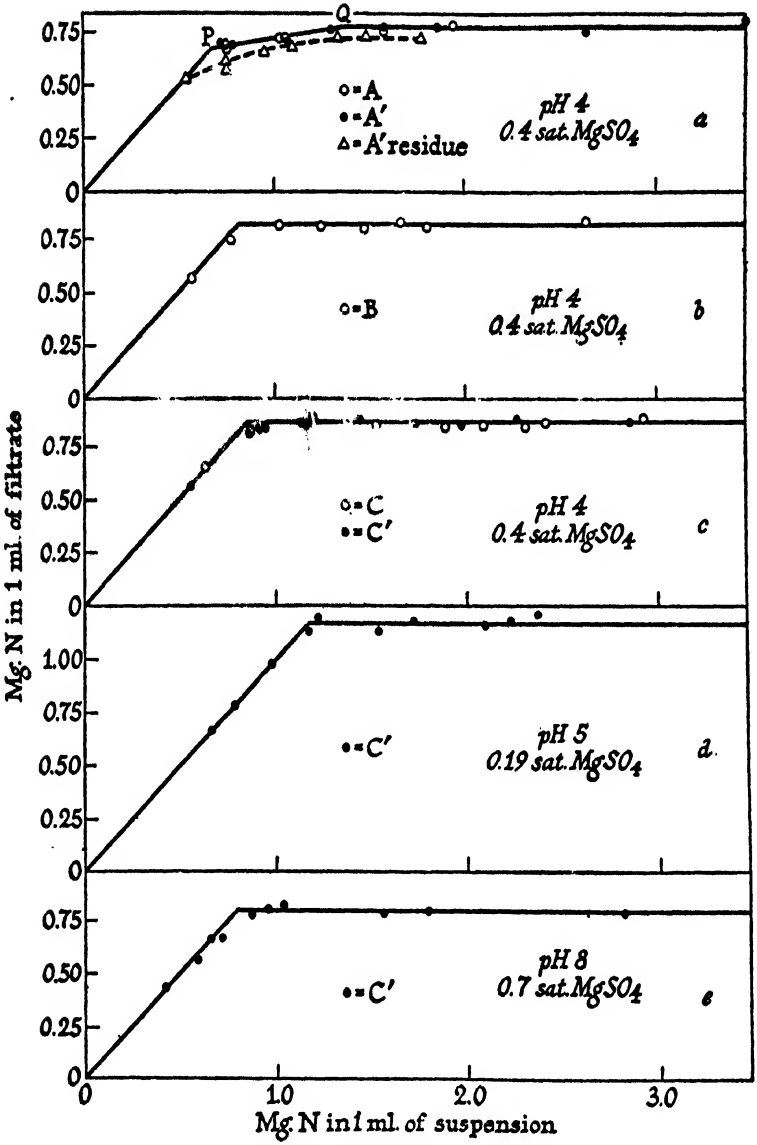


FIG. 38. Solubility curves of chymo-trypsinogen fractions in various solvents.

shows the solubilities of crystals formed in magnesium sulfate solutions at pH 4.0 and 5.0 in successive shakings with fresh portions of two distinct solvents. The crystals made at both pH's ultimately reached the same solubility in either solvent.

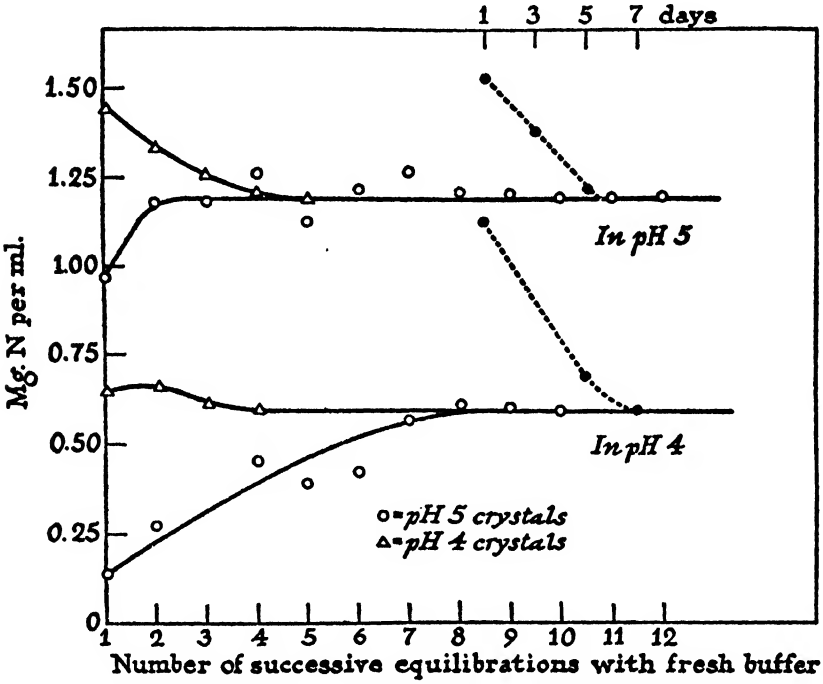


FIG. 39. Solubilities of crystals formed at pH 4.0 and pH 5.0. Triangles refer to crystals formed at pH 4.0, and open circles to crystals formed at pH 5.0. Closed circles represent successive concentrations in supersaturated solutions. The upper set of points is for solubilities in 0.19 saturated magnesium sulfate, pH 5.0 solvent; the lower set of points is for solubilities in 0.4 saturated magnesium sulfate, pH 4.0 solvent.

2. The solubility of the crystals was independent of the concentration of magnesium sulfate in the solution in which they were formed.

Concentration of magnesium sulfate:	0.35 saturated	0.4 saturated	0.5 saturated
Solubility in approximately 0.4 saturated magnesium sulfate at pH 4.0 (mg. N/ml.):	1.01	0.98	0.97

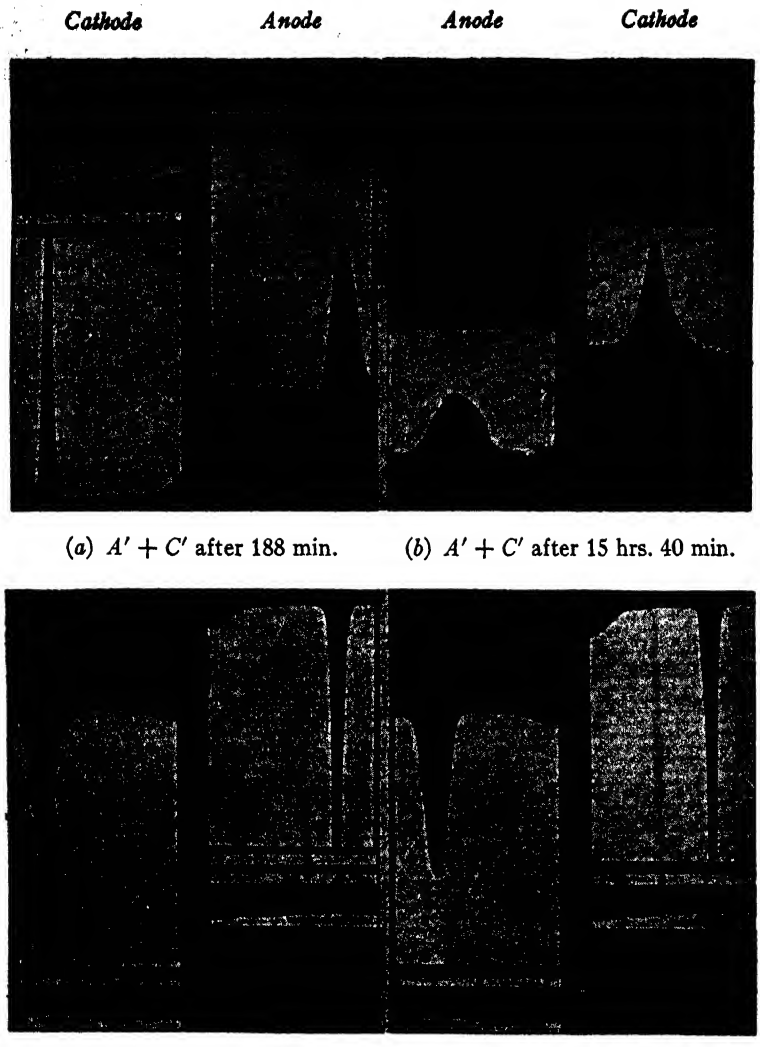
3. The same equilibrium was reached from supersaturated as from undersaturated solutions. The supersaturated solutions were prepared by cooling a saturated solution with excess of the crystals. More dissolves at the lower temperature and when the temperature is raised again a supersaturated solution is obtained. After several days the protein concentration returns to the original value (broken lines of Figure 39).

The material A, which has a complex solubility curve is less soluble than B and the solubility decreases on repeated extractions. It is probable that this material contains some substance which is precipitated at the smaller salt concentration and which forms a solid solution with the chymo-trypsinogen.

Apart from the solubility curves and a rather greater proportion of non-protein nitrogen in A, no significant difference between the fractions A' and C could be detected. When converted by trypsin into chymo-trypsin they had practically the same activities per milligram nitrogen, as measured both by milk clotting and by the digestion of hemoglobin, and the velocity constants of the activation process were the same within the experimental error. Since the accuracy of these measurements is of the order of 5 percent it can be concluded that the impurity in A and A', which is not present in C, does not contribute more than 5 percent of the total nitrogen, unless it is also capable of activation. It must be remembered that if the molecular weight of the impurity is low the molar fraction may be appreciable and sufficient to produce a significant change of solubility.

The impurity in this fraction is probably present in solution in the protein crystals. If it were a separate phase the solid residue in the region PQ, in which the solutions are saturated with the main constituent but the subsidiary constituent continues to dissolve (Figure 38*a*), should consist of the pure protein. Some of this residue was collected and its solubility curve, shown by the dashed line in Figure 38*a*, indicates a greater concentration of the impurity in the residue; as might be expected for a solid solution. The dialysis of the solutions of the fraction A' caused a small decrease in the amount of non-protein nitrogen, but had no significant effect on the solubility curve.

The electrophoresis of solutions A' and C' and of a mixture of A' and C' in an M/15 citrate-hydrochloric acid buffer solution at pH 3.0 after equilibration by dialysis with a large quantity of this buffer, was



(*a*) $A' + C'$ after 188 min. (*b*) $A' + C'$ after 15 hrs. 40 min.

Cathode *Anode* *Cathode* *Anode*

(*c*) A' after 151 min. (*d*) C' after 162 min.

FIG. 40. Electrophoresis patterns of fractions A' and C' and mixtures of both (Dr. A. Rothen).

Solution A'; pH 2.98, velocity in cathode compartment 0.675×10^{-4} cm.^{2}/sec. volt; gradient 4.09 volt/cm.

Solution C'; pH 2.98, velocity in cathode compartment 0.667×10^{-4} cm.^{2}/sec. volt; gradient 4.09 volt/cm.

Solution $A' + C'$: pH 3.0, velocity in cathode compartment 0.608×10^{-4} cm.^{2}/sec. volt; gradient 4.09 volt/cm. for 188 minutes, 2.04 volt/cm. for additional time.

kindly examined by Dr. Alexandre Rothen of the Rockefeller Institute for Medical Research in New York. The electrophoretic patterns obtained by him, using Longsworth's schlieren scanning technique, are shown in Figure 40.

In all cases only a single moving boundary was observed and the cathodic boundaries in every case were extremely sharp. The mixture of A' and C' showed no signs of any resolution even after more than fifteen hours' migration. The migration velocities were approximately the same in all solutions, the small differences being attributable to slight variations of the pH in the dialyzed solutions.

We conclude that no genuine fractionation of the protein has been achieved, but a small quantity of impurity has been concentrated in the fractions first precipitated. This is insufficient in quantity to produce any appreciable difference in the enzymatic properties and it does not produce a visible boundary in the electrophoretic pattern. But if the molecular weight of the impurity is comparatively low, its molar fraction in the crystals may be sufficient to produce the observed diminution of solubility of the protein and the break in the solubility curve. The final fractions satisfy every test of a pure substance which has been applied.

AMINO ACID CONSTITUENTS OF CHYMO-TRYPSINOGEN AND ITS MOLECULAR WEIGHT

Brand and Kassell (1941) have analyzed chymo-trypsinogen for several amino acids which may be accurately determined. The molecular weights calculated from these analyses are remarkably concordant (Table 21), and agree with that found by osmotic pressure.

TABLE 21

MOLECULAR WEIGHT OF CHYMO-TRYPSINOGEN CALCULATED FROM AMINO ACID CONTENT

Amino Acid, Percent	*Methionine*	*Half-Cystine*	*Protein Sulfur*	*Tyrosine*	*Tryptophane*
	1.22	4.59	1.48	2.96	5.51
No. of Residues per Mol	3	14	17	6	10
Molecular Weight	36,600	36,600	36,700	36,700	37,000

These results suggest that the discrepancies frequently found in protein analyses, especially in relation to molecular weight, like many other anomalous properties, are not due to any peculiarity of proteins but merely to the fact that the samples analyzed have not been homogeneous.

The general properties of the various preparations are summarized in Table 22.

The amino acid content of chymo-trypsinogen and of α, β, and γ chymo-trypsin is given in Table 4 (page 26). No characteristic value for the amino acid content, which would differentiate the enzymes from the proteins, is apparent.

FORMATION OF CHYMO-TRYPSIN FROM CHYMO-TRYPSINOGEN

Chymo-trypsinogen after recrystallization has a variable and barely measurable activity equivalent to about 1/10,000 that of chymo-trypsin. This activity is probably due to the presence of traces of chymo-trypsin, since the relative activity on various proteins agrees with that of chymo-trypsin.

Crude chymo-trypsinogen solutions (at pH 7.0–8.0) are rapidly activated by extract of small intestine (enterokinase), but cannot be activated by small amounts of trypsin. After one crystallization the protein can be activated either by enterokinase or trypsin. After repeated crystallization, however, the protein cannot be activated by enterokinase but only by trypsin. These variable and puzzling results are due to the following facts: Crude chymo-trypsinogen solutions contain traces of trypsinogen and also a substance which inhibits trypsin. When small amounts of trypsin are added to such preparations no activation of chymo-trypsinogen occurs since the trypsin added is inactivated, but when enterokinase is added, sufficient active trypsin is formed from the trypsinogen to overcome the inhibiting action of the solution and so activate the chymo-trypsinogen. The same result can be obtained by adding enough trypsin even in the presence of the inhibitor. The effect of repeated crystallization is simply to remove the last traces of these impurities, and the experiment is a good example of the efficiency of recrystallization as a method of purification.

The mechanism outlined above was confirmed by mixing pure

Table 22

Summary of the Properties of Chymo-Trypsinogen, Chymo-Trypsin, and Crystalline Trypsin

		Chymo-trypsinogen	Chymo-trypsin	Trypsin
Crystalline form		*Long, Square Prisms*	*Rhombo-hedrons*	*Short Prisms*
Elementary analysis percent dry weight	Carbon	50.6	50.0	50.0
	Hydrogen	7.0	7.06	7.1
	Nitrogen	15.8	15.5	15.0
	Chlorine	0.17	0.16	2.85
	Sulfur	1.9	1.85	1.1
	Phosphorus	0	0	0
	Ash	0.1	0.12	1.0
Amino nitrogen as percent total nitrogen	By formol	4.7	6.0	9.3
	By Van Slyke	4.75	6.0	
Tyrosine + tryptophane equivalent milli-equivalents/mg. total nitrogen		2.5×10^{-3}	2.7×10^{-3}	3×10^{-3}
Optical activity, 25°C.		In M/10 acetic acid		pH 4.0 in 0.25 sat. ammonium sulfate
$[\alpha]D$ line, per mg. nitrogen		−0.48	−0.40	−0.27
Solubility in distilled water		Slight	Very soluble	Very soluble
		In M/2 K_2SO_4, pH 4.0	*M/10 Acetate*	*In 0.5 Sat. $MgSO_4$*
Diffusion coefficient, 6°C. cm.²/day	By nitrogen	0.039	0.037	0.023
	By hemoglobin		0.039	
	By rennet		0.037	
Molecular volume from diffusion coefficient, cm.³/mol		52,000	52,000	65,000
Molecular weight from osmotic pressure		36,000 (32,000)	41,000	36,500
Hydration, gm. water/gm. protein, from osmotic pressure and diffusion coefficient		0.7	0.5	0.8
By viscosity		0	0.1	0.6
Isolectric point from cataphoresis of collodion particles		5.0	5.4	7–8
Specific activity [T.U.] per mg. protein nitrogen	Substrate			
	Hemoglobin	$<1 \times 10^{-5}$	0.04	0.17
	Casein, sol.	$<1 \times 10^{-5}$	1.0	2.4
	Casein, F.	<0.01	0.08	0.18
	Gelatin V.	<0.1	12.0	100
	Rennet	<0.01	8.5	<0.1
	Clot blood	<2.0	<2.0	1500
	Sturin F.	$<2 \times 10^{-4}$	0.018	0.63
pH optimum for digestion casein			8–9	8–9
Total digestion casein, ml. M/50 sodium hydroxide/5 ml. 5 percent casein			17	9–11

crystalline chymo-trypsinogen with the mother liquor from the first crystallization and adding trypsin or enterokinase. The results of such an experiment are the same as though a crude chymo-trypsinogen solution were used. The inhibiting effect of the mother liquor may also be demonstrated directly by its effect on the digestion of hemoglobin by trypsin.

KINETICS OF ACTIVATION OF CHYMO-TRYPSINOGEN BY CRYSTALLINE TRYPSIN

Effect of pH.—The effect of the pH of the solution on the rate of activation of chymo-trypsinogen by trypsin is shown in Figure 41. The curve resembles that for the effect of pH on the digestion of casein by trypsin and indicates that the reaction is related to the usual hydrolytic action of trypsin. However, no evidence for any actual cleavage of the chymo-trypsinogen molecule could be found (cf. page 112).

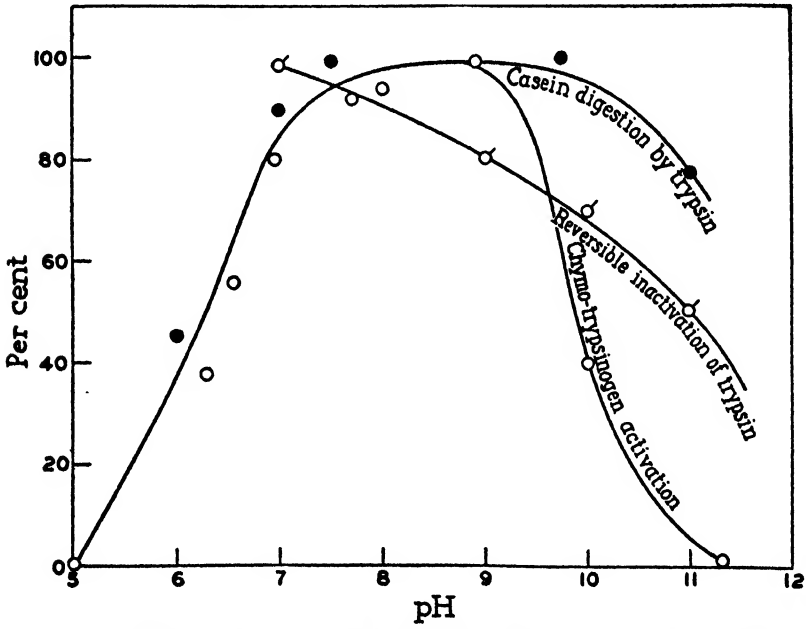


FIG. 41. Effect of pH on rate of activation of chymo-trypsinogen (5°C.) by trypsin compared with the effect of pH on rate of digestion of casein by trypsin and the effect of pH on reversible inactivation of trypsin.

Effect of the concentration of trypsin.—The activation follows the course of a monomolecular reaction, and the rate is proportional to the concentration of trypsin added. This result is shown in Figure 42, in which the logarithm of the percent of the chymo-trypsinogen remaining at any time is plotted against the time. The resulting curves are all straight lines, showing that the reaction is monomolecular. The slopes of the curves are proportional to the concentration of trypsin present, showing that the rate of reaction is proportional to the trypsin concentration.

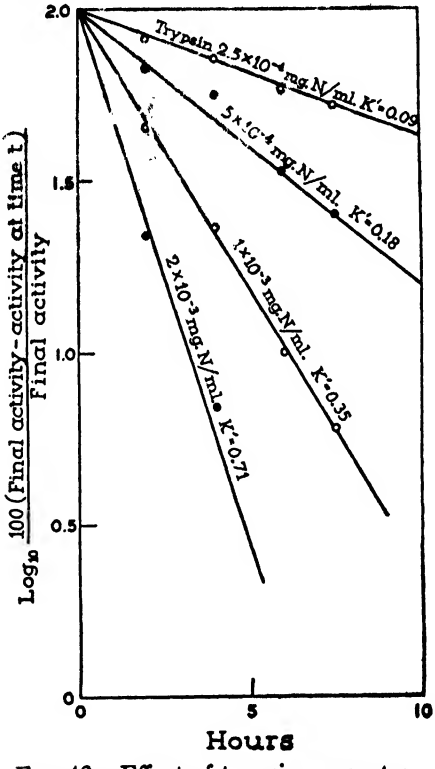


FIG. 42. Effect of trypsin concentration on rate of activation of chymo-trypsinogen.

Effect of the chymo-trypsinogen concentration.—The percent of the chymo-trypsinogen activated at any time is constant and independ-

ent of the concentration of chymo-trypsinogen. The activation of chymo-trypsinogen by trypsin, therefore, is expressed by the equation

$$-\frac{dG}{dt} = KTG$$

in which T equals concentration of trypsin and G equals concentration of chymo-trypsinogen, or on integration

$$KT = \frac{1}{t} \ln \frac{G_0}{G_t}$$

KT is the observed velocity constant (K^1) for any one concentration of trypsin. The value of K for unit trypsin concentration may be calculated and is found to be 2.1×10^6 per hour per mol trypsin per liter or 360 per hour per milligram trypsin nitrogen per milliliter.

In the preceding experiments the activity was determined by the rate of digestion of hemoglobin. The active enzyme clots milk and digests sturin so that the rate of activation was followed also by the rennet action and by sturin digestion. The percent of activation at any time, as determined by these three methods, is the same. This result indicates that these various substrates are all attacked by the same enzyme.

Changes in non-protein nitrogen during formation of chymo-trypsin. —There is a slight increase in non-protein nitrogen during this reaction, but the appearance of this non-protein nitrogen does not parallel the increase in activity. It is probable, therefore, that the production of these non-protein compounds is a secondary reaction caused by the gradual autolysis of the chymo-trypsin. The total amount of non-protein nitrogen found amounts to less than 10 percent of the total nitrogen.

ACTIVATION ENERGY OF THE FORMATION OF CHYMO-TRYPSIN FROM CHYMO-TRYPSINOGEN

(Butler 1941a)

Determinations of the velocity constant have been made at several temperatures from which it is found that the activation energy has the comparatively high value of 16,300 calories. The reaction is abnormally rapid for this energy and the significance of this fact is discussed in connection with the kinetics of trypsin (page 144).

Some experiments designed to elucidate the nature of the reaction also were carried out. There is an increase in the formol titration in the course of the reaction (Figure 43) which parallels the growth of enzymatic activity at the beginning but continues to increase slowly when the conversion is completed. It follows that the conversion is accompanied by a small amount of some secondary action, as has also been found in other experiments. The primary increase of the formol titration is most simply interpreted as being due to the liberation of acid groups produced by the splitting of peptide bonds.

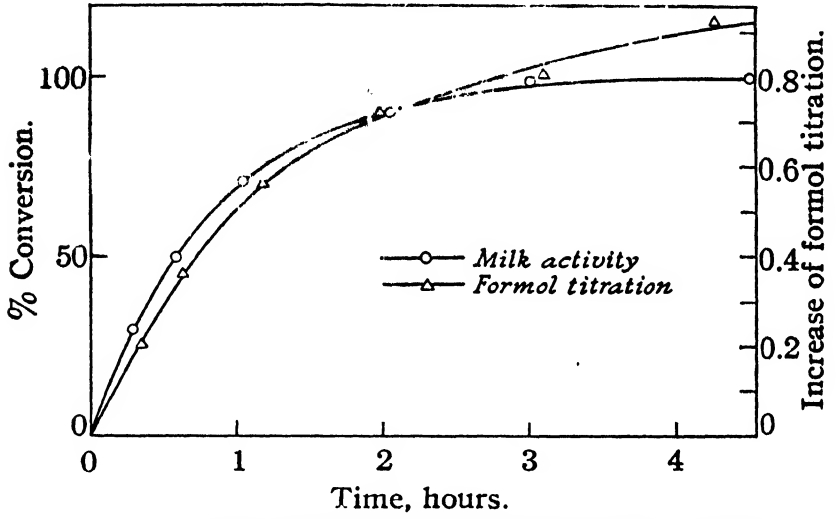


FIG. 43. Increase of formol titration during activation of chymo-trypsinogen.

The number of peptide bonds split can be estimated in two ways. (1) The increase of formol titration during the activation amounts to about 7.5×10^{-6} equivalent in a solution containing 1.3×10^{-6} g. mol per ml. of chymo-trypsinogen, or about 6 equivalents per g. mol, i.e., the conversion produced 6 acid groups in the molecule. (2) The initial rate of hydrolysis as measured by the formol titration can be compared with the rate of formation of chymo-trypsin molecules. The velocity constant of the reaction was 2.0×10^{-2} min.$^{-1}$, i.e., at the outset $2.0 \times 10^{-2} \times 1.3 \times 10^{-6} = 2.6 \times 10^{-8}$ g. mol per ml. of chymo-trypsinogen is converted initially per minute, while the initial

rate of increase of the formol titration is 10^{-7} equivalent per minute. The conversion of each molecule thus involves 4 equivalents. The two methods therefore lead to the conclusion that, in the formation of a chymo-trypsin molecule, from 4 to 6 equivalents of acid are liberated.

There is, however, no evidence that any appreciable quantity of nitrogenous material is split from the chymo-trypsinogen molecule in the course of the reaction. Kunitz and Northrop (1935) found that a slow increase in the amount of non-protein nitrogen accompanies the reaction and continues when the conversion is completed. They regarded this as being mainly formed by a side reaction, leaving open the question whether any part of it was directly associated with the formation of chymo-trypsin. This experiment was repeated using a much higher concentration of trypsin, so that the conversion was completed in less than an hour (Table 23). The increase in the amount of

TABLE 23

FORMATION OF NON-PROTEIN NITROGEN IN CONVERSION OF CHYMO-TRYPSINOGEN TO CHYMO-TRYPSIN

Reaction mixture: 10 ml. dialyzed chymo-trypsinogen solution containing 3.68 mg. nitrogen per cc.; 0.5 ml. trypsin, approximately 1 mg. nitrogen per cc.

Time, Hours	*Conversion, Percent*	*Non-Protein Nitrogen, Percent*
0	0	3.9
1	80	4.5
2	90	3.7
23	100	6.3
47	...	10.6
71	...	10.4

"non-protein" nitrogen during the conversion was certainly less than 1 percent of the total nitrogen present, the accuracy of the determination being of the order of 0.5 percent. The increase to be expected, if each peptide bond broken releases only one nitrogen atom, is from 1 to 1.5 percent. It is therefore probable that no molecules containing nitrogen are split off.

Since trypsin hydrolyzes the terminal amide group of benzoyl-arginine amide (Bergmann, Fruton, and Pollock 1939), it appeared to

be possible that its action causes the hydrolysis of one or more amide groups. It was found that the amount of free ammonia liberated in the conversion was negligible.

It is possible, of course, that an acid containing no nitrogen is liberated, but no acids of this kind have been detected in proteins, and it is much more probable that the reaction is the opening of peptide bonds in closed rings. The fact that the rate of the activation is completely uninfluenced when the hydrogen in the solvent is largely replaced by deuterium (Table 24) shows that the reaction is not similar

TABLE 24

RATE OF REACTION IN PRESENCE OF DEUTERIUM OXIDE

Reaction mixture 8 ml. water or 70 percent deuterium oxide; 0.5 ml. of chymo-trypsinogen solution, 0.5 ml. 1 M pH 7.5 phosphate buffer, 0.2 ml. trypsin (0.05 mg. nitrogen per ml.)

Time, Minutes	30	60	90	120	160
Percent Conversion in Water	30.5	68	76	82	85
Percent Conversion in Deuterium Oxide	41.5	69	74	78	83

to a hydrogen ion hydrolysis and does not involve protons in any direct way. This is in contrast to the behavior of emulsin on glucosides (Bonhoeffer 1937).

The velocity constant of the reaction is independent of the chymo-trypsinogen concentration up to 0.42 mg. of protein nitrogen per ml. (8×10^{-5} mols per liter) (cf. page 109). To find whether a stable complex exists between the enzyme and the substrate, determinations were made at still higher concentrations and it was found that the velocity constant was only 20 percent smaller at a chymo-trypsinogen concentration of 1.3×10^{-3} mols per liter than at low concentrations. This diminution might be due to secondary effects arising from the increased concentration rather than approaching saturation of a complex; but it is clear that the Michaelis constant is greater than 1.3×10^{-3} mols per liter.

Some experiments were made on the effect of salts on the rate of the reaction. All the salts tried depress the rate, the effect being in the order KCl > NaCl > Na_2SO_4 for equal normalities. Up to 1 *N*, the logarithm of the rate varies roughly linearly with the salt concen-

tration, but at higher concentrations the change is smaller and with sodium sulfate the rate reaches a minimum and begins to rise again. This effect is probably produced by the salting-out action of the salt on the reaction complex. If the reaction complex between the chymo-trypsinogen and the trypsin is salted-out to a greater extent than the

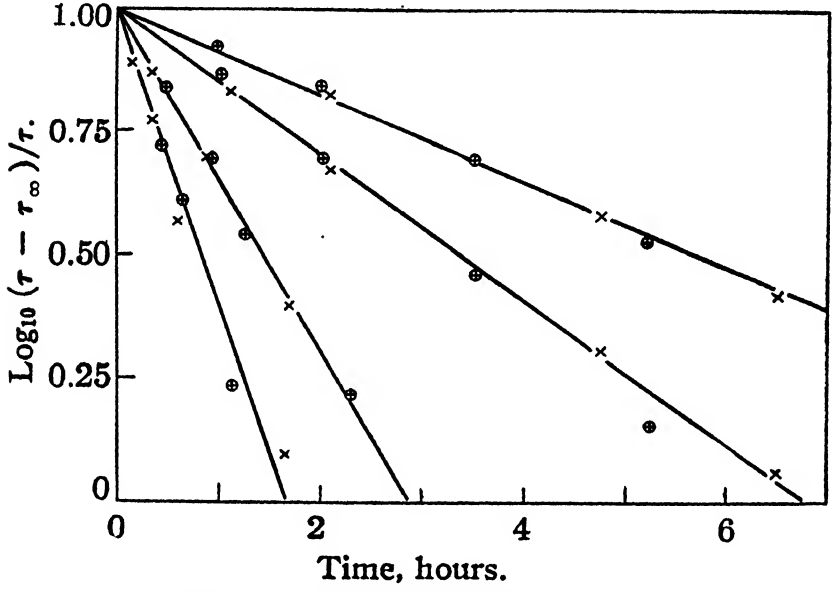


FIG. 44. Conversion of chymo-trypsinogen into chymo-trypsin at various temperatures (two distinct experiments at each temperature).

two constituents, this behavior would occur. The reaction complex is probably negatively charged since the experiments were made at a pH at which chymo-trypsinogen is negatively charged, while trypsin is in the vicinity of its isoelectric point. The marked difference between potassium and sodium salts indicates a marked cation effect which might be expected for a negative complex.

SPONTANEOUS ACTIVATION

The activation experiments just described were all carried out in the presence of trypsin. There is, however, a very slow spontaneous activation. Less than one percent of the chymo-trypsinogen is activated in a month at 5°C. There is no marked pH optimum, but the

reaction appears to go faster in weakly acid or alkaline solutions. It is probable, therefore, that it is an independent reaction and is not caused by minute amounts of trypsin.

RELATION BETWEEN THE PROTEIN AND THE ACTIVITY OF CHYMO-TRYPSIN

Fractional crystallization.—The optical activity and specific enzymatic activity of the chymo-trypsin remain constant through at least three fractional crystallizations as shown in Table 25.

TABLE 25

FRACTIONAL CRYSTALLIZATION OF CHYMO-TRYPSIN

No. of Times Crystallized	Optical Activity M/10 Acetic Acid, 25°C. $[\alpha]^{D}_{mg.\ protein\ nitrogen}$	Specific Activity/mg. Protein Nitrogen			
		Hemoglobin $[T.U.]^{Hb}_{mg.\ protein\ nitrogen}$	*Gelatin V.* $[T.U.]^{Gel.\ V.}_{mg.\ protein\ nitrogen}$	*Casein S.* $[T.U.]^{Cas.\ S.}_{mg.\ protein\ nitrogen}$	*Casein F.* $[T.U.]^{Cas.\ F.}_{mg.\ protein\ nitrogen}$
1	0.386	0.039	11.3	0.98	0.077
2	0.416	0.037	12.0	1.05	0.079
3	0.380	0.038	10.7	1.01	0.073

Solubility.—The purity of the preparation has been further tested by the solubility method with the result shown in Figure 45. Analysis of the curve in this case shows the presence of about 3 percent inert protein. It is probable that this inert protein was formed during the course of the solubility measurements, since, as discussed elsewhere, chymo-trypsin decomposes readily to form partly inert protein and partly slightly different enzymes.

Change in activity with decrease in native protein.—When the chymo-trypsin protein is denatured in M/10 hydrochloric acid, the decrease in activity is proportional to the decrease in native protein concentration, as shown in Table 26.

If the chymo-trypsin is heated to 100°C. in M/400 hydrochloric acid it is very rapidly and completely inactivated with the formation of denatured protein as shown by the fact that the protein is completely precipitated when the hot solution is poured into an equal volume of 2 M sodium chloride, and by the fact that the filtrate from

the salt precipitate is completely inactive. However, if the heated solution is cooled and allowed to stand at 20°C., the solution recovers its original activity and the protein, like unheated chymo-trypsin, is soluble in M/1 sodium chloride. Thus, the denaturation and inacti-

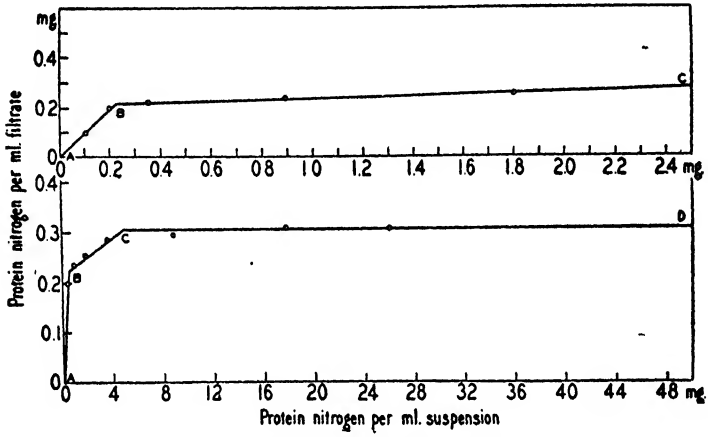


FIG. 45. Solubility of crystalline chymo-trypsin in 0.40 saturated ammonium sulfate in pH 4.0 M/10 acetate buffer in the presence of increasing quantities of solid phase. Upper curve large scale plot of region A–C shown in lower curve.

vation of chymo-trypsin by heat is completely reversible, as is also the denaturation of trypsin. If the native protein is assumed to be

TABLE 26

CHANGES IN ACTIVITY AND NATIVE PROTEIN OF CHYMO-TRYPSIN SOLUTIONS IN M/10 HYDROCHLORIC ACID, 20°C.

10 ml. chymo-trypsin solution (0.8 mg. protein nitrogen/ml.) + 10 ml. N/5 hydrochloric acid, 20°C. 2 ml. samples taken and added to 2.0 ml. N/10 sodium hydroxide, solution (No. 2). Activity by hemoglobin method.

Native protein nitrogen: 2 ml. (No. 2) + 2 ml. 2 M sodium chloride in M/200 hydrochloric acid. Precipitate = denatured protein; filter. Protein in filtrate determined by turbidity method.

Time at 20°C., Hrs.	*0*	*1.3*	*4*	*7*	*16*
$[T.U.]^{Hb}_{ml.}$	0.015	0.011	0.081	0.0052	0.0016
Native protein N/ml., *mg.*	0.390	0.256	0.172	0.144	0.031
$[T.U.]^{Hb}_{mg.\ protein\ nitrogen}$	0.038	0.043	0.047	0.036	0.052

merely a carrier for an hypothetical "active group," it is necessary to assume that the active group becomes inactive when the protein is denatured and then becomes active again when the protein reverts to the native condition. On longer heating this reversibly inactivated and denatured form gradually changes to an irreversibly inactivated and denatured form which does not become active and salt soluble again on cooling and standing at 20°C. (Table 27).

TABLE 27

REVERSIBLE AND IRREVERSIBLE INACTIVATION OF CHYMO-TRYPSIN AT 100°C.

10 ml. chymo-trypsin solution (0.43 mg. protein nitrogen/ml.) in M/400 hydrochloric acid; immersed in boiling water (No. 1).

Activity and native protein in hot solution: 1 ml. (No. 1) + 4 ml. hot M/400 hydrochloric acid, 2 ml. of this solution + 2 ml. 2M sodium chloride (20°C.) filter, activity and protein nitrogen determined on filtrate.

Activity and native protein after reversal by cooling: 1 ml. (No. 1) + 4 ml. cold M/400 hydrochloric acid, 10 minutes 20°C. 2 ml. + 2 ml. 2M sodium chloride. Activity and protein nitrogen determined on filtrate.

Time at 100°C. Min.	*Not Heated*	*1*	*5*	*15*	*30*
ACTIVITY AND NATIVE PROTEIN IN HOT SOLUTION					
Activity $[T.U.]^{Hb}_{ml.}$	0.017	0	0		
Native protein N/ml., *mg.*	0.33	0	0		
Percent total inactivation	0	100	100		
ACTIVITY AND NATIVE PROTEIN AFTER REVERSAL BY COOLING					
$[T.U.]^{Hb}_{ml.}$	0.017		0.014	0.0084	0.0054
$[T.U.]^{Rennet}_{ml.}$	1.90		1.7	1.0	0.54
Protein N/ml., *mg.*	0.33		0.28	0.22	0.15
$[T.U.]^{Hb}_{mg.\ protein\ nitrogen}$	0.052		0.05	0.038	0.036
$[T.U.]^{Rennet}_{mg.\ protein\ nitrogen}$	5.8		6.1	4.6	3.6

If chymo-trypsin solutions are allowed to stand at pH 9.0 and 37°C., there is a loss in protein nitrogen paralleled by the loss in activity. This reaction is probably analogous to the inactivation of trypsin in alkali and is due to the formation and subsequent hydrolysis of denatured protein.

The connection between the protein and the activity may also be tested by pepsin digestion. The hydrolysis of the protein by pepsin is accompanied by a corresponding decrease in activity.

GENERAL PROPERTIES OF CHYMO-TRYPSIN

pH of maximum stability.—Dilute solutions of the enzyme at 37°C. are most stable at pH 3.0–3.5.

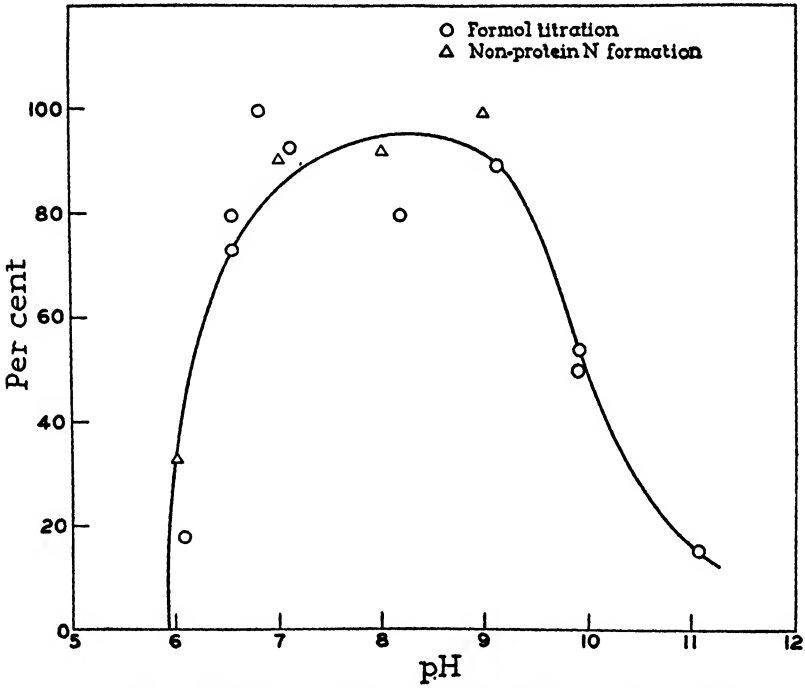


FIG. 46. Digestion of casein at various pH by chymo-trypsin.

Effect of pH on the rate of digestion by chymo-trypsin.—The rate of digestion of casein by chymo-trypsin at various pH is shown in Figure 46. The pH activity curve is similar to that of trypsin.

Digestion of sturin.—Waldschmidt-Leitz and Kollmann (1927) have found that digestion of protamines by pig pancreas, previously considered as a property of "trypsin-kinase," is due partly to a separate enzyme, "protaminase," which may be separated from the proteolytic enzyme by adsorption of the latter on egg albumin. These ex-

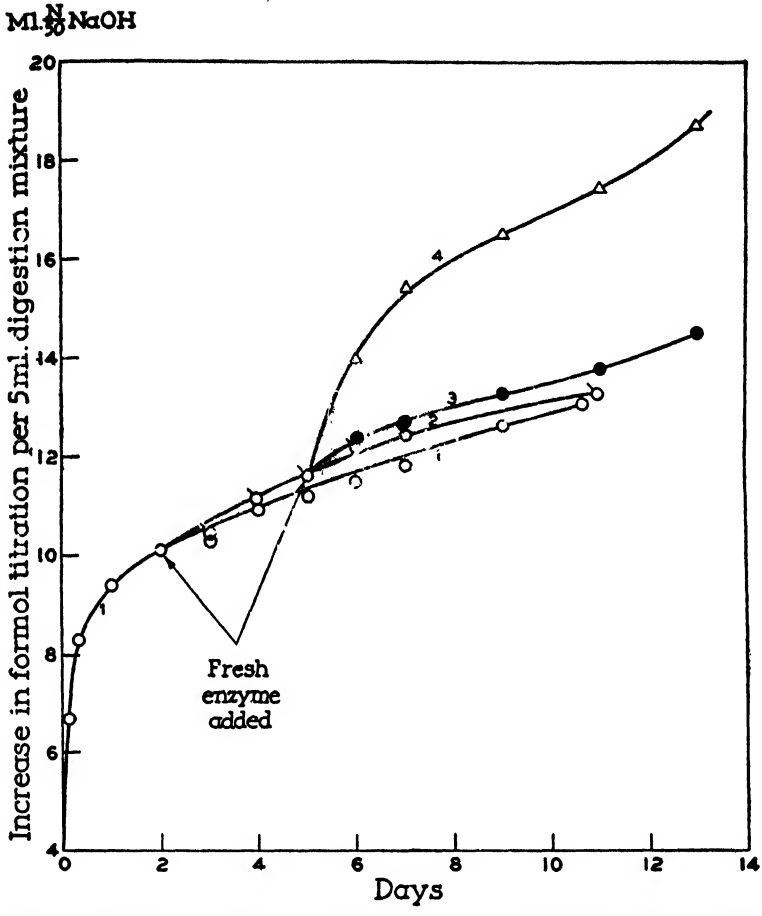


FIG. 47. Digestion at 35°C. of casein by chymo-trypsin followed by trypsin.

100 ml. { 5 percent casein pH 7.6 (M/10 phosphate buffer)
0.08 mg. chymo-trypsin nitrogen/ml. = No. 1

After 2 days 0.08 mg. chymo-trypsin nitrogen/ml. added to 75 ml. No. 1
(Total chymo-trypsin concentration 0.16 mg. nitrogen/ml.) = No. 2

After 5 days 0.08 mg. chymo-trypsin nitrogen/ml. added to 25 ml. No. 2
(Total chymo-trypsin concentration 0.24 mg. nitrogen/ml.) = No. 3

After 5 days 0.08 mg. crystalline trypsin nitrogen/ml. added to 25 ml. No. 2
(Total enzyme concentration 0.16 mg. chymo-trypsin nitrogen/ml. plus
0.08 mg. trypsin nitrogen/ml.) = No. 4

Digestion determined by formol titration.

periments were repeated with chymo-trypsin, but it was not possible to separate the protaminase activity from the proteinase activity by this method. The percent activity removed by the egg albumin was the same as determined by either casein, hemoglobin, or sturin digestion.

Digestion of peptides.—The chymo-trypsin has no measurable effect upon the hydrolysis of many di- and polypeptides. Bergmann and Fruton (1937) have shown that it does hydrolyze polypeptides in which tyrosine or phenylalanine supply a peptide carbonyl group. Benzoyl-tyrosyl-glycine amide is rapidly hydrolyzed by the enzyme.

Specificity of chymo-trypsin and trypsin.—Casein is hydrolyzed more completely by chymo-trypsin than by crystalline trypsin, but the hydrolysis by the two enzymes occurs at different linkages. This is shown by the fact that addition of trypsin to casein previously hydrolyzed with chymo-trypsin (Figure 47), or of chymo-trypsin to casein previously hydrolyzed with trypsin (Figure 48), causes a marked increase in hydrolysis.

Miller (1939) finds that trypsin hydrolyzes 33–35 linkages in lactalbumin, whereas chymo-trypsin splits 47 to 50. Fifteen of the groups are hydrolyzed by both enzymes.

Balls and Lineweaver (1938) state that chymo-trypsin hydrolyzes native egg albumin thirty times faster than does trypsin.

Haurowitz, Tunca, and Yurd (1943) find that globular proteins are digested more rapidly after denaturation, whereas fibrous proteins are digested at about the same rate.

Trypsin digestion, following pepsin, liberates cystine. No cystine is liberated by pepsin alone (Damodaran and Krishnaswamy 1942). Chymo-trypsin destroys the biotic activity of oxytocin, but trypsin does not (Croxatto, Croxatto, Illanes, and Salvestrini 1943).

Isoelectric point.—The isoelectric point was determined by measuring the rate of migration of collodion particles immersed in the enzyme solutions at various pH. This method shows an isoelectric point for chymo-trypsin at pH 5.4 and for chymo-trypsinogen at 5.0.

Diffusion coefficient.—The diffusion coefficient of chymo-trypsin was determined by measuring the rate of diffusion of the protein nitrogen and of the activity. Both methods gave a diffusion coefficient at 6°C. of 0.037 cm^2/day for chymo-trypsin. This result shows that

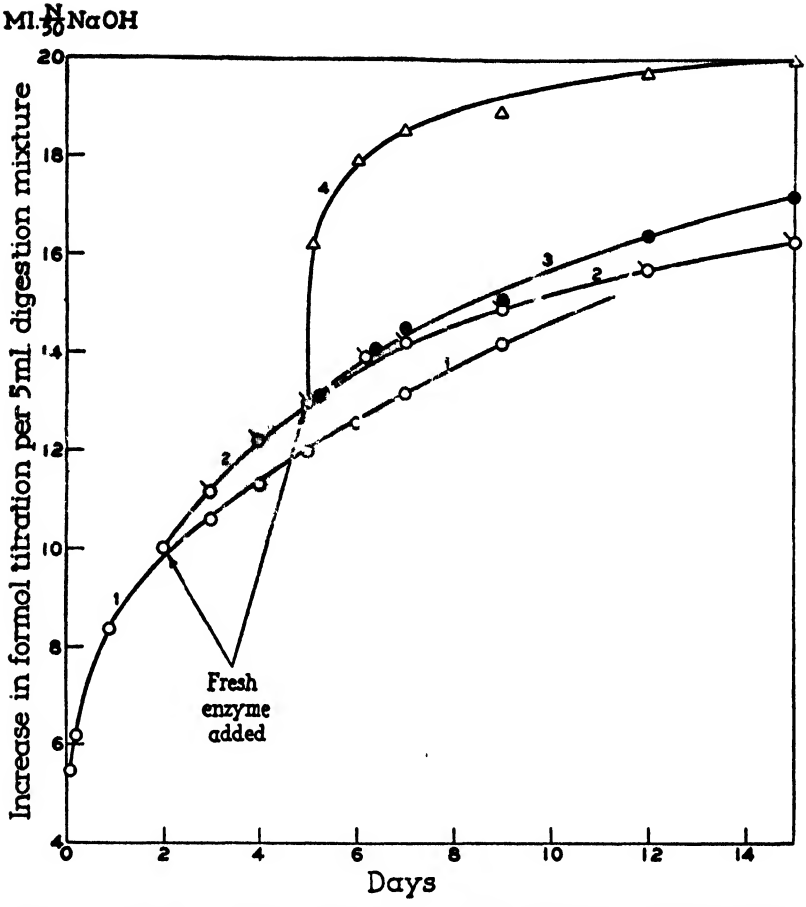


FIG. 48. Digestion at 35°C. of casein by trypsin followed by chymo-trypsin.

100 ml. { 5 percent casein pH 7.6 (μ/10 phosphate)
0.08 mg. trypsin nitrogen/ml. = No. 1

After 2 days 0.08 mg. crystalline trypsin nitrogen/ml. added to 75 ml. No. 1 (Total trypsin concentration 0.16 mg. nitrogen/ml.) = No. 2

After 5 days 0.08 mg. crystalline trypsin nitrogen/ml. added to 25 ml. No. 2 (Total trypsin concentration 0.24 mg. nitrogen/ml.) = No. 3

After 5 days 0.08 mg. chymo-trypsin nitrogen/ml. added to 25 ml. No. 3 (Total enzyme concentration 0.16 mg. trypsin nitrogen/ml. plus 0.08 mg. chymo-trypsin nitrogen/ml.) = No. 4

the active molecule diffuses at the same rate as does the protein and furnishes additional evidence for the identity of the two molecules.

The diffusion coefficient for chymo-trypsinogen was measured by the nitrogen only and is 0.039 cm^2 day. The molecular volume corresponding to this diffusion coefficient is about 52,000 $cm.^3$, on the assumption that the molecules are spheres.

TABLE 28

PREPARATION OF β AND γ CHYMO-TRYPSIN FROM CHYMO-TRYPSIN

Solution of 3 × crystallized chymo-trypsin pH 8.0
↓
15 days at 5°C. then brought to 0.4 saturated ammonium sulfate pH adjusted to 5.6. 3 days at 20°C.
↓
Suspension of γ Crystals
↓
Filtered. Filtrate to 0.7 saturated ammonium sulfate and pH 4.0. Amorphous precipitate dissolved in equal volume 0.01 sulfuric acid. 2 days at 20°C.
↓
Crystals of Solid Solution of β Plus Inert Protein
↓
1 percent solution pH 8.0, 1 hour at 35°C. Acidified to pH 4.0. Reprecipitated in 0.7 saturated ammonium sulfate; precipitate dissolved in 3 volumes water, brought to 0.4 saturated ammonium sulfate. 3 days at 20°C.
↓
β Crystals

The molecular weight of both proteins from osmotic pressure measurements is about 40,000, so that from these measurements the proteins are hydrated to the extent of about ½ gm. of water per gm. protein. Viscosity measurements, however, give much lower hydrations.

FORMATION OF β AND γ CHYMO-TRYPSIN FROM CHYMO-TRYPSIN (Kunitz 1938a)

It was noted during the crystallization of chymo-trypsin that a large portion of the enzymatic activity and considerable protein remained in the mother liquor and could not be induced to crystallize under these conditions. Systematic study of this solution showed that it contained two new enzymes very closely related to chymo-trypsin but differing from the chymo-trypsin in crystalline form, solubility, and rate of inactivation in alkali or acid or by urea. They have been called β chymo-trypsin (Figure 49) and γ chymo-trypsin

FIG. 49. β chymo-trypsin crystals. $\times$ 315.

FIG. 50. γ chymo-trypsin crystals. $\times$ 15.5.

(Figure 50). Up to the present no certain difference has been found in the proteolytic activity of these enzymes. The rate of digestion of the various substrates tried is the same by all three preparations. They are formed under conditions which favor the decomposition of chymo-trypsin and are probably produced by slight hydrolysis of

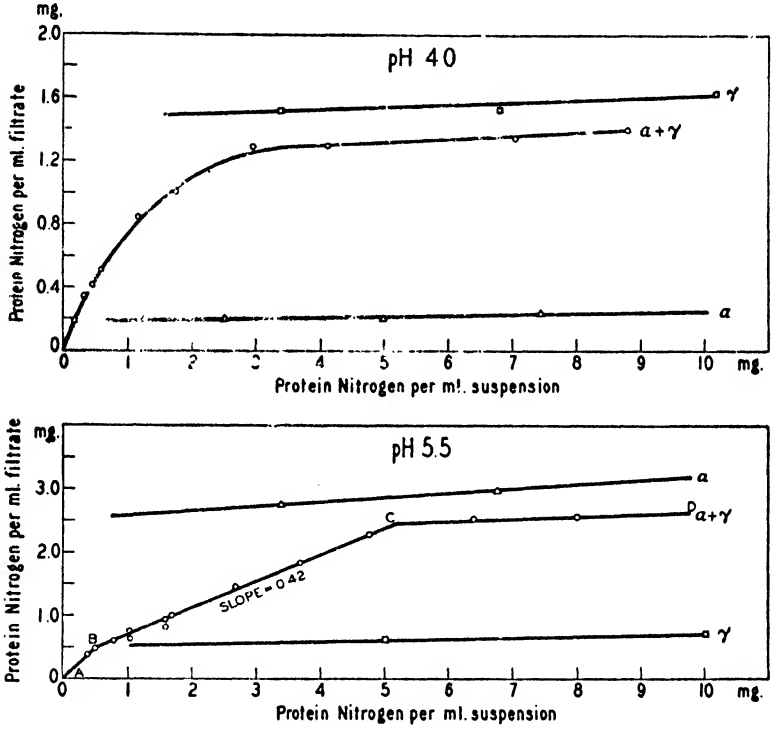


FIG. 51. Solubility curves of artificial mixtures of crystals of chymo-trypsin (α) and γ chymo-trypsin (γ) (40 percent α + 60 percent γ) in 0.4 saturated $(NH_4)_2SO_4$ at 10°C. in presence of increasing quantities of solid phase.

the parent molecule. Osmotic pressure measurements indicate that the molecular weight of γ chymo-trypsin is 27,000, of β is 30,000 while chymo-trypsin itself has a molecular weight of 40,000. Since there is no marked effect on the activity of the enzyme, the change in the molecule is evidently in that part of the structure which is not related to its proteolytic action. An outline of the method of formation and isolation of these proteins is shown in Table 28.

Solubility experiments show that these preparations are distinct proteins with different and characteristic solubilities. They form solid solutions when the pH is less than 5.0, but crystallize separately between pH 5.0 and 6.0 (Figure 51).

Precipitin reaction of chymo-trypsins.—Chymo-trypsin and β and γ chymo-trypsin form antibodies when injected into rabbits, although with some difficulty and the serum is not especially powerful. The precipitin reaction fails to distinguish differences between these three proteins. A similar result was obtained by Landsteiner and Heidelberger (1923) who found that horse hemoglobin could be distinguished from donkey hemoglobin by solubility measurements, whereas the resulting precipitin reactions were inconclusive.

VI: TRYPSINOGEN, TRYPSIN, AND TRYPSIN INHIBITOR

(Kunitz and Northrop 1936)

THE work described in the preceding chapter has shown that a crystalline protein may be obtained from acid extracts of fresh pancreas which, in the presence of minute amounts of trypsin, is changed into an active proteolytic enzyme, chymo-trypsin. The filtrate from the crystallization of this inactive protein still possesses powerful proteolytic activity (after activation by kinase) as measured by digestion of hemoglobin or other proteins. It has little effect upon the clotting of milk as this property is due to the chymo-trypsin. This filtrate, upon further fractionation (cf. Table 20, page 99), yields a further crop of inactive protein crystals. This protein is trypsinogen, the precursor of trypsin.

TRYPSINOGEN

Trypsinogen is obtained as small triangular prisms. When these crystals are dissolved in neutral solution the trypsinogen is rapidly transformed into active trypsin (Figure 52) and it has, therefore, been impossible so far to recrystallize trypsinogen. The original crystallization occurs without activation owing to the presence of the inhibitor and if inhibitor is added to a solution of trypsinogen recrystallization may be carried out without activation. Numerous attempts have been made to recrystallize inhibitor-free trypsinogen under conditions which would not at the same time cause activation, but so far without success. Analyses and properties of this substance are therefore somewhat uncertain and solubility experiments impossible.

Formation of trypsin from trypsinogen.—The transformation of trypsinogen into trypsin is accelerated by the addition of trypsin or enterokinase or concentrated solutions of magnesium sulfate or ammonium sulfate. The addition of inhibitor retards activation by all three methods and a large quantity of inhibitor will completely pre-

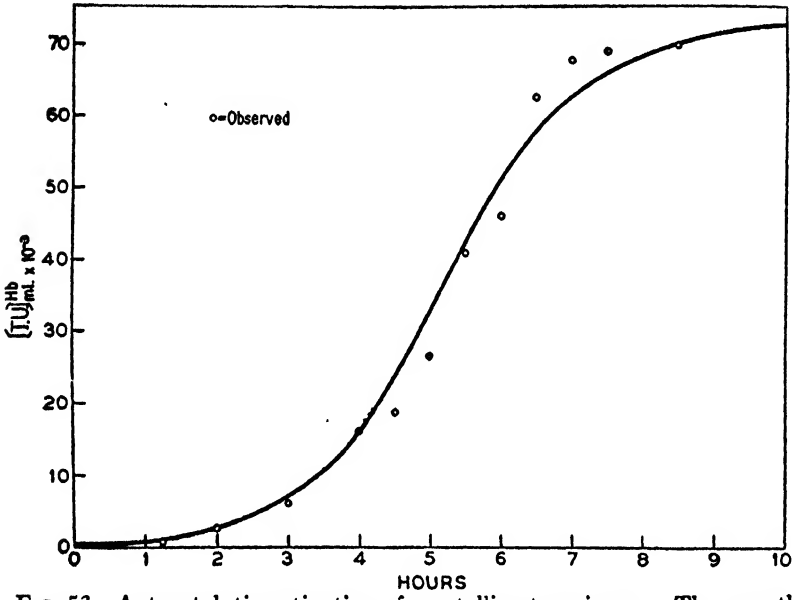


FIG. 53. Autocatalytic activation of crystalline trypsinogen. The smooth curve is calculated from the equation for a simple autocatalytic reaction

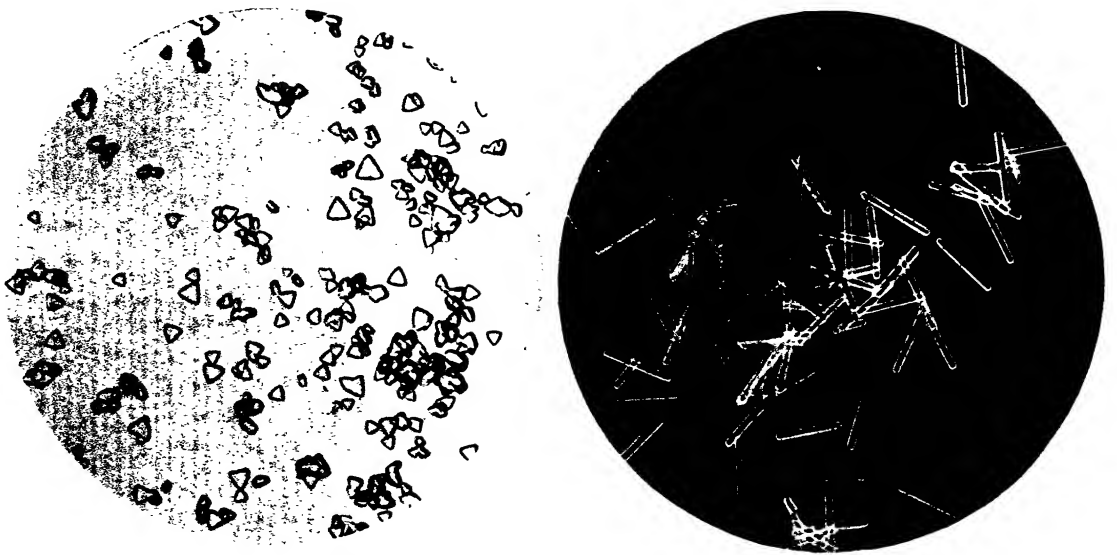
$$KA_e t = 2.3 \log \frac{A(A_e - A_0)}{A_0(A_e - A)}$$

where $K = 14.6$, $A_e = 0.072$ $(T.U.)^{Hb}$ and $A_0 = 0.0003$ $(T.U.)^{Hb}$.

vent activation. A solution of trypsinogen to which inhibitor has been added behaves, therefore, just as the crude trypsinogen solution previously described. The fact that activation is accelerated by the addition of trypsin indicates that activation is autocatalytic and this is borne out by the kinetics of the reaction as shown in Figure 53. Under these conditions the reaction follows quite closely that of a simple autocatalytic reaction. The rate of activation depends on the pH and is maximum at pH 7.0–8.0. The reaction is complicated by the fact that an inert protein is also formed from trypsinogen in the presence of trypsin (Kunitz, 1939b). This reaction is also catalyzed by trypsin so that the entire reaction may be written

$$\text{Trypsinogen} \xrightarrow{\text{Trypsin}} \text{Trypsin} \qquad (1)$$

$$\text{Trypsinogen} \xrightarrow{\text{Trypsin}} \text{Inert protein} \qquad (2)$$



× 220. FIG. 52. Trypsinogen and trypsin crystals. × 202.

or
$$\frac{dA}{dt} = K_1A(G_0 + A_0 - A - I)$$

$$\frac{dI}{dt} = K_2A(G_0 + A_0 - A - I)$$

when A is the concentration of active trypsin
I = concentration of inert protein
G_0 = initial concentration of trypsinogen.

In solutions more alkaline than pH 5.0 the rate of formation of inert protein is greater than that of trypsin and at pH 7.0 a large part of the trypsinogen is transformed into inert protein. In solutions more acid than pH 5.0 the rate of formation of inert protein is much less and most of the trypsinogen is transformed into trypsin. Experiments show that the same substrate may be transformed into two different products by the same enzyme, the course of the reaction being determined by the pH of the solution. The formation of this inert protein under certain conditions undoubtedly accounts for the controversial statements concerning the autocatalytic nature of the reaction to be found in the literature.

It follows from this that if trypsinogen could be prepared completely free from active trypsin it would probably remain inactive. Owing to the extremely minute amounts of trypsin required to activate, however, it has so far been impossible to obtain such preparations. Activation by the addition of concentrated magnesium or ammonium sulfate is also autocatalytic in type. Since the reaction is autocatalytic the velocity decreases as the concentration decreases and in dilute solutions (< 0.01 mg. nitrogen per ml.) the rate of the autocatalytic reaction is so slow as to be negligible.

FORMATION OF TRYPSIN FROM CRYSTALLINE TRYPSINOGEN BY MEANS OF ENTEROKINASE (Kunitz 1939b)

The mechanism of activation of trypsinogen by enterokinase has been a matter of almost continuous controversy since the discovery of enterokinase by Schepowalnikow (1899) in Pavlov's laboratory in 1899. The discoverers considered enterokinase to be a typical enzyme. Studies of the kinetics of activation have shown, however, that while it is true that the rate of activation of a definite concentration of trypsinogen is proportional to the concentration of entero-

kinase used, the ultimate amount of trypsin formed is not independent of the concentration of enterokinase as would be expected if enterokinase were a true enzyme. This led to the suggestion that the formation of trypsin from trypsinogen by enterokinase is essentially a stoichiometric combination between trypsinogen and enterokinase to form an active enzyme, "trypsin-kinase" (Waldschmidt-Leitz 1924).

The isolation of crystalline trypsinogen from fresh beef pancreas, its autocatalytic transformation at pH 7.0–9.0 into active trypsin without the aid of any outside activator, and the isolation of the active trypsin in pure crystalline form offer proof against the assumption that trypsin is a stoichiometric compound of kinase and trypsinogen. It was also found that pure crystalline trypsinogen can be changed into active trypsin at pH 3.0–4.0 by means of a kinase obtained from a mold of the genus *Penicillium* (cf. following section); the trypsin formed has been crystallized and found to be identical in crystalline form, solubility, and specific activity with the crystalline trypsin obtained by spontaneous autocatalytic activation of trypsinogen at pH 8.0. The action of mold kinase was that of a typical enzyme; the process of activation followed the course of a catalytic unimolecular reaction, and the ultimate amount of trypsin formed was independent of the concentration of mold kinase used.

This section deals with the kinetics of activation of crystalline trypsinogen by means of purified enterokinase obtained from pig duodenum contents. Enterokinase acts best in the range of pH 6.0–9.0 where spontaneous autocatalytic activation of trypsinogen occurs readily. The percentage rate of this autocatalytic reaction is proportional to the concentration of trypsinogen. Hence by using very dilute trypsinogen solutions the rate of spontaneous activation may be made negligible compared with that of the activation brought about by the enterokinase. The autolysis of the trypsin produced, which generally occurs in the range of pH 7.0–9.0 (Kunitz and Northrop 1934), is also minimized by using dilute trypsinogen and by employing temperatures not higher than 5°C. A further complication exists at pH 7.0–9.0, since under these conditions trypsinogen in the presence of trypsin is partly changed to an inert protein which can no longer be transformed into trypsin either by enterokinase or mold kinase (Kunitz 1939b). This complication may be avoided by working

at pH 5.5–6.0 where the rate of transformation of trypsinogen into inert protein is greatly reduced. Thus when activation by enterokinase is allowed to proceed at pH 5.8 enterokinase acts like a typical

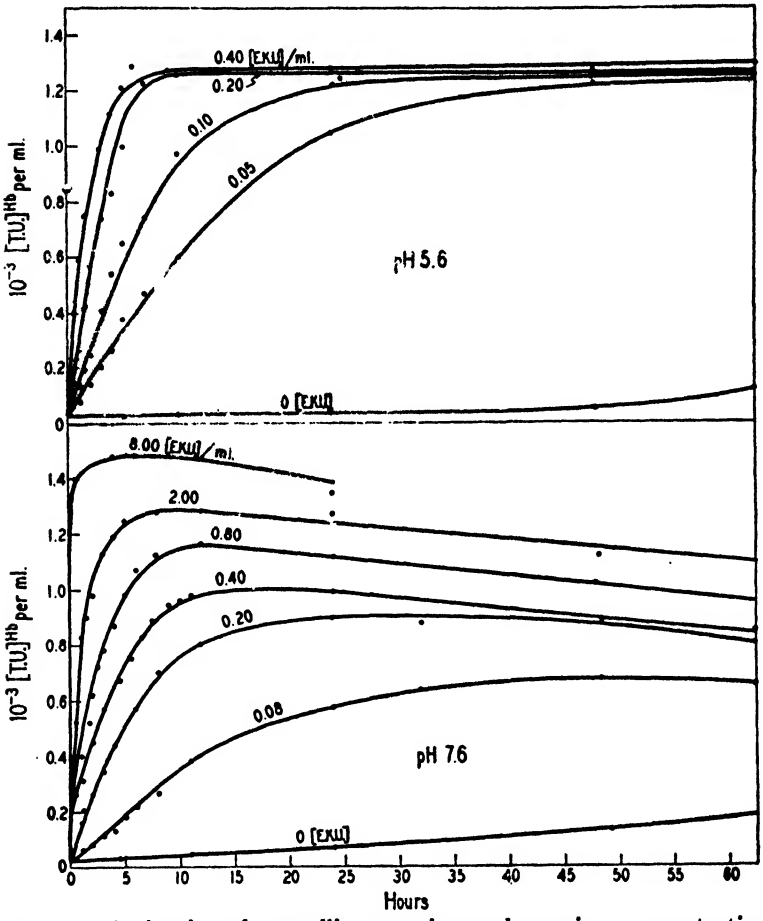


FIG. 54. Activation of crystalline trypsinogen by various concentrations of enterokinase at 5°C.

enzyme and the reaction follows the course of a theoretical unimolecular reaction with a velocity constant proportional to the concentration of enterokinase used, while the ultimate amount of trypsin formed is independent of the concentration of kinase (Figure 54).

If, on the other hand, the activation is allowed to proceed at pH 7.6

a great portion of the trypsinogen is transformed into inert protein, the more so the lower the concentration of enterokinase used, with the result that the ultimate amount of trypsin formed is less as the concentration of enterokinase used is decreased in conformity with the results of other workers. Another result of the complication due to the formation of inert protein is that the apparent rate of activation of crystalline trypsinogen by enterokinase is greater at pH 5.6 than at 7.6 since the trypsinogen is more stable in the acid solution. The kinetics of the formation of trypsin from crystalline trypsinogen by means of enterokinase at pH 7.6 can be expressed mathematically as follows:

Let G_0 = initial concentration of trypsinogen, expressed in terms of activity after conversion into trypsin (by means of excess kinase)

E = concentration of enterokinase added

A = tryptic activity at any time (t)

A_e = final activity

I = concentration of inert protein formed from trypsinogen

I_e = final concentration of inert protein

Assuming (1) that the rate of formation of I at any time is proportional to the concentration of trypsin and the concentration of trypsinogen, and (2) that the rate of formation of trypsin is proportional to the concentration of kinase and to the concentration of trypsinogen, we have the following equations:

1. $\frac{dI}{dt} = K_1A(G_0 - A - I)$

2. $\frac{dA}{dt} = K_2E(G_0 - A - I)$

3. $\frac{dI}{dA} = \frac{K_1}{K_2E}A$ and $I = \frac{K_1}{2K_2E}A^2 + 0$ or $I = bA^2$

where $b = \frac{K_1}{2K_2E}$

hence $\frac{dA}{dt} = K_2E(G_0 - A - bA^2).$

4. At equilibrium $G_0 = A_e + I_e = A_e + bA_e^2$

5. Hence $$\frac{dA}{dt} = K_2E[(A_e - A) + b(A_e^2 - A^2)].$$

From equations (3) and (4):

$$b = \frac{G_0 - A_e}{A_e^2} = \frac{K_1}{2K_2E}$$

or $$\frac{E(G_0 - A_e)}{A_e^2} = \frac{K_1}{2K_2} = \text{constant.}$$

The constancy of the values in the fourth column of Table 29 shows that the incomplete activation of trypsinogen by small amounts of kinase at pH 6.0–7.0 is correctly predicted by the assumptions made in deriving the equations.

TABLE 29

CALCULATION OF ACTIVATION OF TRYPSINOGEN BY SMALL AMOUNTS OF KINASE

E	A_e	$\frac{G_0 - A_e}{A_e^2}$	$\frac{E(G_0 - A_e)}{A_e^2}$
[EKU]	$[\text{T.U.}]^{Hb} \times 10^{-3}$		
5	1.40	51	250
2.5	1.30	118	300
1.0	1.15	265	260
0.5	1.00	500	250
0.25	0.90	740	190
0.10	0.65	2,000	200

The transformation of trypsinogen into trypsin in the presence of kinase is thus a typical enzyme reaction, catalyzed by the enzyme enterokinase, and the anomalous results found under certain conditions are due to a secondary reaction by which trypsin changes trypsinogen to an inert protein.

The kinetics of the reaction outlined above apply only to purified trypsinogen. The activation of crude pancreatic extracts by enterokinase is much more complicated since, as previously noted, these extracts contain a substance which inhibits trypsin and also chymotrypsinogen in addition to trypsinogen. In outline, the activation of such crude extracts proceeds as follows: addition of kinase transforms the trypsinogen to trypsin which is partly inactivated by the inhibitor, thus decreasing the rate of the autocatalytic reaction. The

free trypsin catalyzes the conversion of trypsinogen to form more trypsin and also catalyzes the conversion of chymo-trypsinogen to chymo-trypsin. If the method of activity determinations used determines both trypsin and chymo-trypsin, as is usually the case, the curves obtained when the activity of the solution is plotted against time are S-shaped but asymmetrical and resemble those obtained by Vernon.

Formation of trypsin from trypsinogen by mold kinase (Kunitz 1938b).—It was noticed that some acid solutions of trypsinogen became activated quite rapidly while others did not. This abnormal activation in certain acid solutions was traced to the presence of a minute amount of mold later identified as a *Penicillium*. It was found on cultivating the mold that the culture medium became increasingly powerful in its ability to form trypsin from trypsinogen, and a concentrated and partially purified solution of this activating principle has been obtained. It is associated with a proteolytic enzyme produced by the mold, but whether or not it is identical with this enzyme has not yet been determined. In any case, the substance presents a very useful reagent for the formation of trypsin from trypsinogen since the reaction occurs in acid solution under which condition both trypsin and trypsinogen are stable. The reaction may, therefore, be studied to much better advantage than the activation in neutral or alkaline solution since under these latter conditions trypsin is very unstable and the results are complicated by the formation of inert protein and decomposition products of trypsin.

The reaction is found to be monomolecular in respect to trypsin, and the velocity constant of the reaction is proportional to the concentration of the mold enzyme and independent of the concentration of trypsin. The optimum pH is about 3.0. Trypsin formed in this way is identical with that obtained either from spontaneously activated pancreas or from trypsinogen which has been transformed to trypsin by the autocatalytic reaction at pH 7.0. Since the trypsin formed by any one of these three reactions is identical it follows that the chemical reaction must be the same in all three cases, and since the reaction is catalyzed by trypsin itself, which is not known to attack any group except the peptide link, it seems quite certain that the transformation of trypsinogen to trypsin is due to the rupture of a peptide link.

THE EFFECT OF CALCIUM AND OTHER IONS ON THE AUTOCATALYTIC FORMATION OF TRYPSIN FROM TRYPSINOGEN (McDonald and Kunitz 1941)

The effect of salts upon the formation of trypsin from its inactive precursor, trypsinogen, has been extensively studied. Delezenne (1905), Zunz (1906, 1907), Ayrton (1909), Meyer (1910), and Wohlgemuth (1912) showed that the spontaneous activation of fresh pancreatic juice or extracts of fresh pancreas was hastened upon the addi-

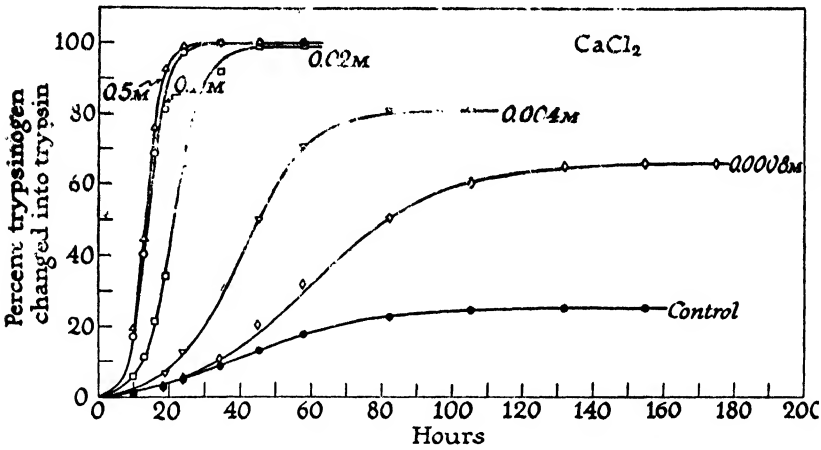


FIG. 55. Effect of concentration of calcium chloride on the formation of trypsin. Activation mixtures: various concentrations of calcium chloride in 0.1 M borate buffer pH 8.0 and containing 0.1 mg. protein nitrogen per ml. Final pH = 7.2. Smooth curves drawn through calculated points.

tion of calcium or other alkaline earth salts, the former being most effective. De Sonza (1908) and Mellanby and Woolley (1913) considered that the accelerating action of calcium salts was due to neutralization since the amount of calcium chloride required corresponded closely with that necessary to precipitate the carbonate of the pancreatic juice. They found that barium and strontium chloride or neutralization with hydrochloric acid was as effective as calcium chloride. Waldschmidt-Leitz (1924), on the other hand, was unable to show any increase in spontaneous activation with calcium salts. More recently Farber and Wynne (1935) found that the activity of impure pancreatic proteinase was definitely stimulated by calcium

salts but thought it possible that this increase in activity was due to the removal of inhibitors. The contradictory results found in the

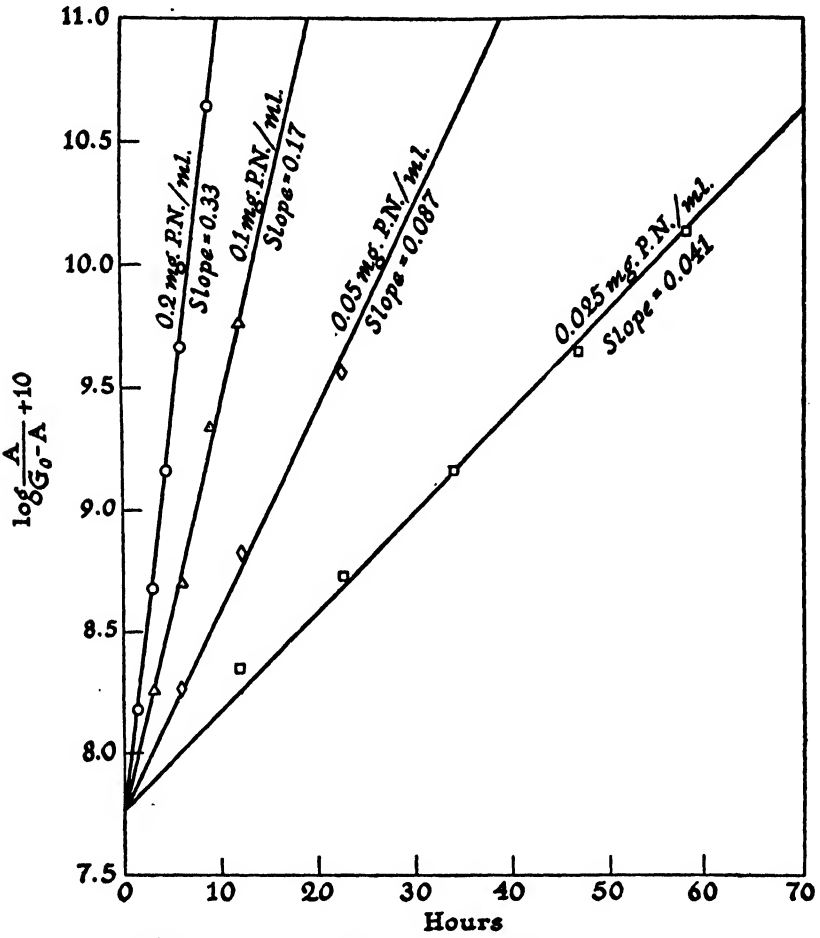


FIG. 56. Effect of concentration of trypsinogen on the formation of trypsin. Activation mixtures: calcium chloride solutions in 0.1 M borate buffer pH 8.0 and containing various amounts of trypsinogen per ml. Final pH = 7.2.

literature are undoubtedly due both to the impure materials and to the diversity of the conditions used.

The present studies show that salts influence the transformation of trypsinogen into trypsin in one of the following ways.

(*a*) Increasing both the rate of formation of trypsin and the rate of formation of inert protein. (*b*) Decreasing both the rate of formation of trypsin and the rate of formation of inert protein. (*c*) Increasing the rate of formation of trypsin and decreasing the rate of formation of inert protein. (*d*) Decreasing the rate of formation of trypsin and increasing the rate of formation of inert protein.

The effect of salts upon the ultimate amounts of trypsin and inert protein formed depends upon the relative influence of the various ions on the rate of both reactions since the final amounts of the products formed depend on the ratio of the velocity constant, K_1, for the formation of trypsin to the velocity constant, K_2, for the formation of inert protein. The influence of salts is due to both the cation and the anion; the chemical nature of the ions is more important than their valency.

The behavior of the calcium ion is particularly striking since it inhibits completely the formation of inert protein even in concentrations as low as 0.02 M with the result that the trypsinogen is converted quantitatively into trypsin. In the presence of calcium salts, therefore, the transformation of trypsinogen into trypsin by means of trypsin follows the course of a simple unimolecular autocatalytic reaction (Figures 55, 56).

The ions studied may be arranged in the following approximate series, the first members in each group having an increasing and the last members a decreasing effect.

A. Effect on the Rate of Formation of Trypsin

Anions	Cations
Sulfate, citrate, oxalate, tartrate, acetate	Calcium
Fluoride, chloride	Strontium
Bromide	Barium, magnesium
Nitrate	Sodium
Iodide	Lithium
	Potassium, ammonium
	Rubidium
	Caesium

B. Effect on the Rate of Formation of Iin Protein

Anions	Cations
Iodide	Barium
Nitrate	Magnesium
Tartrate, citrate, bromide	Lithium
Oxalate, acetate, sulfate	Potassium
Chloride	Sodium
Fluoride	Ammonium, rubidium, caesium
	Strontium
	Calcium

C. Effect on the Ultimate Percentage of Trypsinogen Changed into Trypsin

Anions	Cations
Acetate, sulfate, oxalate, citrate, tartrate, fluoride, chloride	Calcium
	Strontium
Bromide	Magnesium, sodium
Nitrate	Rubidium, ammonium, lithium, potassium
Iodide	Caesium, barium

PURITY OF CRYSTALLINE TRYPSIN

The specific activity of various preparations of crystalline trypsin prepared by the various methods after repeated crystallization is shown in Table 30. The results of solubility experiments agree (Kunitz and Northrop 1938b) quantitatively with a curve for a solid phase of one component (Figure 57) and furnish very good evidence that the preparation represents a pure protein.

EVIDENCE THAT THE TRYPTIC ACTIVITY IS A PROPERTY OF THE PROTEIN MOLECULE

Inactivation and reactivation by heat (Northrop 1932c).—Mellanby and Woolley noted that trypsin can be heated to boiling in dilute acid solution with little loss in activity. This is a very unusual property for either a protein or an enzyme. The crystalline trypsin possesses this peculiar property. It may be heated and cooled rapidly many times without any detectable change in the solution. There is little or no loss in activity and no change in the protein content

TABLE 30

ACTIVITY OF VARIOUS PREPARATIONS OF CRYSTALLINE TRYPSIN

PREPARATION	[T.U.] MG. N DETERMINED BY		
	Gelatin viscosity pH 4.0	*Hemoglobin*	*Casein formol*
No. 50 prepared from crystalline trypsinogen			
1 × crystallized	77	0.10	0.15
3 × "	73	0.12	0.15
5 × "	75	0.12	0.14
8 × "	90	0.14	0.15
3 × crystallized, precipitated + trichloracetic acid		0.16	
Crystals prepared from active pancreatic juice			
1 × crystallized	98	0.17	
3 × "	120	0.15	
5 × "	90 100	0.16	0.16

of the solution. If, however, salt be added to the hot solution, all the protein is precipitated and there is no activity in the filtrate. These results indicate that the protein is denatured at temperatures above 50°C. or 60°C. but returns to its original native condition upon cooling. A detailed study of this behavior confirmed this assumption.

It can be shown that the enzyme is also inactive at 80°C.–90°C. since a mixture of casein and trypsin at this temperature does not digest while if the same solution is cooled to 37°C. rapid digestion occurs.

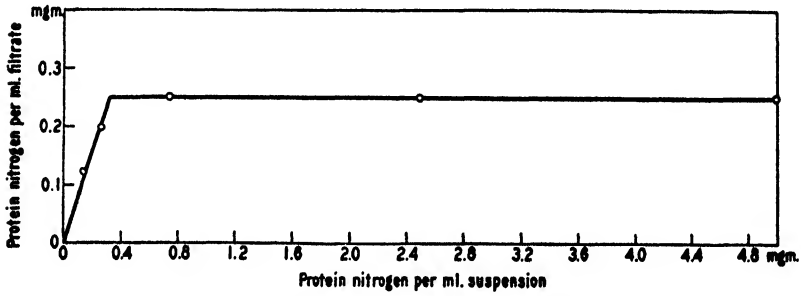


FIG. 57. Solubility of crystalline trypsin in saturated magnesium sulfate pH 4.0 at 10°C. in the presence of increasing quantities of solid phase.

These experiments show that when the protein is denatured the activity is lost, and when the protein reverts to its original native state the original activity is regained. If the activity, therefore, be due to a non-protein molecule it is necessary to assume that this hypothetical molecule is inactivated and reactivated at the same rate and under the same conditions as cause denaturation and reversal of denaturation of the protein.

Effect of temperature on the equilibrium between native and denatured trypsin (Anson and Mirsky 1934).—A study of the effect of temperature on the equilibrium between native and denatured trypsin in 0.01 N HCl shows that the enzyme is almost completely native at 40°C. and almost completely denatured at 50°C. The logarithm of the equilibrium constant, i.e., the ratio of native trypsin to denatured trypsin, is a linear function of the reciprocal of the absolute temperature, as is stated by Van't Hoff's relation between the heat of reaction and the effect of temperature on the equilibrium,

$$-\ln K = \frac{\Delta H}{RT} + C.$$

K equals the equilibrium constant, ΔH is the heat of reaction, and R is the gas constant. Table 31 gives the observed values of the percent denaturation and the values calculated from Van't Hoff's equation, assuming the heat of reaction denatured to native to be −67,000 calories per mol. It will be noticed that this value represents a shift in equilibrium and is not the constant obtained from Arrhenius' equation which represents a rate of reaction. It happens that the numerical value is about the same as that found for other proteins when the rate of denaturation is determined but the significance of the two figures is quite different.

Decrease in activity and protein concentration on heating in dilute acid (Northrop and Kunitz 1932a).—On prolonged heating in dilute acid a secondary change in the protein occurs which is not reversible, and a form of protein which is precipitated by the addition of salt remains in the solution after cooling. There is at the same time a decrease in the activity of the solution corresponding to the decrease in the concentration of soluble protein.

Pepsin digestion (Northrop and Kunitz 1932a).—Trypsin is rapidly digested by pepsin in acid solution, and the decrease in protein nitro-

TABLE 31

EFFECT OF TEMPERATURE ON THE EQUILIBRIUM BETWEEN NATIVE AND DENATURED TRYPSIN IN 0.01 N HYDROCHLORIC ACID

Temperature	*Percentage Denaturation*	*Percentage Denaturation Calculated from* $\ln K = \frac{\Delta H}{RT} + C$ $\Delta H = -67{,}000$ *Calories/Mol*
°C.		
42	32.8	32.8
43	39.2	41.0
44	50.0	50.0
45	57.4	56.4
48	80.4	80.0
50	87.8	87.2

gen under these conditions is proportional to the decrease in activity. This is shown in Table 32. It will be noted that the specific activity of the protein remaining in solution and precipitated by 2.5 percent trichloracetic acid is constant over the first 80 percent of the reaction while the specific activity of the protein, as determined by precipitation with 18 percent trichloracetic acid, decreases from the beginning. The experiment indicates that none of the split products of the protein, including those large enough to be precipitated by strong tri-

TABLE 32

DECREASE IN ACTIVITY AND PROTEIN CONCENTRATION IN TRYPSIN SOLUTIONS DIGESTED BY PEPSIN

	TIME AT 35°C.						
	0 Hour	*1 Hour*	*2 Hours*	*4 Hours*	*8 Hours*	*24 Hours*	*48 Hours*
$[T.U.]^{4g\ V}_{ml.}$	125	99	74	56	40	10	10.3
Protein nitrogen per ml., mgm.							
18 percent CCl_3COOH	1.49	1.24	1.1	0.96	0.79	0.51	0.40
2.5 percent CCl_3COOH	1.21	0.93	0.62	0.51	0.40	0.23	0.20
$[T.U.]^{4g\ V}_{mg.\ P.N.}$							
18 percent CCl_3COOH	84	80	67	58	51	39	26
2.5 percent CCl_3COOH	103	107	119	110	100	87	52

chloracetic acid, has any appreciable activity compared to that of the original protein molecule.

Decrease in activity in dilute alkali.—Trypsin decomposes very rapidly if allowed to stand in solutions more alkaline than pH 6.0. Under these conditions the protein content of the solution decreases with a corresponding decrease in activity in very much the same way as during pepsin hydrolysis.

GENERAL PROPERTIES OF TRYPSIN

Chemical composition of trypsin and trypsinogen.—Trypsin differs from trypsinogen in crystalline form, solubility, and stability as well as in activity. No certain difference, however, can be detected by analysis, and no increase in amino groups can be detected. Trypsin is probably formed from trypsinogen by hydrolysis of a peptide link, and an increase in amino groups would be expected. The experimental method, however, is hardly accurate enough to detect the appearance of a single amino group, but it seems quite certain that the number of amino groups liberated must be small. There is the further possibility that a free carboxyl appears on the molecule instead of a free amino group, but this, again, could not be detected with certainty. While the experiments in this connection are still incomplete, no significant difference has been found in the chemical constitution of the two proteins.

Molecular weight.—The molecular weight of trypsin as determined by osmotic pressure measurements in 0.25 saturated ammonium sulfate in M/10 pH 4.0 acetate, 5°C. is 34,000 ± 1,000.

The diffusion coefficient (Scherp 1933) in 0.50 saturated magnesium sulfate is 0.020 ± 0.001 cm.^{2}/day at 5°C. as determined either by activity measurements or nitrogen determinations. This shows that the activity cannot be separated from the protein by fractional diffusion and, hence, that the size of the molecule possessing tryptic activity must be very nearly the same as that of the protein molecule.

The isoelectric point, as determined by the zone of maximum precipitation on heating or cataphoresis measurements of collodion particles suspended in a solution of trypsin, is about pH 7.0.

Inactivation of trypsin at various pH and temperatures (Kunitz and Northrop 1934).—Trypsin, like other enzymes, becomes inactive when in solution and the rate at which this inactivation occurs de-

pends upon the temperature, the pH, the concentration, and the purity of the solution. Crude trypsin preparations have a maximum stability at about pH 5.0 and the solutions become more stable as the concentration is increased. They are completely and permanently inactivated if heated above 70°C. Solutions of purified crystalline trypsin behave quite differently; they may be heated to boiling (in acid solution) for a short time without permanent loss in activity; in slightly alkaline solution they become more unstable as the concentration of the enzyme increases.

Since the crystalline preparation is apparently a pure protein it is possible to follow changes in the composition of the solution as well as changes in activity. A detailed study has been made of the inactivation of solutions of crystalline trypsin at various pH ranges and at various temperatures below 37°C. in order to determine the nature of the reactions and to see whether the loss in activity under all conditions is proportional to the decrease in trypsin protein.

Reversible inactivation.—The inactivation of trypsin solutions may be either reversible or irreversible. Reversible inactivation is caused by raising the temperature or by making the solution strongly alkaline. This reaction is practically instantaneous. The loss in activity is accompanied by the appearance of reversibly denatured protein which is insoluble in 0.5 M salt solutions at pH 2.0 and which is inactive. The native active protein is soluble even in molar salt solutions at pH 2.0. The reversibly denatured protein is in equilibrium with the active native protein and reverts to the active native protein if the solution is allowed to stand at pH 2.0 and at about 20°C. At 0°C. the reversibly denatured protein becomes demonstrable in the solutions at about pH 8.0. As the alkalinity is increased from pH 8.0 to pH 12.0 the percentage of the trypsin protein present in this reversibly denatured form increases rapidly, and at pH 13.0 practically all of the enzyme is in the denatured form. This decrease in the active form of the enzyme present from pH 8.0–13.0 agrees quite closely with the decrease in the rate of digestion of proteins by trypsin in this range of pH. The formation of this denatured form of the enzyme accounts for the effect of the pH on the digestion of proteins with trypsin on the alkaline side of the optimum and offers experimental evidence to show that changes in the nature of the enzyme protein result in corresponding changes in activity (Figure 2).

Irreversible inactivation.—The rapid loss in activity at higher temperatures or in alkaline solutions, just described, is completely reversible for a short time only. If the solutions are kept under these conditions for a longer time, the loss in activity becomes gradually irreversible. This irreversible inactivation is accompanied by the appearance of various reaction products the nature of which depends upon the temperature and pH of the solution. The loss in activity at various pH is shown in Figure 58. On the acid side of pH 2.0 the trypsin protein is changed to an inactive protein which is irreversibly denatured by heat. The course of the inactivation in this range of pH is monomolecular. The rate of inactivation decreases as the acidity becomes less and is very slow at pH 2.0.

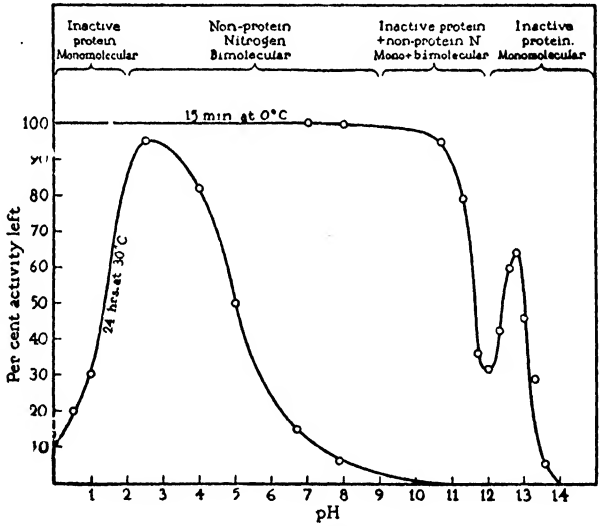


FIG. 58. Loss in activity of trypsin solutions at various pH.

From pH 2.0 to about pH 9.0 the trypsin protein is slowly hydrolyzed and decomposition products which are not precipitated by trichloracetic acid (non-protein nitrogen) appear in the solution. The amino nitrogen content increases but no ammonia is liberated. The kinetics of the irreversible inactivation in this range of pH agree with the assumption that the active native protein hydrolyzes the denatured form with which it is in equilibrium. This reaction results in the formation of non-protein products. The irreversible inactiva-

tion is, therefore, bimolecular in this range of pH and the rate of inactivation increases from pH 2.0 to pH 10.0 and then decreases. Since the inactivation due to the formation of inactive protein increases with increasing acidity, while inactivation due to hydrolysis of the protein increases with increasing alkalinity, there is a pH at which the total inactivation is minimum. This point of minimum irreversible inactivation or point of maximum stability is at pH 2.3.

On the alkaline side of pH 13.0 the reaction is similar to that in strongly acid solution and consists in the formation of inactive protein. No non-protein products are formed during the inactivation and the course of the reaction is monomolecular. The velocity of the reaction increases with increasing alkalinity.

In the intermediate range of pH, from 9.0–12.0, the trypsin protein is partly hydrolyzed and partly changed to inactive protein so that the course of the reaction is represented by the sum of the bimolecular reaction, representing the hydrolysis, and the monomolecular, representing the transformation into inactive protein. As the pH is increased beyond 11.0 the percentage of active trypsin in solution decreases rapidly so that the rate of the reaction resulting in the hydrolysis of denatured trypsin becomes progressively slower. On the other hand, the reaction which results in the formation of inactive protein becomes progressively more rapid, so that as a result of these two reactions there is a second point at about pH 13.0 at which the rate of irreversible inactivation is a minimum.

It was found in general that the decrease in activity under all the various conditions was proportional to the decrease in concentration of the trypsin protein, although in strongly acid or alkaline solutions a small amount of inactive protein is formed at 30°C. which cannot be separated quantitatively from the trypsin protein. As a result the specific activity of the "active native protein" decreases during the experiment. In no case was the specific activity of the protein fraction higher than that of the original trypsin protein. There is, therefore, no indication that protein can be destroyed without a corresponding loss in activity. These results also show that none of the split products of the trypsin protein has any appreciable activity. They are, therefore, similar to the results of the experiments on the inactivation of pepsin. Equations have been derived which agree quantitatively with the results of the various inactivation experiments.

Extent of hydrolysis.—The crystalline trypsin differs from previous trypsin preparations in that it does not carry hydrolysis of gelatin or casein any further than does pepsin, and in fact, with casein, pepsin digestion proceeds further than does trypsin. The pepsin and trypsin hydrolyses take place at different groups in the substrate molecule since the increase in the formol titration caused by the addition of trypsin is the same for gelatin or casein solutions previously digested by pepsin as for the original casein or gelatin solution. Such an experiment is summarized in Table 33 (Northrop and Kunitz 1932b).

TABLE 33

HYDROLYSIS OF GELATIN AND CASEIN WITH CRYSTALLINE PEPSIN AND TRYPSIN

Formol titration per 5 ml. 5 percent protein solution, pH 7.0 ml. N/50 sodium hydroxide

Protein	*Casein Total*	*Increase*	*Gelatin Total*	*Increase*
Original protein solution	9.0		4.0	
After pepsin digestion alone	27.0	18.0	11.5	7.5
After trypsin digestion alone	18.0	9.0	11.0	7.0
After pepsin digestion followed by trypsin	36.0	27.0	19.0	15.0

Trypsin hydrolyzes benzoyl-*l*-arginine-amide (Bergmann, Fruton, and Pollock 1937).

The optimum pH for digestion of casein.—The pH activity curve for digestion of casein with crystalline trypsin is very similar to that with crude trypsin and has a flat maximum from about pH 8.0–9.0. The mechanism responsible for this optimum pH was discussed in Chapter I.

THE MOLECULAR KINETICS OF TRYPSIN ACTION
(Butler 1941b)

Very little reliable information exists about the absolute rates and activation energies of enzyme reactions. In many cases in which measurements have been made, the reaction is sensitive to the hydrogen ion concentration and corrections for the effect of temperature on the pH are necessary before the true activation energy can be

known. Trypsin is particularly suited to a study of this kind because the same enzyme brings about a number of different reactions, e.g., (*a*) the hydrolysis of ammonia from the synthetic peptide benzoyl-*l*-arginine amide (Bergmann, Fruton and Pollock 1939; Hofmann and Bergmann 1941); (*b*) the conversion of chymo-trypsinogen into chymotrypsin (Kunitz and Northrop 1935); (*c*) the autocatalytic conversion of trypsinogen into trypsin (Kunitz and Northrop 1936; Kunitz 1939b and 1939c); (*d*) the digestion of native and denatured proteins. In the region of the optimum pH the effect of changes of pH is comparatively small and the effect of temperature change on the pH can probably be neglected in finding the activation energy of the reaction.

It is known that good unimolecular velocity constants, which are proportional to the trypsin concentration, are obtained for reactions (a) and (b). The action of trypsin on a protein is to be regarded as a group of simultaneous or consecutive reactions and it is not usually possible to find a velocity constant covering the whole course of the reaction. Northrop (1932e) showed that in the action of trypsin on casein the first stage could be distinguished by observing the change of viscosity and a fairly good velocity constant was calculated therefrom. For the comparison of reactions (a) and (b) with (d) we have used as the velocity constant of the latter the initial rate of digestion (measured by the number of acid groups liberated as determined by the formol titration), divided by the molecular concentration of the substrate. This would be identical with the true velocity constant of the reaction when each molecule of substrate gives rise to one equivalent of acid on complete hydrolysis. In other cases it seems a good basis of comparison (on the assumption that the action of trypsin is really primarily the breaking of peptide bonds) as it gives the number of bonds broken by a given concentration of enzyme with equal numbers of substrate molecules. If the protein contains different kinds of bonds which are acted on by the enzyme at different rates, the observed rate will be the sum of the rates of the various actions. In many cases, however, the observed rate would be predominantly that of one particular action and no great error will arise from treating it as such. Considerable caution is required however in the interpretation of the activation energy in such a case since the different processes may have different temperature coefficients. In such a case the Arrhenius equation will not hold.

The procedure can be applied only to the initial stage of the reaction where the protein is mainly intact. As the reaction proceeds new bonds may become accessible to the enzyme, the velocity constants may be influenced by the changes which have taken place in other parts of the molecule, as is well known (Northrop 1922c) the products of the digestion may exert an inhibitory effect and when the protein breaks up the substrate concentration becomes indefinite.

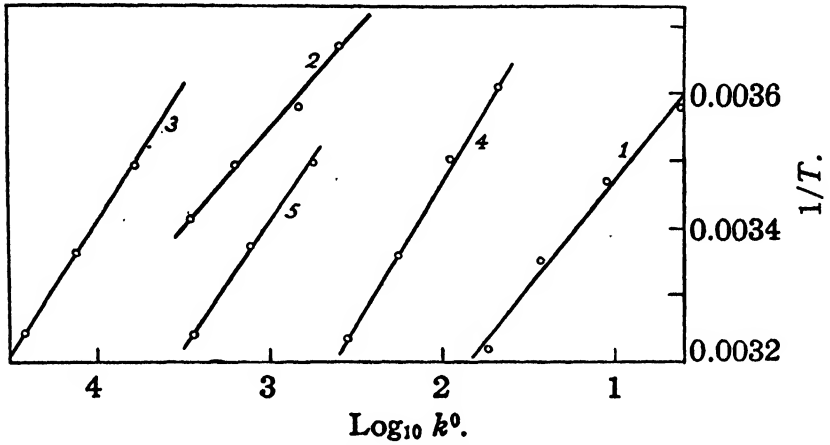


FIG. 59. Plots of $\log_{10}k^0$ against 1/T for: (1) trypsin on benzoyl-*l*-arginine amide; (2) trypsin on chymo-trypsinogen; (3) trypsin on sturin; (4) chymo-trypsin on benzoyl-*l*-tyrosyl-glycyl amide; (5) chymo-trypsin on denatured pepsin.

The choice of a protein substrate presented some difficulty. It is known that trypsin frequently does not act appreciably on native proteins, e.g., egg albumin, but it digests them rapidly when denatured. Casein is acted on rapidly, but it is a mixture of proteins. Pepsin denatured by warming at pH 7.5 was tried as a substrate; trypsin appears to have a real action on it but the amount is so small that the initial rate is very difficult to measure. Trypsin also digests protamines very easily (Waldschmidt-Leitz and Kollmann 1927) and sturin was found to be a very suitable substrate. Experiments were also made with chymo-trypsin acting on the synthetic peptide benzoyl-*l*-tyrosyl-glycyl amide (Bergmann and Fruton 1937 and 1938) and on denatured pepsin.

Discussion.—The evidence previously available appeared to indicate that enzyme reactions are frequently abnormal kinetically. In a

review of the available information Stearn (1938) found that in most cases the entropy of activation of enzyme reactions was considerably more negative than that of the same reactions catalyzed by acids, etc. This means that the increase in the reaction rate brought about by the enzyme is not so great as would be expected from the decrease of activation energy, so that there is a factor which might be a stringent condition of mutual orientation of the substrate and enzyme molecules which "interferes" with the reaction.

This conclusion is not borne out by the data obtained here. Figure 59 shows that over the range of temperatures studied the Arrhenius expression holds within the experimental error. Table 34 summarizes

TABLE 34

VELOCITY CONSTANTS, ENTROPY OF ACTIVATION, AND HEAT OF ACTIVATION OF VARIOUS ENZYME REACTIONS

Enzyme	*Substrate*	$Log_{10}\ k\ (0°)$	$\Delta H‡$	$\Delta S‡$
Trypsin	Benzoyl-*l*-arginine amide	0.40	14,900	−6.2
Trypsin	Chymo-trypsinogen	2.6	16,300	+8.5
Trypsin	Sturin	3.33	11,800	−4.7
Chymo-trypsin	Benzoyl-*l*-tyrosyl-glycyl amide	1.57	10,500	−17.4
Chymo-trypsin	Pepsin	2.34	11,200	−11.5
(Hydrogen ion)	Acetylglycine	−6.47 (60°)	21,200	−24.8

the characteristics of all the reactions and gives the entropy of activation, $\Delta S‡$, and the heat of activation, $\Delta H‡$, calculated by

$$k = \frac{RT}{N_0 h}\, e^{-\Delta H‡/RT} e^{\Delta S‡/R}$$

or, for 0°[1]

$$k^\circ = 5.7 \times 10^{12} e^{-\Delta H‡/RT} e^{\Delta S‡/R}$$

The activation energies of these reactions are rather high for enzymatic processes (10–16 kcal.), although considerably lower than that of the hydrolysis of the peptide bond by acids which is about 21 kcal. (Escolme and Lewis 1927). The entropies of activation are, with one exception, reasonably close to the range (− 5 to − 10) usually taken as representing normal reactions.

[1] In the original paper this equation was given as $K^\circ = 5.7 \times 10^{12}$ . . . , and $\Delta H‡$ was considered identical with E, the Arrhenius constant. Actually $\Delta H‡ = E - RT$. Thus the corrections would increase the values of $AS‡$ in Table 34 by 2.6.

The rates are, in fact, not very different in most cases from that calculated on the simple collision theory, viz.

$$k = Ze^{-\Delta H\ddagger/RT}$$

where Z, the number of collisions between the reacting molecules per cc., is given by

$$Z = n_1 n_2 \left(\frac{\sigma_1 + \sigma_2}{2}\right)^2 \left\{8\pi RT \left(\frac{1}{M_1} + \frac{1}{M_2}\right)\right\}^{1/2}$$

where n_1, n_2 are the numbers of the reacting molecules per cc.; $\sigma_1\sigma_2$ their diameters and M_1, M_2 their molecular weights. Taking for trypsin $\sigma_1 = 6 \times 10^{-7}$ cm., $M_1 = 36{,}000$, we find that the calculated rate, which is not much influenced by the size of the substrate molecule, corresponds to $\Delta S\ddagger = -6$ to -8. The conversion of chymo-trypsinogen, which has a positive entropy, is considerably faster than the other reactions when allowance is made for its high activation energy (cf. page 110).

In reactions involving one or two large molecules it can hardly be expected that every collision having the necessary energy will lead to reaction, since there must inevitably be steric or orientational factors tending to reduce the rate. It follows that even for reactions coming within the normal range for small molecules, there must be a factor producing an enhanced rate. Such a factor may be the complex formation between enzyme and substrate which is a very general feature of enzyme reactions. The formation of a stable complex will evidently increase the rate above that calculated by the collision theory, because, if the molecules spend an appreciable time in union, the chance of reaction is obviously greater than if they separate immediately on collision.

The effect on the reaction velocity of the formation of stable complexes between the reactants does not appear to have been considered closely. If the course of the reaction is formulated as

$$E + S \underset{k'_1 C}{\overset{k_1 ES}{\rightleftarrows}} C \underset{k'_2 C^*}{\overset{k_2 C}{\rightleftarrows}} C^* \xrightarrow{k_4 C^*} X + S$$

where C is the stable complex between enzyme and substrate and C^* the "activated" complex, which gives rise to the reaction products,

we can assume that C is present at an equilibrium concentration (this implies that $k_2 < k'_2$). There are now two cases: (1) The complex C^* is also present in an equilibrium concentration which will be the case if $k_4 < k'_2$. It can, of course, be assumed that the concentration of the energy-rich complex C^* is very small. The rate of reaction will be unaffected by the concentration of C, since the equilibrium concentration of C^* will be the same whether it is derived directly from E or S or from C. (2) If $k_4 < k'_2$ the activated state will not reach an equilibrium concentration and the rate of the reaction will be k_2C, which is the rate of formation of C^*. In this case the concentration of the stable complex may be expected to be a significant factor.

It is not, however, possible at present to make any correlation between reaction rates and the complex stability which is indicated by the Michaelis constant. The formation of a complex has been demonstrated in the action of trypsin on sturin, where the Michaelis constant is of the order of 7.5×10^{-3} mols per liter. In the case of trypsin on chymo-trypsinogen the constant is $> 1.3 \times 10^{-3}$, and it follows that the stability of the latter complex is not so much greater than the former as to account by itself for the greater rate of the latter reaction.[1] A great deal more data will have to be collected before the connection can profitably be discussed in detail.

RELATION OF TRYPSIN TO BLOOD CLOTTING

Effect of trypsin on clotting of blood.—The mechanism of the clotting of blood by trypsin has been further investigated by Eagle and Harris (1937) who found that the trypsin takes the part in the system normally played by tissue extracts or platelets. They consider that the enzyme changes prothrombin to thrombin and suggest that the reaction is analogous to the formation of chymo-trypsin from chymo-trypsinogen by the active trypsin. On the basis of this analogy prothrombin is the inactive precursor of active thrombin which in turn converts fibrinogen to fibrin. Tyson and West (1937) have shown further that trypsin will greatly accelerate the clotting of blood from hemophilic patients *in vitro*.

Tagnon (1944) found that intravenous injection of trypsin accelerated the clotting of hemophilic blood for a short period of time.

Ferguson (1939) and Ferguson and Erickson (1939) also consider

[1] The stability of the complex is inversely proportional to the Michaelis constant.

that the action of trypsin is due to the activation of prothrombin but state that the primary action of trypsin is to liberate cephalin which then combines with calcium and prothrombin to form thrombin.

Milstone (1942), however, found that concentrated partially purified prothrombin solutions changed to thrombin without the addition of any activator. The rate of formation was accelerated by trypsin but was not decreased by the addition of trypsin inhibitor.

The question of blood protease and its relation to trypsin, fibrinolysin, etc., has recently been carefully investigated by Christensen and MacLeod (1945). Their experiments have established the following facts: Blood contains the precursor, "plasminogen," of a proteolytic enzyme "plasmin." This enzyme is identical with Milstone's "fibrinolysin" and also with "serum protease." The plasminogen may be changed to active plasmin by means of an activator, present in hemolytic streptococcus cultures, and also by chloroform. The system as a whole closely resembles the trypsinogen-trypsin system.

Plasmin is distinct from trypsin, since the pH optimum is slightly different and the successive action of the enzymes on casein is almost additive. That is, a solution of casein which has been digested by trypsin is further hydrolyzed by plasmin.

The enzyme is inhibited by pancreatic-trypsin inhibitor but the reaction differs quantitatively from that between trypsin and the inhibitor. Plasmin requires much higher concentrations of inhibitor to decrease the activity than does trypsin.

The fibrinolysin of hemolytic streptococci catalyzes the transformation of plasminogen to plasmin. The rate of the reaction is proportional to the fibrinolysin concentration and the extent of the reaction is independent of the fibrinolysin concentration. The reaction is, therefore, typically enzymatic.

This reaction is complicated by the presence of an inhibitor in much the same way as is the activation of trypsinogen.

Mirsky (1944) has reported that very high concentrations of pancreatic trypsin inhibitor prevent the action of hemolytic fibrinolysin and considers therefore that fibrinolysin itself is similar to trypsin. It is probable, however, as Christensen and MacLeod suggest, that the concentrations of inhibitor were high enough to inactivate the plasmin (serum protease) even after it had been formed.

Rennet action.—The rennet action is very weak and is only about

10 percent of that shown by the original crude material. This weak effect seems to be constant in the preliminary experiments tried so far but it may be due to the presence of a minute amount of some other enzyme.

IMMUNOLOGICAL REACTIONS OF TRYPSIN

Ten Broeck (1934) found that the immunological reactions of various trypsins are similar to those already described for pepsin. In the case of trypsin, however, it is necessary to use the Dale anaphylactic test rather than the precipitin reaction since the anaphylactic reaction is more sensitive and since it was found difficult to obtain precipitins to chymo-trypsin. The results of the test showed that trypsin from swine and cattle and chymo-trypsin and chymo-trypsinogen from cattle all act as distinct antigens in that they will sensitize guinea pigs. Each of the four preparations may be distinguished from the others by the anaphylactic reaction.

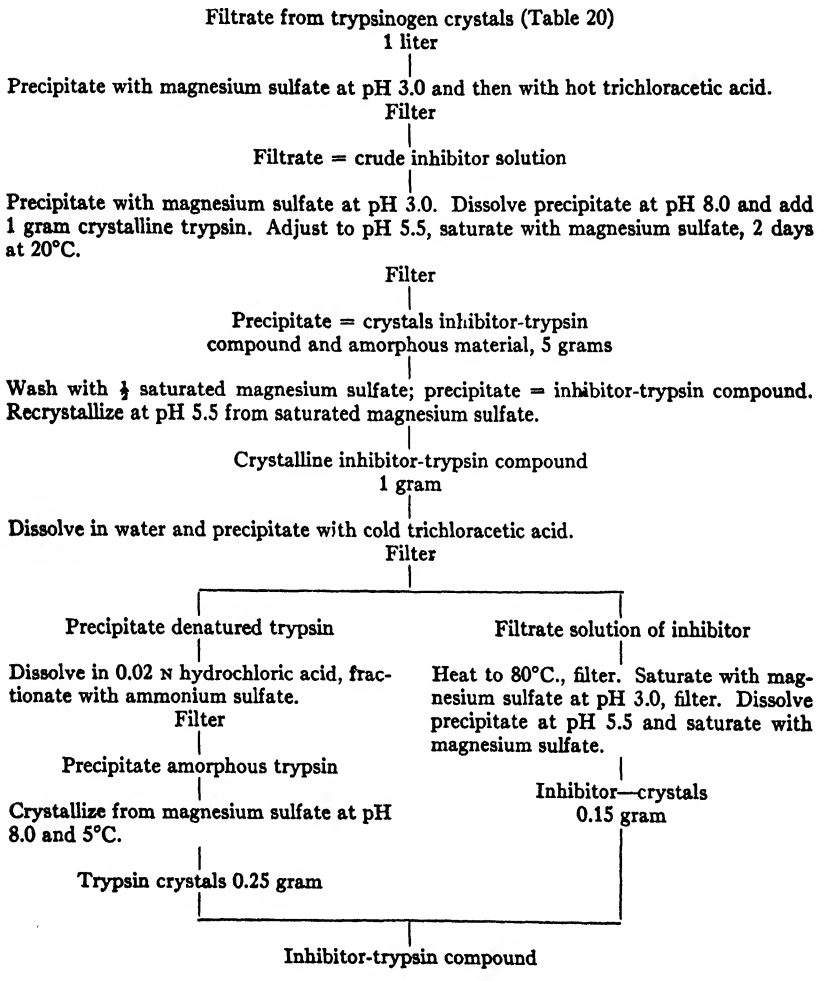
Pharmacological properties.—Rocha e Silva (1939a, 1939b, 1940) has investigated the pharmacological action of purified crystalline trypsin in detail. The enzyme is toxic when injected intravenously. There is an increase in the tone of smooth muscles while the blood pressure drops. The effects are similar to that produced by snake venoms and may be due to the liberation of histamine.

TRYPSIN INHIBITOR

It has long been known that pancreatic extracts contain some substance which inhibits trypsin and this was clearly brought out by Willstätter and Rohdewald. It has been suggested by Dyckerhoff, Miehler, and Tadsen that trypsin exists in fresh pancreas as a compound with the inhibitor so that the formation of trypsin from this inactive compound consists merely in the removal of the inhibitor and that no special inactive form of trypsin, i.e., trypsinogen, exists. The actual isolation of trypsinogen, as described in the present chapter, shows that this explanation is incomplete. The inhibitor, however, does play a very important part in regulating the activation of trypsinogen and there is no doubt that in partly activated pancreatic extracts more or less active trypsin occurs in the form of an inactive compound with the inhibitor. The method of preparation and crystallization of this substance is shown in Table 35 (Kunitz

TABLE 35

ISOLATION OF TRYPSIN INHIBITOR AND INHIBITOR-TRYPSIN COMPOUND



and Northrop 1936). The inhibitor (Figure 60) has the general properties of a polypeptide. It gives a faint biuret test and is precipitated by saturated magnesium sulfate or 0.7 saturated ammonium sulfate, but is not precipitated or changed by 2.5 percent trichloracetic acid, either hot or cold, or by boiling in water. It diffuses slowly

through a collodion membrane and has a molecular weight, by osmotic pressure, of about 6,000. The carbon and nitrogen content appear to be lower than usual for proteins. The amino nitrogen content is low but after acid hydrolysis amounts to 80–90 percent of the total nitrogen so there is reason to believe that the substance is made up largely of amino acids.

REACTION BETWEEN INHIBITOR AND TRYPSIN

When a solution of the inhibitor is mixed with a solution of trypsin of equal molecular strength at pH 7.0, the activity of the mixture decreases rapidly with time and after about half an hour at 6°C. it is completely inactive as measured by the digestion of hemoglobin. If the solution is allowed to stand in the pH range of 7.0–3.0 it remains inactive upon addition to hemoglobin digestive mixture, but if titrated to pH 7.0 before addition to hemoglobin the activity rapidly reappears and in about half an hour will have completely returned. The cycle may be repeated indefinitely. The inhibitor evidently reacts with trypsin to form an addition compound which dissociates in acid solution. Both dissociation and combination require measurable time intervals so that the reaction does not appear to be ionic. An experiment illustrating this inactivation and reactivation is shown in Figure 62. This inactivating effect is apparent also when the activity is measured by the digestion of casein, the clotting of blood, the digestion of sturin, or the activation of chymo-trypsinogen or trypsinogen in the presence of salt. The substance also inhibits chymotrypsin but to a less marked extent.

INHIBITOR-TRYPSIN COMPOUND

This substance is obtained in the form of hexagonal, many-faced crystals (Figure 61). It consists of one molecule of inhibitor combined with one molecule of trypsin. These may be separated by trichloracetic acid which precipitates the trypsin and leaves the inhibitor in solution. As described under "Trypsin Inhibitor," the compound when dissolved in acid solution and added to protein solutions possesses the full tryptic activity, but if allowed to stand for a short time at pH 7.0 and then added to the protein solution it is completely inactive. It differs in one marked respect from trypsin in that it is not adsorbed by egg albumin under the conditions described by Wald-

schmidt-Leitz, whereas trypsin itself is adsorbed under these conditions. The molecular weight by osmotic pressure is 40,000.

CRYSTALLIZATION OF A TRYPSIN INHIBITOR FROM SOYBEAN (Kunitz 1945, 1946, 1947a)

The presence of a protease inhibitor in soybean has been reported by Ham and Sandstedt (1944) and by Bowman (1944). A crystalline material has been isolated from cold-processed defatted soybean meal which acts as a powerful inhibitor of trypsin (Figure 63). The details of the method of preparation are given in the Appendix (cf. page 271).

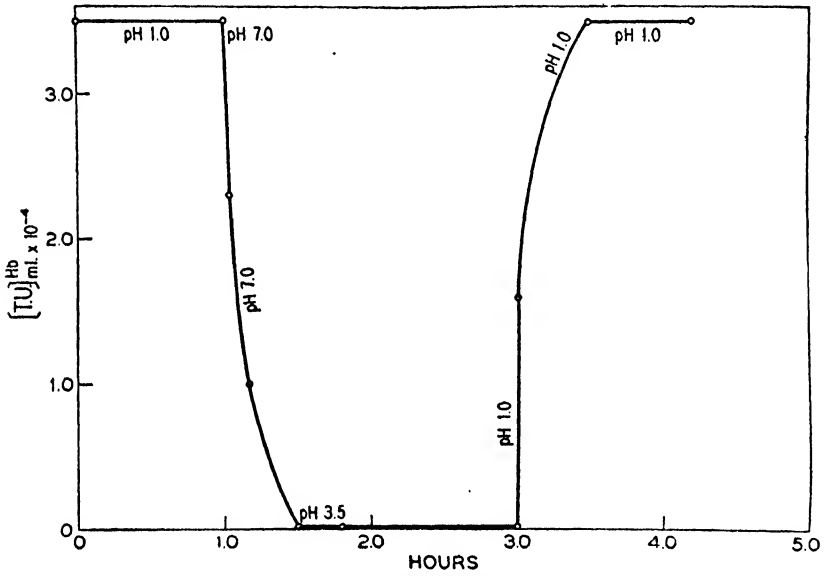


FIG. 62. Effect of time at 6°C. and various pH on the activity of inhibitor-trypsin compound.

General properties.—The soybean inhibitor is a protein of the globulin type. It is precipitated by trichloracetic acid and is nondiffusible through collodion or cellophane membranes. Its light absorption spectrum is that of a typical protein with a maximum at 280 mμ and a minimum at 252 mμ. The protein contains less than 0.01 percent phosphorus and is free of carbohydrate. It acts as an inhibitor only when it is in its native state; denaturation of the soy protein by heat, or acid, or alkali is accompanied by a loss in its inhibiting power.

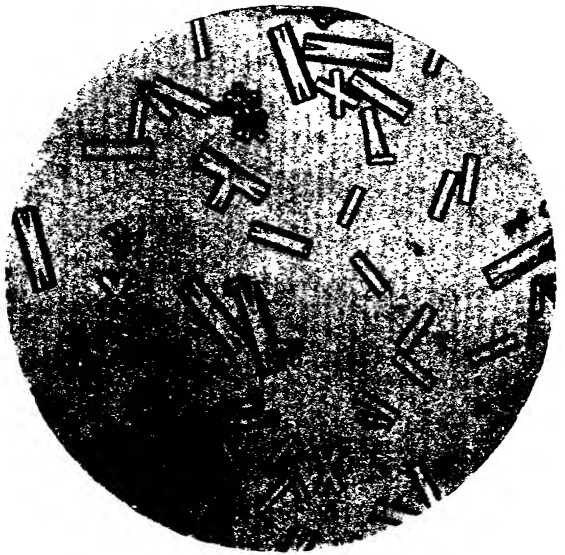


FIG. 60. Trypsin inhibitor crystals. × 225.

FIG. 61. Inhibitor-trypsin compound crystals. × 370.

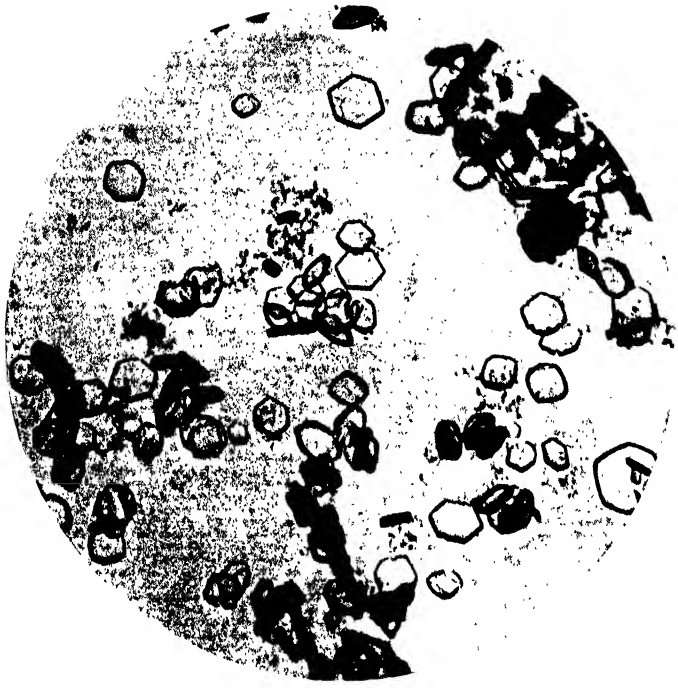


FIG. 63. Soybean trypsin inhibitor crystallized in 20 percent alcohol. × 225.

The action of the native soybean protein as a trypsin inhibitor is due to its combination with trypsin to form an irreversible stoichiometric compound. The combination is apparently instantaneous.

The soy protein inhibits slightly the proteolytic action of chymotrypsin, but unlike that of trypsin the inhibition is due to the formation of a loose reversible compound of the type described by Northrop (1920) for the combination between pepsin or trypsin with crude inhibitors. The reaction between chymotrypsin and the soybean inhibitor was found to agree with the law of mass action for a reversible uni-unimolecular reaction.

Crystalline soybean protein,[1] if denatured, is readily digestible by pepsin, chymotrypsin, and by trypsin.

Crystalline soybean inhibitor has no inhibiting effect either on the proteolytic activity or on the milk-clotting ability of pepsin.

Solubility test for purity.—The purity of a sample of several times crystallized soybean inhibitor was tested by measuring the solubility of the material in the amorphous form in 0.1 M acetate buffer, pH 4.6, at 5°C., in the presence of increasing amounts of solid protein in suspension. The results (Figure 64) are very close to the theoretical for a pure substance.

Reaction between crystalline soybean inhibitor and crystalline trypsin.—Addition of increasing amounts of soy inhibitor to a solution of trypsin decreases the proteolytic activity of the trypsin in direct proportion to the amount of soy inhibitor added. Pure soy inhibitor counteracts approximately an equal weight of pure trypsin. The inhibition is apparently instantaneous and is independent, within a wide range, of the pH of the solution.

The quantitative relationship between the amount of soy inhibitor added and the amount of trypsin inhibited is shown in Figures 65 and 66. For analytical methods, see Appendix, page 310.

The amount of trypsin inhibited is directly proportional to the amount of inhibitor used and is independent of the total concentration of trypsin in the inactivation mixture.

The amount of trypsin inhibited per unit weight of inhibitor when expressed in tryptic units, is independent of the purity of the preparation of trypsin used and it corresponds approximately to a weight

[1] For brevity's sake the terms "soy inhibitor" and "soy protein" are frequently used in the text instead of the full expression "crystalline soybean trypsin inhibitor."

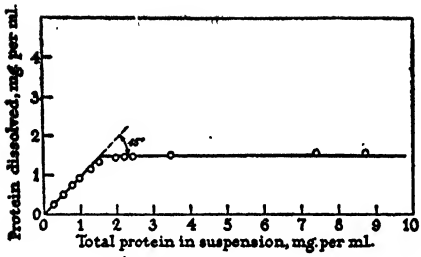


FIG. 64. Solubility curve of amorphous soybean inhibitor.

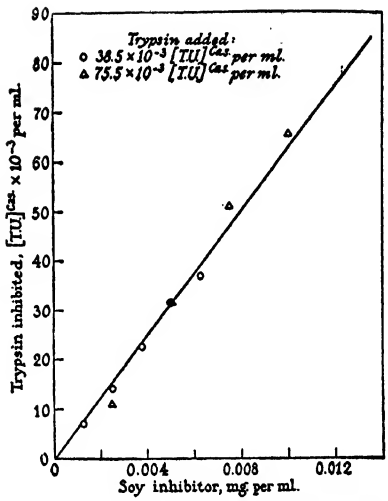


FIG. 65. Effect of soy inhibitor on the digestion of casein by trypsin.

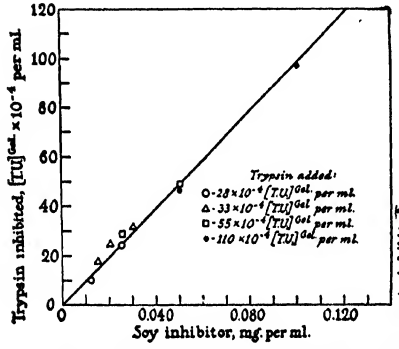


FIG. 66. Effect of soy inhibitor on the digestion of gelatin by trypsin.

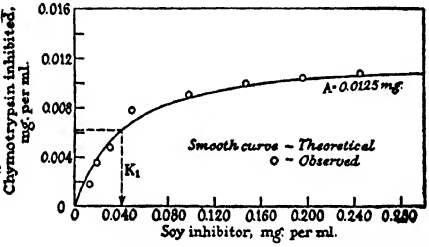


FIG. 68. Effect of soy inhibitor on the digestion of casein by chymo-trypsin.

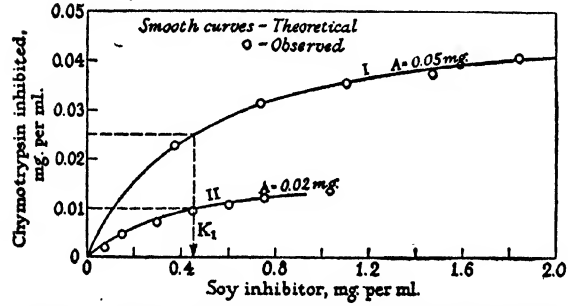


FIG. 67. Effect of soy inhibitor on the clotting of milk by chymo-trypsin.

of pure trypsin equal to the weight of inhibitor used. It appears that the reaction between soy inhibitor and trypsin is of the ionic type similar to neutralization of hydrogen ion by hydroxyl ion. The reaction cannot be reversed either by dilution or by change of pH.

Reaction between chymo-trypsin and soybean inhibitor.—Soy inhibitor exerts a slight inhibiting effect on the proteolytic and the milk-clotting activities of chymo-trypsin. The relationship between the amount of inhibitor used and the amount of chymo-trypsin inhibited as tested on the ability of chymo-trypsin to clot milk is shown in Figure 67. The plotted curves differ strikingly from those obtained for trypsin (Figures 65 and 66). The amount of chymo-trypsin inhibited per unit weight of inhibitor is small compared with that of trypsin and it decreases rapidly with the relative proportion of total inhibitor and chymo-trypsin mixed. The data on the amount of chymo-trypsin inhibited when 20 γ per ml. were used fall on a lower curve than the data for 50 γ chymo-trypsin per ml. The lack of proportionality between the amount of chymo-trypsin inhibited and the soy inhibitor used holds true also for the effect on the digestion of casein, as shown in Figure 68.

The type of curves obtained is similar to those obtained by Northrop (1920) in his studies of the effect of crude inhibitors on pepsin and trypsin and suggest the same mechanism, namely, that the reaction between the soy inhibitor and chymo-trypsin is of the reversible type, obeying the law of mass action, so that there is always an equilibrium between the concentration of the product of the reaction and the concentrations of the reactants in solution.

An analysis of the data is simplified by the fact that the total amount of the inhibitor in all the solutions used is large compared with the amount of inhibitor combined with chymo-trypsin, so that the concentration of free inhibitor equals approximately that of the total inhibitor taken. It is assumed here, as in the case of Northrop's experiments, that the reaction is uni-unimolecular so that one molecule of chymo-trypsin combines reversibly with one molecule of inhibitor to form one molecule of an addition compound.

Let M_a and M_b be the molecular weights of chymo-trypsin and the soy inhibitor proteins respectively. Let also A and B equal the total weights, and a and b equal the weights of the free chymo-trypsin and

inhibitor in solution in volume V, then at equilibrium we have, in accordance with the law of mass action for a reversible reaction,

$$\frac{a}{M_a V} \times \frac{b}{M_b V} = K \frac{A - a}{M_a V} \qquad \text{(Equation 1)}$$

$\frac{A - a}{M_a V}$ being the concentration of the compound formed which is numerically the same as the concentration of the inhibited chymo-trypsin.

K = equilibrium constant

Since $b = B$ (approximately) Equation 1 becomes

$$\frac{a}{A - a} = \frac{V}{B} K_1 \qquad \text{(Equation 2)}$$

where $K_1 = KM_b$ and is equal numerically to the value of

$\frac{B}{V}$ at 50 percent inhibition, i.e., when $\frac{a}{A - a} = 1$

Equation 2 can also be written as $\frac{A - C}{C} = \frac{VK_1}{B}$

Solving for C we get

$$C = \frac{AB}{VK_1 + B} \qquad \text{(Equation 3)}$$

Equation 3 was used to calculate the values of C for the theoretical curves in Figures 67 and 68 for the relationship between C = the weight of chymo-trypsin combined and B = the total weight of inhibitor used, V being equal to 1, since the weights given were expressed per unit volume. The value of K_1 was read in each case at $C = 0.5\,A$ on a preliminary smooth curve drawn between the experimental points in the same region.

The experimental results are in agreement with the theoretical assumption that the mechanism of inhibition of chymo-trypsin by soybean inhibitor consists in the formation of a uni-unimolecular compound in equilibrium with free chymo-trypsin and soy inhibitor in solution. Equation 3 shows that the amount of chymo-trypsin inhibited per unit weight of soy inhibitor is proportional to the total amount of chymo-trypsin in solution and is decreased with the increase in amount of inhibitor used and with dilution; in the case of

trypsin, the amount of trypsin inhibited per unit weight of soy inhibitor is constant and is equal approximately to the weight of inhibitors used, independently of the total concentration of trypsin or inhibitor.

Effect on pepsin.—Soy inhibitor has no inhibiting effect on pepsin, either on its proteolytic activity at pH 2.0 or on its ability to clot milk at pH 5.8. At pH 2.0 soy inhibitor is digestible by pepsin.

Stability and denaturation of soy inhibitor.—Crystalline soy inhibitor is stable in the range of pH of 1 to 12 when dissolved in dilute buffer solution and stored at temperatures below 40°C. At higher temperatures and in stronger acid or alkaline solutions the protein is gradually denatured, as evidenced by a decrease in its solubility at the isoelectric point or in salt solutions. The gradual denaturation of the protein is accompanied by a corresponding loss in its ability to inhibit the action of trypsin.

Denaturation in 0.1 M sodium hydroxide.—A solution of 0.1 M sodium hydroxide containing 2.5 mg. soy inhibitor per ml. was left at 36°C. After various intervals of time, 2 ml. samples were taken, neutralized with 2 ml. of 0.1 M hydrochloric acid, and analyzed for denatured protein and inhibiting activity. The results of the experiment are given in Figure 69. The gradual loss in native protein is accompanied by a corresponding percentage of loss in inhibiting activity.

Denaturation in 0.1 M hydrochloric acid at 50°C., or higher, gave results similar to those obtained on denaturation in 0.1 M sodium hydroxide.

Reversible denaturation by heat.—Soy inhibitor protein, like many other proteins, becomes denatured when heated in dilute acid or alkaline solution at temperatures above 40°C. The denaturation in the absence of salts is not accompanied by any visible precipitation of denatured material. The denatured protein is readily precipitable on addition of salt or on adjusting the pH of the heated solution to that of the isoelectric point. The denaturation is reversed on cooling. Prolonged heating, however, brings about permanent denaturation. The following experiments show that denaturation on heating, and also the reversal of the denaturation on cooling, as well as irreversible denaturation, are accompanied by a corresponding loss or gain in the inhibiting activity.

a. Denaturation (reversible) at 70°C. Samples of 2 ml. 0.1 percent

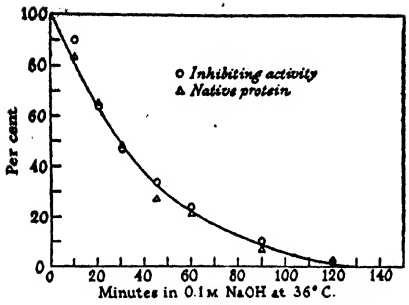


FIG. 69. Denaturation of soy inhibitor in 0.1 M sodium hydroxide and at 36°C.

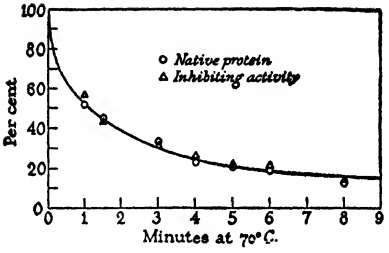


FIG. 70. Reversible denaturation of soy inhibitor at 70°C. and pH 3.0.

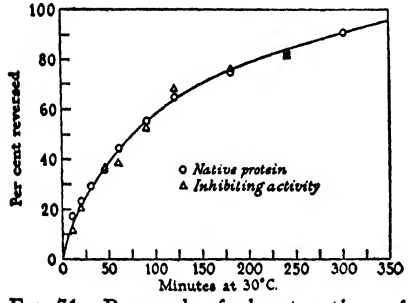


FIG. 71. Reversal of denaturation of soybean inhibitor at 30°C.

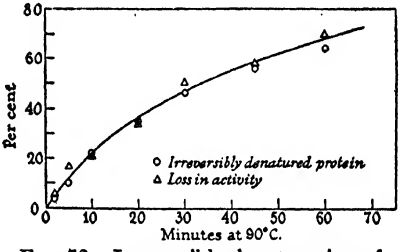


FIG. 72. Irreversible denaturation of soybean inhibitor at 90°C.

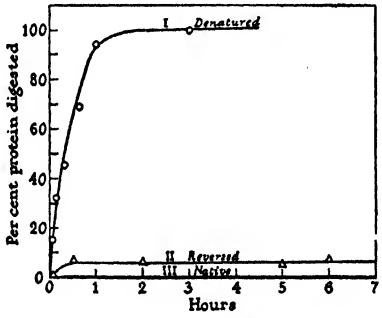


FIG. 73. Digestion of soy inhibitor by pepsin at pH 3.0.

FIG. 74. Compound of trypsin and of soybean inhibitor crystallized in water. × 220.

FIG. 75. Compound crystallized in 20 percent alcohol. × 239.

solution of soy inhibitor of pH about 3.0 (in 0.0006 M hydrochloric acid) were placed in a water bath at 70°C., removed at various times, cooled to about 5°C., and mixed with 4 ml. of 0.15 M acetate buffer, pH 4.5. The precipitates formed were centrifuged after standing one hour at room temperature. The supernatant solutions were analyzed for protein and inhibiting activity.[1] The results are shown in Figure 70.

b. Reversal of denaturation at 30°C. 25 ml. of a 0.25 percent solution of crystalline soy inhibitor in 0.0025 M hydrochloric acid were heated at 80°C. for 5 minutes, then transferred to a water bath of 30°C. Samples of 1 ml. were mixed at various times with 5 ml. 0.06 M acetate buffer, pH 4.5, and treated as described in *a*. The results are shown in Figure 71.

c. Irreversible denaturation at 90°C. Samples of 1 ml. solution of 0.5 percent crystalline soy inhibitor in 0.0025 M hydrochloric acid were heated at 90°C. for various lengths of time and stored at 20°C. for 18 hours. Each sample was then mixed with 5 ml. 0.06 M acetate buffer, pH 4.5, and treated as described in *a*. The results are shown in Figure 72.

The denaturation by heat and also the reversal of denaturation on cooling proceed at a measurable rate. At temperatures above 40°C. and below 60°C. denaturation and the reversal of denaturation proceed until a point of equilibrium is reached between the amount of native and denatured protein in solution. The equilibrium values depend on the temperature and the pH of the solution.

Digestion of Soy Inhibitor Protein by Proteolytic Enzymes

Digestion by pepsin.—Crystalline soy inhibitor protein, if denatured, is readily digestible by pepsin in slightly acid solution. Native soy inhibitor is hardly affected by pepsin at pH 3.0 (Figure 73). On the other hand, in more acid solution, even native soy inhibitor is gradually digested, though only at a rate of less than 1/500 of that of denatured soy protein.[2] The gradual digestion of native soy protein by pepsin at pH 2.0 is accompanied by a proportional loss in trypsin inhibiting activity, so that no significant and definite change in the

[1] Activity measurements when done on the uncentrifuged suspension gave higher values, possibly because of reversal of denaturation in the digestion mixture, pH 7.6.

[2] It is possible that at pH 2.0 or lower the protein becomes gradually denatured to a slight extent, and it is the denatured protein that is digestible by the pepsin.

specific activity of the soy protein is brought about by treatment with pepsin.

Digestion by trypsin and chymo-trypsin.—Soy inhibitor, if denatured, is digestible by trypsin and chymo-trypsin. However, in order to become susceptible to digestion by these enzymes the soybean protein has to be denatured more vigorously than when tested for pepsin digestion, since the range of pH favorable for the action of trypsin and chymo-trypsin is also favorable for the rapid reversal of denaturation of the soy inhibitor, with the resulting inhibition of the proteolytic enzymes. It was found necessary to heat soy protein in 0.1 M sodium hydroxide for 10 minutes at 100°C. in order to make the protein susceptible to the digestive action of small amounts of trypsin or chymo-trypsin. High concentrations of these enzymes undoubtedly digest soy protein even when less vigorously denatured. The measurement of digestion in the presence of relatively high concentrations of the enzymes is complicated by the autolysis of the enzymes, so that the measurements reflect not only on the amount of substrates digested, but also on the digestion of the enzymes themselves.

Chemical and physical properties of soy inhibitor.—A summary of some of the chemical and physical properties of the soy inhibitor protein is given in Table 36.

CRYSTALLINE PROTEIN COMPOUND OF TRYPSIN AND OF SOYBEAN TRYPSIN INHIBITOR (Kunitz 1947b)

Trypsin combines with soybean trypsin inhibitor to form a stable protein compound which has been isolated in crystalline form (Figures 74 and 75).

Some of the Properties of the New Crystalline Compound

Isoelectric point. The compound is a globulin and is least soluble at about pH 5.2. The isoelectric point of the compound is at about pH 5.0 as tested by cataphoresis.

Stability. The compound is stable over a wide range of pH if kept at a temperature below 30°C., and is practically free of either proteolytic or inhibitor activity.

Reversible heat denaturation at pH 3.0. Release of trypsin.—Heating of a solution of the compound at pH 3.0 results in the dena-

TABLE 36

CHEMICAL AND PHYSICAL PROPERTIES OF CRYSTALLINE SOYBEAN TRYPSIN INHIBITOR

Elementary analysis in percent dry weight	C	51.95
	H	7.16
	N	16.74
	S	0.97
	P	0.00
	Ash	0.10
Tyrosine, percent dry weight		4.0
Tryptophane, percent dry weight		2.2
Free amino nitrogen, percent total N		4.0
Total Cu — biuret reagent color value in mg. tyrosine equivalents per mg. protein		0.21
Optical rotation $[\alpha]_D^{25}$ per gm. protein per ml. at pH 3.0		−105.
Extinction coefficient at 280 mμ and at pH 3.0		
Density per mg. protein per ml.		0.91
Isoelectric point		pH 4.5
Molecular weight, by osmotic pressure measurement		24000 ± 3000
Diffusion coefficient		0.07 — 0.08 cm.² per day at 24°C.

turation of the compound. The denaturation is accompanied by an apparent release of the two components, both in denatured form. On cooling there is a reversal of denaturation of both components, the rate of reversal, however, being greater for trypsin than for the soy inhibitor so that the reversed material acts like a mixture of native trypsin and denatured soy inhibitor protein. This results in rapid autolysis, if the solution is made slightly alkaline soon after cooling (Figure 76). There is a striking difference in the rate of autolysis of the heated compound as compared with the autolysis of the non-heated sample. The heated sample is autolyzed initially at a rate approximately equal to that of the same concentration of trypsin. The autolysis of the compound gradually slows down and stops when about 70 percent of the total protein is digested. This is probably caused by the gradual reversal of the denatured inhibitor part of the molecule. There is no striking difference in the rate of autolysis of heated and non-heated trypsin, since the heated trypsin is rapidly reversed to its native state on cooling (Northrop 1932c).

The gradual release of free trypsin on heating at 60°C. in acid solution is shown in Figure 77. The gradual appearance of free trypsin is due to the gradual denaturation of the compound on heating at 60°C. The release of free trypsin reaches, however, only 50 percent of the theoretical value. This may be due to partial reversal of denaturation of the inhibitor in the dilute casein solution at pH 7.6.

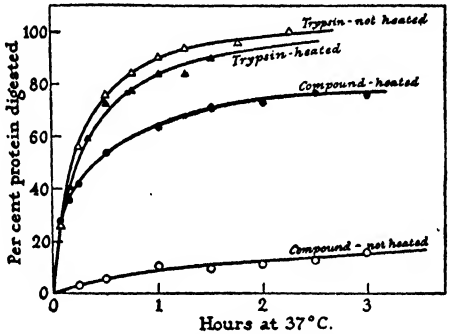


FIG. 76. Autolysis of heat denatured compound compared with autolysis of trypsin.

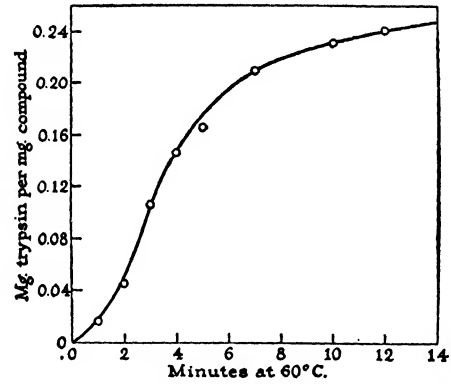


FIG. 77. Release of trypsin from compound on heating at 60°C.

Release of inhibitor from compound on denaturation in sodium hydroxide at 36°C.—It has been shown (Kunitz and Northrop 1934) that trypsin is rapidly and irreversibly denatured at pH above 12.0. Soy inhibitor is likewise denatured in alkaline solution but the denaturation of the inhibitor is reversible if the treatment in alkali is not prolonged. It is thus possible to isolate free inhibitor by exposing the

compound to the action in 0.1 M sodium hydroxide for about 5 minutes at 36°C. and then neutralizing the solution. The appearance of free inhibitor in a solution of the compound in 0.09 M sodium hydroxide is shown in Figure 78.

There is a rapid release of inhibitor, the maximum being reached

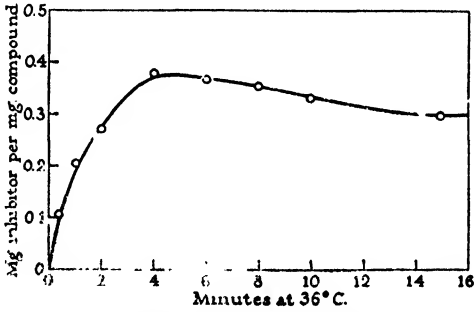


FIG. 78. Release of inhibitor from compound on denaturation in 0.09 M sodium hydroxide at 36°C.

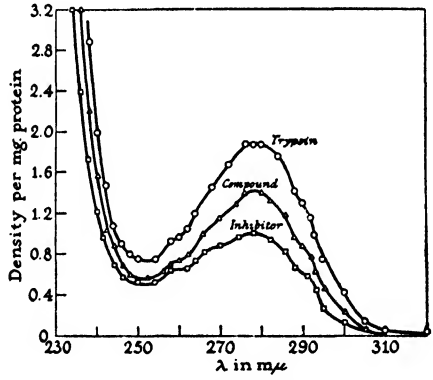


FIG. 79. Ultraviolet light absorption spectra of trypsin, soy inhibitor, and compound.

in about 4 minutes at 36°C. The gradual falling off in the later part of the curve is evidently due to the secondary reaction, namely, the irreversible denaturation of the inhibitor on long exposure to the alkali treatment.

The combining weight of trypsin and soy inhibitor.—It has been shown in the preceding section (cf. page 155) that pure crystalline

soybean inhibitor counteracts the proteolytic activity of about an equal weight of pure crystalline trypsin. The reaction results in the formation of the inert compound. The crystalline compound isolated from a mixture of trypsin and the soy inhibitor does apparently consist of about equal weights of the two components. This is shown by an analysis of some of its "extensive" properties such as tyrosine and tryptophane contents, ultraviolet light absorption, and optical rotation per unit weight of the compound and also of trypsin and the soy inhibitor. The values of these properties per unit weight of compound are approximately equal to the average values of the properties in the two components. See Table 37.

TABLE 37

COMPARISON OF SOME OF THE PROPERTIES OF TRYPSIN, SOYBEAN INHIBITOR, AND OF TRYPSIN-SOY INHIBITOR COMPOUND

	*Tyrosine**	*Tryptophane*	*Optical Rotation* $[\alpha]^{D}_{26}$	*Optical Density at 280 mμ*†	*Free Amino Nitrogen by Formol Titration*‡
	Percent Protein	Percent Protein	Per gm. Per ml.	Per mg. Per ml.	Percent total N
Trypsin	7.8	3.65	−45	1.88	6.90
Soy inhibitor	4.0	2.23	−105	1.05	3.95
Average	5.9	2.94	−75	1.47	5.43
Compound	6.1	2.89	−72	1.40	4.30

* Tyrosine and tryptophane were determined by the methods described by Kunitz (1947a, Table I).
† See Fig. 79.
‡ Northrop (1926b).

Molecular composition of the compound.—No satisfactory data are available at present on the molecular weight of the compound and no definite conclusion can be drawn as yet on the molecular composition of the compound in terms of moles of trypsin and inhibitor.

The nature of the reaction between trypsin and the soy inhibitor.—It has been pointed out in the preceding section that the reaction between trypsin and the inhibitor is apparently instantaneous and that it resembles the ordinary acid-base neutralization. An approximate

estimation of the free amino nitrogen of the three materials indicates a slight loss in free amino nitrogen on formation of the compound (see last column of Table 37). The reaction between trypsin and the soybean inhibitor consists apparently in neutralization of free amino groups of trypsin by free carboxyl groups of the soy inhibitor protein. Further analysis of the amino acid composition of the three materials may elucidate this phase of the problem.

For details of the method of crystallization see Appendix, page 275.

TRYPSIN INHIBITOR IN EGG WHITE
(Balis and Swenson 1934)

Egg white contains a powerful trypsin inhibitor which may be concentrated by precipitation with alcohol and ammonium sulfate. The substance has the properties of a peptone and is probably closely related to the inhibitor protein in the pancreas and in blood.

TRYPSIN INHIBITOR IN BLOOD

Schmitz (1938) found that blood contains an inhibitor which is very similar, if not identical, with that of the inhibitor isolated from pancreas. Glazko and Ferguson (1940) and Horwitt (1940) consider that heparin is a trypsin inhibitor.

PHYSIOLOGICAL EFFECTS OF TRYPSIN INHIBITORS

Mirsky and Foley (1945) state that pancreatic trypsin inhibitor acts as an antibiotic for *Escherichia coli* and other bacteria. The concentrations required are relatively high (1–5 percent).

The inhibition of trypsin has been further studied by Grob (1946). He finds that trypsin and also hemoprotease are slightly inhibited by the reducing substances, sulfonamide and para-aminobenzoic acid. The growth of bacteria is greatly accelerated by the presence of hemoprotease and Grob suggests that the bacteriostatic action of sulfonamide and trypsin inhibitor is due to the suppression of the proteolytic action of hemoprotease and other proteolytic enzymes.

VII: CARBOXYPEPTIDASE

(Anson 1935)

CARBOXYPEPTIDASE splits the amide linkages of certain amino acid compounds such as chloracetyl-tyrosine, tyrosyl-tyrosine, and leucyl-glycyl-tyrosine. In each case an amino acid is liberated which in the intact compound has a free carboxyl group. Since chloracetyl-tyrosine is attacked, it is clear that the substrate of carboxypeptidase need not have a free amino group nor despite the name of the enzyme, need it be a peptide. It has been assumed, but not proven experimentally, that chloracetyl-tyrosine is attacked by only a single enzyme and that the same enzyme which attacks chloracetyl-tyrosine also attacks the other supposed substrates of carboxypeptidase. It may be, however, that what has hitherto been called carboxypeptidase is in reality a mixture of enzymes.

The present chapter describes the preparation from autolyzed beef pancreas of a crystalline, water-insoluble protein (Figure 80) which attacks chloracetyl-tyrosine and peptic digests of proteins. This crystalline carboxypeptidase is active even in the presence of formaldehyde, which abolishes the free amino groups of both enzyme and substrate.

Two sources of material have been used for the preparation of crystalline carboxypeptidase (Anson 1937a): the turbid fluid which exudes when sliced frozen bovine pancreas is thawed overnight at 5°C., and ordinary commercial frozen pancreas which can be obtained from any of the large meat packers. The turbid fluid is by far the more convenient starting material. One liter of this fluid yields roughly a gram of crystals. [Appendix, page 277]

The specific activity of crystalline carboxypeptidase is 0.081 $[CP.U.]^{Ct}_{mg.N}$ measured with chloracetyl-tyrosine and 0.103 measured $[CP.U.]^{PDE}_{mg.N}$ with peptic digest. The specific activity of twice crystallized carboxypeptidase is not changed by repeated or fractional recrystallization. If crystalline carboxypeptidase is partially dena-

FIG. 80. Carboxypeptidase crystals. × 85.

tured and precipitated, the surviving soluble protein has the same specific activity as the original crystalline material.

Recrystallized carboxypeptidase gives no test for proteinase, dipeptidase, amino polypeptidase, or amylase.

The elementary analysis of carboxypeptidase is: C, 52.6 percent; N, 14.4 percent; H, 7.2 percent; S, 0.47 percent; P, 0.00 percent; ash, 0.68 percent.

An amount of carboxypeptidase containing 0.20 mg. N gives the same color with the phenol reagent as 0.15 mg. tyrosine.

Ågren and Hammarsten (1937b) report that carboxypeptidase crystals prepared by them contain trypsin but do not state whether or not the enzyme was recrystallized. The preparation was not homogeneous in the electrophoresis cell.

Putnam, Neurath, Elkins, and Segal (1946) have prepared pure carboxypeptidase by repeated recrystallization. The preparation was homogeneous in the electrophoresis cell. The isoelectric point was at pH 6.0 and the molecular weight was 31,600.

PRO-CARBOXYPEPTIDASE (Anson 1937b)

Extracts of autolyzed pancreas contain carboxypeptidase which even in the presence of formaldehyde can digest chloracetyl-tyrosine and peptic digest of edestin. If fresh pancreas is extracted with cold salt solution, the extract does not attack a formalinized peptic digest of edestin. On standing at 37°C., however, the extract slowly becomes active. The activation is enormously hastened by the addition of trypsin. Thus, fresh pancreas contains not active carboxypeptidase (CP) but an inactive precursor, pro-carboxypeptidase (PCP). The nature of this precursor is not known. It may be a protein which is different from carboxypeptidase. It may be carboxypeptidase combined with an inhibitor.

Pro-carboxypeptidase can be partially purified by fractionation with ammonium sulfate. Most of the pro-carboxypeptidase in the extract is precipitated by 0.35 saturated ammonium sulfate but not by 0.2 saturated ammonium sulfate. The protein can be freed of ammonium sulfate by precipitation with ferric chloride or by dialysis under carefully controlled conditions.

The pro-carboxypeptidase in the partially purified preparation, like the pro-carboxypeptidase in the crude pancreatic extract, is

activated by trypsin. Partially purified pro-carboxypeptidase contains trypsinogen, which trypsin can convert into trypsin. The activation of impure pro-carboxypeptidase by trypsin, therefore, is partially due to the added trypsin and partially due to trypsin formed from the trypsinogen present. Until pro-carboxypeptidase is prepared free from trypsinogen, experiments on the kinetics of activation of pro-carboxypeptidase by trypsin are of dubious significance.

Enterokinase can activate crude pro-carboxypeptidase. This activation may also be due to the trypsin formed from trypsinogen by enterokinase. In the presence of sufficient trypsin-inhibitor no activation takes place. From this result alone one cannot decide whether the inhibitor acts by eliminating activation by trypsin or by interfering with direct activation by enterokinase. The inhibitor does not affect the activity of activated carboxypeptidase.

Pro-carboxypeptidase is not activated by a small amount of chymo-trypsin. If a large amount of chymo-trypsin is used there is in time a partial activation which may be due to a slight impurity of trypsin.

In general the results agree with but do not prove the hypothesis that pro-carboxypeptidase, like chymo-trypsinogen, is activated only by trypsin. More conclusive experiments are not possible with the impure pro-carboxypeptidase now available.

VIII: CRYSTALLINE RIBONUCLEASE[1]

(Kunitz 1940)

THE presence in pancreas of a heat stable enzyme capable of digesting yeast nucleic acid was described by Jones (1920). He found that the digestion was not accompanied by any liberation of free phosphoric acid.

This chapter describes the method of preparation and the properties of a crystalline protein which acts as a powerful digestive enzyme on yeast nucleic acid. The protein was isolated from beef pancreas.

Crystalline ribonuclease (Figures 81, 82) is a soluble protein of albumin type. Its molecular weight is about 15,000. It contains very little, if any, phosphorus. It yields on hydrolysis tyrosine but not tryptophane. Crystalline ribonuclease is very stable over a wide range of pH. The activity is only very slowly diminished irreversibly when the protein is heated at 100°C. at pH 2.0. Heating at pH 5.0 or in more alkaline solution brings about a gradual denaturation of the protein with a corresponding percentage loss of enzymatic activity.

The digestion of yeast nucleic acid by ribonuclease is accompanied by a gradual formation of free acid groups without any significant liberation of free phosphoric acid. The split products, unlike the undigested yeast nucleic acid, are not precipitable by glacial acetic acid or by 0.5 M hydrochloric acid. The products of digestion readily diffuse through collodion or cellophane membranes that are impermeable to the undigested yeast nucleic acid. Crystalline ribonuclease does not appear to exert any significant digestive action on thymus nucleic acid but is reported to hydrolyze thymus nucleohistone (Cohen 1945). It acts as an inhibitor for some oxidation enzymes but not for urease or xanthine oxidase (Potter and Albaum 1943).

[1] Loring and Carpenter (1943) have suggested the name "ribonucleinase" for this enzyme, in conformation with the nomenclature of Levene and Medigreceanu. The name "ribonuclease," however, has been so widely used in the literature that the writers believe a change in nomenclature now would lead to confusion.

Eiler and Allen (1941) consider the enzyme to be a specific phosphatase.

Bolomey and Allen (1942) find that nucleic acid is hydrolyzed much more rapidly by other phosphatases after preliminary hydrolysis with ribonuclease.

Loring and Carpenter (1943) isolated and identified four mononucleotides, guanylic acid, uridylic acid, cytidylic acid, and adenylic acid, after hydrolysis of yeast nucleic acid by crystalline ribonuclease.

METHOD OF ISOLATION OF CRYSTALLINE RIBONUCLEASE

The method of isolation consists essentially in separating the proteins of an acid extract of fresh beef pancreas by means of fractional precipitation with ammonium sulfate. The bulk of the ribonuclease protein is found in that fraction which is soluble in 0.6 saturated ammonium sulfate and insoluble in 0.8 saturated ammonium sulfate solution (see Appendix, page 280).

DIGESTION OF YEAST NUCLEIC ACID BY CRYSTALLINE RIBONUCLEASE

Addition of crystalline ribonuclease to a solution of yeast nucleic acid under appropriate pH and temperature conditions brings about a gradual splitting of the nucleic acid molecules into smaller components. This is shown by an increase in the diffusibility of the nucleic acid. The splitting of the molecules of yeast nucleic acid by the new enzyme is accompanied by formation of titratable acid groups without the liberation of free phosphoric acid.

DIFFUSION MEASUREMENTS

An approximate estimate of the relative molecular size of the split products as compared with the size of the undigested yeast nucleic acid is conveniently obtained by measuring the diffusion coefficient of the material. The method of Northrop and Anson (1929; Anson and Northrop 1937) has been employed for this purpose. The results show that the diffusion coefficient of the digested acid is about twice that of the original nucleic acid. Since the molecular weight is proportional to the third power of the diffusion coefficient, this result indicates that the nucleic acid is hydrolyzed into approximately eight fragments.

FIG. 81. Crystals of ribonuclease. × 248 and 190.

FIG. 82. Crystals of ribonuclease in alcohol. × 190.

EFFECT OF DIGESTION ON PRECIPITATION OF YEAST NUCLEIC ACID WITH GLACIAL ACETIC ACID

Undigested yeast nucleic acid is insoluble in concentrated acetic acid or in dilute hydrochloric or sulfuric acid; hence addition of these acids to a solution of yeast nucleic acid brings about complete precipitation of the nucleic acid. The effect of digestion of yeast nucleic acid by crystalline ribonuclease is to prevent the precipitation by acetic or other acids. This effect is very striking in the case of dilute solutions of yeast nucleic acid. Concentrated solutions of digested nucleic acid continue, however, to give precipitates when mixed with the precipitating reagents even after long digestion with an excess of enzyme. A quantitative study shows that the undigested material which is still precipitable amounts to 10-15 percent of the total nucleic acid in solution. This may be due to the presence of some modified nucleic acid which cannot be attacked by the enzymes.

The nucleic acid in the tobacco mosaic virus protein is not digested by ribonuclease. The enzyme and virus form an insoluble and inactive compound (Loring 1942).

Effect of concentration of enzyme.—Tubes containing 0.5 ml. 2 percent yeast nucleic acid in 0.1 M borate buffer pH 8.0 and 0.5 ml. of various concentrations of ribonuclease in water were placed in a water bath of 25°C. for 10 minutes. Then 10 ml. of glacial acetic acid were added to each tube, mixed thoroughly, and filtered after 5 minutes through No. 42 Whatman paper. The total phosphorus per ml. filtrate was measured and designated as "soluble phosphorus." The results show that addition of even one part of ribonuclease to 2,000 parts of substrate causes formation of "soluble phosphorus" in 10 minutes at 25°C., equal to 73 percent of the total phosphorus in the substrate. The ultimate extent of digestion even in the presence of large excess of enzyme is 87 percent.

Effect of temperature.—The optimum temperature for the rate of digestion as measured by the rate of formation of "soluble phosphorus" is 65°C. The rapid decline in the rate of digestion at temperatures above 65°C. is probably due to the inactivation of the enzyme.

Effect of pH.—Ribonuclease acts best in the range of pH 7.0–8.2, the optimum being at pH 7.7.

Kinetics of the reaction.—The digestion of yeast nucleic acid by crystalline ribonuclease when measured by the rate of formation of "soluble phosphorus" follows the course of a typical enzymatic reaction.

The time required for any amount of digestion is inversely proportional to the concentration of enzyme in solution while the ultimate amount of digestion is independent of the amount of enzyme used.

A mathematical analysis of the kinetics of the process is complicated by the fact that the enzymatic action is always accompanied by a significant amount of spontaneous hydrolysis of the substrate.

Formation of free acid.—The digestion of yeast nucleic acid by crystalline ribonuclease is accompanied by a gradual formation of titratable acid groups. The rate of formation of free acid is much slower than the rate of formation of "soluble phosphorus." The ultimate amount of gram atoms of free acid formed is about one half of the ultimate amount of gram atoms of phosphorus non-precipitable in concentrated acetic acid.

PROPERTIES OF CRYSTALLINE RIBONUCLEASE

Chemical and physical properties.—Table 38 contains data for the elementary analysis and for other chemical and physical properties of ribonuclease. The material is a protein with a molecular weight of about 15,000.

Ultraviolet absorption.—The ultraviolet absorption spectrum of ribonuclease has been determined by Uber and Ells (1941). The spectrum resembles that of most proteins and shows no indication of a special group.

X-ray and crystal structure (*Fankuchen 1941*).—The crystals gave very sharp diffraction spots down to 2 A, which is the lowest value found with any proteins. The unit cell was found to be: $a = 36.6$ Å, $b = 40.5$ Å, $C = 52.3$ Å. There appear to be four molecules per unit cell. On this basis the cell volume is 77,300 A^3 and the molecular weight of the hydrated molecule 15,700 ± 300.

Antigenicity (*Smolens and Sevag 1942*).—The enzyme is a good antigen and reacts with antibody at a dilution of 1:1,000,000.

Stability.—An aqueous solution of crystalline ribonuclease is quite stable over a wide range of pH when kept at temperatures below 25°C.

Heating to higher temperatures causes gradual loss in enzymatic activity. The rate of inactivation varies, however, with the pH of the solution. Ribonuclease is more stable in acid than in neutral or alkaline solutions. The region of maximum stability is between pH 2.0 and 4.5.

TABLE 38

CHEMICAL AND PHYSICAL PROPERTIES OF CRYSTALLINE RIBONUCLEASE

Elementary analysis in percent dry weight*	C	48.7
	H	6.2
	N	16.1
	S	3.6 (partly inorganic)
	P	Trace
	Residue	0.1
Amino nitrogen as percent of total nitrogen†		6.95
Tyrosine equivalent in millimols per mg. total nitrogen‡		2.65×10^{-3}††
Tryptophane§		0
Optical rotation of 5 percent solution in water $[\alpha]_{25}^{D}$ per mg. nitrogen		−0.47
Molecular weight by osmotic pressure measurement** at 5°C. of 2.5 percent solution in 0.5 M and 1 M ammonium sulfate (average of 9 determinations)		15,000 ± 1,000
Diffusion coefficient at 20°C. in 0.5 M ammonium sulfate by the method of Northrop and Anson (1929; Anson and Northrop 1937)		0.092 cm.² per day
Molecular volume calculated from diffusion coefficient		14,850
The following measurements were reported by Dr. Rothen (1940)		
Isoelectric point by electrophoresis		About pH 8.0
Specific volume at 25°C.		0.707
Sedimentation constant at 25°C. in 0.5 M ammonium sulfate		1.84×10^{-13}
Molecular weight calculated from sedimentation and diffusion data		13,000
Diffusion coefficient in 0.5 M ammonium sulfate at 25°C.		0.116 cm.² per day
Protein tests	Biuret	Positive
	Xanthoproteic	Positive
	Millon	Positive

* Analyses carried out at the Arlington Laboratories, Arlington, Virginia.

† Amino nitrogen measured by Van Slyke's manometric method.

‡ 1.0 ml. of dialyzed solution containing 0.13 mg. total nitrogen plus 1.0 ml. 1 M hydrochloric acid plus 3.0 ml. water plus 10 ml. 0.5 M sodium hydroxide plus 3.0 ml. of Folin and Ciocalteu's phenol reagent (Folin and Ciocalteu 1927) diluted twice with water. Color read after 10 minutes against a similar mixture containing 1×10^{-6} millimols tyrosine.

§ Colorimetric method of R. W. Bates (1937).

** Northrop and Kunitz method (1926, page 354).

†† The figure of 5.3×10^{-3} which appeared in the original paper was an error.

RELATION OF THE ENZYMATIC ACTIVITY TO THE NATIVE PROTEIN

Pepsin digestion of ribonuclease.—Crystalline ribonuclease is readily digestible by pepsin in acid solution. The rate of digestion of ribonuclease protein by pepsin is accompanied by a corresponding percentage loss in the enzymatic activity of the ribonuclease.

Denaturation by heat.—The gradual inactivation of ribonuclease when heated at 100°C. is accompanied by gradual denaturation of the protein. The rate of denaturation can be measured by the change in the solubility of the protein in ammonium sulfate solution.

Inactivation of ribonuclease at 100°C. is accompanied by a corresponding proportional loss in the concentration of native protein in the ribonuclease solution.

Inactivation by alkali.—When ribonuclease is exposed to the action of alkali of pH 12 or higher it gradually loses its enzymatic activity. The loss in activity is also accompanied by a change of the native protein into denatured protein which, like the denatured protein produced by heat, is insoluble in 0.66 saturated ammonium sulfate. Experiments showed repeatedly that the rate of inactivation by alkali is proportional to the rate of change of the native protein into denatured protein.

It is thus evident that changes brought about in the protein molecule by various agents such as heat, alkali, or pepsin, are reflected in every case by a corresponding change in the enzymatic activity of the molecule. This suggests that the enzymatic activity is directly related to the protein molecule.

TESTS OF PURITY OF CRYSTALLINE RIBONUCLEASE

Repeated crystallization.—Crystalline ribonuclease becomes relatively pure after two or three crystallizations and it retains through further repeated crystallization a constant activity per unit dry weight.

Fractional crystallization.—The material after purification by two or three recrystallizations does not show any difference in the properties of the various crops of crystals obtained through fractional crystallization in various concentrations of ammonium sulfate. The specific activity of the first small crop of crystals does not differ from the specific activity of the succeeding crops and even from the specific

activity of the last small amount of material left in solution in the mother liquor.

Solubility test.—The theory as well as the technique of the solubility test for the purity of a protein has been described elsewhere (Kunitz and Northrop 1938b). Measurements were made here of the solubility of crystalline ribonuclease in 0.6 saturated ammonium sulfate pH 4.0 in the presence of increasing amounts of crystals of ribonuclease in suspension.

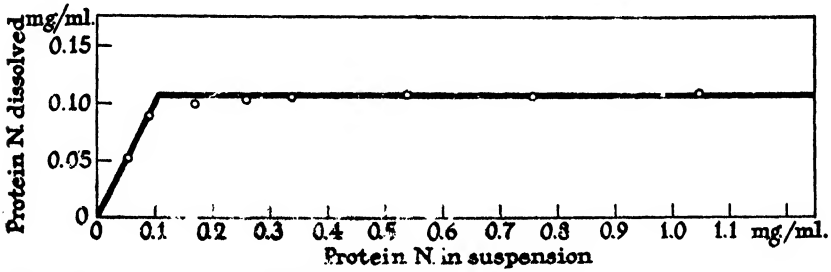


FIG. 83. Solubility of three times crystallized ribonuclease in 0.6 saturated ammonium sulfate pH 4.0 in the presence of increasing quantities of solid phase.

The results are shown in Figure 83. The solid lines represent the theoretical solubility curve of a pure substance. The experimental points fall on the theoretical lines except for one or two points. This indicates the possible presence of a small amount of impurities in the material used. The analytical data for the concentration of protein nitrogen were used. The same result would be obtained if the activity data were used for plotting the curve since the ratio of activity to protein nitrogen was found to be practically constant in all cases.

Amino acid content.—The amino acid content of ribonuclease is given in Table 4 (page 26). No distinctive values, which would differentiate the enzyme from other proteins, are apparent. The value for the molecular weight calculated from the amino acid content appears to be somewhat higher than that obtained by direct physical measurements.

MOLECULAR WEIGHT AND ELECTROPHORESIS OF CRYSTALLINE RIBONUCLEASE (Rothen 1940)

Electrophoresis studies.—Experiments were carried out from pH 4 to pH 10 in a Tiselius apparatus at 0°C. using the Longsworth scan-

ning method for the observation of the boundaries (Longsworth 1939). 25 to 30 ml. of solutions containing 1 percent of three times crystallized ribonuclease were made up in appropriate buffers and dialyzed in collodion bags for several days at 5°C. against 2 liters of the corresponding buffer solution. The outside buffer solutions were used to fill the upper compartments of the Tiselius apparatus.

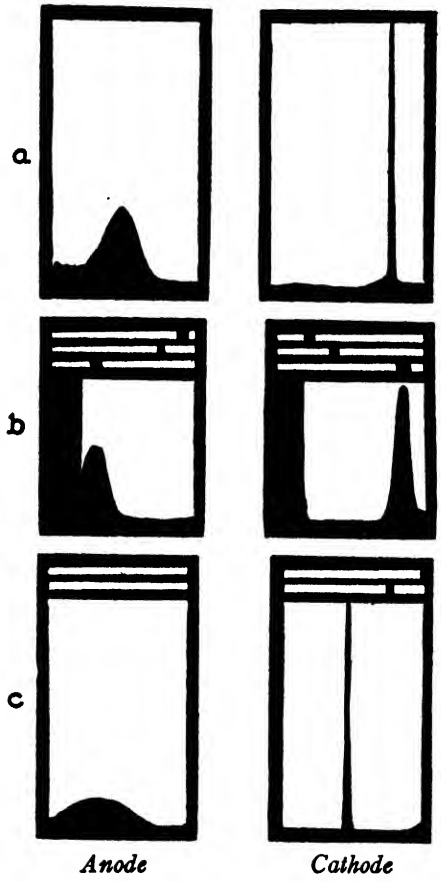


FIG. 84. Electrophoretic patterns of ribonuclease.

The electrophoretic patterns indicated the presence of only one moving component. The boundaries showed the phenomenon of reversible boundary spreading to a considerable extent. The boundaries

moving towards the cathode were much sharper than the boundaries moving towards the anode. A few patterns have been reproduced in Figure 84. The boundaries at pH 4.1, 7.8, and 8.9 can be seen in Figures 84a, b, c, respectively. The isoelectric point is at pH 7.8.

Diffusion measurements.—Diffusion measurements were carried out at 25°C. in the apparatus used for the electrophoresis experiments. A 1 percent solution of ribonuclease in 0.5 M ammonium sulfate, pH 5.8 was used.

From these data the value of the diffusion constant was computed to be $D^{25} = 1.36 \times 10^{-6}$ (in 0.5 M ammonium sulfate)

Ultracentrifuge studies.—The apparatus used was an air-driven centrifuge of the turret type described by Bauer and Pickels (Svedberg 1940, page 191). Three methods were used to follow the course of sedimentation: the light absorption method for which a resonance mercury arc lamp served as source of light, the scale method of Lamm, and the "schlieren method" as used by Svensson (1939) in electrophoretic studies. The speed of the centrifuge was measured by a 631 A Strobotac built by the General Radio Company.

Rate of sedimentation.—On account of the relatively low molecular weight of this enzyme, long time intervals were needed to obtain a sufficient displacement of the boundary. During that time a considerable amount of diffusion took place, making it difficult to determine accurately the maxima of the displacement curves obtained by the scale or schlieren methods as well as the position of 50 percent concentration range when the absorption method was used. Only one moving component was observed, and the symmetry of the curves (absorption, schlieren, or "scale" curves) indicated the fair homogeneity of the material.

Different preparations of crystalline ribonuclease were used. No difference in the rate of sedimentation could be detected in material recrystallized from ammonium sulfate solution or from dilute alcohol. Solutions of 1 percent ribonuclease and cells 3 and 6 mm. thick and 15 mm. long were used.

Specific volume.—The specific volume was calculated from accurate determination of densities of solutions of known concentration.

The value 0.709 was used for calculation. It should be noted that it is a very low value compared to that found for most proteins with the exception of cytochrome.

Molecular weight from rate of sedimentation.—Taking $S^{25} = 1.85 \times 10^{-13}$ (in 0.5 M ammonium sulfate) and $D = 1.36 \times 10^{-6}$ (in 0.5 M ammonium sulfate), the molecular weight according to the expression

$$M = \frac{RTs}{D(1 - V\rho)} \text{ becomes } M = 12{,}700.$$

Molecular weight from sedimentation equilibrium.—A sedimentation equilibrium run was made in 0.5 M ammonium sulfate solution in a cell 6 mm. thick and 15 mm. long. The speed was 14,400 R.P.M. and the temperature 21.8°C. The scale method was used for the determination of the distribution of the refraction gradients with a scale distance of 10 cm. Total time of centrifugation was 66 hours.

The average value for the molecular weight was 13,000. This low value found for the molecular weight of a protein shows how much caution should be exerted before accepting the idea of a universal protein building-stone of a weight of 17,600 (Svedberg 1940, page 406).

Dissymmetry factor.—Since the molecular weight has been determined from parameters which do not include the assumption of Stokes' law (equilibrium measurements or sedimentation plus diffusion measurements) it is possible to calculate the dissymmetry factor which is found to be 1.04. This low value shows the high degree of symmetry of the shape of the molecule of ribonuclease, the most symmetric of all proteins investigated. With the aid of the formula of Herzog, Illig, and Kudar, and of Perrin, the ratio of the two main axes of the molecules is calculated to be about two.

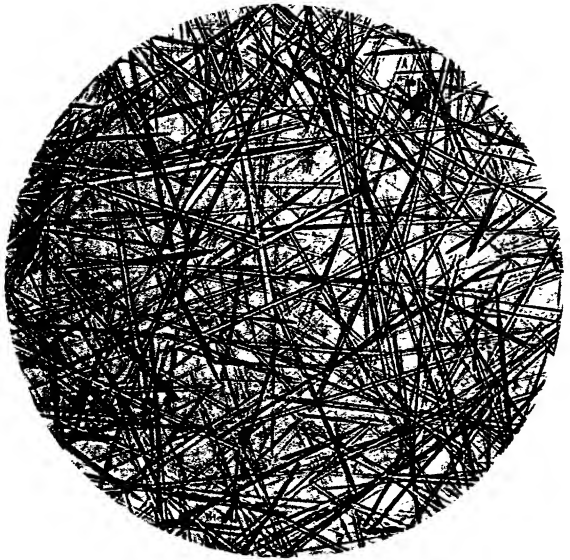


FIG. 85. Crystals of yeast protein No. 2. × 129.5.

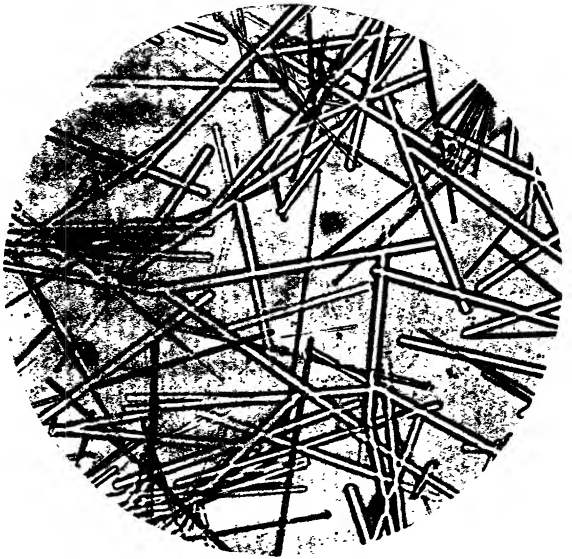


FIG. 86. Crystals of yeast protein, No. 3. × 129.5.

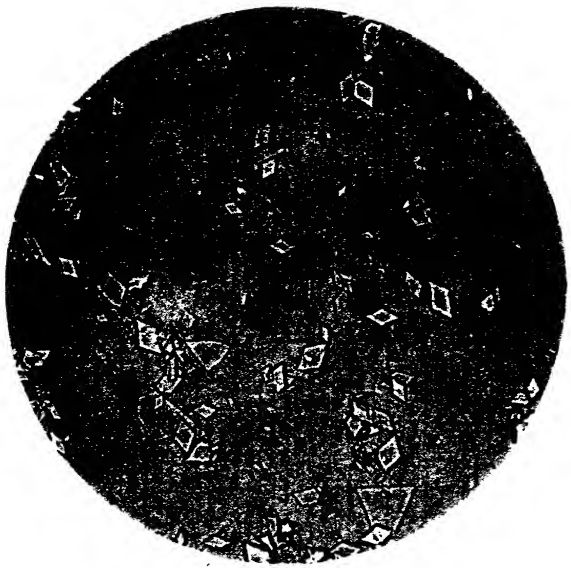


FIG. 87. Crystals of yeast yellow protein. × 128.

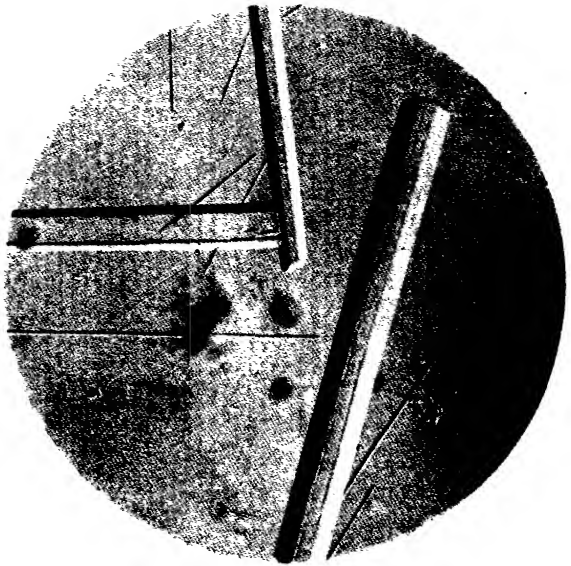


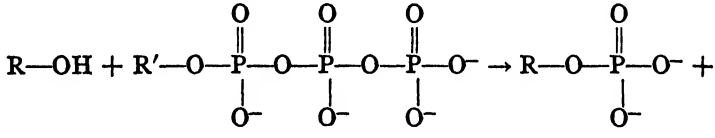
FIG. 88. Large crystals of hexokinase. × 116.

IX: CRYSTALLINE HEXOKINASE (HETEROPHOSPHATESE)[1]

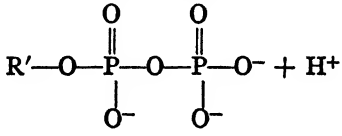
(Kunitz and McDonald 1946b)

MEYERHOF in 1927 obtained an "activator" from yeast which restored to aged muscle juice its lost ability to ferment glucose. In 1935 Euler and Adler found an enzyme in yeast which catalyzed the transfer of phosphorus from adenosine triphosphate to hexoses. They named the enzyme "heterophosphatese." Meyerhof (1935) then established that his "activator" of 1927, which he renamed "hexokinase" had the same enzymatic property as Euler's "heterophosphatese."

Colowick and Kalckar in 1941 showed that hexokinase catalyzes the transfer of one phosphate group from adenosine triphosphate to glucose with the liberation of one hydrogen equivalent of acid. The reaction is represented as follows:



Glucose Adenosine triphosphate Glucose-6-phosphate



Adenosine diphosphate

This chapter describes the isolation from baker's yeast of pure hexokinase in crystalline form. Crystalline hexokinase is a protein of

[1] Berger, Slein, Colowick, and Cori (1946) have reported the isolation of hexokinase from baker's yeast. Their method was based mainly on fractionation with alcohol. The final product could be crystallized from ammonium sulfate without change in specific activity under the conditions described in this chapter by Kunitz and McDonald.

the albumin type. It is crystallized at 5°C. in the presence of ammonium sulfate and dilute phosphate buffer pH 7.0. The crystals become relatively pure after two or three crystallizations as tested by solubility, electrophoresis, and ultracentrifuge measurements. The hexokinase activity of the crystals is associated with the protein nature of the material.

Solutions of crystalline hexokinase in dilute buffers of pH 4.5–7.5 are stable for several days when kept at a temperature of 5°C. or lower. At higher temperatures the hexokinase activity is rapidly lost. The loss in activity is accompanied by denaturation of the protein. The point of maximum stability is around pH 5.0 which is near the isoelectric point of the material. The elementary composition of crystalline hexokinase is that of a typical protein. It contains 0.11 percent phosphorus which would indicate a minimum molecular weight of about 30,000. Sedimentation and diffusion measurements in acetate buffer pH 5.5 at 1°C. gave a molecular weight of 96,600.

METHOD OF PREPARATION OF CRYSTALLINE HEXOKINASE FROM BAKER'S YEAST

Meyerhof's original method (1927) for preparing hexokinase from yeast consisted essentially in plasmolyzing baker's yeast by means of toluene and extracting the plasmolyzed yeast with water at 35°C. The active material was then precipitated in 50 percent alcohol at 0°C. Berger, Slein, Colowick, and Cori (1946) reported that purification of crude hexokinase by means of alcohol is more effective if carried out in a solution containing 1 percent dextrose and 0.05 M acetate buffer pH 5.2–5.4

Meyerhof's method of plasmolysis and of extraction with water was used as a starting step in the present work on the preparation of hexokinase. Advantage was also taken of Meyerhof's method of purification by means of alcohol as modified by Colowick and associates. The basis of the present method, however, is the technique of purification and crystallization of proteins by means of ammonium sulfate from concentrated protein solutions as developed by Northrop, Kunitz, and others for the isolation of crystalline enzymes.

The method consists essentially of the following steps:

1. Plasmolysis with toluene and extraction of the plasmolyzed yeast with water.

2. Concentration and fractionation by means of ammonium sulfate.
3. Removal of "inert" crystalline proteins (Figures 85, 86). Several crystalline proteins, including a crystalline yellow protein (Figure 87), appear during the process of fractionation with ammonium sulfate.[1] These proteins do not possess hexokinase activity. The separation of these crystalline proteins, however, leads to a considerable improvement in the hexokinase activity of the remaining fractions.
4. Dialysis.
5. Purification by fractional precipitation with alcohol.
6. Crystallization in the presence of ammonium sulfate (Figure 88).
7. Recrystallization.

All operations, except when mentioned otherwise, are done at temperatures of 8–10°C. The pH of the preparations is tested by the drop method, by mixing 1 drop of solution with 1 drop of 0.01 percent Clark indicator on a test plate and comparing the colors with those of drops of 0.1 M standard buffers mixed on the plate with the same indicators. The saturated ammonium sulfate is prepared at about 20°C.

For details of preparation see Appendix, page 282.

TESTS OF PURITY OF CRYSTALLINE HEXOKINASE

Repeated crystallization.—The new protein has been recrystallized five times. The hexokinase activity per milligram of protein reached a constant value after two recrystallizations, while the activity of the mother liquors approached the same constant value after the third crystallization (see Table 39).

Solubility curve.—The purity of a crystalline protein is conveniently tested by measuring the solubility of the crystals in a suitable solvent in the presence of increasing amounts of crystals in suspension. A curve is plotted of the amount of protein dissolved *vs.* the total amount of protein in suspension. The plotted curve is compared with the theoretical phase rule curve for a pure substance (Kunitz and Northrop 1938a and b).

Figure 89 shows the result of a solubility test on the crystals of the hexokinase protein which has been recrystallized five times.

[1] Some of the properties of the yeast yellow protein have been investigated by Ball (1946). He found the protein to contain two prosthetic groups, one of which is a flavin. The nature of the other is unknown, though some of its properties are described. As yet no catalytic function has been found for the yellow protein.

TABLE 39

SUMMARY OF HEXOKINASE PREPARATION

Preparation	*Filter Cake, Gm.*	*Total Hexokinase Activity Units*	*Specific Activity. Hexokinase Units per Mg. Protein*	*Yield, Percent* 6,000,000 = 100
Original aqueous extract + washing from 25 lb. yeast		4–8 million	About 20	
Fraction 0.6	400–600	2–3 million	25–30	40
Fraction 0.7	150–250	1–2 million	25–30	25
Fraction 0.63	5–20	200,000–300,000	100–170	4
Fraction 0.72	30–70	400,000–500,000	50–120	8
Fraction 0.63	After alcohol fractionation	100,000	250	1.7
Fraction 0.72	"	120,000	300	2.0
	Mg. protein			
First crystals (combined 0.63 and 0.72)	167	150,000	900	2.5
First mother liquor		33,000	100	
Second crystals	80	90,000	1,100	1.5
Second mother liquor		22,000	300	
Third crystals	38	54,000	1,400	0.9
Third mother liquor		27,000	950	
Fourth crystals			1,350	
Fourth mother liquor			1,200	
Fifth crystals			1,440	
Fifth mother liquor			1,320	

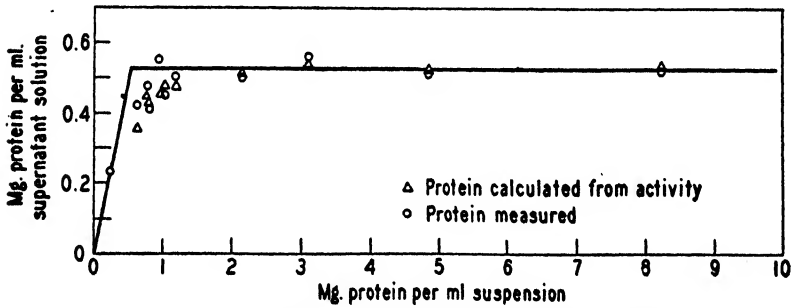


FIG. 89. Solubility curve of crystalline hexokinase.

The solid lines in Figure 89 represent the theoretical solubility curve of a pure substance. The experimental points fall close to the theoretical lines except near the point of their intersection. The irregularity in that region may be due to the presence of a small amount of impurities or denatured protein formed during the stirring as evidenced by the presence of fine strings and broken films of protein.

Electrophoresis.—The mobility of five times recrystallized hexokinase protein in the Tiselius apparatus was measured by Dr. A. Rothen. Measurements were made at pH 5.6 and 6.8 In both cases the protein was negatively charged and moved as a single electrophoretic component as shown by the uniformity and sharpness of the moving boundary.

Sedimentation studies by means of the ultracentrifuge.—Tests by Dr. A. Rothen show that when centrifuged at pH 5.6 the protein is homogeneous to a high degree. At pH 6.0, however, a double boundary appears. This abnormality may be due to an effect of ultracentrifugation on the homogeneity of the hexokinase protein.

THE PROTEIN NATURE OF CRYSTALLINE HEXOKINASE

That the hexokinase activity of the crystals is associated with the protein nature of the material follows directly from the fact that the hexokinase activity per milligram of *protein* remains constant on repeated crystallization. Also, the solubility experiment showed that the dissolved material consisted of protein of the same specific activity as the bulk of the material. Additional evidence on the protein nature of the hexokinase is shown by studies on the stability of crystalline hexokinase. Inactivation is accompanied by denaturation of the protein. Hexokinase is inactivated in the presence of a small amount of trypsin. The inactivation is a gradual process and is accompanied by a loss of protein as tested by precipitation in 2.5 percent trichloracetic acid. The inactivation, however, proceeds at a rate faster than the rate of digestion of the protein. Crystalline chymo-trypsin does not appear to affect crystalline hexokinase.

STABILITY OF CRYSTALLINE HEXOKINASE

Effect of temperature and pH.—Crystalline hexokinase dissolved in dilute buffers of pH 4.5–7.5 is stable for 2 to 3 days when kept at a temperature of 5°C. or lower. At temperatures above 5°C. the enzy-

matic activity is gradually lost. The rate of inactivation increases rapidly with increase in temperature.

The rate of inactivation varies also with the pH of the solution. Hexokinase is most stable at about pH 5.0 which is near its isoelectric point. The inactivation in the region of pH 4.5–7.5 is not accompanied by any significant hydrolysis of the protein. The protein is denatured, however, and precipitates out when the solution containing the inactivated material is brought to pH 5.0

Effect of various substances on the stability of hexokinase at pH 7.0 and 27°C.—The stability of a 0.0025 percent solution of hexokinase in 0.02 M phosphate buffer pH 7.0 when stored at 26–27°C. is increased in the presence of certain sugars and also in the presence of glycine.

The order of the effectiveness of sugars as stabilizers of hexokinase does not correspond to the order of their effectiveness as acceptors of phosphorus from adenosine triphosphate.[1]

PHYSICOCHEMICAL PROPERTIES OF CRYSTALLINE HEXOKINASE

Elementary analysis.—An aqueous solution of five times recrystallized hexokinase protein was dialyzed in a collodion bag with stirring against slowly running distilled water for 24 hours at about 5°C. The dialyzed protein solution was then frozen in dry ice-methyl cellosolve mixture and evaporated to dryness under vacuum while frozen.

The chemical analysis was carried out by Dr. A. Elek of the Rockefeller Institute. The results of analyses are as follows:—

Carbon	52.16	percent
Hydrogen	7.08	"
Nitrogen	15.62	"
Phosphorus	0.11	"
Sulfur	0.91	"
Ash	0.36	"

Isoelectric point of crystalline hexokinase by cataphoresis.—Measurements were made of the rate of cataphoretic migration (Northrop and

[1] Berger, *et al.* (1946), found that the stability of a dilute solution of crystalline hexokinase is greatly enhanced in the presence of small amounts of other proteins, insulin being most effective.

Cysteine and glutathione exerted no protective action.

The inactivation of the crystalline enzyme on incubation with trypsin could be prevented to a marked degree by glucose.

Kunitz 1925) of collodion and of quartz particles which had been soaked for a few minutes in 0.5 percent hexokinase solution and then suspended in 0.02 M buffer solutions. The region of minimum mobility was found to be at pH 4.5–4.8.

Ultracentrifuge data by Dr. Rothen.—Diffusion constant (D^0_{20}) at 1°C. measured in acetate buffer pH 5.5 = 2.9×10^{-7} cm.^{2} sec.$^{-1}$.

Sedimentation constant (S^0_{20}) at same pH and temperature = 3.1×10^{-13} cm. sec.$^{-1}$ dyne^{-1} gm.

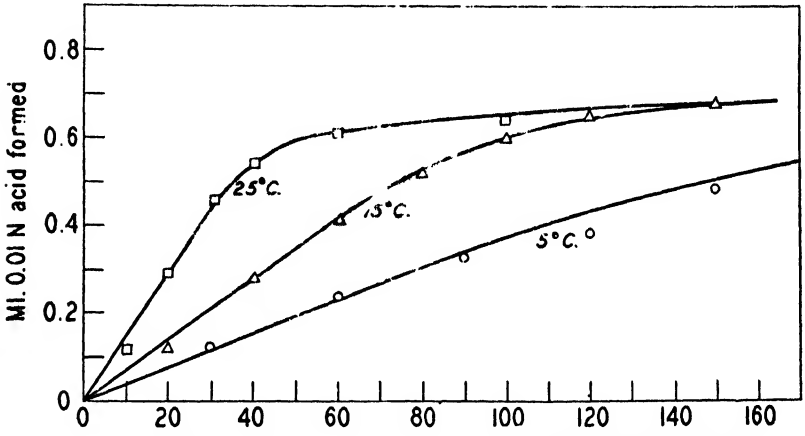


FIG. 90. Effect of temperature on rate of reaction of hexokinase.

Molecular weight at pH 5.5 calculated from the diffusion and sedimentation constants = 96,600.

The specific volume of the material was assumed to be 0.740 at 1°C. which is the usual value for a protein.

STUDIES OF THE ENZYMATIC ACTION OF CRYSTALLINE HEXOKINASE

The effect of temperature.—Figure 90 shows the curves for the rate of the reaction between adenosine triphosphate and dextrose at 5, 15, and 25°C. in the presence of a constant amount of crystalline hexokinase (2.5 hexokinase units). The rate of reaction was measured by the rate of formation of free acid in the reaction mixture. The temperature coefficient was estimated from the initial slopes of the curves and is about 2 per 10°C.

Effect of crystalline hexokinase protein on the reaction between adenosine triphosphate and various sugars.—The dextrose in the reaction mixture was replaced by the following sugars:

Pentoses: *l*-arabinose, *d*-xylose, *l*-rhamnose
Hexoses: *d*-dextrose, *d*-fructose, *d*-mannose, *d*-galactose
Disaccharides: sucrose, *d*-lactose, maltose, trehalose
Trisaccharides: raffinose.

It was found that only dextrose, fructose, and mannose react with adenosine triphosphate. The rate of reaction in the presence of 15 hexokinase units is the same for dextrose and fructose and only about half as much for mannose. The samples of sugars used were mostly Pfanstiehl, C. P.

Effect of magnesium ions on hexokinase activity.—Crystalline hexokinase requires for its catalytic action the presence of magnesium ions. This is shown in Table 40. The usual reaction mixture was used except for varying the concentration of magnesium chloride in solution.

Effect of inorganic phosphate ions.—Crystalline hexokinase does not require the presence of inorganic phosphate ions. The usual reaction mixture was used except for varying the concentration of phosphate buffer in solution.

TABLE 40

EFFECT OF MAGNESIUM IONS ON HEXOKINASE ACTIVITY

Molar concentration of magnesium chloride in solution	0.04	0.02	0.01	0.005	0.0025	0.00125	0
0.01 N acid formed in 30 min. at 5°C., *ml.*	0.50	0.49	0.47	0.31	0.25	0.16	0.06

Effect of varying the concentration of the substrates.—Within certain limits of concentrations of adenosine triphosphate and of dextrose the rate of reaction is practically independent of their concentrations.

The extent of reaction.—The extent of the reaction, as determined by the final amount of acid formed, varied slightly with the samples of adenosine triphosphate used, depending on their percentage content of pure ATP. The rate of the reaction, however, was found not to vary with the sample used. The final amount of acid formed is equiv-

alent approximately to one third of the phosphorus content of the adenosine triphosphate in the reaction mixture, thus confirming the findings of Colowick and Kalckar (1941, 1943). Crude hexokinase preparations gave rise to the formation of acid equivalent to about two thirds of the phosphorus content of the ATP.

X: CRYSTALLINE DIPHTHERIA ANTITOXIN

(Northrop 1941a)

VON BEHRING and Kitasato in 1890 found that the sera of animals which had received repeated small doses of diphtheria toxin developed the property of neutralizing the poisonous effects of the toxin. This neutralizing substance was called antitoxin. Diphtheria antitoxin was the first such substance discovered and is still one of the most powerful therapeutic agents known. Further study of diphtheria and other antitoxins has shown that they are proteins closely related to the normal serum proteins. They must possess some characteristic chemical structure, however, not present in the normal proteins since they neutralize toxin whereas normal serum proteins do not. Knowledge of the structure responsible for the antitoxic power may eventually lead to the synthesis of antitoxins of greater therapeutic value. The first step in the attempt to identify the structure responsible for this therapeutic value is the isolation of the antitoxin in pure form. Preparations of pneumococcus antibody, which are pure in the sense that they are completely precipitated by antigen, have been obtained by Chow and Goebel (1935), Chow and Wu (1937) Heidelberger and Kendall (1936) and their co-workers. Petermann and Pappenheimer (1941) prepared diphtheria antitoxin which was homogeneous by electrophoresis or ultracentrifuge but was not completely precipitated by toxin. Highly purified preparations of diphtheria antitoxin have also been obtained by Pope and Healey (1939) and co-workers.

Ramon (1922) showed that under certain conditions diphtheria toxin and antitoxin combine to form an insoluble precipitate. Evidently recovery of the antitoxin from this precipitate would result in a very considerable purification. Ramon was able to recover some antitoxin from this precipitate by heating in slightly acid solution (Ramon 1923). Sandor (1939, 1942) found that most of the antitoxin

could be recovered from such precipitates by treatment with pepsin in slightly acid solution. Pope (1939) obtained the same results. The work has been confirmed and extended by Pappenheimer and coworkers. Pepsin destroys both toxin and antitoxin to some extent and it seemed possible that better yields might be obtained by using trypsin. It was found that trypsin has no effect on the toxin-antitoxin in neutral solution but if the complex is dissolved in dilute acid and then brought back to neutral solution the toxin is digested and 30–60 percent of the antitoxin may be recovered

It was thought at first that this result was due to dissociation of the toxin-antitoxin complex in acid followed by digestion of the toxin. It appears, however, that this is probably not the case since diphtheria toxin is very unstable in acid (pH 3.5) as Pappenheimer (1937, 1938) showed and would be inactivated and precipitated were it present in solution. This does not occur when the toxin-antitoxin complex is allowed to stand in acid. The action of trypsin does not take place in acid but during and immediately after neutralization. It seems probable that the toxin-antitoxin complex dissolves as such in acid and the toxin is then split off by the trypsin during and after neutralization while the complex is still dissolved. If the toxin-antitoxin complex is allowed to precipitate again in neutral solution before the addition of trypsin, the toxin is no longer digested.

The "crude antitoxin" recovered after removal of the toxin contains 30–60 percent of the original total antitoxin.

The preparation is 95 percent or more precipitated by toxin and has an antitoxin titer of 300–400 units per mg. protein nitrogen. This preparation may be further purified by precipitation with ammonium sulfate. The fraction precipitating between 0 and 35 percent saturated ammonium sulfate has an antitoxic value of about 550 units per mg. protein nitrogen while that precipitating between 0.45 and 0.65 saturated ammonium sulfate has an antitoxic value of 700–800 units per mg. protein nitrogen by the flocculation test.

The antitoxin value of this purified antitoxin, by animal protection tests, has been determined by Dr. W. E. Bunney at E. R. Squibb and Sons, New Brunswick, New Jersey. Two preparations were tested and found to contain about 700 antitoxin units per mg. of protein nitrogen.

This fraction probably corresponds to a similar preparation ob-

tained by Pope and Pappenheimer by means of pepsin digestion. It is homogeneous by electrophoresis and ultracentrifuge measurements. The solubility of this fraction, however, is not constant but varies with the quantity of solid present showing that it consists of two or more proteins which are indistinguishable by electrophoresis, rate of sedimentation or precipitation by toxin. The fact that the solubility varies with the amount of solid present shows that the solid phase in the dilute suspension is different from that in the more concentrated. A partial separation of the system has therefore been accomplished and, on paper at least, all that is necessary is to repeat the process until no difference in solubility is observed. This is the theoretical basis for the fact that fractional precipitation has been of such great practical value in the purification of proteins.

In practice it is not always possible to complete the separation in this way owing to the fact that insufficient material is available or because the protein is too unstable and changes during the experiment. In the present case both difficulties are encountered. Nevertheless, it is possible to obtain a much more homogeneous protein by removing only the most soluble portion of the preparation. This is best done by extracting the solid precipitate with half-saturated ammonium sulfate at pH 7.2. The protein obtained in this way is much more nearly homogeneous but still does not have constant solubility. Repetition of the extraction with this fraction would be expected to result in further improvement but the yield is so small that this cannot be done in practice. If this saturated solution is titrated to pH 3.5, however, a precipitate appears. After removal of this precipitate the protein has constant or nearly constant solubility and crystallizes readily in rather poorly formed thin plates (Figure 91). These plates are slightly double refractive. They are slightly less soluble than the amorphous protein and their appearance in solution is greatly accelerated by inoculation of a supersaturated solution with some of the plates. They resemble very closely the first crystals of ribonuclease obtained by Kunitz. Ribonuclease crystals, however, become beautifully formed on recrystallization or on long standing while the antitoxin crystals do not improve and may become less well formed. This difference is due to the fact that the antitoxin is very unstable. Even after 24 hours at 20°C. some less soluble protein has been formed and after a few days the solubility curve of the preparation resembles that

FIG. 91. Crystalline diphtheria antitoxin. × 129.

of the crude fraction from which it was derived. At the same time the flocculation time, when the antitoxin is mixed with toxin, increases although the final end point remains the same. The formation of this less soluble protein interferes with crystallization and soon prevents it altogether.

Similar changes in the rate of flocculation with aging have been noted by Glenny and others with antitoxic sera. It is possible that the pure antitoxin represents the protein in its original form and that the more insoluble proteins are derived from this unstable protein. So far no conditions have been found which prevent these changes.

PROPERTIES OF THE ANTITOXIN

Purity.—The electrophoretic pattern has been determined by Dr. A. Rothen. There is only one boundary but there is considerable "reversible spreading" in dilute phosphate buffer at pH 7.3. A possible cause of this phenomenon, which has been noted with several proteins, is the migration of water in the cell due to electroendosmosis. If this were the cause the spreading should not be observed under conditions which prevent the electroendosmosis. The experiment was therefore repeated in the presence of calcium chloride at pH 7.3 and at pH 3.0. Glass has no charge when in contact with these solutions and hence there is no electroendosmosis. There is no reversible spreading of the protein under these conditions.

The protein is strictly homogeneous in the ultracentrifuge with a sedimentation constant of $S_{20}^{water} = 5.3 \times 10^{-13}$. The diffusion coefficient is $D^{20} = 5.56 \times 10^{-7}$ cm.^{2}/sec. and the molecular weight calculated from these figures is 90,500 (Rothen 1942). This is close to the figure obtained by Pappenheimer for a sample of antibody obtained by the action of pepsin. Purified antibody prepared by heating the toxin-antitoxin complex in acid has a sedimentation constant of 6.8×10^{-13}. Apparently, therefore, trypsin hydrolyzes part of the antitoxin molecule as well as the toxin. This agrees with Pappenheimer's experiments with pepsin.

The most highly purified fraction has nearly constant solubility. All the preparations of this fraction give better solubility curves than most proteins but only one shows really constant solubility. These curves, however, were all made on amorphous preparations and hence are open to some uncertainty as it is not possible to be sure that they

represent equilibrium values. The crystalline preparations do not give as good curves owing to the fact that some less soluble material is formed while the crystallization is taking place.

The protein contains about 2.5 percent carbohydrates determined as glucose by Sørenson's method.

PRECIPITATION OF PURIFIED ANTITOXIN AND TOXIN

Solutions of various samples of purified antitoxin were mixed with an equivalent of crude toxin, obtained from E. R. Squibb and Company, or of purified toxin. The solutions were kept at 50°C. for 20 hours, centrifuged, and protein nitrogen determined in the supernatant. Of the total protein nitrogen present 4 to 10 percent remains in the supernatant with both the pure antibody having an antitoxic 800 L_f/mg. P.N. and with the fraction precipitated between 0.35–0.45 saturated ammonium sulfate (400–500 L_f–500 L_f/mg. P.N.) and with either crude or purified toxin. Therefore 90–95 percent of the total protein nitrogen precipitates out. It is not possible to say at present whether the protein nitrogen which fails to precipitate comes from the toxin or antitoxin.

This result indicates the presence of at least two antitoxins which combine with different amounts of toxin. Evidence for the existence of two such antitoxins has been obtained by Kekwick and Record (1941).

Precipitation zone with crude and pure antitoxin and toxin.—Mixtures of immune serum and crude toxin precipitate only over a range of concentration in which the two substances are present in the proportion of less than 2 to 1; that is, if complete flocculation occurs with a 1 to 1 mixture the precipitation will be incomplete if more than two equivalents of either component is present.

With pure antibody and crude toxin the range is about doubled and precipitation occurs over a range of 4 to 1.

With pure antibody and purified toxin (kindly supplied by Dr. A. M. Pappenheimer, Jr.) the range of complete precipitation is very wide and the components may be varied in the ratio of at least 64 to 1.

IMMUNOLOGICAL REACTIONS

Some of the immunological properties of the purified toxin have been determined by Dr. Carl Ten Broeck. The serum of a rabbit

immunized against normal horse serum gave a precipitate with 1/4,000 ml. normal horse serum (containing about 0.002 mg. protein nitrogen) but gave no precipitate with 1 ml. of a solution of purified antibody containing 1/10 mg. protein nitrogen.

Guinea pigs sensitized with purified antibody reacted on injection of 0.05 mg. antibody protein nitrogen, whereas 5 mg. normal horse serum protein nitrogen was required to cause a reaction.

The purified antitoxin is, therefore, antigenically distinct from the normal serum proteins.

The toxin and the anti-diphtheria horse plasma used in this work were obtained from the Biological Laboratory of E. R. Squibb and Sons, New Brunswick, New Jersey.

XI: BACTERIOPHAGE

TWORT and D'Herelle observed that certain cultures of bacteria grew normally at first but that later the cells suddenly dissolved and the whole culture became clear. If a drop of this clear culture was now added to a second normal culture the process of clearing occurred in this inoculated culture and this process of inoculation could be continued indefinitely. This experiment showed that there is present some active substance which causes the bacterial cells to disintegrate and which increases in quantity during the growth of the bacterial culture. The process of disintegration of the cells was called "lysis" and the active agent was named "bacteriophage" by D'Herelle. The general properties of bacteriophage have been studied in detail by D'Herelle (1926), Gratia (1938), Bronfenbrenner (1928), and many other workers.

The bacteriophage is specific, i.e., any one phage usually affects only a particular strain or closely related strain of bacteria. It is possible to adapt phage from one strain of bacteria to another. An antiserum may be produced by injecting rabbits with solutions of phage, and the antibodies obtained in this way are also specific. It has been further established that there are a large number of distinct phages that differ from each other in many respects.

It is not certain that bacteriophage can be obtained from completely inactive material, although such results have been reported. It is usually necessary to obtain phage from a solution which is already active so that the ultimate origin of the active agent is unknown.

In general, the biological properties of bacteriophage are closely analogous to those of bacteria, with one important exception: no evidence that phage has any metabolism has been found although careful measurements have been made by Bronfenbrenner (1928), Schüler (1935), and others. It will be seen that an equally good analogy exists between the bacteriophages and the enzymes.

MECHANISM OF THE BACTERIA-BACTERIOPHAGE REACTION

The main phenomena which occur during the process of lysis were established by D'Herelle and have been confirmed by all subsequent workers. When bacteriophage is added to a culture of susceptible organisms most of the phage is taken up by the cells. The cells then divide at the same (or faster) rate than do cultures containing no phage but after a longer or shorter time interval the cells disintegrate. During the time the cells are dividing the phage is increasing rapidly. The time required for lysis to occur is longer the smaller the amount of phage used to inoculate the culture. A slightly higher final concentration of phage is reached with a small initial inoculation than with larger ones.

D'Herelle assumed that the phage particles multiply inside the bacterial cell and are liberated when lysis occurs. Since the usual "phage count" method of determining phage counts "active centers," and not individual phage particles this mechanism leads to steplike increase in phage. The phenomenon has recently been studied in detail by Delbrück and his co-workers and D'Herelle's results confirmed. Krueger and Northrop (1930) found that the phage concentration could be determined by noting the time required for lysis. This method determines total phage present, whether in the bacterial cell or not. When concentrated suspensions are used this method yields smooth logarithmic curves. The subject has been reinvestigated by Krueger (1946), using both methods. It was found that small steps are present even with the time method, if dilute solutions are used. In concentrated solutions of high phage content these steps overlap and so are lost.

These different results are due to the fact that the time method of Krueger and Northrop in concentrated suspensions represents a statistical average similar to that obtained in growth curves of bacteria or in following the course of chemical reactions. In the plaque count method in dilute suspension the results represent the changes in very few particles, and hence the reaction progresses in steps. The same result is obtained if cell counts are made of very dilute bacterial suspensions or of chemical reactions in which it is possible to follow the fate of single molecules. In all these cases there is a sudden increase

in the number of particles or cells at certain times, whereas if the reaction rate of a very large number of units is determined smooth curves are obtained.

Under these conditions the growth of phage is related to the growth of bacteria by the equation $\frac{dP}{P} = \frac{K}{G}\frac{dB}{B}$ when K and G are the growth constant for the rate of growth of phage (K) and bacteria (G). It is further assumed that lysis occurs when the ratio of the phage concentration to the bacteria reaches a certain critical value. From these two assumptions it is possible to derive the following equation, which predicts the time of lysis (T lysis) in terms of the initial phage (Po) and bacteria (Bo) concentration and the growth constants for the phage (K) and for the bacteria (G).

$$\text{T lysis} = \frac{2.1 - \log \text{P}_\text{o} \log \text{B}_\text{o}}{\text{K} - \text{G}}$$

This equation agrees with the experimental results and makes it possible to determine the initial phage concentration by noting the time required for lysis.

The relation of phage increase to bacterial increase $\frac{dP}{P} = K\frac{dB}{B}$ is the same as that found for Huxley (1932) to express the relation between the growth of various organs in higher animals.

INCREASE IN BACTERIOPHAGE AND GELATINASE CONCENTRATION IN CULTURES OF BACILLUS MEGATHERIUM (Northrop 1939)

A formal analogy also exists between the production of phage and the formation of enzymes. If this analogy has any real physical significance the simultaneous formation of phage and of an extracellular enzyme in the same culture should follow very similar curves. In order to test this prediction the rate of formation of phage and of a gelatinase by lysogenic and "sensitive" strains of *B. megatherium* has been determined under a variety of conditions. In general the curves for the increase of phage and of gelatinase are very similar. There is a rapid increase in phage and enzyme concentration during the growth stage of the bacteria. The increase in all these quantities is logarithmic (autocatalytic); i.e., the amount formed is proportional to the amount already present. The concentration of phage and gelatinase reaches a

maximum and then decreases. The decrease may or may not be accompanied by a decrease in the number of cells depending upon the conditions and strain of bacteria used. The production of phage may be separated from that of gelatinase by varying the calcium concentration. In the presence of high calcium concentration little phage is produced while the gelatinase increases to a measurable extent, but less than it does in low calcium concentration. In the absence of calcium no phage is produced and the production of gelatinase is decreased.

EXPERIMENTAL RESULTS

Megatherium culture and phage.—The culture of *megatherium* (899 T) used was isolated originally by De Jong and further studied by De Jong (1933), Gratia (1936a–d), Wollman (1936), and others. The organism forms two types of cultures: (1) a smooth strain (899 S) which does not spore and, (2) a spore-forming strain (899 R). Both strains produce a bacteriophage which causes lysis of a sensitive strain (Megatherium 36 S) but does not cause lysis of the lysogenic culture 899 which produces it.

Two types of phage are formed (Gratia 1936c), one of which causes complete lysis of Megatherium 36. This "C" phage was used in the present work. A second type of phage causes only partial lysis of Megatherium 36 (Phage T).

Increase in number of bacteria, bacteriophage, and gelatinase in growing lysogenic cultures of B. megatherium (899 T) in yeast extract media at 35°C.—The results of an experiment in which the bacteria concentration, the plaque count, and the gelatinase activity were determined are shown in Figure 92.

There is a lag period of about one half hour in which no change is detectable. The cell concentration then increases continuously while the phage concentration and gelatinase concentration increase much more rapidly than the cells at first, reach a maximum, and then decrease. The curves are all logarithmic during the stage of rapid increase. The cell concentration remains constant after about 14 hours. There is no demonstrable lysis. The maximum number of plaques per ml. is about 1000×10^6 or about 2–3 times that of the maximum bacteria concentration of 40×10^7. The plaque count in the filtrate is nearly the same as that of the suspension. The maximum gelatinase

concentration is 100–200 $\times 10^{-4}$ gelatin units per ml. The relation of gelatinase production to cell growth is the same as that found by Kocholaty, Weil, and Smith (1938) with *Clostridium histolyticum.*

PURIFICATION OF BACTERIOPHAGE (Northrop 1938)

The most successful of many previous attempts to purify bacteriophage was carried out by Schlesinger and Bechhold. These workers

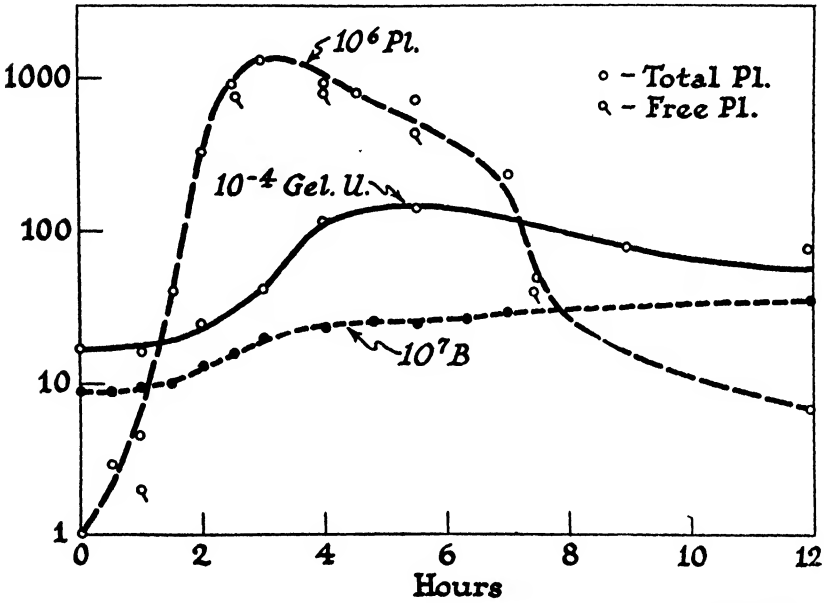


FIG. 92. Increase in plaque count, number of bacteria, and gelatinase concentration during growth of resistant strain, 899 T.

obtained from lysed *Bacillus coli* cultures, by repeated centrifuging, a suspension of particles which they considered to be the active agent. The particles were free from bacterial protein and corresponded in elementary composition to nucleic acid. Bechhold, however, considered them to be living cells.

Preliminary experiments in this laboratory showed that in order to obtain appreciable amounts of phage it would be necessary to handle large quantities of culture, since the quantity of active material present was very small, probably less than a milligram per liter. It was also necessary to develop a synthetic medium, since the ordi-

nary culture media contained large quantities of inert protein, and, further, to have a method of titration which could be carried out in the presence of contaminating organisms so as to avoid the necessity of handling large quantities of material without contamination. The organism used in this work was a culture of non-pathogenic staphylococcus isolated by Glaser and its accompanying phage isolated by Shope from houseflies. It was found that an extract of dried yeast in boiling water formed a good culture medium and that a slight modification of the titration method described by Krueger could be carried out in the presence of contaminating organisms. Conditions were worked out for the growth and lysis of the culture in 200-liter quantities.

The lysed solution obtained in this way was slightly yellowish and clear. It contained about 10 [illegible] ml.,[1] about 0.02 mg. protein nitrogen per ml., and some mucin-like material. It could not be precipitated with neutral salts or fractionated by any of the usual protein methods. This is usually the case with such dilute solutions, especially if they contain mucin. Some of the mucin-like material was removed with lead acetate and the filtrate concentrated *in vacuo* at 30°C. to 1/20 its volume. There was no loss in activity during this operation and the solution was now sufficiently concentrated so that the usual methods of protein fractionation could be used with some hope of success. A good deal of mucin and inert protein could be removed by filtration with Filter-Cel but the resulting solution still could not be fractionated. In the meantime it had been found that the active principle was not attacked by trypsin. Trypsin was therefore added to this filtrate and the solution allowed to stand at 10°C. for several days. At the end of this time a large part of the inert protein had been digested and the solution could now be readily precipitated with 0.6 saturated ammonium sulfate. The precipitate obtained in this way is brownish. The color is removed by charcoal in alkaline solution and the protein reprecipitated with 0.6 saturated ammonium sulfate. The precipitate is filtered with Filter-Cel to remove traces of charcoal and some inert protein. The yield varies from 10 to 80 percent of the original total phage activity. Through the first five steps there is a rapid decrease in protein but no loss in activity and at the end of the

[1] In this section one phage unit (ph.u.) is defined as the quantity of phage which will cause lysis of 5 ml. standard suspension in 1 hr.

first five steps the specific activity of the preparation (ph.u./mg. protein nitrogen) is the same as in the final preparation. In succeeding steps, however, there is, at each precipitation, a loss in activity without loss of protein, i.e., inert protein is formed. This loss of activity during the process of purification has greatly increased the difficulty of the work. Crude phage solutions are extremely stable and may be kept for years at 0°C. without loss of activity. As purification proceeds the preparation becomes increasingly unstable; so much so that many of the usual methods cannot be used. Thus, filtration of any active precipitate results in loss of much activity, and if the filtration be carried on until the precipitate is dry the activity is completely destroyed. Prolonged centrifuging inactivates the material and concentration in the ultracentrifuge also causes more or less complete inactivation. On the other hand, settling by gravity causes very little loss, and this method is therefore used as much as possible.

The original concentration of active phage present in the crude culture may be calculated from the specific activity of the final product and is found to be about 1 mg. per liter. This is less than that found by Schlesinger with coli phage. The difference may be due to the greater activity of the present preparation, or coli phage may actually be found in much larger quantity than is the staphylococcus phage.

GENERAL PROPERTIES OF THE PREPARATION

Composition.—The material obtained in this way has the composition of a nucleoprotein. It contains 40 percent carbon, 5.3 percent hydrogen, 14.3 percent nitrogen, 4.8 percent phosphorus, less than 1/10 percent glucosamine and about 1 percent glucose. The phosphorus content is higher than that reported by Schlesinger. There is a marked increase in amino nitrogen after acid hydrolysis. Hydrolyzed solutions give a precipitate with alkaline silver nitrate, indicating the presence of purine basis.

The activity is destroyed by chymo-trypsin, but not by trypsin or pepsin; by heating to 50°C. for 5 minutes, by acidity greater than pH 5.0, and by glycerin, alcohol, and acetone. It is most stable in solution in about 25 percent saturated ammonium sulfate at pH 7.0, but even under these conditions it loses activity at the rate of about 10 percent a day at 10°C.

Concentrated solutions of the purified phage cause immediate lysis of living bacteria but will not cause lysis of dead bacteria. This confirms Bronfenbrenner's observation that lysis of dead bacteria requires some other substance. About 1×10^{-16} gm. protein nitrogen of the purified phage will cause lysis. If this quantity is assumed to be one molecule then the gram molecular weight of the substance is about 30×10^7 gm. This is about the same order of magnitude as that found for the molecular weight by the sedimentation velocity method in concentrated solution (Wyckoff 1938) but is larger than that found by diffusion. Sedimentation determinations in the ultracentrifuge showed the preparation to be homogeneous in size and to have a sedimentation constant of 650×10^{-13} cm. dyne^{-1} sec.$^{-1}$ corresponding to a molecular volume of about 300,000,000. It was also found that the total protein sedimented at the same rate as the bacteriophage, as measured by activity determinations. Diffusion experiments in concentrated solution gave approximately the same value, but in more dilute solution it was found that the diffusion was much faster and corresponded to a molecular weight of about 500,000. Under these conditions the protein also diffused at the same rate as the active material.

Sedimentation rate by direct observation cannot be determined in such dilute solutions, but if a series of increasingly dilute solutions is centrifuged at the same time it was found that the ratio of phage concentration at the top to that in the bottom of the tubes increases as the solution becomes more dilute. This result confirms qualitatively the results of the diffusion experiments.

Tobacco mosaic virus protein was added to the phage solution in some experiments and the relative sedimentation of the phage and of the tobacco mosaic protein was determined. In phage solution containing more than 50 gamma phage protein per ml. the phage sedimented more rapidly than did the tobacco mosaic, whereas in more dilution solution the phage sedimented more slowly than the mosaic virus. Bronfenbrenner and Hetler previously had found by filtration experiments that some of the activity could pass through much smaller filters than those corresponding to a molecular weight of about 300,000,000.

Hershey, Kimura, and Bronfenbrenner (1947) have repeated these experiments, using the rate of sedimentation in the ultracentrifuge.

They find the preparation to be homogeneous in size at all concentrations and consider the results obtained by diffusion to be due to errors in the method. In this laboratory the diffusion method with ordinary proteins has consistently given slightly larger figures for the molecular volumes, and not smaller figures as found by Bronfenbrenner and his collaborators. The existence of phage particles of varying size, however, rests on uncertain evidence and requires further investigation.

The present experiments indicate that phage dissociates into smaller molecules upon extreme dilution, as Svedberg and his collaborators have found to be the case with certain proteins. Hemocyanin dissociates upon making the solution alkaline, and there appears to be an equilibrium between the large and small molecules. If such an equilibrium exists then there must be some small molecules present at all degrees of acidity, and at infinite dilution the protein would be present entirely as small molecules.

RELATION OF PHAGE ACTIVITY TO THE PROTEIN

If the nucleoprotein is really the active agent it would be expected that denaturation or digestion of the protein would result in a corresponding loss in activity. The protein is not digested by trypsin nor is the activity destroyed by trypsin. Chymo-trypsin inactivates the preparation, however, and at the same time causes the appearance of insoluble protein. The amount of denatured protein formed corresponds to the loss in activity. The action of chymo-trypsin on the protein is similar to its action on casein. In solutions more acid than pH 5.0 and at 20°C. denatured protein is formed and corresponding loss in activity occurs. At pH 7.0 and 55°C. there is also rapid loss in activity and corresponding formation of denatured protein.

Cohen (1946) has found that desoxyribonuclease splits off 30 percent of the phosphorus from T_2 *coli* phage without causing loss in activity. This result indicates that some of the nucleic acid present in phage preparations is not an essential part of the phage particle and also predicts that active particles of different sizes must exist.

Krueger and Mundell (1936) have shown that such heat-inactivated phage may be reactivated under certain conditions. Lominski (1936) has reported similar results. This is probably a case of the reversal of denaturation of a protein and may furnish the explanation for Kendall's (1936) experiments in which it was found that active phage

may be obtained from autoclaved solutions. It has been objected that phage was not produced from inert material in these experiments but that an amount of phage too small to detect remained after the heat treatment and that this residual active phage then increased. The dilution experiments described above show that it is possible to detect very few or even a single phage molecule and similar results have been reported by Feemster and Wells (1933). If this conclusion is correct it is evidently impossible to account for the regeneration results by assuming that a quantity of phage too small to detect remained in the solution.

ABSORPTION OF PHAGE ACTIVITY AND PROTEIN BY BACTERIA

Susceptible living or dead bacteria remove phage activity from solution. These reactions are specific, since nonsusceptible bacteria do not exhibit this property, and furnish a sensitive test for the relation of the phage activity and the protein. Two phage preparations were used; one (60–16) contained some inert protein while the second (94–25) contained less. The bacteria removed relatively more activity than protein from the impure preparation, so that the solution left showed a lower specific activity than the original. With the more active preparation the loss in activity was proportional to the protein removed. The bacteria, therefore, appear to take up only the active protein. Nonsusceptible staphylococci or coli do not remove either protein or activity.

COMPARISON OF ULTRAVIOLET ABSORPTION SPECTRA AND INACTIVATION BY ULTRAVIOLET LIGHT

The relative efficiency of various wave lengths of ultraviolet light for the inactivation of bacteriophage has been determined by Gates (1934b). From these measurements the relative absorption spectrum can be calculated. The direct measurement of the ultraviolet absorption spectrum of purified bacteriophage results in a curve which agrees with that found by Gates from 2,600–2,900 Å, but below 2,600 the absorption measured directly is greater than that indicated by Gates's experiments. This discrepancy may be due to the fact that Gates's measurements were carried out with a slightly different phage or to the presence in this material of some inert protein which absorbs

strongly in this region. It is also possible that light may be absorbed in this region without causing inactivation. Similar discrepancies have been found in the case of both pepsin (Gates 1934a) and urease (Kubowitz and Haas 1933).

The discrepancy may also be due to scattering of light since this effect increases rapidly as the wave length decreases. No correction has been made for this effect so that the light absorbed, as determined by direct measurement, also includes scattered light.

SOLUBILITY DETERMINATIONS

The preceding experiments show that the protein and activity are certainly very closely related and that the activity appears to be a constant property of the protein. Experience in this laboratory has shown, however, that a protein may behave in many respects like a pure chemical compound and yet, when tested by the solubility method, prove to be a mixture or solid solution of several different, although probably closely related, proteins. There is some indication in the experiments reported above that the preparation contains some inert proteins, since the specific activity varies between 5,000 and 10,000 ph.u./mg. nitrogen and since it was found impossible to obtain quantitative separation of denatured and native protein. Solubility determinations were therefore made and it was found that the solubility increased rapidly as the total concentration increased, showing that the solid phase consisted of more than one component. It was further found that the specific activity of the solution was slightly higher than that of the precipitate.

These results indicated a further method of purification. In the earlier work the protein was precipitated from dilute solution with 0.5 saturated ammonium sulfate. It appeared from the results of the solubility experiment that in more concentrated solution a large amount of activity would remain in solution in this concentration of ammonium sulfate and that this fraction would be more active. Accordingly, attempts were made to prepare a concentrated solution which could then be precipitated with 0.5 saturated ammonium sulfate. The difficulty lies in the fact that at each precipitation more or less denatured protein is formed which can be removed only by redissolving in water, precipitating at 0.45 ammonium sulfate to remove the inert protein, and filtering. The precipitate formed under

these conditions carries with it more or less of the active protein so that again a dilute solution is obtained. However, two preparations were obtained which had a specific activity of 20,000 ph.u./mg. nitrogen, which is two to three times that of the material used in the earlier experiments. This material was soluble to the extent of at least 0.10 mg./ml. in 0.45 saturated ammonium sulfate, and its solubility in 0.53 saturated ammonium sulfate varied only slightly with the total concentration (Figure 93). This is a very rigorous test of purity, and the results show that this preparation consisted principally of only one component.

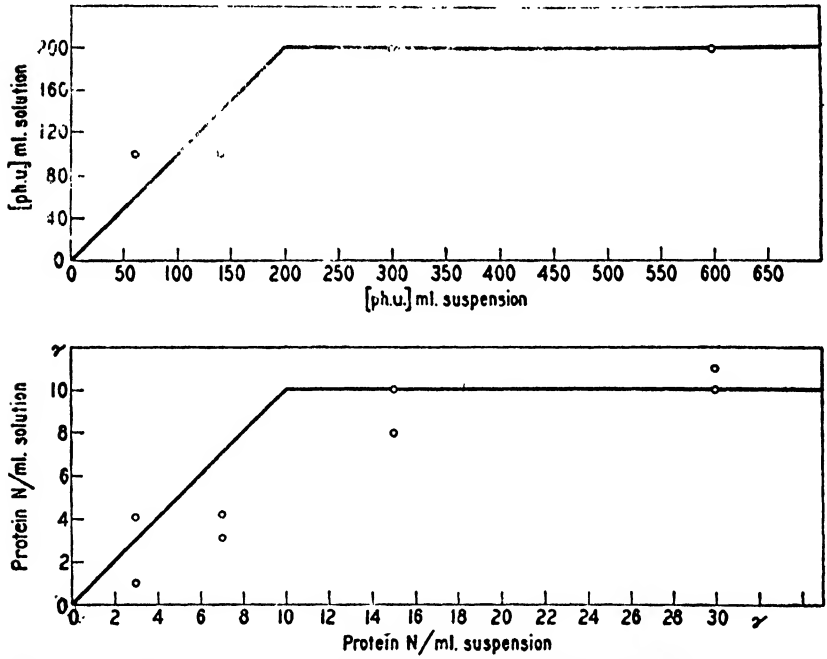


FIG. 93. Solubility of phage protein (specific activity 20,000 ph.u. mg. N) in { 0.53 saturated ammonium sulfate / 0.02 M sodium phosphate } in the presence of increasing quantity of solid phage.

The preceding experiments show that a substance with the chemical properties of a nucleoprotein possesses the biological properties characteristic of bacteriophage.

Recent photographs with the electron microscope reveal that some

phage particles (after drying) are tadpole-shaped and this result has convinced some workers that they are cells. It has also been suggested that they are motile, but the fact that the size as measured by diffusion or sedimentation agrees with that found by photography shows that they are not motile since calculation of the size by diffusion includes the assumption that no motion, other than that of thermal diffusion, exists. The possibility of motion is also pretty well ruled out by the lack of respiration, since motion would require the use of energy which, in turn, would require respiration. Many molecules, of course, also have tails, and would presumably appear as tadpoles when photographed. Detergents in general have a hydrophylic group attached to a hydrophobic one and are among the few known chemicals which cause complete lysis of bacteria, very similar to the action of phage. On the whole the answer to the question as to whether phage particles are cells or molecules cannot yet be answered with certainty and it still appears necessary to know whether they use energy to synthesize themselves, as do cells, or whether they are formed by an autocatalytic reaction as are some enzymes.[1]

[1] Recent experiments by Bang (1947) are of interest in this connection. The virus of chicken, "Newcastle disease," is round in allantoic fluid but is tadpole-shaped in normal saline and becomes diffuse and irregular in distilled water. There is no loss in infectivity during these changes. These results suggest that the form obtained when the preparation is dried may be altered by the composition of the solution.

XII: REACTION OF ENZYMES AND PROTEINS WITH MUSTARD GAS (Bis (*β chlor ethyl*) *sulfide*)

(Herriott, Anson, and Northrop 1946)

RATE OF REACTION OF MUSTARD GAS (H) WITH DIFFERENT ENZYMES AND PROTEINS

THIRTEEN different proteins[1] have been treated with mustard (H) in aqueous solution.[2] In every instance the H reacted with the protein as shown by either an increase in bound sulfur or a change in enzymatic activity, or both. We have failed to find any protein which did not react. Some enzymes were more rapidly inactivated than others, as shown by the value of their inactivation constants. Dixon, Van Heyningen, and Needham (V17890) have concluded that enzymes fall into two classes; those which react and those which fail to, but our results would indicate a scale of reaction rates with chicken pepsin near the faster end and chymo-trypsin at the slower end. Crystalline yeast hexokinase is intermediate.

Effect of the method of addition of H.—Two methods have been used for the addition of H to the reaction mixture. In one case—Needham and Dixon (1941)—the H was dissolved in alcohol or a similar organic solvent and added all at once to the reaction mixture. In the stirring method, liquid H was stirred with the protein solution. It has been suggested (Dixon Y7483) that variation in the results has been due to the fact that the reaction proceeds differently in the presence of liquid H, even though the concentration of dissolved H is the same.

[1] Crystalline swine pepsin and pepsinogen, chicken pepsin, crystalline chymo-trypsinogen and crystalline chymo-trypsin, yeast sucrase and crystalline yeast hexokinase, crystalline egg albumin, human serum albumin, human serum globulin, human fibrinogen, gelatin and zein.

[2] The H used in the experiments was a highly purified sample prepared from thiodiglycol (TDG). Technical preparations give quite different results, reacting much faster with SH groups.

The rate of inactivation of pepsin and of hexokinase by H using both methods were therefore determined. No significant differences were found, as may be seen in Figure 94. The different results obtained in this laboratory and in Professor Dixon's laboratory are therefore not due to the different methods. The differences are probably due to differences in the enzyme preparation or in the hydrogen ion concentration.

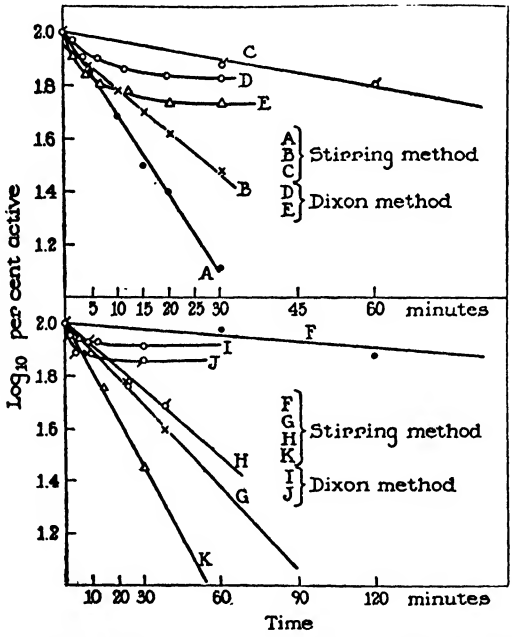


FIG. 94. Inactivation of swine pepsin and yeast hexokinase by various concentrations of mustard at 25°C. in M/25 pH 5.8 acetate buffer using the Dixon or the stirring method. Curves A,B,C,D,E—pepsin; curves F,G,H—purified hexokinase; curves I,J,K—crystalline hexokinase.

Experimental results.—In the Dixon method small amounts of alcohol or ethyl cellosolve solutions of H were added to the aqueous enzyme solution accompanied by rapid stirring to minimize the separation of the free H oil. The quantity of H in the organic solvent was such that when mixed with the protein the H concentration was less than saturated, 6×10^{-3} molar. In general a final concentration of $1 - 2 \times 10^{-3}$ molar H has been used. The H concentration decreased

with time due to hydrolysis as well as through action with the protein and was nearly zero in 30 minutes at 25°C. if no inhibiting substances, such as chlorides were present. In this method there was only one phase, i.e., the system was homogeneous.

The Dixon method is much simpler to carry out but the results are not so readily interpreted kinetically.

In the stirring method an excess of H is stirred at a constant rate in the protein solution. The concentration of the dissolved H can be varied from a saturated solution ($6 \times 10^{-3}M$) to practically zero by varying the rate of stirring but during any given experiment the dissolved H concentration can be kept relatively constant. In holding the H concentration constant the mathematical treatment of the kinetics is simplified, as will be seen from the discussion of kinetics in the next section. Using the stirring method one may treat a protein with large quantities of H by merely extending the time of stirring. Ball (personal communication) has found similarly that the results on different enzymes by the two methods are about the same.

Thus, as long as other conditions are comparable the same relative sensitivity of enzymes to H is obtained by either method.

KINETICS OF INACTIVATION OF ENZYMES BY H

Kinetics using the stirring method.—In comparing the effect of H on the activity of different enzymes it was necessary to choose conditions in which all the enzymes were stable and also to keep at a minimum the possible complicating reactions. Because of its physiological importance it would have been desirable to work at pH 7.0–8.0. However, both swine pepsin and crystalline yeast hexokinase are rapidly inactivated above pH 7.0 so that pH 6.0, where these and other enzymes are stable, was used. The buffer was M/25 acetate throughout. In a few experiments at pH 7.8, 0.014 M veronal buffer was used. The stirring method holds the H concentration fairly constant but analyses were usually made.

Under the above conditions the drop in activity of all the enzymes tested was logarithmic when plotted against time. This is illustrated in Figure 94 by experiments on crystalline swine pepsin and crude or crystalline yeast hexokinase. It was also true for crystalline chymotrypsin, chicken pepsin, yeast sucrase and swine pepsinogen. The slope of this logarithmic curve is practically independent of the pro-

tein concentration but is directly proportional to the dissolved H concentration.

Derivation of a bimolecular equation.—The bimolecular equation derived below fits all the facts just described when the H concentration is held constant throughout the experiment, i.e., when the stirring method is used.

In order to prevent confusion between the symbol for hydrogen ion and for activated H the symbol DH is used for mustard gas and DH^* for the activated form of it.

It seems probable that the active mustard unit is not pure DH but an "activated" or "cyclized" compound DH^* formed by a reaction with water. Thus: $DH + H_2O \rightarrow DH \cdot H_2O \rightarrow DH^* + Cl^-$.

When $[DH]$ is held constant the reaction to the right will reach a steady state and $[DH^*]$ will be constant, or

$$[DH^*] = C\,[DH]$$

With protein P, n molecules of DH^* may react, then

$$n\,[DH^*] + P \rightarrow P\,[DH^*]_n \quad \text{and}$$

$$\frac{-dP}{dt} = K\,[P]\,[DH^*]^n \qquad \text{or}$$

$$\frac{-dP}{P} = K\,[DH^*]^n\,dt$$

If, as in our experiments, the $[DH]$ is held constant then $[DH^*]^n$ is also constant and on integrating between P at 0 time and P at time t the following equation is obtained

$$K = \frac{1}{t\,[DH^*]^n} \ln \frac{P_o}{P_t}$$

but $$[DH^*] = C\,[DH]$$

therefore $$KC = \frac{1}{t\,[DH]^n} \ln \frac{P_o}{P_t}$$

KC has the dimensions of mols P reacting per mol P per liter per mol of $[DH]^n$ per liter per minute.

This equation predicts that:

1. The inactivation of an enzyme or the decrease in concentration of any reactant other than DH will be logarithmic. This relationship

would not hold if $[DH]$ decreased during the reaction as it does in the Dixon method.

2. The fraction of the protein inactivated at a given time will be independent of the concentration of the protein.

3. The fraction inactivated per unit time will be proportional to $[DH]^n$.

4. The relative rates of reaction of two different compounds cannot be predicted unless n (the number of mols of H combined per mol of other reactant) is known.

The rate of inactivation of crystalline swine pepsin, chicken pepsin, swine pepsinogen, crude and crystalline yeast hexokinase, sucrase, and crystalline chymotrypsin were determined in the presence of constant H concentration and with varying time and protein concentrations. The results showed that the equation predicts the results quite well. The decrease in activity is logarithmic (Figure 94—curves A,B,C,F,G, H,K), and the rate of inactivation is proportional to the H concentration.

Since many mustard molecules react with a given protein molecule, i.e., $n > 1$, it was expected that the fraction of enzyme inactivated would vary as some higher power of the DH concentration. However, the fraction inactivated varies directly with the gas concentration as though $n = 1$. Any of the following assumptions will predict this result.

1. The first DH molecule to react with the enzyme causes complete inactivation.

2. The reaction of the enzyme with the first mol of DH is the slowest or pace-setting reaction.

3. Each group of the enzyme reacts independently with DH, due probably to the fact that the groups are far apart in the protein molecule. In this case n is the number of DH mols which react with one *equivalent* of protein, i.e., $n = 1$.

It is not possible from the present data to decide among these possibilities, although 3 is probably correct.

Rates of action of H with enzymes compared to the rate with water.—It is sometimes desirable to compare the rates of action of H on various materials to that on some one common substance such as water. This was the purpose of the competition theory (Holiday, Ogston, Philpot, and Stocken, #1). If the reaction of H with water is really monomolecular, as is usually assumed, then comparing the bimolecular protein reactions to the water reaction has no particular physical

significance. Holiday, Ogston, Philpot, and Stocken have concluded that the reaction of H with water is purely monomolecular and there is considerable evidence for this assumption. It fails, however, to predict the decrease in hydrolysis rate when the water concentration is decreased by the addition of alcohol or other non-aqueous solvents. Thus, in terms similar to those used above for enzymes the bimolecular constant for water at 25°C. is

$$KC = \frac{0.13}{55} = 0.0024$$

The rate of action of H on an enzyme such as swine pepsin compared to its action on water may, therefore, be expressed as a ratio of the bimolecular constants or

$$\frac{KC \text{ (pepsin)}}{KC \text{ (water)}} = \frac{37}{0.0024} \text{ or } 15{,}000.$$

Kinetics using Dixon's method.—In this method the reaction goes to completion and the percent of the enzyme inactivated varies with the product of the active enzyme left and the initial gas concentration. This holds over a considerable range of gas concentration for many enzymes, as may be seen in Figure 95. The formula

$$k = \frac{\frac{E_o}{E} - 1}{[H_o]} \quad \text{or} \quad \frac{(E_o - E)}{[H_o]\,E}$$

describes the reaction. E is the active enzyme left after reaction with $[H_o]$, the initial gas concentration.[1] In this formula k is the slope of the plot of $\frac{E_o}{E}$ against $[H_o]$. No theoretical derivation of this equation has been found. The expected relation is

$$k = \frac{a}{[H_o]} \ln \frac{E_o}{E}$$

where a = the hydrolysis constant of H under the conditions of the experiment. This relation does not hold as well as the simple reciprocal equation above.

[1] The value of k increases very gradually with decreasing concentrations of proteins. In the experiments shown graphically in Figure 95, the protein nitrogen was 0.1/ml.

The values reported by Dixon (Y7483) for hexokinase have also been plotted in Figure 95. It may be seen that his values do not give a straight line. His experiment was carried out at pH 7.8 and 39°C. whereas ours was at pH 6.0 and 25°C. Our crystalline preparation was rapidly inactivated at pH 7.8 in the absence of H so it was decided to avoid these conditions. We have carried out the inactivation of chicken pepsin and yeast sucrase by the Dixon method at pH 7.8 as well as pH 6.0. With chicken pepsin the value of *KC* at pH 7.8 was about twice that at pH 6.0, while with sucrase it was thirty times. Thus, no general quantitative prediction can be made about the effect of pH on the rate of inactivation of enzymes by H.

H inactivation constants of yeast enzymes in vivo *and* in vitro.—It was shown by Nelson, Palmer, and Wilkes (1932) that Fleischmann's yeast sucrase (invertase) would hydrolyze sucrose solutions while the enzyme was still a part of the living yeast cell. They found the kinetics of this enzyme reaction to be the same *in vivo* and *in vitro*.

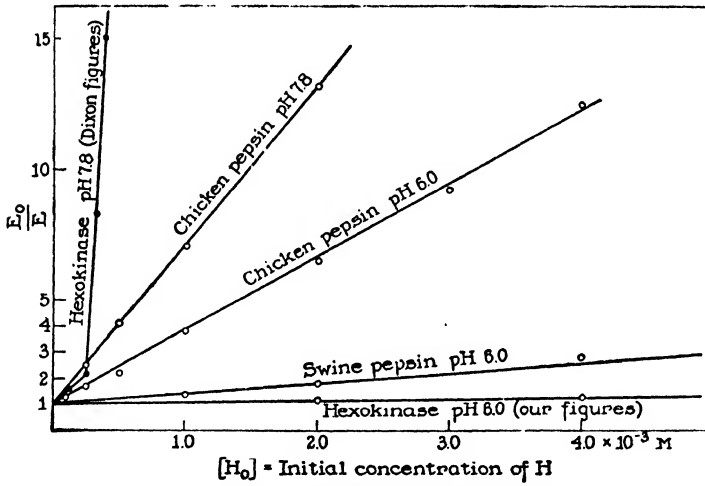


FIG. 95. Inactivation of various enzymes by mustard using the Dixon method.

Figure 96 shows the effect of H on the *in vivo* and *in vitro* enzymatic activity of yeast sucrase at pH 6.0. Freshly washed Fleischmann's bakers' yeast was used. The bimolecular equation has been applied and the constants for the two cases were of the same order of magnitude, being 2–3 for the *in vitro* reaction and 4–5 for the *in vivo* reaction.

Anerobic glycolysis of yeast was similarly examined and a constant of 9 was obtained. This is not far from the value of 12–15 obtained *in vitro* with yeast hexokinase which is supposed to be the enzyme involved in anerobic glycolysis.

Inhibition of yeast glycolysis by mustard and other war gases has been observed by Massart and Peeters and reported for them by Bacq (1941).

PROTEIN GROUPS REACTING WITH H

The groups in proteins that may be available for chemical reaction are carboxyl, amino, tyrosine phenol, tryptophane indol, histidine imidazole, arginine guanido, aliphatic hydroxyl of serine, threonine, hydroxy-proline, etc. *SH* of cysteine, the disulfide of cystine and the thiomethyl ether linkage of methionine.

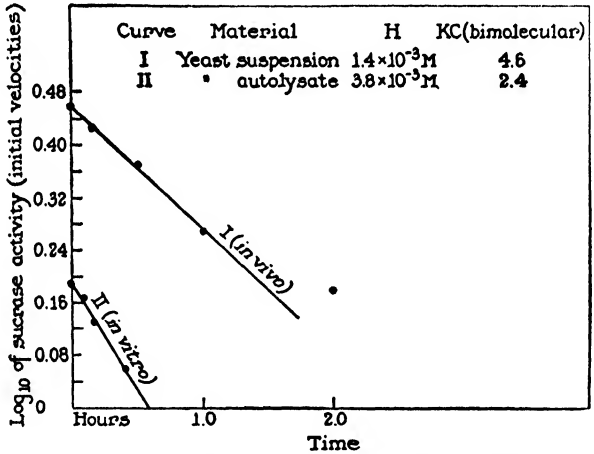


FIG. 96. Inactivation of yeast sucrase (invertase) *in vivo* and *in vitro* by mustard.

The work in this paper is primarily concerned with the carboxyl, amino, tyrosine phenol, and tryptophane indol groups of enzymes and proteins. Preliminary experiments with amino acids and peptides indicated that all four of the groups react with mustard. Ball, Doering, and Linstead (1942) have found that carboxyl groups of many acids react with H.

Other workers (Ball, Davis, and Ross—1943; Ball, Ormsbee, and Henriques—1943; Mann and Pirie—V12478) have suggested that

carboxyl groups of proteins react with H. Since our preliminary report containing titrimetric evidence that H had reacted with carboxyl groups of pepsin and gelatin, Ball, Davis, and Ross (1943) have shown that hemoglobin when treated with H at pH 5.5 loses many of its carboxyl groups as evidenced by the change in titration curve. When the reaction was carried out at pH 7.5 then, in addition to carboxyl groups, the imidazole group of histidine reacted.

Hellerman (1942), Bergmann (1942b), and Bacq (1942) have shown that H reacts with the *SH* group of cysteine. Moritz, Henriques, *et al.* (1942), Ball, Davis, and Ross (1943), and Du Vigneaud, Stevens, and McKennis (1942) have found the imidazole of histidine to react if the reaction media was held between pH 6.0–8.0. Bergmann (1942a) has reported that a sulfonium compound is formed by the action of H on the sulfur of methionine.

Cogan, Grau, and Kinsey (1943a) fed rats a hydrolysate of H-treated casein. The results of these feeding experiments indicated that the histidine, lysine, methionine, and threonine were not "available." In another paper (1943b) they suggest that in the case of threonine the lack of "availability" is not due to a direct reaction of the threonine residue with H.

Voegtlin and collaborators (1943) found that H combined with *SH* and NH_2 groups. These experiments were carried out in "slightly alkaline" solution. The H-carboxyl group compound might not be formed under these conditions since in some instances this linkage has been hydrolyzed in solutions more alkaline than pH 9.0.

Hellerman's experiments (1944) revealed that the *SH* groups of denatured egg albumin and certain *SH* groups of native urease react with chemically pure mustard at pH 7.0. Bacq and his associates, Fischer and Desreux, have also found that H reacts with *SH* groups of denatured egg albumin at pH 8.0 (Bacq and Fischer 1943), with crystalline lens protein (Bacq and Desreux 1942), and urease (Fischer 1943). Bacq attributes the inactivation of enzymes by H to this reaction. Banks, Boursnell, *et al.* (1942), also reported that H reacts with the *SH* groups of egg albumin.

Two other laboratories have analyzed the action of H on proteins. Bergmann, Fruton, Irving, Moore, and Stein (1943) studied the action of H on some amino acids and peptides as well as on some intracellular and serum enzymes. Ball and Ormsbee (1943) using pig skin

found that about half the fixed radioactive mustard was alkali labile. This, they suggested, was attached to protein carboxyl groups.

In the present experiments various aqueous protein solutions were stirred with H at pH 6.0. Samples were removed at various time intervals. These samples were dialyzed to remove thiodiglycol (TDG), salts, etc., and analyzed for activity (in case of enzymes), alkali labile sulfur, carboxyl, amino, tyrosine plus tryptophane groups and, in some instances, total sulfur.

In four of the five different proteins the change in the number of free carboxyl groups resulting from treatment with H equalled within the experimental error the increase in number of alkali labile H residues bound. In most instances the labile sulfur was nearly the same as the total sulfur. In these same four proteins there was no decrease in amino nitrogen content. There was a decrease in the Folin phenol color value which, although indicating that some of the tyrosine moieties have reacted with H, is probably a secondary effect; perhaps due to steric hindrance. This will be discussed more fully in a later section.

The results on crystalline yeast hexokinase stand out as being different since they indicate a reaction at the NH_2 groups. This result depends, however, on a single analysis. Unfortunately this experiment could not be repeated owing to lack of material. With gelatin, pepsin, egg albumin, and chymo-trypsinogen large quantities of protein permitted several runs and duplicate analyses involving large differences but the measurements and differences obtained with hexokinase were small. On the other hand, it may well be that hexokinase is different from other proteins and that the H reacts with its amino groups even at pH 6.0.

Effect of H on phenol color of proteins.—Proteins which contain tyrosine or tryptophane give a blue color with Folin's phenol reagent. These proteins gave less color after they were treated with H. The color returned when the H-protein complex was allowed to stand in dilute alkali. As stated earlier, the loss of carboxyl groups when these proteins were treated with mustard gas was approximately equivalent to the number of H residues attached. Thus, not enough H groups were attached to the protein to combine with both tyrosine and carboxyl groups.

In the case of pepsin the carboxyl groups decreased at first in pro-

portion to the number of H residues attached. During this stage of the reaction little change occurred in the phenol color. As more carboxyl groups reacted, the change in phenol color became greater.

Gelatin contains no tyrosine or tryptophane and hence gave no color with the phenol reagent. Hexokinase, on the other hand, yields a color with the reagent but no change occurred on treatment with H.

Reaction of H with peptides.—Ball, Davis, and Ross (1943) found that H does not react with phenol groups of tyrosyl tyrosine. Du Vigneaud, McKennis, *et al.* (1943), reported that the Folin's reagent color value of tryptophane was lowered by forming the methyl ester of the carboxyl group and was increased again when the ester was hydrolyzed.

Tyrosyl-glycine and glycyl tyrosine on the other hand, gave about the same phenol color so that the effects were not constant but depended on the nature of the linkage formed.

Herriott and Northrop found in preliminary experiments that glycyl tyrosine on treatment with H lost over 75 percent of its pH 8.0 phenol color and that little recovery was observed even after 20 hours in N/10 alkali (pH 13.0). Du Vigneaud and Stevens (1942) have reported similar findings, and they consider that this might be expected because of the ether type linkage between the phenolic group and H. Recently we have prepared an H-glycyl tyrosine compound which also has only a fraction of the original phenol color value but in this case the color was almost completely recovered by treatment with dilute alkali for ten minutes. Thus, this second H-glycyl tyrosine compound behaved like H pepsin and many other H proteins on treatment with dilute alkali. The method of preparing these two H peptides differed only in the concentration of peptide. In the earlier work the concentration was 5 mg. per ml. while for the new derivative the concentration was 100 mg. per ml. The pH was 7.5–8.0 in both cases.

Rate of recovery of phenol color of various proteins in dilute alkali.—Mann and Pirie (V12478), Peters and Wakelin (V14978), Moritz, Henriques, *et al.* (1942), and Ball and Ormsbee (1943) have shown that H proteins are unstable in dilute alkali and that some of the H is hydrolyzed even at pH 9.0–10.0.

There is a great difference in the rate of recovery of the phenol color with different proteins. Pepsin recovers all its color in a few seconds at pH 11.0 while zein shows very little recovery at pH 13.0 in many

hours and there are many rates in between these extremes. Unfortunately, we do not have the corresponding rates of hydrolysis of the H residues or the liberation of carboxyl groups from these H proteins. It appears, however, that the rates of reaction of similar groups in different proteins may vary enormously.

Discussion of the change in phenol color.—It is evident from the preceding experiments that interpretation of the observed results is complicated since both phenol groups and carboxyl groups have been affected, whereas less than an equivalent number of H residues were combined. Several suggestions have been made to account for this anomaly.

1. One mol of H reacts with both OH and COOH groups. This reaction is unexpected from kinetic theory; also, benzyl-H ($C_6H_5—CH_2—S—C_2H_5—Cl$), which has only one reacting group has the same effect on the phenol color as does H itself. In this case also the loss in carboxyl groups is equivalent to the combined benzyl-H.

2. It was suggested by Du Vigneaud and Stevens that the change in phenol color is due to denaturation. It is true that the rate of denaturation of protein-H compound is different from that of the original protein. This cannot account for the present results, however, since pepsin, denatured before reaction with H behaves just as does native pepsin. Also, egg albumin, serum albumin, and chymotrypsinogen are not denatured during the reaction and yet show the same results as pepsin. Evidently these results cannot be explained by denaturation.

3. Esterifying the carboxyl groups affects the phenol color indirectly. Du Vigneaud, McKennis, *et al.* (1943), have shown that this is experimentally true in the case of tryptophane but our experiments showing that tyrosyl glycine had the same molar color as glycyl tyrosine indicate that esterifying the carboxyl group *per se* will not account for the results. The nature of the ester must be considered. There is not enough tryptophane present in some proteins to account for the observed color change and hence tyrosine residues must be involved. With other derivatives of tyrosine, such as the diiodo (Herriott 1941a) and N-acylated (Tracy and Ross 1942b; Miller 1942), the phenol color value is appreciably lower than tyrosine yet there is probably no direct chemical union with the phenol group. A decrease in color,

therefore, is not necessarily evidence that some of the tyrosine phenol groups have been linked with mustard molecules.

4. H reacts with the OH groups and the reaction changes the pK of carboxyl groups so that they are not titrated. Gelatin which contains neither tyrosine nor tryptophane would have to be considered a special case if this proposal were valid. We were unable to find carboxyl groups of H proteins ionizing below pH 2.0.

5. A sulfonium salt of H and protein phenolic OH, COOH groups is formed (Rydon 1943; Crane and Rydon 1943). An H molecule is assumed to combine directly with protein COOH or OH groups. A second H molecule then combines with the first to form a sulfonium salt. Such a compound would be expected to be split from the protein by alkali more rapidly than an H-phenol compound and in this respect the results with protein agree with the facts. In other respects the experimental results do not agree with this hypothesis.

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XIII: ENZYMES AND THE SYNTHESIS OF PROTEINS

(Northrop 1946b)

EVIDENCE is rapidly accumulating to show that all enzymes and at least some viruses are proteins. As a result, three fundamental problems, which previously appeared unrelated, may now be considered as one general problem—the synthesis of proteins.

Solution of the problem requires the explanation of a large number of very precise experimental facts on the one hand, and is confined by strict theoretical limits, on the other. Each tissue of each species of plant or animal can form its own special proteins and these proteins are characteristic of the organ as well as of the species. It is probable, therefore, that millions of different proteins exist. In some cases, as in the production of antibodies, or adaptive enzymes, the presence of a foreign compound may cause the organism to develop a new protein having a very definite and specific relation to the compound which caused its production. In other cases the presence of a foreign protein (viruses) results in the formation of more of the foreign protein. The formation of the type-specific nucleic acid (Avery, MacLeod, and McCarty 1944) is an example of a similar reaction.

Whatever the mechanism of protein production, there is every reason to believe that it is controlled in one way or another by enzymes, and there is good experimental evidence (cf. pages 7–9) that many enzymes behave as theoretical catalysts. The synthesis of proteins, therefore, must presumably obey the laws of catalysis, and any hypothesis purporting to account for the synthesis of proteins by means of enzyme reactions must conform to the general theory of catalysis.

This theory states that a catalyst cannot change the equilibrium point of a reaction but only the time required to reach this point. It follows that if any catalyst accelerates a reaction in one direction, no catalyst can cause the reaction to go in the opposite direction, under the same conditions.

The assumption of special synthesizing enzymes is therefore contradictory to the theory of catalysis, unless some other condition is also changed.

VARIOUS POSSIBLE SYNTHETIC REACTIONS

The various methods which have been suggested to account for the synthesis of proteins may be roughly classified, as follows:

1. Purely catalytic synthesis—no energy added.
   a. Equilibrium point shifted in favor of protein by change of concentration or other condition (plastein formation).
   b. Removal of protein as soon as formed. Insoluble proteins—plastein (?); synthesis of anilides (Bergmann and Fruton 1941). Protein surface films (Langmuir and Schaefer 1938).
2. Energy added so as to shift equilibrium in favor of protein.
   a. Coupled reaction (cf. Borsook and Dubnoff 1940, hippuric acid).
   b. Synthesis from building stones other than amino acids.

PURELY CATALYTIC SYNTHESIS (Wasteneys and Borsook 1930)

This mechanism is the simplest and the most thoroughly investigated.

Theory.—Proteins are hydrolyzed by enzymes and therefore they must also be synthesized by the enzyme which caused hydrolysis, since catalysts must accelerate the reaction rate equally in both directions. In order actually to obtain proteins in this way, therefore, it is only necessary to find conditions under which significant quantities of protein will exist at equilibrium. Theoretically, more protein will exist in concentrated than in dilute solution. This follows from the law of mass action which states that the equilibrium constant for the reaction $A = nB$ is defined by the equation $K = \frac{[B]^n}{[A]}$ or $[A] = \frac{[B]^n}{K}$. $[A]$ and $[B]$ are concentrations at equilibrium, and n is the number of molecules of B formed from A. If $[A]$ is very small compared to $[B]$ then the concentration of A at equilibrium will increase in proportion to the nth power of the total concentration. Thus if it be assumed that a protein is hydrolyzed to 100 amino acids, increasing the total concentration 10 times should increase the concentration of unhydrolyzed protein 10^{100} times. The fact that there are undoubtedly a number of intermediate steps in the hydrolysis does not change the results since according to thermodynamic reasoning the condition at

equilibrium must be independent of the path by which the equilibrium is reached.

Effect of pH.—It is possible to assume that the equilibrium is shifted by changes in pH of the solution since the energy change is small in any case and could be significantly affected by the energy of ionization of the products of the reaction.

Experimental results.—Danilewsky found that a precipitate (plastein) was formed when a preparation of pepsin was added to a concentrated solution of the products of pepsin hydrolysis of protein. The problem was carefully investigated by Wasteneys and Borsook (1930). They confirmed the importance of the concentration although the experimental result is not exactly as expected by theory since it is necessary also to change the pH. In dilute acid solution the plastein is digested, whereas the synthetic reaction had an optimum rate of pH 4.0. There is a decrease in amino and carboxyl groups.

The amount of plastein formed is greater when greater amounts of enzyme are added. This is apparently contrary to theory but it is very difficult to be sure that equilibrium is reached, especially with low concentrations of enzyme in the presence of reaction products. The products act as inhibitors and greatly decrease the reaction rate.

Wasteneys and Borsook also found that the reaction was accelerated by the presence of some emulsifying agents (benzaldehyde) and took place to some extent without the enzyme. This observation is difficult to understand from the point of view of synthesis unless these substances help remove the protein from solution. Wasteneys and Borsook consider that the protein is synthesized as a soluble protein which soon changes to an insoluble (denatured ?) form and precipitates from the solution. If this is correct then the emulsifying agents may act by accelerating the denaturation reaction since many proteins are rapidly denatured at interfaces.

Plastein is also formed by the action of trypsin or papain on a solution of insulin which has been previously digested by pepsin (Haddock and Thomas 1942).

There seems little doubt that some synthetic reactions occur during plastein formation, but whether proteins are produced is still uncertain. According to Folley (1932) no substance having a molecular weight of over 1000 is formed.

Taylor (1907 and 1909) reported the synthesis of protamine sulfate

from a completely digested solution of the compound. The enzyme preparation was a glycerine extract of liver. The substance formed was identical, or very similar, to the original protamine.

The relation of the plastein to the original protein may be tested by hydrolyzing enzyme proteins. Plastein is formed in concentrated solutions of trypsin or pepsin which have been hydrolyzed by pepsin. The plastein, however, has no enzymatic activity nor does it have the general properties of the enzyme protein from which it is derived. In these cases, therefore, the plastein obtained is not related to the protein originally hydrolyzed (Northrop 1947b). Ecker (1947) has examined the plastein formed in these solutions in the ultracentrifuge. No evidence for the existence of any molecules larger than 1000 molecular weight could be found. These results confirm those of Folley.

There is some experimental evidence, therefore, that proteins may be formed by enzymatic synthesis under the proper conditions.

Removal of protein from reaction mixture.—The preceding section summarizes attempts which have been made to synthesize proteins by changing the concentration of the reaction mixture so as to favor the presence of the protein.

Protein could also be accumulated if the quantity in equilibrium with the hydrolysis products could be constantly removed. This operation would require energy but would result in the formation of indefinite quantities of protein.

The synthesis of anilides discovered by Bergmann and Fruton (1944) is probably an example of this type of reaction.

Robertson (1926) suggested that protein might be synthesized at liquid interfaces since the protein is concentrated there. The experiments of Langmuir (1939) and of Gorter (1937) have shown that native proteins, although soluble in bulk, form an extremely insoluble surface layer at the air-water interface. If this layer were constantly removed a new one would form and in this way protein would be obtained. Langmuir has suggested (Langmuir and Schaefer 1938) that the molecules already present on the surface could act to regulate the formation of more identical molecules. This mechanism also predicts that the synthesis of proteins would be associated with some type of structure such as is present in cells which would provide the necessary surface.

This hypothesis solves both the energy and the specificity problem

and appears to the writer to be the most reasonable and simplest mechanism so far suggested. It deserves the most careful consideration. The accumulation of protein in the surface layer is an undoubted fact so that only the specific synthesis is in doubt. Unfortunately, no experimental verification has been obtained as yet for this step. It must be remembered, however, that negative results are very unconvincing in experiments of this type, since even after positive results have been reported it is sometimes necessary for the problem to be studied for years before conditions are sufficiently well established so that the results may be obtained at will.

SYNTHESIS BY THE ADDITION OF ENERGY

Coupled reactions.—Many cases are known in which a reaction which requires energy and hence does not occur alone, will do so if another reaction which liberates energy takes place simultaneously. Such reactions control carbohydrate metabolism (Meyerhof 1944 and Kalckar 1944). In these reactions part of the energy liberated by hydrolysis or oxidation is used for a synthetic reaction so that, although the total change results in the liberation of energy some steps occur which require energy. Each step is controlled by a special enzyme. Phosphoric acid esters are concerned in these energy exchanges and it is reasonable to suppose that similar esters may take part in the synthesis of proteins as Bergmann and Fruton (1944) have suggested. Many other possible sources of energy exist but no direct experimental evidence has been found to connect the synthetic and energy-providing reaction. The synthesis of hippuric acid from glycine and benzoic acid, in the presence of liver slices is perhaps the nearest approach to such a reaction. Borsook and Dubnoff (1940) found that rapid synthesis occurred in the presence of intact liver cells. Extracts or even minced tissue failed to cause the reaction.[1] It seemed evident that the energy was obtained from respiration and hence that the reaction would stop if the respiration were stopped, and hydrolysis should then occur instead of synthesis. Actually, addition of HCN which poisons the respiratory enzymes, did stop synthesis but it also stopped hydrolysis. This disconcerting result may be due to poisoning of the enzyme which catalyzes the benzoic acid reaction, although such an effect would not

[1] It is perhaps significant that the three fundamental reactions, protein synthesis, photosynthesis, and fixation of nitrogen all require energy and all are so far inseparable from intact cells.

be expected, since only the respiratory enzymes are known to be inactivated by HCN. The result might be due to the fact that hydrolysis and synthesis are both caused by the same enzyme.

Schoenheimer's beautiful experiments (1942) have shown that proteins are continuously being synthesized and hydrolyzed so that it is quite possible that part of the energy for synthesis of proteins is obtained from the hydrolysis of other proteins or peptides.

The enzymes which hydrolyze proteins (pepsin, trypsin, papain, cathepsin, etc.) do not carry digestion very far. The hydrolysis is completed by the various peptidases Part of the energy liberated by the hydrolysis of the peptides could, therefore, be returned to the protein ⇆ peptide system and so result in the synthesis of proteins. Such a system is highly organized, and, hence, would be difficult to isolate in good working order. It could not function indefinitely without an external source of energy.

An interesting reaction which may be an example of this mechanism has been studied by Behrens and Bergmann (1939). They found that neither acetyl-*di*-phenylalanyl glycine nor glycyl-*l*-leucine alone are hydrolyzed by papain, but that glycine and leucine are formed when both peptides are present. Similar results have been reported by Abderhalden and Ehrenwall (1933). Behrens and Bergmann were also able to show that an intermediate compound, acetyl phenylalanyl glycyl glycyl leucine, is formed and then hydrolyzed. They consider that the tripeptide acts as "co-substrate" for the hydrolysis of glycyl leucine and that the energy required for the formation of the intermediate compound is obtained from the hydrolysis of glycyl leucine. The tripeptide appears to be a "co-enzyme," however, since its concentration is unchanged at the end of the reaction rather than a "co-substrate" which is a substance changed by the reaction.

The reaction is an excellent sample of a catalytic reaction in which the acceleration is due to the formation of an intermediate compound. The over-all change is glycyl-leucine ⇆ leucine and glycine and hence the formation of the pentapeptide does not enter into the energy relation at all, since thermodynamic equilibria are entirely independent of the path by which they are reached. As Petrie (1943) has pointed out, the reaction is actually an hydrolysis and does not by itself serve as a source of the synthetic substance. Nevertheless, the reaction is of interest since it shows that the enzyme does catalyze the synthetic

reaction. If a mechanism were present which would remove the pentapeptide the reaction could be used as a source of the substance. In this case the energy for synthesis would be furnished by the mechanism which removed the peptide.

Synthesis of proteins from compounds other than amino acids.— Several reactions are known (cf. Bergmann and Fruton 1944) which could give rise to proteins. Alcock (1936) states that plants can form proteins from sources other than amino acids. He claims, with some justification, that two entirely different mechanisms for protein synthesis would not be expected and suggests that animals first change the amino acids to the hypothetical building stones, which are common to both plants and animals. An "ur-protein" molecule is then synthesized and the other proteins derived from this "ur-protein." This hypothesis is logically attractive but must be amplified somewhat to take into account the energy changes required (cf. page 232).

If it could be shown that the compounds taking part in these reactions are formed from amino acids by the addition of energy and that these compounds then reacted to form proteins with the liberation of energy, the mechanics of synthesis would be greatly clarified. Synthesis of proteins from such high energy compounds has been discussed by Delbrück (1941) and Gulick (1944). Such a reaction would account for the fact that N or C isotopes supplied in any amino acids are very soon found in all amino acids (except lysin) (Schoenheimer 1942). This is difficult to understand if the proteins are synthesized from amino acids, but would be expected if amino acids are first changed to other intermediates before synthesis occurs.

At present, however, there does not appear to be any experimental evidence for the existence of such compounds, or reactions, or catalysts therefor in biological material. The enzymatic synthesis of glutamic acid from NH_3 and the keto acid (Euler, Adler, Günther, and Das 1938) is probably the nearest approach to such a reaction so far discovered. The polymerization of amino acid anhydrides recently reported by Woodward and Schramm (1947) is of interest in this connection. The reaction occurs in benzene solution containing a trace of water and results in the formation of long, high molecular weight peptide chains. The reaction occurs without the addition of energy so that, under these conditions, the large molecules must be more stable and contain less energy than the anhydrides. If this reaction

can be made to occur under biological conditions it may very well represent the actual course of protein synthesis by cells.

Glutamic acid can be converted into several other amino acids by transamination (Braunstein and Kritzmann 1937; Braunstein 1939). The transmigration of the methyl groups in certain amino acids, studied by Du Vigneaud and others (reviewed in Borsook and Dubnoff 1943) furnishes another partial mechanism for equilibria between amino acids. These reactions can account in part for transformation of one amino acid into another but do not supply a complete synthetic reaction for proteins.

FORMATION OF NATIVE AS DISTINCT FROM DENATURED PROTEINS

It was stated earlier in this discussion that the reaction, protein and water $\rightleftharpoons$ amino acids is nearly complete and very little protein remains at equilibrium. This is experimentally true in the case of denatured proteins but merely an assumption as far as native proteins are concerned. Most native proteins are digested very slowly by proteases and it is possible to assume, as Linderstrøm-Lang and Jacobsen (1941) have suggested, that the first step in the hydrolysis is the formation of denatured protein from the native protein. Linderstrøm-Lang attempted to answer the question by a study of the temperature coefficient of hydrolysis of native and denatured protein but the results were inconclusive. The hydrolysis of a protein like trypsin or chymo-trypsin, which exists in an equilibrium between native and denatured forms (cf. pages 117, 136) should furnish evidence in this connection.

Haurowitz and collaborators (1945) have reported that globular proteins, such as egg albumin and serum globulin, are not hydrolyzed by trypsin unless they are denatured, whereas "fibrous" proteins, such as fibrin and myosin, are hydrolyzed at about the same rate in either native or denatured forms.

If enzymatic hydrolysis does require preliminary denaturation then it could be assumed that *native* proteins can be synthesized without adding energy, i.e., by a purely catalytic reaction. Energy is required to denature the protein which then hydrolyzes with the liberation of energy. The entire cycle would require three reactions:

1. Amino acids $\overset{\text{enzyme}}{\rightleftharpoons}$ native protein + energy.
2. Native protein + energy $\rightleftharpoons$ denatured protein.
3. Denatured protein $\overset{\text{enzyme}}{\rightleftharpoons}$ amino acids + energy.

It is also possible to assume that *denatured* protein is formed first from the amino acids and that the native proteins are then formed from this denatured protein. This cycle would be as follows:

Amino acids + energy $\overset{\text{enzyme}}{\rightleftharpoons}$ denatured protein.

Denatured $\rightleftharpoons$ native protein + energy.

The direction in which the reactions proceed is determined by the energy changes and the rate of the reaction by the enzymes present.

SPECIFICITY OF SYNTHESIS

The preceding outline shows that several mechanisms are known which could result in the synthesis of proteins without coming into conflict with any of the accepted theories of chemical reactions. Even if some of these over-all mechanisms are correct, there still remains the problem of the regulation of the reaction so that the desired protein only is obtained and not simply a random assortment of proteins.

Nothing is to be gained, in the absence of experimental evidence, by assuming a series of enzymes, each controlling the synthesis of a single protein, since we are then faced with the mechanism of synthesis of the enzymes themselves. These are presumably also proteins and we are therefore back at the beginning again.

The known proteases hydrolyze practically all (denatured ?) proteins and hence must catalyze the synthesis of all proteins, so that these enzymes can hardly account for the formation of any one protein. For instance, horse hemoglobin and pig hemoglobin are both presumably hydrolyzed completely to amino acids by the autolytic enzymes of either the horse or pig. If, therefore, conditions were found in which the reaction were reversed, addition of either of these enzymes would result in the formation of at least two hemoglobins: horse and pig. In the animal, however, this does not occur. Horse hemoglobin only is formed in the horse and pig hemoglobin only is formed in the pig.

It is possible that the specificity of protein synthesis is not due to the specific action of the enzyme but simply to the chemical nature of the protein itself. Most chemical reactions are specific in that, as a rule, very few and often only one of a large number of possible reaction products actually appear. Thus, if the reaction between sodium hydroxide and hydrochloric acid is treated purely statistically, there are a great many possible reaction products. Actually, however, only

two products are formed—sodium chloride and water. This "specificity" is not due to any catalyst but simply to the fact that the products, sodium chloride and water, are the only stable ones under the condition of the reaction. It is possible, therefore, that the formation or synthesis of specific proteins is regulated by the difference in stability of the many structurally different proteins rather than by specific catalysts.

AUTOCATALYSIS

Autocatalysis is the only known chemical reaction by which indefinite quantities of a required substance may be obtained without assuming the existence of several other substances, the origin of which must also be accounted for. Autocatalysis, therefore, offers the only escape from the dilemma of the preformationists and has been invoked by a number of workers. Troland (1917) developed a general theory of cell formation on this basis. He concluded that complicated molecules reproduced themselves by a process similar to crystallization. The molecular structure of the underlying layer determined the structure of the superimposed layer. Troland also reviews earlier and less definite suggestions of autocatalysis. Koltzoff (1928) developed a similar theory. He concluded that antibody formation was a special example of such a reaction. Koltzoff also realized the fact that an autocatalytic reaction cannot start itself, but must have at least one molecule of the reaction product present at the beginning (cf. Northrop 1937). He assumed that the "genonema" contains at least one molecule of each protein of the species. It is difficult to avoid the necessity for this assumption but it may be that, if the total number of different kinds of protein molecules present in the individual were known, genonema and even the sperm and ovum would be too small to contain them. Gulick (1938) suggests that protein molecules are biscuit-shaped discs and that this structure is impressed on each succeeding molecule somewhat as a coin is stamped by a die. He points out, however, that the contact must be "front to back" as otherwise the impression of the disc and not the replica would be obtained. Langmuir and Schaefer (1938) have also suggested the "template" method of regulating synthesis. Pauling's (1940) theory of antibody formation is somewhat similar. Darlington (1944) ascribes the formation of "plasma germ" and viruses to a similar autocatalytic reaction.

Stanley (1938a) suggests that a protein-nucleic acid complex is the simplest structure capable of autocatalytic synthesis. He ascribes the specific synthesis to a crystallization-like reaction similar to that suggested by Troland.

A WORKING HYPOTHESIS FOR THE SYNTHESIS OF PROTEINS

The preceding section has summarized briefly theories and experimental results relating to protein synthesis. These theories account more or less satisfactorily for the specificity of the reaction but (with the exception of the system of Langmuir and Schaefer) fail to provide for the necessary energy. Since there is every reason to believe that energy is required, the synthesis cannot be accounted for by a simple autocatalytic reaction, nor can it be said that any of the various types of proteins or viruses "synthesize themselves," unless it be further assumed that they are formed from unknown, high energy compounds. They may be able to direct the synthesis so that the proper protein is formed but the energy must come from some other reaction, so that at least two reactions, and not a single autocatalytic reaction, are necessary.

The working hypothesis outlined in the rest of this discussion is an attempt to formulate the simplest assumption that is adequate to account for the known facts. It is quite probable that future experiments will force modification to be made and may render the entire mechanism untenable. If the hypothesis leads to the discovery of new facts it will have served its purpose even though the new facts destroy the assumptions which led to their discovery.

In general the hypothesis may be stated as follows:

The formation of proteins occurs in two steps. The first step consists in the synthesis of one or more "type" proteins ("proteinogens") ("ur-protein" of Alcock) which are specific for the species and perhaps for the organ. This step requires energy which may be obtained from a coupled reaction or from preliminary formation of high energy building stones (not amino acids). It is "autocatalytic" in that the structure of this proteinogen is determined by itself. No experimental evidence for this step exists at present. It probably takes place in the cell since respiration, and similar reactions that liberate energy, occur usually in the cell.

Since even the existence of such a protein is purely hypothetical,

discussion of its properties is hardly warranted. The only essential properties are that stable proteins may be formed from it without the addition of energy and that its own formation is autocatalytic. There may be several such proteinogen structures, one for each general group of proteins. They probably contain nucleic acids. The virus proteins may be special examples of these proteinogens.

Spiegelman and Kamen (1946) have suggested that nucleoproteins are the controlling factor in protein synthesis and present evidence which indicates that the energy may be supplied by the phosphoric acid of the nucleoprotein. Muller (1945) has made a similar suggestion.

The possibility that the proteinogen has the characteristics of a denatured, rather than a native protein, was suggested by Kunitz. This assumption would account for the observed immunological reactions as well as the energy requirements (cf. page 226), since denatured proteins do not cross-react with the native proteins, nor do they show such marked specificity. Therefore cross-reactions between the proteinogen and the native proteins derived from it would not be expected.

It is probably unnecessary to assume that the molecular weight of the proteinogen is equal to or greater than that of the largest molecules which are to be formed from it since Svedberg (1937) and others have found that very large protein molecules like the hemocyanins dissociate very easily and reversibly. The formation of these very large molecules from molecules the size of normal proteins, therefore, requires little or no energy (Northrop 1938, page 363).

It is also unnecessary to assume that any appreciable quantity of this protein exists at any one time. It could be decomposed as rapidly as formed and still act as an intermediate between the energy-requiring step and the specific step in protein synthesis.

In the second step the individual proteins are formed by a catalytic or an autocatalytic reaction from this proteinogen. This reaction does not require energy and may occur anywhere.

The formation of an enzyme from its precursor is an example of such a reaction. It is the only mechanism so far discovered whereby proteins may actually be produced *in vitro*. The reactions are specific and are adequate to account for the formation of proteins in general, provided the precursor is present. The exact chemical changes involved in these reactions are not known.

The proposed mechanism accounts for the group specificity of proteins from the same organ and species. Assumption of one (or a few) synthetic reactions is simpler than assumption of a separate synthetic reaction for each individual protein. It has the further advantage that it is in keeping with the gradual development of the cell, since, once the proteinogen is formed, other proteins can readily be derived from it (cf. Troland 1917). If each protein must be synthesized individually, each one requires an energy source and the evolution of a series of related proteins is more difficult.

Madden and Whipple (1940) have pointed out that partial synthesis of all proteins in one organ (the liver ?) would greatly simplify the mechanism of tissue metabolism since it would then be unnecessary for every cell of every tissue to contain the complete protein-synthesizing system. According to the present mechanism, the proteinogen could well be synthesized in one organ and distributed from there to the various other organs and tissues where it would be modified into the specific proteins needed.

It may be noted in this connection that many autocatalytic reactions are known, and many coupled reactions, but no autocatalytic coupled reactions have been described so far as the writer is aware. If every cell in every tissue had such coupled reactions occurring for each protein, the chances of the discovery of the reaction would be far greater than if it occurred only in a special organ or organs.

In the following sections an attempt is made to describe the formation of enzymes, antibodies, normal proteins, and viruses in terms of this working hypothesis.

FORMATION OF NORMAL PROTEINS

It is becoming increasingly evident that some sort of equilibrium exists between the various amino acids and the various proteins or both (Madden and Whipple 1940; Schoenheimer 1942). Schoenheimer demonstrated by means of isotopes that both the N and C atoms of an ingested amino acid appear in nearly all the proteins and amino acids of the body in the course of a few days. This result is totally unexpected from the viewpoint of the classical theory of protein synthesis and indicates the existence of a dynamic equilibrium (cf. Borsook and Keighley 1935), since such equilibria would result in a rapid distribution of the atoms. It is probable that, as Schoenheimer

has suggested, some unknown building stones take part in the equilibria as otherwise it is difficult to account for the transfer of the C atoms from one amino acid to another.

Whipple and Madden's results are extremely interesting in this connection. These workers find that dogs may be kept in health when the only source of nitrogen is plasma injected into the vein. The animal must, therefore, be able to form any required protein from the protein present in plasma without preliminary hydrolysis by the enzymes of the digestive tract. Whipple and Madden consider the results to show that an equilibrium exists between plasma protein and the cell proteins. They suggest that large polypeptides may be the intermediate compounds in the equilibrium.

A similar equilibrium between the serum proteins, themselves, has often been suggested and there is considerable evidence to show that these proteins can be transformed into each other.

Pederson has found by ultracentrifugal studies that a lipoid-containing protein of enormous molecular weight, which he calls X-protein, exists in serum. He assumes that this protein is in equilibrium with the albumin and globulin fractions. It is true that albumin and globulin are not in equilibrium with each other when isolated from serum, but this fact by no means proves that they are not in equilibrium with each other in the circulating blood.

Moore, Shen, and Alexander (1945) have found a special protein in chicken embryos which is not found in adult chicken serum.

The various equilibria indicated by these experimental results may be described in terms of the present hypothesis as follows:

Amino Acids ⇵ (Intermediate ?) ⇵ Proteinogen	Energy added somewhere between amino acids and proteinogen. Autocatalytic reaction.
± Enzyme ⇵ ⇵ Protein A Protein B Protein C, etc.	Autocatalytic reaction.

Since all the proteins are in equilibrium with the proteinogen they are in equilibrium with each other.

The quantity of proteinogen present at any time may be very small and hence difficult to detect.

The proteinogen is probably synthesized only in cells. The formation of the usual proteins from proteinogen may occur anywhere.

No experimental evidence for the formation of normal proteins in this way is at hand. It is possible that such a reaction would be observed if an organism could be completely freed from one of its normal proteins. The re-addition of this protein to the organism should then result in renewed production.

The formation of enzymes from their precursors is exactly analogous to the hypothetical reaction assumed for the formation of all normal proteins, and hence one of interest in this connection. The precursors of the enzymes must themselves be formed from the proteinogen molecules and an indication of such a reaction has been noted by Allen, Ray, and Bodine (1938).

FORMATION OF ENZYMES FROM THEIR PRECURSORS

Trypsin from trypsinogen.—This reaction agrees quantitatively with the theory for a simple autocatalytic reaction. The velocity is affected by acidity and temperature in the same way as is the activation of chymo-trypsinogen or hydrolysis of other proteins by trypsin.

Trypsin is also formed from trypsinogen at pH 5.0 by a proteolytic enzyme secreted by a mold (*Penicillium*) (cf. page 132). Trypsin is only slightly active at this pH, so that the activation curve is no longer auto-catalytic but simply logarithmic as is the activation of chymo-trypsinogen. Trypsin obtained in this way is identical with that formed by autocatalytic activation. The mold enzyme, therefore, must attack the trypsinogen molecule at the same place as does trypsin.

The formation of trypsin from trypsinogen is also accelerated by enterokinase (cf. page 127). The reaction trypsinogen → trypsin is therefore catalyzed by three different catalysts: trypsin itself, mold kinase, and enterokinase.

Pepsin from pepsinogen (cf. page 83).—The reaction at pH 4.65 is autocatalytic and hence is caused by pepsin itself. About 15 percent of the nitrogen is split off during this reaction. So far as is known, pepsin attacks only peptide linkages so that there is reason to believe that the rupture of one or more peptide links in the precursor leads to

the formation of the active enzyme. If swine pepsinogen is activated by chicken pepsin, swine pepsin is formed (cf. page 86). The structure responsible for the species specificity of the enzyme therefore is present in the precursor.

Relation of protein to precursor.—The exact changes in chemical structure which occur during these reactions are not yet known. It is known, however, that the protein formed may be quite distinct in chemical, physical, and immunological properties from its precursor.

It is probable that all amino acids present in the enzyme must also be present in the precursor although, in view of the remarkable reactions discovered by Schoenheimer, even this conclusion may be questioned.

Immunological relationship of chymo-trypsin, chymo-trypsinogen, and beef and pig trypsin (cf. p. 151).—Chymo-trypsinogen was found to be distinct from chymo-trypsin.

Trypsin from beef pancreas was distinct from trypsin from pig pancreas and both were distinct from chymo-trypsin and chymo-trypsinogen.

Pepsinogen and pepsin (cf. p. 81).—These tests were carried out by the precipitin reaction.

Pepsinogen antisera reacted with pepsinogen but did not react with pepsin or with serum protein from the homologous species.

Antipepsin sera reacted with pepsin and also to a small extent with pepsinogen. They do not react with serum proteins of the homologous species.

These results show there is at best only a faint cross-reaction between the enzyme and its precursor, even when the experiments were carried out with concentrated solutions of the two pure proteins. Had the tests been carried out with crude tissue extracts of the precursors it is probable that no cross-reaction between the enzyme and its precursor would have been observed, owing to the small quantities present.

Specificity of the reactions.—Each enzyme, as a rule, has its own precursor, but Bodine (1945) has reported that different tyrosinases may be formed from protyrosinase by different methods. The enzyme precursors isolated so far, therefore, do not represent the hypothetical proteinogen, but must be considered a separate step between this and the active enzyme. The formation of the precursor of tyrosinase has

been studied by Allen, Ray, and Bodine (1938) and found to be an autocatalytic reaction. The enzymes whose precursors have been isolated are secretory enzymes and are required in high concentration at special times. It is possible that relatively large quantities of the precursors of these enzymes are to be found in the organs for this reason, since they serve as a convenient source of supply from which the active enzymes can be obtained rapidly at any time. The cellular enzymes and the normal proteins, on the other hand, are not required suddenly and there is no necessity for a rapidly available reserve. This difference in the requirements may account for the fact that the precursors for the secretory enzymes only have been found in quantity.

FORMATION OF VIRUSES

There is good reason to believe that some viruses, at any rate, are proteins (for a review of this work see Stanley 1940).

No experimental evidence exists, so far as the writer is aware, to distinguish the formation of virus from the formation of normal proteins. Both are found only in or on living cells and virus production is accompanied usually, if not always, by protein production. Experimentally the only difference between the two processes is that "normal" proteins are always produced by the cells but that the production of some virus proteins may be started by introduction of the protein from outside the organism (Northrop 1937; Darlington 1944). Phage produced by lysogenic strains of bacteria and "indigenous virus" of plants and animals are always produced just as are "normal" proteins. It can, of course, be assumed (cf. Gratia 1938) that infection occurred sometime in the past, but there is no direct evidence for this assumption. No means is known to free lysogenic strains from their viruses.

It would be expected, then, that if an organism could be completely freed from a protein, it would no longer produce that protein until reinoculated with it. The suggestion was made (Northrop 1938) that the substance producing type-specificity in pneumococci was an example of such a reaction. The analogy between virus and the substance regulating type-specificity in pneumococci was recognized by Gratia (1936c). Since then the substance has actually been isolated (Avery, MacLeod, and McCarty 1944). It turns out that it is a desoxyribonucleic acid instead of a protein, but the mechanism of formation may well be the same.

Virus proteins possess the following characteristics which must be accounted for by any hypothesis for their formation:

1. They increase only in the presence of appropriate living cells.

2. Production of virus is usually associated with growth and active metabolism of the host cells (Bordet, Gratia, and Jaumain 1921; Krueger and Northrop 1930; Zinsser 1937; Gratia 1938; Howe and Mellors 1945—this paper contains other relevant references).

This correlation between metabolism and virus production is strong evidence that the synthetic part of the reaction is carried out by the cell in conjunction with the synthesis of normal proteins, since the viruses themselves do not contain the enzymes necessary to carry out these reactions.

All hypotheses concerning the production of virus proteins (other than those which consider the virus to be a cell) assume some sort of autocatalytic reaction. Some writers (Troland 1917; Stanley 1938b; Caspersson 1939; Jordan 1944) assume that the virus can synthesize itself from simple building stones. This mechanism requires energy and entails a series of assumptions as to the source of energy and the manner in which it is made available (cf. page 226).

Bordet (1931) pointed out that a simpler and equally adequate hypothesis is to suppose that the virus is formed from a precursor. This assumption does not involve any energy mechanism in addition to that already present in the host cells and has the further advantage that there is a close experimental analogy in the formation of enzymes (Gratia 1922; Northrop 1938). Darlington (1944) has recently suggested a similar mechanism. Potter and Albaum (1943) consider viruses to be "misplaced enzymes."

Viruses have also been considered as obligate parasites. This point of view is ably presented by Fraenkel (1945), by Rivers (1939), and by Burnet (1945), who consider that a virus is a fragment of a degenerate bacterial cell. This fragment has maintained or developed the power of multiplying in the presence of the host cell. Actually the original source of the virus is the only significant difference between these points of view and that of Bordet who considered that the virus was originally part of a host cell, rather than of a parasitic bacterial cell. It is quite possible that both ideas are correct since the definition of virus is at present partly based on size. Vaccinia, for instance, is similar to bacteria in many ways, and would not be considered a

protein even though its peculiar physiological and pathological properties were not known.

On the other hand the well-defined crystalline viruses, tobacco mosaic, and especially bushy stunt of tomatoes, are typical proteins in many respects and would never be considered as related to parasites were it not for the pathological changes which they cause.

Final decision between the two possibilities probably cannot be made until the reaction can be produced *in vitro* and in the absence of host cells (cf. Seifriz 1939).

3. Viruses are formed as a rule only in the presence of cells from related species, but may sometimes increase in the presence of cells of widely separated species.

4. Viruses are immunologically distinct from the host proteins.

5. The virus protein produced in the presence of appropriate living cells is usually identical with that used for inoculation but may be different.

6. Inoculation with two viruses results in production of only one, as a rule. In the case of bacteria (Delbrück and Luria) the virus which is inoculated first is produced and not the second.

7. No evidence of metabolism has been found.

8. Viruses are of high molecular weight and contain nucleic acid. Neither of these characteristics, however, suffices to distinguish them from normal proteins.

9. Some viruses at least (bacteriophage, Northrop 1939; tobacco mosaic, Loring 1940) can form saturated solutions. This result shows that the virus can exist in solution or, more precisely, that the liquid containing the virus is a single phase.

Such solubility experiments afford a decisive test of the question as to whether the virus molecules are "in solution" or not. From the kinetic point of view any particle is a molecule and there is no distinction experimentally or theoretically between particles and molecules (cf. Taylor 1925, page 1279). For this reason the word molecule seems appropriate until evidence is found contradicting its use.

From the point of view of thermodynamic equilibria as stated in Gibb's phase rule, however, there is a sharp and definite distinction between suspensions and solutions. A suspension is a two-phase system and a solution is a single-phase system. It is perfectly possible to

decide, therefore, whether a virus "solution" consists of one or two phases.[1]

Quantitative measurements have not been made on other viruses, but any virus which crystallizes must exist as a saturated solution, in equilibrium with the crystals.[2]

Such solubility experiments have not been carried out with larger viruses such as vaccinia, but it appears very unlikely that any indication of a saturated solution would be obtained in these cases.

The formation of viruses may be formulated in the same way as that outlined for normal proteins in the preceding section (Northrop 1946b). Recent results by Cohen (1947) and by Price (1947b), however, cannot be interpreted from this point of view without additional assumptions. Cohen has reported that the phosphorus and nitrogen in *coli* phage are derived entirely from the culture medium and not from the host cell. Price finds that staphylococcus phage requires some substance present in yeast extract for its production, whereas the host cell does not require this substance.

These observations pretty well rule out the existence of a phage precursor in the normal host cell, and it becomes necessary to assume

[1] There is some uncertainty as to the application of the phase rule to colloidal solutions but the results obtained with proteins (cf. page 288) show clearly that the rule may be applied to these compounds. The following experimental results are possible in a study of the solubility of viruses:

1. The concentration of virus in the liquid is zero or variable and no equilibrium can be shown to exist between the solid and liquid. Such systems are suspensions but no further conclusion can be drawn since the phase rule cannot be applied to systems which are not in equilibrium.

2. The concentration of virus in the liquid is constant and in equilibrium with the solid virus.

a. The concentration of virus in the solution is independent of the quantity of solid virus present. The system consists of two phases and two components and the virus, therefore, is a single component. The virus is in solution in the liquid phase. No such results have been reported.

b. The concentration of virus in solution varies with the amount of solid. There are two phases and three or more components. The virus is in solution in the liquid phase. The virus preparation consists of two or more components.

This is the result obtained with bacteriophage (Northrop 1938) and tobacco mosaic preparations (Loring 1940), although the bacteriophage curve is as good as that obtained with many normal proteins.

[2] The fact that the solutions may be concentrated in the centrifuge does not affect this conclusion, since this simply amounts to increasing the force of gravity and any solution may theoretically be concentrated in this way. The gravitational field simply represents another degree of freedom.

that the precursor is formed only by the infected cell. These additional assumptions destroy the simplicity of the precursor hypothesis and hence remove its principal advantage. The facts may be more simply explained by assuming that the host cell supplies the energy required for virus formation, as suggested by Levaditi (1946).

Price's results (1947a and c) are of interest in this connection. Price finds that *Staph. muscae* phage is formed in cell suspensions, the growth of which has been prevented by the addition of penicillin. Growing cells are therefore not necessary for phage production. If sodium azide, iodo-acetate, or sodium fluoride is added to the system, no phage is formed. Iodo-acetate and sodium fluoride are known to prevent the formation of adenosine triphosphate, and the quantity of the compound present in cultures containing the inhibitors is much less than in the control cultures. Adenosine triphosphate is a high energy compound, and it is possible that this substance is the source of the energy required for the synthesis of phage. Somewhat similar results have been reported by Spiegelman and Kamen (1946) in connection with the formation of adaptive enzymes (cf. page 243).

FORMATION OF ADAPTIVE ENZYMES

The formation of normal proteins and enzymes outlined in the preceding section is purely autocatalytic and results in the formation of more of the same molecules. The formation of adaptive enzymes or antibodies, however, is not purely autocatalytic since the molecules formed are different from those originally present. The hypothesis must be modified, therefore, to account for the formation of these compounds.

Enzymes are sometimes formed as a specific response to the presence of the corresponding substrate or its decomposition products. Enzymes formed in this way are called "adaptive enzymes" (Karström 1930).

The production of galactase by yeast is the best known example but many similar cases are known (cf. Yudkin 1938; Knight 1936; Dubos 1939). "Normal" yeast cannot ferment galactose but Dienert (1900) found that yeast acquires the power to ferment it if grown in the presence of this sugar. It was thought at first that this result was due to selection of yeast cells during growth of the culture. Later work established the fact that the enzyme can be formed without any in-

crease in the number of cells. If this observation is correct the fermentation cannot be due to selection of yeast cells, but must be due to production[1] of a new enzyme.[2] The formation of the enzyme is always associated with the formation of protoplasm, even though no cell division occurs (Dubos 1939).

Spiegelman and Kamen (1946) find that growth of yeast and formation of adaptive enzymes is accompanied by a decrease in nucleoprotein phosphorus. Sodium azide prevents this change in phosphorus and also prevents the formation of adaptive enzymes and growth of the cell.

In some cases, as the polysaccharide-splitting enzyme of Dubos (1939), the enzyme displays extreme specificity, in fact the specificity of this enzyme is more precise than the immunological tests.

Formation of the enzymes may be caused by the decomposition products of the substrate. The production of yeast invertase is brought about by glucose as well as by sucrose (Euler and Cramer 1913).

The production of the enzyme is not inherited but stops it if the cells are grown without the substrate.

The potential ability to produce the enzyme, however, is inherited as a Mendelian character (Lindegren, Spiegelman, and Lindegren 1944).

Yudkin suggests that the new enzyme is always present in minute amounts in all cells and is in equilibrium with its precursor. Addition of the substrate or products of the reaction or other compounds which react with the enzyme will result in decreasing the enzyme concentration. This disturbs the equilibrium and hence more enzyme will be formed. This explanation is adequate and also accounts for the fact that substances which combine with the enzyme, other than the substrate, may bring about its production.

It includes the assumption that minute amounts of all possible "adaptive" enzymes exist in normal cells and also that the equilibrium

[1] There does not appear to be any advantage in distinguishing between "production" and "activation" of an enzyme. An inactive enzyme, strictly speaking, is not an enzyme at all.

[2] Sevag (1946) considers that no new enzymes are actually formed but that the new substrate increases the activity of the normal enzyme in some unexplained way. According to this point of view the phenomenon is simply a change in the specificity of the reaction similar to that which occurs when various amino acids are added to peptidase systems (cf. page 227).

is such that very little active enzyme is present. It is further necessary to assume that equilibrium is reached slowly since the new enzyme does not appear at once. The hypothesis predicts that the new enzyme should be obtainable *in vitro* if the precursor were available.

Actually there is no experimental evidence of an equilibrium between an enzyme and its precursor since the known reactions of this type run to completion (*in vitro*) as far as can be determined. It is possible to assume equilibrium, but it must be very far in the direction of the active enzyme.

Monod (1943) has suggested that the substrate acts by changing the course of formation of the normal (closely related) enzyme. This assumption has the advantage that closely analogous cases are known and also that it may be applied to the formation of antibodies, a similar reaction. It has the disadvantage that it does not explain the specificity of the new enzyme quite so definitely.

According to this hypothesis, the normal enzymes are continually being formed from their precursors in the cell by an autocatalytic reaction. When a new substrate or other compound which combines with the enzyme is added, it may act as a coenzyme and change the course of the reaction so that a slightly different enzyme is formed as well as the usual enzyme. When the new substrate is no longer present the reaction returns to its normal course and the normal enzyme alone is formed. Examples of similar reactions in which the course of an enzyme reaction is affected by the presence of substances other than enzyme and substrate are well known. Thus either trypsin or an inert protein may be formed during the autocatalytic formation of trypsin from trypsinogen; the nature of the product depends on the pH of the solution (cf. page 127). Different tyrosinases may be formed from protyrosinase under different conditions (Bodine 1945). Abderhalden and Ehrenwall (1933) found that addition of certain peptides caused glycyl-leucine to be hydrolyzed by trypsin, whereas the reaction did not occur when trypsin and glycyl-leucine alone were present. The experiments were extended by Bergmann and his collaborators (cf. page 227). The added peptides appear to act as coenzymes.

The specificity of the amino acid oxidases is completely changed by the addition of other proteins or amino acids to the reaction mixture (Edlbacher 1946).

The products of an enzyme reaction usually combine with the enzyme so that the fact that a reaction occurs between the enzyme produced and the substance which caused its production does not differ qualitatively from many enzyme reactions. The distinguishing factor in the present case is that one of the products of the reaction is itself an enzyme.

FORMATION OF ANTIBODIES

Antibodies are considered to be modified serum globulins which are changed in such a way as to react with the homologous antigen, cf. Marrack (1938); Zinsser, Enders, and Fothergill (1939); Burnet, Freeman, Jackson, and Lush (1941), Landsteiner (1945) and Sevag (1945). There is no doubt that antibodies are proteins, but their relationship to the serum globulins appears to be somewhat uncertain owing to the fact that neither antibodies nor serum globulins have been obtained in pure form[1] (cf. Roche, Derrien, and Mandel 1944). Any hypothesis for the formation of antibodies must account for the following facts (Burnet, Freeman, Jackson, and Lush 1941; Landsteiner 1945):

1. They are proteins which react specifically with their antigens.
2. They may be produced by so many different antigens that it is extremely unlikely that even one molecule of all possible antibodies exists in the normal animal.
3. They are produced in great quantity as compared to the quantity of antigen which causes their formation.
4. Antibody continues to be formed after the antigen has disappeared. This statement unfortunately cannot be considered an experimental fact since the evidence, although strong, is not conclusive (cf. Burnet, Freeman, Jackson, and Lush, 1941; Landsteiner 1945).

[1] Crystalline diphtheria antitoxin (Northrop 1941a) is probably a pure protein and is not a globulin, although it cannot be separated from the globulin fraction if mixed with it by any known procedure except precipitation with the homologous antigen. This result would prove that antibodies are distinct chemically and immunologically from the normal serum proteins, were it not for the fact that the toxin-antitoxin complex used to prepare the crystalline antibody was digested with trypsin. It is possible to assume, therefore, that the pure crystalline antibody does not represent the natural antibody but is an hydrolysis product.

Antibodies are reported to be associated with different serum proteins in different animals and have been found in all serum fractions (cf. Landsteiner 1945, page 133). These results also indicate that the antibody itself may be a distinct protein rather than a modified globulin. On the other hand, young animals which do not form antibodies readily are said to possess very small amounts of serum globulin (Orcutt and Howe 1922).

There is no doubt that antibody continues to be formed long after any antigen can be detected experimentally in the circulation, but it is possible to assume that minute quantities of antigen are still present in the cells. The best evidence for the continued formation of antibodies, after complete disappearance of antigen, are the results obtained by Dougherty, White, and Chase (1945). These workers find that malignant lymphocytes from mice contain antibody. If these lymphocytes are injected into a normal animal, this animal also produces antibodies. Injection of a third animal also results in antibody formation. These results have been confirmed by Price (unpublished experiments) in this laboratory. If this process can be repeated indefinitely, or even for one or two more generations, it must be considered proof that antibodies can continue to be formed, once the process has been started, without the presence of antigen or its decomposition products. If this is true then as Burnet, Freeman, Jackson, and Lush point out, the various theories proposed by Ostromuislenskii (1915), Koltzoff (1928), Mudd (1932), Pauling (1940), Pauling and Campbell (1942), and Sevag (1945) require modification since these hypotheses all include the assumption that antibody is formed as a result of some type of reaction between antigen and globulin.

The same objection applies to the hypothesis outlined below.

5. The chemical nature of antibodies may be different in different species or in animals of different ages.

This statement is open to question since no antibodies have been isolated in pure form.

6. Antibodies are formed only in the animal receiving the antigen. Injection of another animal with cell-free antisera (passive immunization) results in rapid disappearance of antibody and not in the formation of more antibody. This fact precludes any purely autocatalytic mechanism for the formation of antibodies, unless secondary hypotheses are added.

7. Antibodies are formed in cells, probably in the lymphocytes (Dougherty, Chase, and White 1944, 1945; Dougherty and White 1945; Harris and Ehrich 1946).

A number of reports of the formation of antibodies *in vitro* have been made (Ostromuislenskii 1915; Pauling 1940) but the identity of the substance prepared in this way with the natural antibody has not

been established. It must be remembered in connection with these experiments that, owing to experimental difficulties, *whole blood* cannot be used *in vitro*. Failure of the experiment when carried out with serum, plasma, defibrinated blood, or in the presence of anti-coagulants, therefore, does not prove that the reaction cannot occur in the circulation.

Burnet's hypothesis.—Burnet, Freeman, Jackson, and Lush (1941) consider the formation of antibodies to be analogous to the formation of adaptive enzymes and there are many points of similarity.

Antibodies and enzymes may both be divided into two general classes—normal or physiological, and acquired or adaptive. The "normal" enzymes or antibodies are present in all individuals of the species and are inherited, cf. Landsteiner (1945, page 132). The ability to form adaptive enzymes also appears to be inherited (Lindegren, Spiegelman, and Lindegren 1944). "Acquired" antibodies or adaptive enzymes are formed in response to the presence of a foreign substance and their formation stops sooner or later after removal of this substance.

Both antibodies and adaptive enzymes have a specific relation to the substance which caused their formation. In some cases adaptive enzyme reactions are more specific than the antibody reactions (Dubos 1939).

The reaction of enzymes with substances formed during the reaction is quantitatively similar to the reaction between antigen and antibody (Northrop 1922d; Zinsser 1923). The antigen-antibody complex is less dissociated than most enzyme-product compounds.

According to Burnet, normal globulin is synthesized by a special proteinase. Injection of antigen results in a reaction between this proteinase and the antigen, which destroys the antigen and modifies the proteinase. This modified proteinase causes the formation of antibody instead of normal globulins, and also causes replicas of itself to be formed. The modified proteinase resulting from reaction with the antigen decreases gradually when antigen is no longer present, as do adaptive enzymes.

The qualitative and quantitative differences in antibody formation are therefore accounted for. The hypothesis also accounts for the fact that small quantities of antigens can cause the production of very

large quantities of antibody and predicts that antibody formation may continue for an indefinite length of time after the antigen and even its decomposition products have disappeared.

It appears to the writer that this hypothesis predicts that injection of an animal with antiserum should lead to the production of more antibodies, since the production of the antibody-forming proteinase is assumed to be autocatalytic. These proteinases are assumed to be present in the serum and so should cause formation of antibody when serum containing them is injected into a different animal. Actually, the injection of antiserum does not lead to the production of more antibodies but, on the contrary, the antibody injected disappears quite rapidly. Sevag (1945) has also pointed out this difficulty in connection with Burnet's assumption. It is possible to avoid this discrepancy by means of secondary hypotheses, but it appears to be a serious objection to any purely autocatalytic mechanism.

A slight modification of Burnet's hypothesis, however, allows it to fit the experimental facts adequately, including the results of Dougherty, White, and Chase (1945). It is only necessary to assume that the modified proteinases which Burnet assumes form the antibody, exist in the lymphocytes, and not in the serum. This modification does away with the objection that injection of antiserum does not cause the formation of new antibodies as predicted by Burnet's original hypothesis, while it now predicts that the injection of "immune" lymphocytes will result in the formation of more antibodies, even though the antigen is no longer present.

Sevag's hypothesis.—Sevag (1945) has recently reviewed the various hypotheses for the formation of antibodies and has carefully and thoroughly discussed the relation of antigens to enzymes. There is no doubt that many points of similarity exist between the action of enzymes on their substrates and the formation of antibodies after injection of antigens. Sevag concludes that all proteins are enzymes and that antibodies are formed as a result of the action of the antigens (enzymes) on the normal globulins. According to this hypothesis antibodies should be readily formed by the action of antigens on normal serum *in vitro*. Actually, this is not the case. This discrepancy, however, cannot be considered conclusive owing to the difficulty of carrying out such an experiment under the conditions which exist in the blood vessels (cf. page 247).

Sevag's hypothesis also predicts that antibodies can be produced only so long as antigen is present.

Antibody formation from proteinogen.—The writer agrees with Burnet that antibodies and adaptive enzymes are closely related and are probably formed by the same mechanism. The hypothesis describing the formation of adaptive enzymes outlined above is simpler than that of Burnet and agrees as well with the known facts of antibody formation.

The reaction may be summarized as follows:

1. The normal serum proteins are formed from the "proteinogen" by an autocatalytic reaction.

2. Antigen (or its decomposition products) acts as a coenzyme[1] in this reaction and causes the formation of a slightly different protein, in the same way that the substrate causes the formation of the adaptive enzymes (page 242). The resulting protein reacts with the coenzyme (antigen) which led to its formation since products of enzyme reactions in general react with the enzyme which produced them.

3. The antibody is formed almost entirely in the cells where proteinogen is synthesized since the quantity of proteinogen present in the blood at any time is probably small.

These assumptions account for the following facts:

1. Antibodies are related to normal serum proteins and react with the antigen which led to their formation.

2. Small quantities of antigen can produce indefinite amounts of antibody.

3. Antibody production may continue after all antigen has disappeared from the circulation but not after all decomposition products of the antigen have disappeared. In this respect it differs from Burnet's and Sevag's hypotheses. If antibodies can be formed by continued serial injection of "immune" malignant lymphocytes, as indicated by the results of Dougherty, White, and Chase, this assumption cannot be made and the hypothesis must be abandoned in favor of a modification of Burnet's hypothesis.

4. The antibody may be related to one or several normal proteins, depending on which autocatalytic reaction is affected by the antigen.

5. More antibodies will not be formed when antiserum is injected into an animal, since antibody formation, itself, is not assumed to be

[1] Sevag (1945) assumes that the antigens themselves act as enzymes.

autocatalytic. This prediction also differs from Burnet's hypothesis.

6. Antibodies are not formed in any appreciable amount in the absence of cells. The proteinogen is formed in the cells since it is only in the cells that the necessary mechanism exists for the energy supply required for synthesis. It will be transformed into the various special proteins of the circulation, as soon as it comes in contact with them. The formation of antibodies will, therefore, occur principally in or near the synthesizing cells. The various special proteins, including antibodies, are all assumed to be in equilibrium with the proteinogen and hence are in equilibrium with each other. It should be possible, therefore, to prepare antibodies *in vitro* by adding antigens to blood. The quantity of proteinogen actually present is assumed to be very small, so that the rate of reaction may be slow. There is the further technical difficulty that whole blood cannot be used (cf. page 247). Production of antibodies in this way has been frequently reported (cf., for instance, Ostromuislenskii 1915; Pauling 1940).

The difficulty in the way of the production of antibodies by adding antigen to serum *in vitro* may also be due to the fact that the spleen appears to be of special importance in connection with antibody formation. Extracts of spleen from immunized animals give rise to antibodies when injected into normal animals and Topley (1930) has suggested that the spleen contains an intermediate derivative of the antigen and that this intermediate is responsible for the actual antibody formation. This assumption is confirmed by Price's (1947a) recent results. Price used hexokinase as antigen and could not detect the presence of the enzyme in spleen extracts, although 1 gamma of the enzyme gives a positive test. The spleen extracts, nevertheless, gave rise to antibody formation when injected into a normal animal.

The hypothesis does not account in any simple way for the observation that antibody formation is sometimes accompanied by an increase in normal serum globulins.

Rate of appearance of antibodies in the circulation.—Injection of antigen gives rise, after a few days, to a rapid increase of antibody in the circulation. In some cases a second injection of antigen gives rise to a second and larger increase in antibody, especially if a small amount of antigen were injected the first time. The curves are logarithmic in form and Burnet considers this to be evidence for the existence of some process of biological multiplication. Actually the

curves have long "lag periods" and are not strictly logarithmic except for a small part of the reaction. They agree just as well or better with the integral of the usual probability curves (Northrop 1946b). (For the mathematical relation between logarithmic and probability curves, see Yule 1910). The case is very similar to the rate of death curves of bacterial cultures or of insects (Loeb and Northrop 1917). The data may be made to fit a log curve if the "lag period" is neglected. From the point of view of probability curves, the results indicate that the cells in which the antibody is produced die or are ruptured when antibody is released into the blood stream. The rapid and large rise sometimes noted on a second injection of antigen may thus be related to the phenomenon of anaphylaxis. The cells are sensitized by the first small injection and injured by the second. This mechanism agrees with the process of antibody formation suggested by Sabin (1939).

APPENDIX

PREPARATION AND CRYSTALLIZATION OF THE ENZYMES

ALTHOUGH no one method of purification has been found which can be applied to various enzymes, still several general guiding principles have been of great value in all the work.

In the first place, only those reagents and those conditions of temperature, acidity, etc., which are known not to injure proteins have been found to be of value. Adsorption methods have been found useful to remove inert material or coloring matter. Adsorption of the active material usually results in too great loss. Several grams or, if possible, several hundred grams of material for each step in the purification are prepared before proceeding with the purification. This is of the greatest importance since it is the only way in which it is possible to work with concentrated solutions in fairly large volumes. Concentrated solutions (i.e., 1–10 percent) are essential in protein work since proteins in dilute solution can be separated only with difficulty, if at all, while the same proteins in concentrated solution may sometimes be separated with ease. The question of volume is important because many of the conditions for crystallization, such as pH, salt concentration, and protein concentration, are very sharply defined, and it is technically impossible to adjust these conditions with the required accuracy when working with small volumes of solution. Finally, care must be taken, in attempts at crystallization, to see that the solution is not too highly supersaturated since this condition favors the appearance of the amorphous form. The amorphous form of pepsin, for instance, is many times more soluble than the crystalline form. A solution which is saturated with respect to the amorphous material is therefore many times supersaturated with respect to the crystals.

The usual procedure of adding the precipitating reagent until a precipitate just appears is therefore frequently a failure since it results in a solution which is too highly supersaturated for crystallization. The more dilute the solution the greater the danger of too high a degree of supersaturation. In general, the most favorable conditions are concentrated solution and very slight supersaturation.

In regard to experimental procedure, the most important point is thorough and complete filtration. For this reason, large funnels must be used so that the filter cake is not more than 1–5 mm. thick. The cake must be pressed with a spatula, as cracks appear, until no further foam or

liquid is drawn through. This filtration is important since the composition of the solution is determined by the weight of the filter cake, in many cases. The vacuum must be removed before the cake dries, since drying is injurious to all proteins and completely denatures some of them. A filter cake prepared even under the above conditions still contains 30–60 percent mother liquor while one which is several cm. thick may contain 50–80 percent and sediment obtained in the centrifuge or a precipitate filtered without suction contains 90 percent and more mother liquor. One careful filtration with suction is therefore as efficient as several centrifugalizations or filtrations without suction.

The most troublesome impurity to be contended with is mucinlike material. This substance will completely prevent crystallization even when present in very small amount. In larger concentrations it prevents any fractionation procedure. Strong acid, Filter-Cel,[1] copper hydroxide, and acetone are the best reagents so far found for removing it, but each solution has presented a new problem.

Method of Defining the Concentration of Solutions

Many of the operations described in the following procedure depend for their success upon the protein concentration of the solutions. These solutions are prepared frequently by dissolving a more or less wet filter cake in the solvent. The most convenient way of reaching this concentration is to weigh the filter cake and add it to a measured volume of solvent. The resulting solution then may be defined as containing g grams filter cake per ml. solvent. For the sake of brevity the specific gravity of the filter cake is assumed to be 1.0 and the composition of the solution is expressed in terms of volume. Thus (Table 41) "Precipitate 1. Wash twice with equal volume . . ." means that 1 ml. of solution was used for each gram of filter cake.

Dry Preparations

All the enzymes are more or less unstable in solution in water. They are much more stable in glycerine, and some may be kept indefinitely when dry. The drying must be done at low temperature. The following procedure has been found satisfactory with all the preparations. A 5–10 percent solution of the protein is dialyzed at 0°C. at the pH range of maximum stability. The solution is then placed in a distillation flask and frozen. The flask is attached to a good mechanical air pump, and the frozen solvent is evaporated with the use of a condenser cooled with solid carbon dioxide in monomethyl ether of ethylene glycol. Evaporation is rapid enough to keep the material in the flask frozen without further cooling. Storage over anhydrous calcium sulfate (Drierite) or some other agent is essential for certain enzymes.

[1] Several grades of this material are made by Johns-Manville Corporation, New York.

Table 41

Preparation of Crystalline Pepsin

Procedure	Activity per Milligram Protein N			
	Gel. V.	*Cas. S.*	*Rennet*	*Hemoglobin*
500 grams Parke, Davis pepsin U.S.P. 1:10,000 dissolved in 500 ml. H_2O and 500 ml. 1 Normal H_2SO_4 added. 1,000 ml. saturated $MgSO_4$ added with stirring. Solution filtered through fluted paper (S. and S. No. 1,450½) and then with suction. This precipitate must not be allowed to stand at room temperature more than about 24 hours	7.5	0.08	0.14×10^4	0.04
Filtrate discarded				
Precipitate 1. Wash twice with equal volume ¾ saturated $MgSO_4$, filter with suction				
Filtrate discarded				
Precipitate 2. Stir with water to thick paste and M/2 NaOH run in until complete solution. Great care must be taken to avoid local excess of NaOH pH never more than 5.0)	15.0	0.21	0.25	0.10
M/2 H_2SO_4 added with stirring until heavy precipitate forms (pH about 3.0), 3 to 6 hours at 8°C., filter with suction				
Filtrate discarded	15.0	0.30	0.50	0.15
Precipitate 3. Stir with ½ volume H_2O to thick paste at 45°C., M/2 NaOH added carefully until precipitate dissolves, pH about 4 (filter if cloudy and discard precipitate). Beaker containing filtrate placed in a vessel containing about 4 liters of H_2O at 45°C., inoculated and allowed to cool slowly, cooling should require 3 to 4 hours and heavy crystalline precipitate should form at about 30 to 35°. Solution kept at 20°C. for 24 hours. Thick crystalline paste, filter with suction				
Precipitate 4. Wash with small amount of cold H_2O and then with ½ saturated $MgSO_4$ and store under saturated $MgSO_4$ at 5°C.	15.0	0.42	0.71	0.20
Filtrate. M/2 H_2SO_4 added to pH 3.0, amorphous precipitate filtered off and treat as Precipitate 3				

Recrystallization

Method 1. Crystalline paste filtered with suction on large funnel so as to form a thin layer of crystals and washed 3 times with cold M/500 HCl. Filter cake stirred to a paste with ½ its weight of water, the suspension warmed to 45° and M/2 NaOH run in slowly with constant stirring until the precipitate dissolves (pH < 5.0). M/2 H_2SO_4 is then run in until the solution is faintly turbid, a few crystals added and the solution allowed to cool slowly as before. A heavy drop of crystals should separate in about 24 hours. The suspension is then warmed to 45° again and more H_2SO_4 added until the pH of the suspension is about 3.0. It is then allowed to cool slowly again, and filtered after 24 hours. The crystals may be washed with M/500 HCl until free of SO_4.

Ammonium sulfate may be used in place of $MgSO_4$. Sodium acetate may be used in place of sodium hydroxide.

Method 2. Crystals dissolved with NaOH and treated as described for Precipitate 2.

Pepsin (Northrop 1930)

An outline of the method of purification and crystallization of pepsin is shown in Table 41. Most commercial preparations can be used, except those which contain much mucin; such preparations cannot be purified by this procedure. Some preparations contain an inactive protein which crystallizes with the enzyme and may represent 10–40 percent of the crystals. This protein can be removed by many times repeated crystallization or more easily by allowing a concentrated solution of the crystals to stand at pH 2.0 and 30°C. for some time. Under these conditions the inert protein is digested.

A slightly different method of crystallization, which works better with some samples of commercial pepsin, is described by Philpot (1935).

Fractionation of Pepsin (Herriott, Desreux, and Northrop 1940b)

Preparation of crystalline pepsin of constant solubility and activity from Cudahy pepsin is shown in Table 42.

Table 42

Preparation of Crystals 22–29 from 1:10,000 U.S.P. Cudahy Pepsin

	No.	*Quantity, Gm.*	*N.P.N., Percent*	*[P.U.] Hb P.N.*
500 gm. Cudahy pepsin dissolved in 1 liter 0.5 M pH 5.0 acetate buffer, 2 liters saturated magnesium sulfate solution and 200 gm. Filter-Cel added. Filtered. Precipitate washed 3 times with 500 ml. solvent and stirred 2 hrs. in 15 liters solvent.* Filtered. Filtrate saturated with solid magnesium sulfate, filtered.				0.28
Precipitate	1	200	20	0.32
Precipitate No. 1 stirred plus 200 ml. water. 0.5 M sodium hydroxide stirred in until solution clear. 0.5 N sulfuric acid stirred in until pH was 2.5. Stood 6°C. 24 hours, filtered, and crystallized. (Procedure as for Precipitate No. 3, Table 41.)				
Precipitate	19	40	10	0.34
40 gm. No. 19 plus 500 ml. (0.5 saturated magnesium sulfate, 0.1 M pH 4.0 acetate), stood 6°C. for 24 hrs. Filtered.				
Precipitate	29	35	4	0.33

* 0.6 saturated magnesium sulfate, 0.2 M pH 5.0 acetate.

Preparation of Needle Crystals of Pepsin by Crystallization from Dilute Alcohol (Northrop 1946a)

See Table 43.

Table 43

Preparation of Needle Crystals of Pepsin from Cudahy Pepsin

1000 gm. Cudahy 1/10,000 U.S.P. soluble pepsin dissolved in 5 liters 20 percent alcohol. Titrated to pH 3.0 with 10 M sulfuric acid. Kept at 5°C. for 20 hours. Slight flocculent precipitate (mostly mucin).

50 gm. Filter-Cel added and filtered. 5 liters saturated magnesium sulfate added slowly to the filtrate and the suspension allowed to stand about 20 hours at room temperature. Filtered with suction on large funnel. Precipitate (about 350 gm.). Stirred with 1 liter 25 percent alcohol and pH adjusted to 3.8–4.0. Nearly clear. 20 gm. Filter-Cel added and filtered.

Filtrate titrated to pH 1.8–2.0 with 10 M sulfuric acid, inoculated with needles, and left at about 20°C. for 2 or 3 days, stirred occasionally. Heavy crop of plates and needles.* Filtered. Precipitate about 150 gm.

Recrystallization

150 gm. precipitate stirred with 5 liter 20 percent alcohol and pH adjusted to about 4.0 (bromcresol green). Crystals dissolved leaving some amorphous material. 20 gm. Filter-Cel added and filtered.

Filtrate adjusted to pH 1.8 with 10 M sulfuric acid and kept at 20°C. for 1 or 2 days. Heavy precipitate of good plates and needles. Filtered. Precipitate about 100 gm. A further crop of crystals may be obtained from the mother liquor of the first crystallization by keeping it at 5°C. for several days. A heavy amorphous precipitate forms. This is filtered off and crystallized as described under *Recrystallization*.

* Magnesium sulfate also crystallizes as needles from alcohol solution and some magnesium sulfate crystals may appear if the magnesium sulfate precipitate is not filtered as dry as possible. Magnesium sulfate crystals are strongly doubly refractile under the polarizing microscope, while the pepsin crystals are weakly so.

Pepsin from Gastric Juice (Northrop 1933a)

An outline of the method of obtaining crystalline pepsin from cattle gastric juice is shown in Table 44.

Pepsinogen (Herriott 1938a)

An outline of the method of preparing pepsinogen is shown in Table 45.

Experimental Methods of Isolation from Beef Pancreas of Crystalline Chymo-Trypsinogen, Chymo-Trypsin, Trypsinogen, Trypsin, a Trypsin Inhibitor, and an Inhibitor-Trypsin Compound (Kunitz and Northrop 1936)

The methods of preparing these compounds are given in the following sections. The yields reported represent average figures and may vary

Table 44

Preparation of Crystalline Pepsin from Bovine Gastric Juice

	No.	Vol.	N/ml.	[P.U.]^{Hb}		
				Per ml.	Total	Per mg. N
		ml.	mg.			
Contents of 4th pouch removed immediately after death and filtered through fluted paper (S. and S. No. 1450½) 48 hrs. 6°C.	1	5500	1.0	.0025	14	.0025
Filtrate saturated with ammonium sulfate, decanted and filtered with suction, ppt. dissolved in 200 ml. N/500 hydrochloric acid	2	200				
Sol. No. 2 cooled to −10°C. and 300 ml. cold acetone added, centrifuged, supernatant	3	500		.018	9	
Sol. No. 3 cooled to −10°C. and 500 ml. cold acetone added, filtered and precipitate dissolved in 300 ml. N/500 hydrochloric acid	4	310		.025	7.8	
Sol. No. 4 + 1 vol. sat. magnesium sulfate, centrifuged, precipitate dissolved + 100 ml. N/500 hydrochloric acid	5					
Sol. No. 5 + 1 vol. sat. magnesium sulfate, centrifuged, precipitate dissolved + 100 ml. N/500 hydrochloric acid	6	114	.53	.065	7.4	.12
Sol. No. 6 + 1 vol. sat. magnesium sulfate, centrifuged, precipitate dissolved + 70 ml. N/500 hydrochloric acid	7					
Sol. No. 7 + 1 vol. sat. magnesium sulfate, centrifuged, precipitate dissolved + 75 ml. N/500 hydrochloric acid	8	80	.42	.078	6.3	.186
240 ml. of sol. No. 8, equivalent to 15 liters gastric juice + 1 vol. sat. magnesium sulfate, filtered, precipitate	9	3 gm. filter cake			20	
Precipitate No. 9 + 10 ml. N/10 sodium acetate, titrated to pH 3.0 + N/2 sulfuric acid, slight precipitate in dark viscous liquid, could not be filtered nor centrifuged, 1 vol. sat. magnesium sulfate added and suspension filtered with suction. Precipitate dissolved in 8 ml. N/10 sodium acetate, clear yellow solution	10	12	5.6	1.0	10	.18
Titrated to pH 3.0 + N/2 sulfuric acid, stood 18 hrs, 6°C., filtered with suction (slow). Precipitate dissolved at 45°C. with minimum quantity of water	11	10	2.8	.5	5	.18
Cooled slowly while stirring. Crystals formed after about 1 hr. Kept at 20°C. for 24 hrs. and filtered. Precipitate normal pepsin crystals slightly yellowish—about 0.1 gm.	12					
Crystals dissolved in N/10 sodium acetate	13	20	.77	.146	2.8	.19

TABLE 45

PREPARATION OF CRYSTALLINE PEPSINOGEN FROM SWINE GASTRIC MUCOSAE

PROCEDURE, DETAILS OF PREPARATION, ETC.	No.	TIME OF HANDLING	VOL.	PROTEIN NITROGEN	POTENTIAL [P.U.]^{Hb*}				CARBOHYDRATE (AS GLUCOSE)	
		Hrs	*Ml.*	*Mg./ml.*	*1/ml.*	*1/mg. P.N.*	*Total*	*Percent of Original*	*Mg./ml.*	*1/[P.U.]^{Hb}*
3,400 gm. of twice minced, prepared, frozen swine gastric fundus mucosae mixed with 4 times its weight of 0.45 sat. ammonium sulfate in M/10 sodium bicarbonate; stirred 1 hr.	1	1.5	16000	0.67	0.042	0.07	670	100	0.68	14.0
To No. 1 was added 100 gm. fine "Filter-Cel" and 50 gm. coarse (Hyflow) Cel per liter of 0.45 sat. ammonium sulfate solution used. Stirred 15 min. and filtered on 30 cm. Buchner funnel with Whatman No. 3 paper covered with a thin cake of coarse Cel. Filtrate clear. When cake was nearly dry it was washed on funnel twice with 300–500 ml. of 0.45 sat. ammonium sulfate in M/10 sodium bicarbonate, residue discarded; filtrate and washings	2	2.0	13000	0.37	0.048	0.13	630	94	0.24	5.0
No. 2 was brought to 0.68 sat. by addition of 180 gm. solid ammonium sulfate, per liter. Solid ammonium sulfate allowed to stand 10 min. in solution before stirring and then stirred slowly. The suspension allowed to settle overnight or filtered immediately after addition of 50–100 gm. coarse Cel. Discard filtrate	3	0.5	14000		0.0022		30	5	0.16	73.0
Above residue dissolved in water and Cel filtered off. Cel was washed on the funnel until a sample of the washing showed no protein present. Filtrate and washings were combined	4	0.3–0.5	3500	1.3	0.15	0.12	530	79	0.3	2.0

TABLE 45 (Cont.)

PROCEDURE, DETAILS OF PREPARATION, ETC.	No.	TIME OF HANDLING	VOL.	PROTEIN NITROGEN	POTENTIAL [P.U.]$^{Hb^+}$				CARBOHYDRATE (AS GLUCOSE)	
		Hrs.	*Ml.*	*Mg./ml.*	*1/ml.*	*1/mg. P.N.*	*Total*	*Percent of Original*	*Mg./ml.*	*1/[P.U.]^{Hg}*
No. 4 was titrated to pH 6.0 ± 0.2 (yellow to methyl red and to bromthymol blue) with 4 M pH 4.65 acetate buffer and mixed with equal vol. M/1 copper hydroxide suspension previously titrated to pH 6.0. Mixture stirred a few minutes and filtered on large Buchner funnels. Filtrate contained no protein as shown by heating 3 ml. of filtrate with 3 ml. of 10 percent trichloracetic acid. If protein is found more copper suspension must be added and refiltered. Final filtrates were discarded. Filtrate	5	2–3	6500		0.003		20	3	0.053	17.0
Copper residue from No. 5 was stirred to a smooth paste with a vol. of M/10 pH 6.8 phosphate equal to that vol. occupied by the protein solution just before mixing with the copper suspension. This is also the vol. of copper suspension used. In this experiment it was 3,500 ml. The residue was filtered and washed twice with 250 ml. M/10 pH 6.8 phosphate. Residue discarded and the filtrates and washings combined. Filtrates	6	1.5	5000	0.33	0.075	0.23	375	55	0.03	0.4
To No. 6 was added 5 percent fine (Filter-Cel) Cel, stirred and filtered, residue washed twice with 1 vol. M/10 pH 6.8 phosphate equal in ml. to the weight in gm. of fine Cel used. Residue discarded. Filtrate	7	0.25	5100	0.33	0.071	0.20	360	54	0.017	0.24

TABLE 45—concluded

No. 7 brought to 0.7 sat. by addition of 474 gm. of solid ammonium sulfate per liter. 50–100 gm. coarse Cel added and suspension filtered. Filtrate discarded and residue stirred with water and separated from the Celite as described in No. 4. Solution should be diluted with water until the concentration of P.N./ml. is about 1 mg. Solution titrated to pH 6.0 as before and copper hydroxide treatment repeated as well as the treatment with 5 percent fine Cel as described in Nos. 5, 6, and 7. Solution brought to 0.4 sat. by adding 242 gm. solid ammonium sulfate per liter. An equal vol. of sat. ammonium sulfate solution was added through a capillary tubing. Solution should be stirred slowly but constantly. Suspension allowed to stand 2 days at 10°C. and the protein was filtered *without Celite filter aids* on a Buchner with hardened filter paper. (A sample of residue was analyzed and figures were calculated for the entire quantity.)	8	6–8 and 8 standing				0.25	320	48		0.03
No. 8 as a 0.7 sat. ammonium sulfate filter cake was stirred with 9 vol. 0.40 sat. ammonium sulfate solution in M/10 pH 6.25 phosphate at 10°C. The solution was almost clear, containing dirt and shreds of filter paper and was therefore filtered. Solution on stirring slowly at 10°C. for a few hours became opalescent and a silkiness appeared which grew heavier. After stirring 2 days suspension filtered. Filtrate	9		460	2.1	0.43	0.21	200	30		
Crystal cake dissolved	10		126	3.5	0.74	0.21	94	14		

* These values represent the pepsin activity after complete activation of aliquots of pepsinogen.

considerably in individual preparations, but the proportion of precipitate to solvent specified in the text must be accurately adhered to. Weights of precipitates refer to the weight of the filter cake that is removed from the Buchner funnel. It is essential for the success of the preparation that these filtrations be as complete as possible. Large Buchner funnels must be used, the filter cake pressed with a spatula so as to fill all the cracks, and the filtration continued until little or no foam is drawn through the funnel. As a rule, the dry precipitate should form a layer not more than 2–3 mm. thick. The preparations are all quite stable in the form of a moist filter cake if kept in the icebox. Permanent dry preparations may be prepared as described above (page 254).

Determinations of pH were made on a test plate by mixing 1 drop of the Clark indicator with 1 drop of the solution. Standards were prepared by mixing 1 drop of the indicator with 1 drop of the standard buffer. This method, of course, gives only apparent pH values, which may be considerably removed from the true pH of the solution. The method, however, is perfectly adequate for reproducing the necessary conditions.

The saturated magnesium sulfate and ammonium sulfate solutions were prepared at 20°C. The buffer solutions were made up according to Sørensen.

The method of preparation of the enzymes varies somewhat, depending upon the desired product. The first method describes a complete fractionation whereby all of the substances may be obtained, starting from fresh inactive cattle pancreas. This is the best method for obtaining chymotrypsinogen and fair yields of trypsinogen but does not always give good yields of inhibitor-trypsin compound. The second method is the most convenient and reproducible method for obtaining trypsin and inhibitor-trypsin compound but yields no trypsinogen. The third method is the most efficient for the preparation of inactive trypsinogen. The fourth method describes the conversion of trypsinogen into active trypsin, and the fifth method describes the isolation and crystallization of trypsin from active pancreas.

I. Isolation of Crystalline Chymo-Trypsinogen, Chymo-Trypsin, Trypsinogen, and Trypsin Inhibitor from Fresh Cattle Pancreas

1. Preliminary Purification and Concentration

Remove pancreas from cattle immediately after slaughter and immerse at once in enough ice-cold 0.25 N sulfuric acid to cover the glands. Remove fat and connective tissue and mince in a meat chopper within a few hours. Suspend 3 liters of minced pancreas in 6 liters of 0.25 N sulfuric acid at 5°C. and allow suspension to stand at about 5°C. for 18–24 hours. Strain the suspension through gauze, resuspend the residue in an equal volume of cold 0.25 N sulfuric acid, and strain through gauze immediately. Reject residue. Dissolve 242 gm. of solid ammonium sulfate in each liter of com-

bined filtrate and washings. Filter through fluted paper (S. and S. No. 1450½) in cold room. Reject precipitate. Dissolve 205 gm. of solid ammonium sulfate in each liter of filtrate. Heavy precipitate forms. Remove foam and allow to settle for 2 days at 5°C. Decant supernatant solution and filter residue with suction through hardened paper (S. and S. No. 575) on a large funnel. Yield about 100 gm. precipitate. Reject filtrate. Dissolve each 100 gm. precipitate in 300 ml. water, add 200 ml. saturated ammonium sulfate, stir in 5 gm. Standard Super-Cel (Celite Corporation)[1] and filter *with suction* through soft paper (S. and S. No. 1450½). Reject precipitate. Add slowly 205 gm. solid ammonium sulfate to each liter of filtrate and filter with suction through hardened paper (S. and S. No. 575). *Precipitate A* about 90 gm. (mixture of crude chymo-trypsinogen, trypsinogen, and inhibitor). Reject filtrate.

2. Crystallization of Chymo-Trypsinogen

Dissolve each 90 gm. of precipitate A in 135 ml. water, add 45 ml. saturated ammonium sulfate, then adjust to pH 5.0 (brick-red color with 0.01 percent methyl red solution on test plate) by addition drop by drop of about 2 ml. 5 N sodium hydroxide. Allow to stand for 2 days at 20–25°C. A heavy crop of chymo-trypsinogen crystals gradually forms. Filter with suction through hardened paper (*Filtrate Tg*). Wash crystalline filter cake with 0.25 saturated ammonium sulfate and finally with saturated ammonium sulfate, and store at 5°C. Yield about 25 gm.

Recrystallization.—The crystalline filter cake is suspended in 3 volumes of water and 5 N sulfuric acid added from a burette, 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. An equivalent amount of 5 N sodium hydroxide is then added, with stirring, and the solution is inoculated and allowed to stand at 20°C. Crystallization should be practically complete in an hour.

Isolation and crystallization of chymo-trypsin.—The final method adopted for the preparation of chymo-trypsin from chymo-trypsinogen is as follows:

The chymo-trypsinogen should be recrystallized five times. 10 gm. of crystalline chymo-trypsinogen filter cake is suspended in 30 ml. water and dissolved by the addition of a few drops of 5 N sulfuric acid. 10 ml. M/2 pH 7.6 phosphate buffer is added and a quantity of molar sodium hydroxide equivalent to the acid is also added. About 0.5 mg. crystalline trypsin is added and the solution left at about 5°C. for 48 hours. Any active trypsin preparation (of equivalent activity) may be used instead of the crystalline trypsin. After 48 hours the solution is brought to pH 4.0 by the addition of about 5 ml. N/1 sulfuric acid, 25 gm. solid ammonium sulfate is added, and the precipitate filtered with suction.

Crystallization.—The filter cake is dissolved in 0.75 volumes N/100 sulfuric acid and filtered if the solution is not clear. The clear filtrate is inocu-

[1] This useful material was suggested by Dr. M. L. Anson.

lated and allowed to stand at 20°C. for 24 hours. About 5 gm. of crystalline filter cake should form.

Recrystallization.—The crystalline filter cake is dissolved in 1.5 volumes N/100 sulfuric acid; about 1 volume of saturated ammonium sulfate is added cautiously until crystallization commences. The solution is allowed to stand at room temperature and practically complete crystallization should take place in about one hour.

A further crop of crystals may be obtained by precipitating the mother liquors with saturated ammonium sulfate and treating the precipitate obtained in this way as described under crystallization.

3. Crystallization of Trypsinogen

Adjust filtrate and washings from chymo-trypsinogen crystallization (Filtrate Tg) to pH 3.0 (pink with 0.01 percent methyl orange on test plate) with about 1 ml. 5 N sulfuric acid per 100 ml. filtrate. Dissolve 30.4 gm. of solid ammonium sulfate in each 100 ml. of filtrate and filter with suction through hardened paper. Reject filtrate. Dissolve precipitate (40 gm.) in 120 ml. water, add 80 ml. saturated ammonium sulfate and 2 gm. Filter-Cel, and filter with suction through soft paper. Wash paper with 0.4 saturated ammonium sulfate. Reject precipitate. Add slowly 100 ml. saturated ammonium sulfate to each 100 ml. of combined filtrate and washings. Remove foam and filter with suction through hardened paper, size 18.5 cm. or larger. Reject filtrate. Wash precipitate on funnel with saturated magnesium sulfate in 0.02 N sulfuric acid to remove excess of ammonium sulfate. The washing with saturated magnesium sulfate must be done rapidly, otherwise the precipitate is partly dissolved. The saturated magnesium sulfate is poured on the precipitate to a height of about 5 mm. and allowed to filter for a few minutes; then the excess of saturated magnesium sulfate is decanted, and filtration is continued until complete. Dissolve precipitate (30 gm.) in 30 ml. 0.4 M borate[1] buffer pH 9.0 at 2–5°C. (in an ice-water bath), add saturated potassium bicarbonate drop by drop to pH 8.0, measure solution, and add equal volume of saturated magnesium sulfate. Mix and allow solution to stand in icebox at about 5°C. (*Solution B*). Short triangular prisms of trypsinogen appear in the course of 2–3 days. If the solution is inoculated with crystals of trypsinogen, crystallization is much more rapid but the crystals are not so well formed. (If crystallization is delayed more than 4–5 days, or if the material has become partly active during the preparation, crystals of trypsin may appear.)

Filter the crystals with suction at 5°C. (*Filtrate C*). The precipitate (about 10 gm.) is washed on the funnel several times with cold 0.5 saturated magnesium sulfate made up in 0.1 M borate buffer pH 8.0 and finally with

[1] Stock borate solution contains 49.6 gm. boric acid and 80 ml. 5 N sodium hydroxide per 1000 ml. solution. 0.4 M borate buffers, pH 8.0 and 9.0, are mixtures of 100 parts stock borate and 78.6 and 17.6 parts 0.4 M hydrochloric acid respectively.

saturated magnesium sulfate made up in 0.1 N sulfuric acid at room temperature. The crystals are then dried in an electric refrigerator at 5°C. and stored in the icebox. The dried material generally contains about 40 percent of trypsinogen protein and 60 percent magnesium sulfate. For activation into trypsin see Section IV, below.

Purification by means of trichloracetic acid.—10 gm. filter cake of trypsinogen crystals is dissolved in 200 ml. N/400 hydrochloric acid and 200 ml. 5 percent trichloracetic acid added. The solution is left at 20°C. for 1 hour and then filtered with suction and washed several times with small amounts of 2.5 percent trichloracetic acid and finally with water. The semi-dry precipitate is dissolved in 25 times its weight of N/50 hydrochloric acid, allowed to stand about 30 minutes. Ammonium sulfate is added to 0.4 saturation. The precipitate is filtered off and rejected. The filtrate is brought to 0.7 saturation with solid ammonium sulfate and filtered with suction.

4. Crystallization of Inhibitor-Trypsin Compound

Combine Filtrates C and washings from several trypsinogen crystallizations (1000 ml.) Adjust to pH 3.0 with 5 N sulfuric acid and saturate with magnesium sulfate by stirring for 15 minutes at 25°C. with an excess of crystals of magnesium sulfate. Filter with suction through hardened paper. Reject filtrate. Dissolve precipitate (150 gm.) in 750 ml. water and add 750 ml. 5 percent trichloracetic acid. Heat the mixture at 80°C. for 5 minutes, cool to 25°C., and filter with suction through hardened paper. Reject precipitate. Adjust filtrate to pH 3.0 with 5 M sodium hydroxide (about 3 ml. per 100 ml. of solution). Saturate with crystals of magnesium sulfate. Filter with suction through hardened paper. Reject filtrate. Dissolve precipitate (15 gm.) in 45 ml. 0.02 M hydrochloric acid, add 1.5 gm. of crystalline trypsin, allow to stand until the trypsin is dissolved, adjust to pH 8.0 by addition of 0.4 M borate pH 9.0. Allow to stand for 1 hour at 5°C. Adjust to pH 5.5 by addition of 5 N sulfuric acid, saturate with crystals of magnesium sulfate at 25°C., allow to stand for 2 days at 20–25°C. Hexagonal crystals of inhibitor-trypsin compound gradually appear, mixed with amorphous precipitate. Filter with suction. Wash precipitate on paper with 0.5 saturated magnesium sulfate. This dissolves the amorphous precipitate. Residue on paper—crystals of inhibitor-trypsin compound. Yield about 0.5 gm. Filtrate and washings, when saturated with crystals of magnesium sulfate, on standing may yield more crystals of inhibitor-trypsin compound.

Recrystallization of the inhibitor-trypsin compound.—Dissolve the filter cake of crystals (0.5 gm.) in 5.0 ml. M/10 acetate buffer pH 5.5, filter through Whatman No. 42 fluted paper. Saturate with crystals of magnesium sulfate and allow to stand 1 day at 20–25°C. Hexagonal crystals of the inhibitor-trypsin compound rapidly appear. Yield about 0.3 gm. filter cake.

5. Isolation of Crystalline Trypsin Inhibitor and of Crystalline Trypsin from a Solution of Crystalline Inhibitor-Trypsin Compound

Dissolve 1 gm. crystalline filter cake of 3 times recrystallized inhibitor-trypsin compound in 10 ml. water and add 10 ml. 5 percent trichloracetic acid; allow to stand at 20°C. for 30 minutes, until precipitation is about complete. Filter with suction (*Filtrate In*). The *precipitate* (*Ts*) is worked up for trypsin, as described below.

(*a*) *Crystallization of trypsin inhibitor.*—Heat the trichloracetic acid filtrate (In) for 5 minutes at 80°C., cool, and filter through fluted Whatman No. 42 paper. Reject precipitate. Adjust filtrate to pH 3.0 with 5 M sodium hydroxide. Dissolve 5.6 gm. of solid ammonium sulfate in every 10 ml. of filtrate. Filter with suction. Reject filtrate. Dissolve precipitate (0.25 gm.) in 2.5 ml. water, adjust to pH 5.5 with 0.4 M borate pH 9.0. Add saturated ammonium sulfate to slight turbidity. Filter through No. 42 filter paper. Wash paper with 0.5 saturated ammonium sulfate. Add more saturated ammonium sulfate to filtrate and washings combined until slight precipitate forms. The amorphous precipitate gradually changes into long hexagonal prisms. Allow to stand for 2 days at 20°C. Filter with suction. Filter cake 0.15 gm. of inhibitor crystals. Wash filter cake with saturated magnesium sulfate if it is desired to have the crystals free from ammonium salt, and recrystallize with magnesium sulfate.

Recrystallization.—Dissolve crystals (0.15 gm.) in 1.5 ml. of M/10 acetate buffer pH 5.5. Add 7.5 ml. saturated ammonium sulfate (or 7.5 ml. saturated magnesium sulfate plus a few crystals of solid magnesium sulfate). Allow to stand at 20°C. for 1 day. Crystals of inhibitor gradually appear. Yield about 0.1 gm. filter cake.

(*b*) *Crystallization of trypsin.*—Wash the trichloracetic acid precipitate (Ts) on the filter paper with water to remove the free acid. Dissolve precipitate (0.7 gm.) in 20 ml. 0.02 M hydrochloric acid. Allow to stand 30 minutes at 20°C. Add 5 gm. solid ammonium sulfate. Filter through fluted Whatman No. 42 paper until clear. Dissolve 5 gm. of solid ammonium sulfate in the filtrate and filter with suction through 5½ cm. hardened paper. Reject filtrate. Wash precipitate on paper with saturated magnesium sulfate in 0.02 N sulfuric acid. Dissolve precipitate (0.5 gm.) in 0.25 ml. water. Cool to 5°C., add about 0.5 ml. borate buffer pH 9.0 until the solution reaches pH 8.0 (pink to 0.01 percent phenol red but not to 0.01 percent cresol red on test plate). Add 0.5 ml. saturated magnesium sulfate. Allow to stand at 5°C. Square prismatic crystals of trypsin rapidly appear. Filter with suction. Yield about 0.25 gm.

II. Isolation of Crystalline Trypsin and Inhibitor-Trypsin Compound from Chymo-Trypsinogen Free Activated Pancreatic Extract

Preliminary purification and concentration is the same as described under I for preparation of Solution B (Section I, 3).

1. Crystallization of Trypsin

Inoculate Solution B (about 100 ml.) with 50 mg. of trypsin crystals, let stand at 5°C. for several days. Precipitate of very small crystals of trypsin gradually forms. Occasionally a few triangular trypsinogen crystals may also appear. Filter with suction at 5°C. Filtrate E. Filter cake is washed on paper several times with 0.5 saturated magnesium sulfate at 5°C. and finally with saturated magnesium sulfate in 0.1 N sulfuric acid at room temperature. Yield 8 gm. of filter cake.

2. Recrystallization of Trypsin

Dissolve filter cake (8 gm.) in 6 ml. 0.02 N sulfuric acid. Add a few drops of 5 N sulfuric acid if solution is incomplete. Cool to 5°C., add 12 ml. saturated magnesium sulfate and 6 ml. 0.4 M borate pH 9.0. Adjust to pH 8.0 with saturated potassium bicarbonate or 5 N sulfuric acid if necessary. Inoculate. Allow to stand for 1 day at 5°C. Yield 3 gm. filter cake.

3. Purification of Trypsin by Trichloracetic Acid

When first crystallized trypsin sometimes has a slightly low specific activity due partly to the presence of some inhibitor or trypsinogen. The activity may be raised to the maximum value by repeated recrystallization or, more easily, by precipitation with trichloracetic acid followed by crystallization. The procedure for the latter method is the same as that described under Section I, 5, for the preparation of trypsin from inhibitor-trypsin compound except that the starting material is trypsin instead of inhibitor-trypsin compound.

4. Crystallization of Inhibitor-Trypsin Compound

Adjust Filtrate E (Section II, 1) to pH 3.0 with 5 N sulfuric acid and saturate with crystals of magnesium sulfate at 25°C. Filter with suction through hardened paper. Reject filtrate. Dissolve precipitate (10 gm.) in 50 ml. M/16 hydrochloric acid and pour solution with stirring into a large beaker containing 250 ml. M/16 hydrochloric acid at 90°C. Cool after 1 minute in running cold water to 25°C.[1] Dissolve 24.2 gm. solid ammonium sulfate in each 100 ml. of solution, filter through fluted paper; dissolve 20.5 gm. of solid ammonium sulfate in each 100 ml. of filtrate, refilter with suction. Dissolve filter cake (3 gm.) in 12 ml. water, cool in ice water, add about 3 ml. 0.4 M borate pH 9.0 in order to bring the solution to pH 8.0, and pour with stirring into a large beaker containing 75 ml. boiling

[1] The heating and cooling of large quantities of solution may be most conveniently done by running the solution through a glass coil immersed in boiling water and through a coil in cold water. The rate of flow is regulated so that the solution leaves the hot coil at a temperature of 80–85°C. The coil used in these experiments was of 5 mm. (inside diameter) thin-walled tubing, about 2 m. long.

distilled water. Heavy precipitate forms. Cool after 1 minute in running cold water to 25°C. Dissolve 24.2 gm. of solid ammonium sulfate in each 100 ml. of suspension and filter with suction through hardened paper. Reject precipitate. Adjust filtrate to pH 3.0 by addition of several drops of 5 N sulfuric acid and then dissolve 20.5 gm. of solid ammonium sulfate in each 100 ml. of solution. Filter with suction on a large funnel. Reject filtrate. Wash precipitate with saturated magnesium sulfate.

Dissolve precipitate (1 gm.) in 5 ml. M/10 acetate buffer pH 5.5. Adjust to pH 5.5 with about 1 ml. 0.4 M borate pH 9.0. Filter through Whatman's No. 42 paper into a flask containing enough crystals of magnesium sulfate to saturate the solution. Wash filter paper with 4 ml. M/10 acetate buffer pH 5.5. Stir the solution after completion of filtration. Hexagonal crystals of inhibitor-trypsin compound rapidly appear. Allow to stand for 1 day at 20°C. to complete crystallization. Yield about 0.25 gm. filter cake. For recrystallization see Section I, 4.

III. Isolation of Trypsinogen from Fresh Beef Pancreas

Preliminary purification and concentration the same as described for preparation of Precipitate A (Section I, 1).

Wash Precipitate A with saturated magnesium sulfate on the filter paper. Dissolve precipitate (80 gm.) in 80 ml. 0.4 M borate buffer pH 9.0 at 5°C., add 136 ml. saturated magnesium sulfate. Adjust to pH 8.0 with a few drops of saturated potassium bicarbonate or 5 N sulfuric acid, if necessary. Inoculate with trypsinogen crystals. Allow to stand 2–3 days at 5°C. Filter with suction and wash crystals of trypsinogen with magnesium sulfate as described in Section I, 3. Yield about 10 gm. filter cake. Adjust filtrate to pH 3.0, saturate with magnesium sulfate at 25°C. filter with suction, wash with saturated ammonium sulfate, and proceed for isolation of chymo-trypsinogen (partly active), trypsin, and inhibitor-trypsin compound as described in Section I, 2, 3, up to preparation of Solution B and then in Section II.

IV. Conversion of Trypsinogen into Active Trypsin and Crystallization of Trypsin (McDonald and Kunitz 1946)

The yields of crystalline trypsin prepared by the original method (Kunitz and Northrop 1936) are generally far below the values to be expected if all of the trypsinogen were changed into trypsin. The specific activity of the enzyme also tends to be low. This is due to a second reaction (Kunitz 1939b) which occurs simultaneously with the transformation of trypsinogen into trypsin—part of the trypsinogen is transformed into an "inert protein" which cannot be changed into trypsin by any known means. The formation of "inert protein" can be completely prevented (McDonald and Kunitz 1941), however, by allowing the autocatalytic conversion of trypsinogen to trypsin to proceed in the presence of calcium ions. Under these conditions, the trypsinogen is converted quantitatively into trypsin which can then be

easily crystallized. Both the yield and quality of the crystalline trypsin are greatly improved.

The new method for the preparation of crystalline trypsin is as follows:—

1. Conversion of trypsinogen into trypsin in the presence of calcium chloride.—Dissolve 30 gm. dried crystalline trypsinogen[1] (or 50 gm. semidry filter cake) in 200 ml. 0.005 M hydrochloric acid. Add the solution to a previously prepared ice-cold mixture of 100 ml. 1 M calcium chloride, 250 ml. 0.4 M borate buffer,[2] pH 8.0, and 400 ml. distilled water. Adjust the volume of the mixture with water to 1000 ml., and leave in cold room at about 5°C. for 18 to 24 hours.

2. Removal of calcium ions.—Add 2 gm. Standard Super-Cel (Johns-Manville Corporation) and filter in cold room with suction through soft paper (Eaton-Dikeman No. 303). Reject filter cake. Adjust filtrate to pH 3.0 (tested with methyl orange on spot plate) with 5 N sulfuric acid (about 4 ml.). Add solid ammonium sulfate (242 gm. per 1000 ml.) to 0.4 saturation and leave in cold room for 2 days. A heavy sediment of calcium sulfate crystals is formed. Filter with suction through soft paper (Whatman No. 3). Reject precipitate.

3. Precipitation of amorphous trypsin.—Add solid ammonium sulfate to filtrate to 0.7 saturation (202 gm. per 1000 ml.) and filter with suction through hardened paper (Schleicher & Schüll No. 575). Reject filtrate. Dissolve filter cake (about 50 gm.) in 150 ml. distilled water and reprecipitate the trypsin by the *slow* addition, while stirring, of 350 ml. saturated ammonium sulfate from a dropping funnel. Filter with suction through hardened paper (S. & S. No. 575), size 18.5 cm. or larger, and wash filter cake, as described by Kunitz and Northrop (1936), with saturated magnesium sulfate in 0.02 N sulfuric acid to remove excess ammonium sulfate. Yield about 50 gm.

4. Crystallization of trypsin.—Dissolve semidry filter cake in ice-cold 0.4 M borate buffer, pH 9.0 (10 ml. per 10 gm. filter cake), at 2–5°C. (in an ice water bath). Crystals of fine needles appear almost immediately. Leave in cold room for 24 hours, then filter (cold) with suction on hardened paper (S. & S. No. 575). Wash the crystals several times with cold 0.5 saturated magnesium sulfate in 0.1 M borate buffer, pH 8.0, and then, at room temperature, with saturated magnesium sulfate in 0.1 N sulfuric acid. Yield 15 to 20 gm.

5. Recrystallization of trypsin.—Dissolve the semidry filter cake in 0.02 N sulfuric acid (10 ml. per 10 gm. filter cake), mixing the filter cake gradually into the acid to avoid foam. Filter clear on small fluted No. 3 Whatman paper. Wash paper with several milliliters of 0.02 N sulfuric acid. Cool filtrate to about 5°C. and then adjust to pH 8.0 (tested with cresol red on spot plate) with cold 0.4 M borate buffer, pH 9.0 (about 8 ml. per 15 ml.

[1] The crystalline trypsinogen prepared by the method of Kunitz and Northrop generally contains about 50 percent anhydrous magnesium sulfate.

[2] See footnote on page 264.

solution). Crystallization begins almost immediately. Leave in cold room for 24 hours, then filter and wash as described for the first crystallization. Yield 10 to 15 gm. Dry filter cake for several days in a dry refrigerator, grind to fine powder, and store in cold room.

V. Isolation of Crystalline Trypsin from Active Pancreatic Extracts

The starting point for this method may be either frozen pancreas which has been stored for some time until it is active or fresh pancreas which has been allowed to stand in the cold for several days until the extracts are active. The active pancreas is minced and extracted with two volumes of 0.25 N hydrochloric or sulfuric acid for 18–24 hours at 5°C. The acid extract is filtered at 5°C. through fluted paper. Reject residue. Dissolve 242 gm. of ammonium sulfate in each liter of filtrate and refilter through fluted paper at 5°C. Reject precipitate. Dissolve 205 gm. of ammonium sulfate in each liter of filtrate. Filter with suction. Reject filtrate. Dissolve each 100 gm. of precipitate in 2500 ml. 0.5 M hydrochloric acid, heat rapidly[1] to 90°C. and cool at once to 20°C. Dissolve 242 gm. of ammonium sulfate in each liter of solution, filter through fluted paper. Dissolve 205 gm. of ammonium sulfate in each liter of filtrate and filter with suction on large funnel. Wash precipitate with saturated magnesium sulfate in 0.02 M sulfuric acid.

Dissolve 30 gm. of precipitate in 30 ml. 0.4 M borate buffer pH 9.0 at 2–5°C. and adjust pH to 8.0 with a few ml. of same borate solution. Add an equal volume of saturated magnesium sulfate. This solution corresponds to Solution B (II, 1) and is then treated as described under II, 1, for the crystallization of trypsin from Solution B.

VI. Method for Isolation of Beta and Gamma Chymo-Trypsin Crystals (Kunitz 1938a)

As a starting material, either chymo-trypsin or the mother liquor from chymo-trypsin crystallizations can be used. In the latter case the protein is first salted out in 0.7 saturated ammonium sulfate, and the precipitate is then used in the following operations in the same manner as the crystal cake of chymo-trypsin.

Isolation of gamma crystals.—Suspend 100 gm. of crystal cake of chymo-trypsin in 100 ml. water. Add 50 ml. 0.5 M phosphate buffer pH 8.0 and store the clear solution for 3 weeks at about 5°C. Then add 120 ml. saturated ammonium sulfate, adjust the pH to about 5.6 by means of 5 N sulfuric acid, added drop by drop, and allow the mixture to stand at 20°C. for crystallization of gamma chymo-trypsin. Filter after 3 days with suction. The filtrate (first gamma mother liquor) is stored at 5°C. The yield is about 30 gm. gamma filter cake.

[1] See footnote on page 267.

Recrystallization of gamma.—Recrystallize the gamma crystals by dissolving 10 gm. in 30 ml. water and adding 20 ml. saturated ammonium sulfate. Filter after 24 hours with suction. Residue: second crystals of gamma; stored at 5°C.

Isolation of crude beta crystals.—Combine filtrate with the first gamma mother liquor, adjust pH to 4.2 with 5 N sulfuric acid, and salt out the protein by adding 21 gm. solid ammonium sulfate to each 100 ml. of solution. Filter with suction. Dissolve 10 gm. of amorphous precipitate in 7.5 ml. N/100 sulfuric acid and allow solution to stand for several days at 20-25°. until a heavy precipitate of fine needle crystals of crude beta is formed. The solution frequently turns completely into a thick fibrous gel of crystals. Filter with suction. The filtrate on standing may yield another crop of needle crystals. The total yield is about 50 gm. crude beta filter cake per 100 gm. of original chymo-trypsin filter cake.

Recrystallization of crude beta crystals.—Dissolve 10 gm. of crystal cake in 30 ml. of water and add 30 ml. saturated ammonium sulfate. Adjust pH to 5.6 by means of a few drops of 5 N sodium hydroxide and after inoculation with gamma crystals allow the solution to stand for several days at 20°C. Filter off any gamma crystals formed and adjust the pH of the filtrate to 4.2. Crude beta crystals gradually appear. Filter after several days and repeat crystallization of the crude beta in the same manner.

Isolation of pure beta crystals.—Dissolve 10 gm. of three times recrystallized crude beta crystal cake in 250 ml. of water, add 10 ml. 0.4 M borate buffer pH 9.0. Heat solution to 37°C. and then let it stand at this temperature for 1 hour Cool solution to 20°C. and adjust pH to 4.2 by means of 5 N sulfuric acid. Add with stirring 65 gm. of ammonium sulfate and if a precipitate is formed add 5 gm. Standard Super-Cel and filter with suction through 9 cm. No. 3 filter paper. Dissolve 21 gm. ammonium sulfate in each 100 ml. of the clear filtrate and filter the formed amorphous precipitate of protein with suction on hardened paper. Reject filtrate. Dissolve 1 gm. of precipitate in 3 ml. water and add 2 ml. saturated ammonium sulfate. Adjust solution to pH 4.2 and let it stand at 20°C. An amorphous precipitate forms which gradually changes into very fine crystals. Filter after several days with suction; the yield is about 2 gm. of pure beta crystal cake.

The procedure for recrystallization of the "pure" beta crystals is the same as for the recrystallization of the crude beta.

Crystalline Soybean Trypsin Inhibitor
(Kunitz 1946)

1. Washing with 80 percent alcohol.—1000 gm. cold-processed, defatted soybean meal[1] is added to a mixture of 2400 ml. 95 percent alcohol cooled

[1] Soybean meal, Nutrisoy XXX, in the form of flakes, supplied by the Archer-Daniels-Midland Co., Chicago, Illinois, was used throughout this work. According to the manufacturer's statement, "the flakes were processed by the solvent extraction method of manufacture and no heat was used."

to 5°C. and of 450 ml. distilled water. The suspension is stirred well and left at 20–25°C. for 30 minutes. It is then filtered with suction on a 32 cm. Buchner funnel through filter cloth.[1] The filtrate is rejected.

2. Extraction in 0.25 N *sulfuric acid.*—The semidry meal is resuspended in 5000 ml. 0.25 N sulfuric acid (7 ml. concentrated H_2SO_4 per liter of water) at 20–25°C. and left for 1 hour at room temperature with occasional stirring. The suspension is refiltered with suction on the same filter cloth. The meal residue is rejected.

3. Removal of inert protein by means of Bentonite.—20 gm. of a stock mixture of one part by weight of Bentonite (U.S.P. Powder, Amend Drug and Chemical Co., New York) and an equal part of Hyflo Super-Cel (Johns-Manville Corporation) is added to the acid filtrate and stirred for 10 minutes. The suspension is filtered with suction on 32 cm. No. 303 E. & D. filter paper (The Eaton-Dikeman Co., Mt. Holly Springs, Pennsylvania). The residue on the paper is washed twice with portions of 125 ml. of water. The residue is rejected.

4. Adsorption of the inhibitor on Bentonite.—100 gm. of the stock of Bentonite-Super-Cel mixture is added gradually to the combined filtrate and washings from step No. 3. The solution is stirred gently while the Bentonite mixture is added and the stirring is continued for 10 minutes. The suspension is filtered and the residue is washed, as described in step No. 3. The filtrate and washings are rejected.

5. Elution with pyridine and dialysis.—The Bentonite residue is stirred up with 270 ml. of water. At this stage the suspension can be stored in the refrigerator overnight. The suspension is warmed to 25°C. and 30 ml. of pyridine (Stock No. 214, Eastman Kodak Co.) is added with stirring. The thick suspension is filtered with suction on 24 cm. No. 303 E. & D. filter paper in a hood. The filtration generally requires several hours. The residue on the funnel is washed once with 200 ml. 5 percent pyridine in water. The combined filtrate and washing is dialyzed overnight in 12 inch long cellophane tubings[2] placed in a tall jar with running tap water, in the hood, if possible.

6. Removal of inert material at pH 5.3.—The dialyzed solution, free of any gummy residue adhering to the dialysis tubes, is adjusted to pH 5.3 with the aid of about 2 ml. of 1 N hydrochloric acid. (The pH is tested by the drop method on a plate using 0.1 M acetate buffers as standards and 0.01 percent methyl red as an indicator.) 4 gm. of Bentonite-Super-Cel mixture is stirred into the solution which is then filtered with suction on a 15 cm. No. 303 E. & D. filter paper and the residue is washed several times with 15 ml. portions of water. The washings, if not clear, are refiltered. The residue is rejected.

[1] Filter cloth, 146 T W, supplied by the Filter Media Corporation, Irvington-on-Hudson, New York.

[2] 27/32 "Nu Jax Visking Cellulose Sausage Casing," manufactured by the Visking Corporation, Chicago, Illinois.

7. First precipitation of the inhibitor at pH 4.65.—The combined filtrate and washings of step No. 6 is cooled to 5°C. and then titrated with 1 N hydrochloric acid to pH 4.65 (tested carefully with 0.05 percent bromcresol green on a drop plate). A heavy precipitate is formed which is filtered off at 5–8°C. on 15 cm. No. 303 E. & D. filter paper on a Buchner funnel without suction. The filtration is completed with very light suction. Weight of filter cake 10 to 12 gm. The filtrate is rejected.

8. Second precipitation at pH 4.65.—The filter cake is suspended in 100 ml. of water cooled to 5°C., the water being added gradually to the precipitate and incorporated thoroughly with a porcelain spatula. 1 N sodium hydroxide is added drop by drop with stirring until the precipitate is dissolved. Care should be taken, however, not to raise the pH of the solution above 6.4. The clear solution is warmed to 25°C. and titrated slowly with 1 N hydrochloric acid until a slight permanent precipitate is formed. Two gm. of Standard Super-Cel is stirred into the solution which is then filtered with suction on 7 to 11 cm. No. 303 E. & D. filter paper. The residue on the paper is washed with several milliliters of water. The combined filtrate and washing is cooled to 5°C. and titrated to pH 4.65. It is filtered with light suction on 15 to 18 cm. No. 612 E. & D. filter paper at 5–8°C. Yield about 8 to 10 gm. of filter cake which is stored in refrigerator. The filtrate is rejected.

9. Crystallization.[1]—The filter cake of step No. 8 (about 10 gm.) is ground up to a uniform suspension with 10 ml. of cold water and then warmed to about 35°C. 0.5 N sodium hydroxide is added drop by drop with careful stirring until the precipitate is almost completely dissolved and the pH of the solution is about 5.2. The clear solution is decanted into a 50 ml. centrifuge tube. Any residue in the beaker is stirred up with 1 to 2 ml. of cold water, dissolved with the aid of a drop of 0.1 N sodium hydroxide, and added to the main bulk of solution in the centrifuge tube which is then placed at 35–37°C. for crystallization. A heavy sediment of crystals is obtained within 5 to 6 hours. Inoculation with a few crystals greatly facilitates the process of crystallization. The suspension is centrifuged for 10 minutes at about 3000 R.P.M. The residue is stored in the refrigerator, while the supernatant liquid is either stored or, if time permits, titrated with a few drops of 0.2 N hydrochloric acid to pH 5.1 at 36–37°C., inoculated, and left at that temperature. Another crop of crystals is gradually formed which is centrifuged off after several hours and added to the first crop of crystals. The supernatant liquid is rejected.

It is preferable to begin step No. 9 in the morning, so as to be able to centrifuge before the end of the day. The crystals, as well as supernatant solutions, should be stored overnight in the refrigerator.

10. Recrystallization.—The combined crystal residues of step No. 9 (about 7 ml.) are stirred up with twice the volume of cold water and titrated with

[1] It is advisable to begin the crystallization (Step No. 9) with at least 50 gm. of amorphous precipitate collected from several preparations.

0.5 N sodium hydroxide to clearing, the final pH being about 6.0. The clear solution is warmed to 35°C. and titrated with 0.5 N hydrochloric acid to pH 5.1 when a slight permanent precipitate is formed. The solution is mixed with 2 gm. Standard Super-Cel and filtered clear with suction on a small No. 303 E. & D. filter paper. The filtrate is inoculated and left at 36–37°C. A heavy suspension of crystals forms gradually and is centrifuged after 5 to 6 hours. The residue is stored at 5°C. The pH of the supernatant liquid is readjusted with 1 to 2 drops of 0.2 N hydrochloric acid to pH 5.1, inoculated, and left for several hours longer at 36–37°C. when another crop of crystals is formed which is centrifuged off and added to the first crop. The final supernatant solution may yield still more crystals by cooling it to 5°C. and then adding one quarter of its volume of cold 95 percent alcohol as described in the following section. Repeat crystallization three times.

11. Crystallization in dilute alcohol.—Centrifuged crystals are stirred up with five times the volume of cold water, and 0.5 N sodium hydroxide is added drop by drop until the crystals are all dissolved. The pH of the solution is not allowed, however, to rise above 6.6. The clear solution is titrated with 0.2 N hydrochloric acid to pH 5.2. Any precipitate formed is filtered off with suction on No. 303 filter paper with the aid of 4 gm. of Standard Super-Cel per 100 ml of solution. The residue on the funnel is washed with several milliliters of water. The volume of the filtrate and washings is measured and the solution is then cooled in an ice-water bath to about 5°C. A quarter of its volume of 95 percent alcohol, cooled to 5°C., is added slowly to the cold solution. A heavy precipitate is formed. The pH of the mixture is adjusted with 0.2 N hydrochloric acid to 5.0 and left at 30°C. The amorphous precipitate changes within 2 hours into well formed hexagonal and rhomboid crystals and plates (Fig. 63) which settle rapidly to the bottom of the vessel. The supernatant solution is decanted every hour, adjusted with 0.2 N, or more dilute hydrochloric acid to pH 5.0, and returned to the original vessel containing the settled crystals. This is continued for several hours until no formation of precipitate is noticed on adjusting the pH of the supernatant solution to 5.0. The crystallization mixture is then allowed to stand at 30°C. for 30 minutes longer and then filtered with suction on hardened paper, washed on the funnel several times with cold acetone, and allowed to dry in the room for 24 hours. It is stored in the refrigerator.

12. Recrystallization in alcohol.—The dry crystals are suspended in thirty times their weight of cold water, allowed to soak for 5 to 10 minutes, and then treated exactly as in step No. 11.

Yield

The yield of trypsin inhibitor crystals varies considerably with the stock of soybean meal used. Nutrisoy XXX generally yields about 1 gm. of four times crystallized inhibitor per 1000 gm. of meal.

Method of Crystallization of Compound of Trypsin and of Soybean Trypsin Inhibitor (Kunitz 1947b)

The compound crystallizes readily at pH 6 to 8 from a salt-free solution of a mixture of trypsin and soybean inhibitor. The crystallization is accelerated in the presence of about 20 percent alcohol.

The details of isolation of the crystals of the new protein are as follows:

1. Preliminary step.—One gm. of dry soy inhibitor crystals is suspended in 40 ml. of distilled water at about 5°C. The mixture is titrated with 0.2 M sodium hydroxide to about pH 7.5. This brings about complete solution of the inhibitor crystals. 1 gm. of a preparation of dry crystalline trypsin containing about 50 percent anhydrous magnesium sulfate is then added slowly with stirring. The mixture is thus made to contain an excess of inhibitor in order to avoid any proteolysis by trypsin.

The pH, if necessary, is readjusted to 7.5 with several drops of 0.1 M borate buffer pH 9.0. The solution, if turbid, is filtered with suction with the aid of 1 gm. Super-Cel on a small Buchner funnel. The residue on the funnel is washed with about 5 ml. of cold water. The washings if clear are added to the main bulk of the filtrate which is titrated with 0.1 M hydrochloric acid to about pH 6.0 and dialyzed overnight against slowly running distilled water at 5–10°C., preferably with stirring.

A granular precipitate gradually forms in the dialysis bag.

The dialyzed suspension is titrated with a few drops of 0.1 M hydrochloric acid to pH about 5.4 (tested on a drop plate with 0.01 percent solution of methyl red). The suspension is centrifuged. The residue yields the crystalline compound; the supernatant solution (designated as "first supernatant solution") contains the excess of soy inhibitor used which can be partly recovered.

2. Crystallization of the compound.—The residue is suspended in about 20 ml. cold water and recentrifuged. The washed residue is resuspended in 40 ml. of water at about 5°C. and titrated drop by drop with 0.2 or 0.5 M sodium hydroxide to pH 9.0 (pink to 0.1 percent phenolphthalein on test plate), when complete solution generally occurs. The solution is then titrated with a few drops of 0.2 M hydrochloric acid to very slight opalescence and stored at about 20°C. Fine crystals in the form of small rosettes or bundles of needles and plates gradually appear (Figure 74). The suspension of crystals is centrifuged after a day or so; several drops of 0.1 M hydrochloric acid are added to the supernatant solution until a slight turbidity is formed. The solution is stored for several hours at 20°C. A crop of fine crystals generally appears which is centrifuged on the top of the first crop of crystals. More acid is added to the supernatant solution and the process is repeated until pH 5.8 is reached, or until the final acidified supernatant solution no longer yields crystals. It is rejected or is combined with the "first supernatant solution" to be worked up for soy inhibitor, as described in step 7.

3. Recrystallization of the compound.—The crystals are suspended in about 20 volumes of cold water and titrated with several drops of 0.5 M sodium hydroxide to incipient clearing. The solution is allowed to stand for 5–10 minutes at 5°C. and then filtered, if turbid, on fluted No. 3 paper moistened with cold water, pH 9.0. The filter paper is washed once with cold water. The clear filtrate and washing is titrated with several drops of 0.2 M hydrochloric acid to very slight opalescence. It is seeded and left at 20°C. Crystallization is generally complete within 24 hours. The crystals are centrifuged. The supernatant solution is titrated with 0.2 M hydrochloric acid to slight turbidity and left at 20°C. for several hours. A second crop of crystals is obtained which is centrifuged in the flask containing the first crop of crystals. The operation is repeated several times until no further yield of crystals is obtainable. The supernatant solution is treated as described in step 5.

4. Drying of crystals.—The combined crystals are resuspended in a small amount of distilled water and filtered with suction on hardened paper. The crystals are then dried for 24 hours in a mechanical refrigerator at about 5°C. and then in a desiccator over anhydrous calcium sulfate (Drierite) at 20°C. The dried material is ground fine in a mortar and stored in a refrigerator.

5. Crystallization in dilute alcohol.—The final supernatant solution in step 3 may yield further crystals if it is cooled to 5°C., ¼ of its volume of cold 95 percent alcohol is added, and the pH of the solution is adjusted with 0.2 M hydrochloric acid to 5.8. A precipitate forms which, when left at 20°C. changes gradually into rosettes of fine plates (Fig. 75). The crystals are filtered on hardened paper and dried first in a refrigerator and then in a desiccator over anhydrous calcium sulfate (Drierite).

6. Recrystallization in dilute alcohol.—The dry crystalline powder is suspended in about 50 times its weight of water. (The centrifuged residue of crystals, not dried, is suspended in 25 times its volume of water.) The suspension is titrated with several drops of 0.5 M sodium hydroxide to pH 9.0. The crystals gradually dissolve when left for about 10 minutes at 5°C. The solution is filtered, if turbid, and then ¼ of its volume of cold 95 percent alcohol is added. The pH of the solution is adjusted to about 5.8. A heavy precipitate is formed which changes into crystals on storing for a day or two at 20°C.

The crystallization in alcohol is more rapid and the yield of crystals is greater than in the absence of alcohol. There is also the advantage that the alcohol keeps the solution sterile. There is, however, the possibility that alcohol causes slight denaturation of the protein.

7. Partial recovery of excess of inhibitor.—The "first supernatant solution," of step 1, is titrated with 0.5 M hydrochloric acid to pH 4.65 at 5°C. and centrifuged at about the same temperature. The supernatant solution is rejected. The residue is suspended in about 5 volumes of cold water and is titrated with 0.2 M sodium hydroxide to pH 5.2. Any precipitate left

undissolved is centrifuged off and is rejected (or worked up for compound, as described in step 2).

The supernatant solution is cooled to 5°C. and ¼ of its volume of 95 percent alcohol of 5°C. is added. The solution is adjusted with several drops of 0.1 M hydrochloric acid to pH 5.0, is seeded with soy inhibitor crystals, and left at 30°C. Crystals of inhibitor gradually form. The crystals are filtered after several hours, washed with cold acetone and dried in room. Yield 0.1 to 0.2 gm. of dry soy inhibitor crystals.

Preparation of Carboxypeptidase (Anson 1937a)

1. Preparation from Pancreatic Fluid

The starting material.—Commercial frozen pancreas, which may be obtained from the packing companies, is sliced as thin as possible and spread on racks laid on trays in a cold room at about 5°C. As the tissue thaws a turbid fluid drains into the trays and is collected. If commercial frozen pancreas is used this fluid usually amounts to about 10 percent of the weight of pancreas taken. If freshly frozen pancreas is used the yield is much less. The fluid usually contains about 0.7 $[CP.U.]^{PDE}_{ml.}$.

The first globulin precipitate.—Five N acetic acid is added, with stirring, to the crude fluid until the solution is definitely green to bromcresol green. The acidified solution in four 1-liter Erlenmeyer flasks is left in a 37°C. bath for 2 hours and then filtered. The filtrate contains all the carboxypeptidase. Ten volumes of tap water are added to each volume of filtrate in large glass vessels. A precipitate forms which settles. At the end of the day the supernatant fluids are siphoned off and rejected and the precipitates are collected in a single vessel. The next morning the supernatant is again siphoned off and rejected and the precipitate is filtered on Schleicher and Schüll folded filter paper No. 588, 50 cm. diameter. The smaller and also thinner paper of the same number is less satisfactory. Finally the precipitate is evenly suspended in enough water to give a volume roughly one fifth of the original volume of pancreatic fluid. A sample is dissolved with a little di-potassium phosphate and sodium chloride and its activity is measured. From 35 to 90 percent of the original activity is recovered in the first globulin precipitate.

Barium hydroxide extraction.—The suspension of the first globulin precipitate is diluted to have roughly 0.25 $[CP.U.]^{PDE}_{ml.}$ and 0.2 M barium hydroxide is added, with vigorous stirring, until the solution is definitely pink to phenolphthalein and the suspension is immediately filtered with Standard Super-Cel[1] on a Buchner funnel or centrifuged. Barium hydroxide dissolves only a part of the globulin precipitate whereas sodium hydroxide at the same pH dissolves it completely. If not enough barium hydroxide has been added the supernatant solution obtained by centrifugation is turbid and does not contain all the carboxypeptidase. If too

[1] See footnote on page 254.

much barium hydroxide is added the carboxypeptidase activity is slowly destroyed. As a precaution, when a large batch of material is being worked up, the barium hydroxide extraction is first carried out on a small scale, the amount of barium hydroxide added is recorded, and the activity of the solution is measured after removal of the undissolved protein. The solution should be clear and its activity should be the same as that of a complete solution of the globulin precipitate obtained by adding di-potassium phosphate and sodium chloride instead of barium hydroxide.

Crystallization.—Immediately after centrifugation, 1 N acetic acid is added to the barium hydroxide solution, with vigorous stirring, until the solution becomes just turbid (orange to phenol red). Crystals appear slowly if the solution is simply allowed to stand. In practice the solution is heavily seeded, allowed to stand the rest of the day at room temperature with occasional stirring, and finally left in the refrigerator overnight. The next morning the supernatant solution is siphoned off and rejected and the crystals which have settled to the bottom of the vessel are centrifuged. The supernatant solution is rejected, and the crystals are again suspended in water and again centrifuged. Forty to 80 percent of the carboxypeptidase of the barium hydroxide extract is obtained in crystalline form.

At the pH used for crystallization carboxypeptidase is stable, its crystalline form is insoluble, and its amorphous form is soluble. At a more acid pH (green to bromcresol green) both the crystalline and amorphous forms are insoluble and so, since precipitation of the amorphous form is rapid compared with crystallization, carboxypeptidase is immediately and completely precipitated in the amorphous form. If the amorphous precipitate is dissolved at the acid pH with a solution of sodium chloride, the carboxypeptidase slowly coagulates and is inactivated. It is clear that carboxypeptidase is insoluble over a wide range of pH and that the proper pH for crystallization is far on the alkaline side of the pH of minimum solubility.

Recrystallization.—To a suspension of crystals having about 1.0 $[\text{CP. U.}]^{\text{PDE}}_{\text{ml.}}$ (the exact concentration is not important) 0.1 N sodium hydroxide is added slowly, with vigorous stirring, until almost all the material is dissolved. The undissolved material is removed immediately by centrifugation and 1 N acetic acid is immediately added to the supernatant solution until the first turbidity appears. The solution is then seeded and allowed to stand several hours and the crystals centrifuged. The total activity of the recrystallized protein is 80 to 100 percent of the activity of the protein used for recrystallization. The crystals are stored in the cold with toluol as a preservative and appear to be stable indefinitely in aqueous suspension. If pure carboxypeptidase is dried, more or less of the protein is converted into an insoluble, inactive form, even if the protein is dried while frozen or if the water is removed with sodium sulfate.[1] Impure carboxy-

[1] When an alkaline solution of carboxypeptidase crystals is frozen and dried soluble active carboxypeptidase is obtained.

peptidase, however, can be dried with sodium sulfate without any loss of solubility or activity. This protective action of impurities has been noted with pepsin and many other proteins.

It is important, when adding sodium hydroxide, not to make the solution too alkaline. The crystals sometimes dissolve very slowly and the process of solution should not be hurried by adding sodium hydroxide in excess. After the carboxypeptidase has been once recrystallized it usually dissolves completely when the solution is definitely alkaline to phenolphthalein. This pH is entirely safe. It is not important that the crystals be completely dissolved. Undissolved crystals can be saved and added to the supply of once crystallized material.

2. Preparation from Frozen Pancreas

Frozen beef pancreas is ground and stirred up with 3 times its weight of 2 percent sodium chloride and 20 percent of its weight of toluol (Eastman practical), and allowed to stand at room temperature. The next morning the fat and toluol are skimmed off and the suspension is filtered through gauze; 5 N acetic acid is added to the filtrate until it is green to bromcresol green. The acid solution is filtered on a Buchner funnel with the aid of Standard Super-Cel (Johns-Manville) and 390 gm. ammonium sulfate are added to each liter of filtrate. The precipitate formed is filtered off and dialyzed overnight against cold water in a shaking dialyzer (Kunitz and Simms 1928). The dialyzed solution is centrifuged, the supernatant is discarded, and the precipitate is then treated like the precipitate obtained from the fluid which exudes when frozen pancreas is thawed.

Recrystallization and fractional crystallization.—A sample of twice-crystallized carboxypeptidase had a specific activity of 0.082 $[CP.U.]^{Ct}_{mg.N}$ of six times crystallized, 0.083 $[CP.U.]^{Ct}_{mg.N}$. A sample of twice-crystallized carboxypeptidase had a specific activity of 0.095 $[CP.U.]^{PDE}_{mg.N}$. It was recrystallized. The first 25 percent which crystallized out had a specific activity of 0.104 $[CP.U.]^{PDE}_{mg.N}$.

Other enzymes.—Six times crystallized carboxypeptidase contains a trace of proteinase which can be detected by long digestion of hemoglobin with a large amount of carboxypeptidase. This slight activity could be accounted for by an impurity of 1 part trypsin to 30,000 parts carboxypeptidase.

Thrice-crystallized carboxypeptidase is free of dipeptidase and amino polypeptidase. Fruton found that 1 mg. carboxypeptidase per ml. produced no detectable digestion of 0.05 M *dl*-leucyl-glycine or *dl*-leucyl-glycyl-glycine at pH 7.8 in 23 hours at 40°C.

Six times crystallized carboxypeptidase is free of amylase. A 1 percent neutral solution of starch gave almost the original color with iodine after 24 hours at 37°C. despite the presence of 0.7 mg. per ml. of carboxypeptidase.

Method of Isolation of Crystalline Ribonuclease (Kunitz 1940)

The details of the method are as follows:

Preliminary treatment.—Beef pancreas (about 20 pounds) is removed from the animals immediately after slaughter and immersed at once in enough ice-cold 0.25 N sulfuric acid to cover the glands. It can then be stored at 5°C. for a day or so, or worked up immediately. The pancreas is removed from the acid, cleaned of fat and connective tissue, and then minced in a meat chopper. The minced pancreas is suspended in one or two volumes of cold 0.25 N sulfuric acid and is stored at about 5°C. for 18–24 hours. It is then strained through cheese cloth. The strained fluid is brought to 0.6 saturation of ammonium sulfate by dissolving 390 gm. of salt in each liter of strained fluid. The mixture is filtered through 50 cm. fluted filter paper (No. 612 Eaton and Dikeman Co., Mt. Holly Springs, Pa., or No. 1450½ Schleicher and Schüll). The clear filtrate (0.6 F) is used for the preparation of ribonuclease while the residue on the paper (0.6 P) can be used for the isolation of chymo-trypsinogen, trypsinogen, trypsin, and trypsin inhibitor compound.[1]

The clear filtrate (0.6 F) is brought with solid ammonium sulfate to 0.8 saturation (140 gm. per liter of filtrate) and the precipitate formed is allowed to settle for 2 days in the cold room. The settling is greatly facilitated by occasional stirring and removal of foam during the first day of standing. The clear supernatant fluid is siphoned off and rejected, while the remaining suspension is filtered with suction through hardened paper; yield about 30 gm.

Isolation of ribonuclease crystals.—Each 10 gm. of the semidry precipitate is dissolved in 50 ml. distilled water, the pH of the solution is adjusted by means of a few drops of 5 N sodium hydroxide to pH 4.8, and then 50 ml. of saturated ammonium sulfate is added with stirring.[2] The solution is filtered with suction through soft paper with the aid of about 1 gm. of Filter-Cel.[3] The clear filtrate is brought to pH 4.2 (tested with bromcresol green) by means of a few drops of 1 N sulfuric acid and then 14 gm. solid ammonium sulfate is added per 100 ml. of filtrate. The solid ammonium sulfate is added slowly with stirring. The precipitate formed is filtered with suction through hardened paper; yield about 8 gm. Each 10 gm.

[1] The precipitate (0.6 P) is scraped off the filter paper and suspended in about 3 volumes of water. The procedure for further treatment is the same as described by Kunitz and Northrop (1936, page 1002; also chapter on trypsin in the present work) for the treatment of the original acid extract of fresh beef pancreas in the preparation of chymotrypsinogen, etc.

[2] The pH is determined approximately by mixing on a test plate 1 drop of 0.01 percent neutralized methyl red (or any other indicator depending on the pH range) with 1 drop of the solution and the color is compared with the color of 1 drop standard buffer solution mixed with the indicator on the plate.

[3] Supplied by Johns-Manville Corporation, New York.

of final filter cake is dissolved in 10 ml. of water and is refiltered with suction through soft paper on a small Buchner funnel with the aid of 0.5 gm. Filter-Cel. The residue is washed several times with 2–3 ml. water. The combined filtrate and washings are made up with water to a volume of 20 ml. and 7 ml. saturated ammonium sulfate is added with stirring. The clear solution is left at 20–25°C. Crystals of ribonuclease in the form of thin, long plates or fine needles gradually appear (Figure 81). If crystals fail to appear within 24 hours then saturated ammonium sulfate is added to trace of turbidity and the solution is left at 20–25°C. The crystals are filtered after 2 or 3 days; yield 1–2 gm. More saturated ammonium sulfate is added to the filtrate until a slight turbidity is formed. A second crop of crystals appears after several days; yield 2–4 gm.

Alternate method of isolation of ribonuclease crystals.—The following method, described in the preliminary publication (Kunitz 1939a), is more reproducible but it yields fewer crystals. The 0.25 N acid extract of the minced pancreas is brought to 0.7 saturation with solid ammonium sulfate and filtered. The filtrate is then brought to 0.8 saturation with more ammonium sulfate and is refiltered with suction.

Crystallization.—10 gm. of the semidry precipitate is dissolved in about 10 ml. of water. The solution is filtered with the aid of about 0.5 gm. of Filter-Cel through soft filter paper on a small Buchner funnel; the residue on the paper is washed with water. The combined filtrate and washings are brought to a final volume of 20 ml. Saturated ammonium sulfate is then added slowly with stirring until a very faint turbidity appears. The pH of the solution is adjusted first to about pH 5.0 with the aid of a few drops of 1.0 N sodium hydroxide and then to pH 4.2 by means of 1.0 N sulfuric acid. The solution is allowed to stand at about 20°C. An amorphous precipitate rapidly forms. This changes within 1 or 2 days into a mass of fine needles or aggregates of long thin plates. The crystals are filtered after 2 or 3 days. The filtrate on further addition of saturated ammonium sulfate yields more crystals.

Recrystallization.—Each 10 gm. of semidry filter cake of crystals is dissolved in 20 ml. of water. This solution is filtered with suction through soft paper with the aid of 1 gm. of Filter-Cel. The residue is washed with water. The combined filtrate and washings are made up to 30 ml. with water. 10 ml. saturated ammonium sulfate is added. Rapid crystallization takes place at 20–25°C.[1] The crystals are filtered off after 1 or 2 days; yield about 5 gm. The filtrate on further addition of saturated ammonium sulfate gives more crystals; yield about 2 gm.

Recrystallization in alcohol.—Ribonuclease is readily recrystallizable in dilute alcohol. The material has to be quite pure, however, and salt free. The procedure for crystallization from alcohol is as follows: Ribonuclease

[1] If crystals fail to appear after a day of standing then more saturated ammonium sulfate solution is added drop by drop until a very slight turbidity is formed. Inoculation with a few crystals greatly facilitates the process of crystallization.

is first recrystallized twice by means of ammonium sulfate as described in the preceding section. 10 gm. of the crystal cake from the final crystallization is dissolved in 15 ml. of water and is dialyzed in a collodion bag for 24 hours against cold distilled water by the method of Kunitz and Simms (1928). The dialyzed solution is made up with water to 50 ml., is cooled to about 5°C., and then 60 ml. 95 percent alcohol of the same temperature is added with stirring. A heavy amorphous precipitate is formed which on standing at about 20°C. gradually changes into a mass of fine fan-shaped rosettes (Figure 82) of rectangular or needle-shaped crystals. The crystals are filtered with suction after 2 days. They are dried for 24 hours in a mechanical refrigerator at about 5°C. and then in a desiccator over calcium chloride for 24–48 hours. The dry powder can be stored in a cool place indefinitely; yield is about 3 gm. of dry crystals.

Preparation of Crystalline Hexokinase
(Kunitz and McDonald 1946b)

The details of the method of preparation of crystalline hexokinase are as follows:

1. Plasmolysis and extraction.—25 pounds of fresh Fleischmann's baker's yeast is broken by hand into small fragments and then macerated by means of a wooden paddle in a large aluminum or enameled vessel with 6 liters of warm toluene of about 40°C. The vessel is placed in a water bath of about 45°C. The maceration is continued until the yeast is heated to 37°C. at which temperature the yeast rapidly liquefies and begins to "work." A rapid liberation of carbon dioxide takes place and the volume of the mixture increases considerably. It is left in the room for 2 to 3 hours and then cooled to 10°C. in an ice water bath. The thick mixture of plasmolyzed yeast and toluene is distributed in four 10 liter jars, 3 liters of distilled water of about 5°C. is added to each jar and mixed. The jars are left for 18 hours at 5°C. A layer of an emulsion of the toluene with yeast stromata gradually forms above the yeast-water suspension.

The yeast suspension is siphoned off from under the toluene-stromata emulsion and then filtered in the cold room at 8–10°C., with suction on four 32 cm. Buchner funnels with the aid of 100 gm. Hyflo Super-Cel[1] per liter of fluid using Eaton-Dikeman No. 303 paper. The residue on each funnel is washed once with 1 liter of cold water. (The toluene is partly recovered by filtering the toluene-stromata emulsion with the aid of 100 gm. Hyflo Super-Cel per liter.)

2. Fractionation with ammonium sulfate.—The clear filtrate and washings are brought to 0.5 saturation with solid ammonium sulfate (314 gm. per liter of filtrate) and the precipitate formed is filtered with suction with the aid of 10 gm. of Standard Super-Cel, plus 10 gm. Filter-Cel per liter of solution. The residue is discarded. The clear filtrate is brought to 0.65 satu-

[1] Supplied by Johns-Manville Corporation, New York.

ration by further addition of 99.3 gm. of solid ammonium sulfate to each liter of filtrate. The precipitate formed (called 0.6 fraction) is filtered with suction on No. 612 E. & D. paper on large funnels. The filtrate is brought to 0.7 saturation by the addition of 33.8 gm. of solid ammonium sulfate to each liter of filtrate. The precipitate formed (called 0.7 fraction) is filtered with suction after standing for 16 to 18 hours at a temperature not higher than 10°C. Both 0.6 and 0.7 fractions are used for isolation of hexokinase. The purification of the 0.7 fraction will be described first since it involves fewer steps and the yield of crystalline hexokinase obtainable is often greater than in the 0.6 fraction.

3. Removal of "inert" crystalline proteins.—(*a*) Isolation of crystals of "yeast protein, No. 2." Each gram[1] of the 0.7 fraction is dissolved in 2 ml. cold water, the pH adjusted to 7.4 with 1 M sodium hydroxide, then enough saturated ammonium sulfate added with stirring to trace of turbidity. It generally requires about 2.5 ml. ammonium sulfate per gm. filter cake. The solution is kept in an ice bath during this operation and then stored at about 5°C. for 6 to 8 days. A gel of very fine crystals of yeast protein, No. 2, is gradually formed. This is filtered off with the aid of 5 gm. Standard Super-Cel per 100 ml. of solution.[2] The filtrate is brought to 0.85 saturation with solid ammonium sulfate, added slowly with stirring (21.5 gm. per 100 ml. of filtrate). The precipitate formed is filtered with suction. Filter cake = fraction 0.71.

(*b*) Isolation of crystals of "yeast protein, No. 3." Each gram of filter cake of fraction 0.71 is dissolved in 0.5 ml. cold water at 2 – 5°C. Saturated ammonium sulfate is added to trace of turbidity, then 1 M sodium hydroxide to pH 7.5. The solution is left at 5°C. for 5 to 6 days. Prismatic crystals of yeast protein, No. 3 (Figure 86), are gradually formed. The solution is centrifuged at 5–10°C.[3] The supernatant solution is diluted with 2 volumes of cold 0.65 saturated ammonium sulfate pH 7.2 (containing 2 ml. 5 M sodium hydroxide per liter), 10 gm. of Standard Super-Cel and 43 ml. saturated ammonium sulfate are then added for each 100 ml. of original supernatant solution. The ammonium sulfate solution is added slowly with

[1] This expression is used to denote the relative volume of solvent in which the precipitate is dissolved. It does not mean that each gram is dissolved separately.

[2] Recrystallization of yeast protein, No. 2.: The protein is extracted from the Super-Cel with cold water of about 5°C. and is reprecipitated with solid ammonium sulfate at 0.85 saturation. The precipitate is dissolved in about 3 volumes of cold water, 8.0 ml. saturated ammonium sulfate is added for every 10 ml. of water, and the solution is titrated with 0.5 N sodium hydroxide to pH 7.4 and stored for 7 days or longer at about 5°C. Gradual crystallization of long needles takes place (Figure 85).

Yeast protein, No. 2, can also be recrystallized at pH 4.3 in 0.5 saturated ammonium sulfate at 20–25°C. Under these conditions the crystals appear in the form of hexagonal and rhomboid plates.

[3] Recrystallization of yeast protein, No. 3.: The centrifuged residue of crystals is dissolved in about 2 volumes of cold water and 2.5 volumes of saturated ammonium sulfate is added; the clear solution is titrated with 0.5 N sodium hydroxide to pH 7.4 and stored for several days at 5°C. Gradual crystallization of well-formed prisms takes place.

stirring. The precipitate formed is filtered with suction, resuspended twice in a volume of 0.65 saturated ammonium sulfate of pH 7.2, equal to that of the first filtrate, and refiltered each time with suction. Combined filtrates are brought with solid ammonium sulfate to 0.85 saturation (143 gm. per liter) and filtered with suction. Filter cake = fraction 0.72.

(*c*) Isolation of crystals of "yeast yellow protein." Each gram of 0.6 fraction is dissolved in 1 ml. of cold water and 1 ml. saturated ammonium sulfate is added; a slight precipitate generally forms. The turbid solution is stored for 20 to 24 hours at 5°C. It is then centrifuged and the residue is discarded. The filtrate is titrated with 0.5 N sodium hydroxide to pH 7.2 and stored at 5°C. for 5 to 7 days. The gelatinous precipitate formed is removed by filtration with the aid of 10 gm. Hyflo Super-Cel per 100 ml. of solution. (The residue may yield No. 2 crystals when treated as described in footnote 2 on page 283). The clear filtrate is brought to 0.85 saturation by the slow addition of 233 ml. saturated ammonium sulfate per 100 ml. filtrate, and filtered with suction. Yield 200 to 300 gm. filter cake. This is dissolved in one half volume of water, saturated ammonium sulfate is added to trace of turbidity. The solution is then titrated with 1 N sodium hydroxide to pH 7.5, and stored at 6°C. Crystals of a yellow protein (Figure 87) gradually form. These are centrifuged after 1 to 2 weeks.[1]

The supernatant solution is diluted with 2 volumes of cold 0.65 saturated ammonium sulfate of pH 7.2. 10 gm. of Standard Super-Cel and 43 ml. saturated ammonium sulfate are added for each 100 ml. of the original supernatant solution. The suspension is filtered with suction on No. 3 paper. The precipitate is resuspended twice in a volume of 0.65 saturated ammonium sulfate of pH 7.2, equal to that of the first filtrate, and refiltered each time with suction. Combined filtrates are brought with solid ammonium sulfate to 0.85 saturation (143 gm. per liter) and filtered with suction. Filter cake about 50 gm.—fraction 0.62—is treated exactly as described in section *3b* to yield fraction 0.63. A certain amount of No. 3 crystals are generally formed during this step.[2]

4. Dialysis in dextrose solution.—Each gram of filter cake of fraction 0.72, 0.73, or 0.63, as the case may be, is dissolved in 1 ml. of 1 percent dextrose. The solution is transferred into a collodion bag provided with a large glass bead and is dialyzed in a rocking machine for 18 hours at about 5°C. against slowly running 1 percent dextrose.

5. Fractional precipitation with alcohol.—(*a*) The dialyzed solution is

[1] Recrystallization of the yeast yellow protein: The centrifuged residue of crystals is dissolved in an equal volume of cold water and one half volume of saturated ammonium sulfate is gradually added. The solution is titrated with 0.5 N sodium hydroxide to pH 7.5 and stored at 5°C. for several days. Rhombohedral crystals of the yellow protein gradually form.

[2] If the activity of fraction 0.72 is less than 50 hexokinase units per mg. protein, then step *3b* should be repeated. The final precipitate, fraction 0.72, 0.73, or 0.63, as the case may be, is used for dialysis and alcohol fractionation as described in steps 4 and 5.

diluted with cold 1 percent dextrose to a volume equal in milliliters to 4.75 times the original weight in grams of the filter cake in step 4, and 1 ml. 1 M acetate buffer pH 5.4 is added per 19 ml. of solution. The solution is cooled in a freezing-mixture bath to about −2°C. and 35.7 ml. cold 95 percent alcohol added gradually with stirring to each 100 ml. of solution to a concentration of 25 percent. The precipitate formed is centrifuged off at about 5°C. The supernatant fluid is measured, cooled again to −2°C., and more 95 percent alcohol added to a final concentration of 50 percent (55.5 ml. per 100 ml. supernatant). The suspension is centrifuged, and the supernatant discarded. The residue is resuspended in a volume of cold 1 percent dextrose equal to 2 times the weight of the filter cake in step 4 and recentrifuged. The clear supernatant is brought to pH 5.4 by the addition of 5 ml. 1 M acetate buffer of that pH to 100 ml. of solution and the alcohol fractionation is repeated. (This repetition is unnecessary if the activity of the material before dialysis is above 100 units per mg. protein. In that case the residue left after centrifugation of the 50 percent alcohol is suspended not in dextrose but in a volume of cold water equal to 5 times the weight of the filter cake in step 4. The suspension is then centrifuged and the supernatant solution is treated with ammonium sulfate as described in step 5*b*.)

(*b*) The final residue left after centrifugation of the 50 percent alcohol mixture is resuspended in a volume of cold water equal to twice the weight of filter cake before dialysis in step 4. The suspension is recentrifuged. The clear supernatant fluid is brought with solid ammonium sulfate to 0.90 saturation (66 gm. per 100 ml.). The precipitate formed by the addition of ammonium sulfate is filtered with suction. The filtration generally takes 1 or 2 days.

6. Crystallization.—Each gram of filter cake obtained from step 5*b* is dissolved in 1 ml. 0.1 M phosphate buffer pH 7.0 at about 3°C. and 1 ml. saturated ammonium sulfate is added slowly. If a heavy precipitate is formed then a few drops of phosphate buffer are added to incipient clearing. The solution is centrifuged. The clear supernatant solution is left at about 5°C. Crystals in the form of long prisms or fine needles (Figure 88) gradually appear. Seeding hastens the crystallization, as usual.

7. Recrystallization.—The suspension of crystals is centrifuged after 7 to 10 days. The residue is dissolved in a minimum amount of cold 0.1 M phosphate buffer pH 7.0 and a volume of saturated ammonium sulfate is added equal to 1.4 volumes of the buffer used. The solution is left at 5°C. for crystallization which is usually completed in 2 or 3 days. The crystals are centrifuged or filtered with suction.

The yield of crystalline hexokinase varies considerably with the individual lots of yeast delivered to the laboratory, perhaps because of differences of age of the yeast. Some lots even fail to yield any hexokinase crystals. It is advantageous to carry through the purification of lots of 25 pounds of yeast to step 4 and to store the filter cakes (fractions 0.63, 0.72, and 0.73) at 5°C. until an accumulated stock of about 100 gm. is obtained for further treat-

TABLE 46

PREPARATION OF PURE DIPHTHERIA ANTITOXIN

	No.	*Vol.*	*PN ml.*	*Lf ml.*	*Total Lf*	*Lf mg. PN*
33 liters diphtheria toxin + 4 liters antidiphtheria plasma, 25°C. 24 hrs.	1	37 liters	3	60	2×10^6	20
Siphon off supernatant, precipitate suspension + 25 gm. Hyflo per liter, filter and wash precipitate 3 times with 1 liter M/10 pH 7.4 phosphate. Precipitate stirred + 5 liters 0.05 M KH_2PO_4, titrate to pH 3.5, + 2.5 gm. crystalline trypsin. Stand 25°C. 24 hrs. Filter. Titrate filtrate to pH 7.4, cloudy.	2	6.3 liters	0.3	100	6×10^5	350
No. 2 + 700 ml. saturated ammonium sulfate (0.1 saturated) + 60 gm. Hyflo, filter.						
Filtrate clear	3	6.8 liters	0.17	70	5×10^5	400
Precipitate	3P					
No. 3 + 2.3 liters saturated ammonium sulfate (0.33 saturated) + 50 gm. Hyflo. Filter. Precipitate (0.1–0.3 fraction)	4				1×10^5	350
Filtrate	5	8.6 liters	0.10	60 50	4×10^5	500
No. 5 + 2.1 liters saturated ammonium sulfate (0.45 saturated) 2 hrs. 25°C. + 50 gm. Hyflo. Filter. Precipitate (0.33–0.45 fraction)	6				2×10^5	450
Filtrate	7	11 liters	0.03	20	2×10^5	700
No. 7 + 1500 gm. ammonium sulfate (0.65 saturated) 10°C. Settle. Decant supernatant. Precipitate suspension + 20 gm. Filter-Cel. Filter.						
Precipitate	8				2×10^5	700
Precipitate 8, stir + 3 liters (0.5 saturated ammonium sulfate, 0.05 M PO_4 pH 7.4), filter.						
Filtrate	9	3 liters	0.07	45 50	1.5×10^5	650 700
3 liters No. 9 + 60 ml. (2.5 N H_2SO_4, 0.5 saturated ammonium sulfate) 25°C. 1 hr. + 20 gm. Filter-Cel. Filter.						
Filtrate	10	3 liters	0.05	30 35	1×10^5	600 700

TABLE 46 (Continued)

	No.	Vol.	PN ml.	Lf ml.	Total Lf	Lf mg. PN
			m.g.			
Titrate No. 10 to pH 6.8, stir in saturated ammonium sulfate slowly until silky precipitate forms (about 200 ml. saturated ammonium sulfate). Stand 6°C. 24 hrs. Decant and centrifuge. Precipitate suspension, needles, plates and some amorphous. Suspend in 50 ml. 0.5 saturated ammonium sulfate.	11	50 ml.	1.0	650 700	3×10^4	650 700
No. 11 + 10 ml. water (clear) + saturated ammonium sulfate till slightly turbid. Stand 5°C. 2 hrs. centrifuge, thin plates, no amorphous. Stand 25°C. 24 hrs., centrifuge. Precipitate + 20 ml. 0.5 saturated ammonium sulfate. Precipitate suspension.	12	20 ml.	1.0	600 700	1.2×10^4	600 700
No. 12 + 5 ml. water + saturated ammonium sulfate slowly until slightly turbid. Stand 25°C. 1 hr., heavy precipitate thin plates. Centrifuge. Dissolve precipitate + 10 ml. water.	13	10 ml.	1.0	650 700	6×10^3	650 700

ment. In case hexokinase crystals fail to appear in the crystallization mixture within 7 to 10 days the solution is then brought to 0.85 saturation with saturated ammonium sulfate and filtered with suction. The filter cake is reworked through steps *3b* to 7.

The total yield of hexokinase can be increased by reworking the various inert protein crystals and Super-Cel residues of step 3, each according to its place in the general scheme.

The extent of purification of an average lot of 25 pounds of yeast is given in Table 39. The specific activity of the various protein fractions is gradually raised on purification. The greatest rise, however, takes place on crystallization. The specific activity of the first crystals is more than double that of the protein solution from which they were crystallized, while the protein left in the first mother liquor has a specific activity of only 11 percent of that of the crystals. On further recrystallization the specific activity of the crystalline protein is gradually raised. It reaches a constant value after 2 or 3 recrystallizations. The specific activity of the protein in the mother liquor is lower than that of the crystals, the difference, however, becoming less and less as crystallization is repeated.

Preparation of Crystalline Diphtheria Antitoxin (Northrop 1942)

See Table 46.

Constant Solubility as a Criterion of Purity (Northrop and Kunitz 1930; Kunitz and Northrop 1938b; Butler 1940; Herriott 1942)

The rigorous experimental proof of the purity of a substance and even the definition (Lunn and Senior 1929) of a pure substance presents great difficulties. If, instead of "pure," the term "consisting of one component" is used then a definite and unequivocal criterion theoretically and experimentally is furnished by Gibb's phase rule. The phase rule is usually applied to melting or boiling points and in this form is used as the classical test of purity in organic chemistry. Unfortunately, proteins and many other biological compounds do not melt or boil so that the conventional methods cannot be used. Determination of the solubility of such substances, however, furnishes an exactly analogous test.

Other tests of purity, such as homogeneity in the ultracentrifuge or in the electrophoresis cell (cf. Shedlovsky 1943; Cohn 1939; Pirie 1940), have been proposed. These tests furnish very valuable information and if they disclose the presence of two or more components it is certain that the sample is not homogeneous. Failure to detect more than one component, on the other hand, is not proof of purity since a number of cases have been described in which artificial mixtures could not be resolved by these measurements (Butler 1940; Herriott, Desreux, and Northrop 1940a; Walker and Schmidt 1944; Northrop 1942; Herriott 1941a). The solubility test has the further advantage that it is applicable to any molecular species, including those of entirely unknown character, provided only that they may be determined quantitatively. The electrophoretic and, especially, the ultracentrifuge tests, on the other hand, are limited to certain types of molecules. The principal advantage of the solubility test, however, is due to the fact that the solubility of even closely related compounds is additive, whereas the rate of sedimentation or electrophoresis is not.

The solubility (or its analog, the melting point) is thus the only test which will distinguish between optical isomers. The rate of movement of such isomers in the electric or gravitational field is precisely the same and hence they cannot be distinguished by these means. Nevertheless, a solution which is saturated to the L isomer is not saturated to the D and hence a very simple test will distinguish the two forms. No such distinction could be made by electrophoresis or ultracentrifuge, no matter how accurately or in what (optically inactive) solvent the measurement was made.

Closely related proteins are almost as alike in these properties as optical isomers and it is for this reason that their separation, or detection, is such a difficult problem.

Application of the Phase Rule to Protein Solutions

Hardy (1905) and Mellanby (1905) found that the solubility of serum proteins increased as the amount of solid protein increased and their observations were confirmed and extended to other proteins by Sørensen and Høyrup 1917, (Sørensen 1923, 1925, 1926, 1930). Sørensen realized that this behavior was contrary to that required by the phase rule for a pure substance and concluded that proteins were "reversibly dissociating systems" of which the real components are the polypeptides. From this point of view, no protein would have constant solubility and hence the method would be of no value as a test of purity.

Precisely the same experimental result would be obtained, however, if the sample of protein was a solid solution of two or more proteins (Northrop and Kunitz 1930; Bonot 1934). From this point of view the solubility of a protein consisting of one component (i.e., pure) should be absolutely constant and independent of the quantity of solid present.

A number of proteins which actually have constant solubility have now been isolated but they are exceptional and difficult to prepare.

Chymo-trypsinogen has been most carefully studied. Butler prepared samples of this protein by repeated fractional crystallization which gave precisely constant solubility, independent of the quantity of solid, in two different solvents. The system was in equilibrium since the same value was obtained from the super- or undersaturated side. Butler's experiments show that chymo-trypsinogen agrees in every respect with the phase rule criteria for a single component ("pure" substance). These samples of chymo-trypsinogen are probably the purest protein preparations ever obtained. The following is a partial list of protein preparations which are reported to have constant solubility:

Trypsin (Kunitz 1938b), swine pepsin (Herriott, Desreux, and Northrop 1940b), salmon pepsin (Norris and Elam 1940), ribonuclease (Kunitz 1940), luteinizing hormone (Shedlovsky, Rothen, Greep, Van Dyke, and Chow 1940), lactogenic hormone (Li, Lyons, and Evans 1941), oxytocic hormone (Van Dyke, Greep, Rothen, and Chow 1941–42), horse serum albumin (McKeekin 1939), diphtheria toxin (Northrop 1942), rennin (Berridge 1945), flaxseed protein (Vassel and Nesbitt 1945).

Theory of the Phase Rule as Applied to Protein Solutions

The usual expression for the phase rule is $P + F = C + 2$, when P = number of phases, C = number of components, and F = degrees of freedom. A phase is defined (Findlay 1938) as an homogeneous, physically distinct, and mechanically separable part of the system. The components are defined as the smallest number of individually variable constituents by means of which the composition of each phase participating in the equilibrium can be expressed in the form of a chemical equation. Degrees of freedom are the conditions, usually "temperature, pressure, and concentration of each

component which must be arbitrarily fixed in order that the condition of the system be perfectly defined." Gravitational or centrifugal fields are conditions which define the system and may also be considered degrees of freedom. Under usual conditions these forces are constant but if they were not they would have to be taken into account. The fact that molecules in solution may be concentrated by centrifugal force, therefore, does not invalidate use of the phase rule but simply adds another degree of freedom.

In the case of most protein solutions the number of components will usually be four—protein, salt, acid, and water.

TABLE 47

PHASE RULE ANALYSIS OF 4 OR 5 COMPONENT SYSTEMS

Components	*Phases*	*Degrees of Freedom at Constant Temperature, Pressure, and Concentrations of Acid and Salt*	*Examples*
4 = water, salt, acid, protein	1	1	A homogeneous solution
	2	0	A solid phase + saturated solution
	3	(−1)	Two solid phases + saturated solution. (Only possible at a particular concentration of acid or salt.)
5 = water, salt, acid, two proteins	1	2	A homogeneous solution
	2	1	One solid phase containing (a) a single protein, or (b) a solid solution of two proteins, in a solution of variable composition.
	3	0	Two solid phases in solution of constant composition

Additional components may of course be introduced into the system at will, e.g., there may be two salts, such as the buffering salt and a neutral salt like ammonium sulfate. But it is unnecessary to regard a salt formed by the combination of the protein and the acid, even if the solid is present partly or completely as such, as a separate component since its composition can be completely specified by the amounts of protein and acid contained in it. The protein in a specified invariable condition can be taken as the component protein and the amount of protein in any phase is determined by a suitable analysis; e.g., of the amount of "protein nitrogen."

By the phase rule, this system of four components has five degrees of freedom in one phase, and four degrees of freedom when present in two phases. Four of these are the temperature, pressure, and concentrations of

salt and acid respectively. When these four variable factors are fixed there remains one degree of freedom for a single phase and none for a system of two phases. In a homogeneous solution of a single protein the concentration of protein may vary, but if a solid phase is present the concentration of the dissolved protein is fixed. If it is not fixed then an additional component must be present. If it should happen that two distinct solid phases are present (e.g., if a new phase were formed by reaction of the protein with the salt solution) the number of degrees of freedom would be further reduced by one; i.e., it could only occur at a particular acid or salt concentration.

The characteristics of the possible systems which may be formed from one or two proteins together with water, acid, and salt are shown in Table 47. When two proteins are present, a single solid phase may consist of

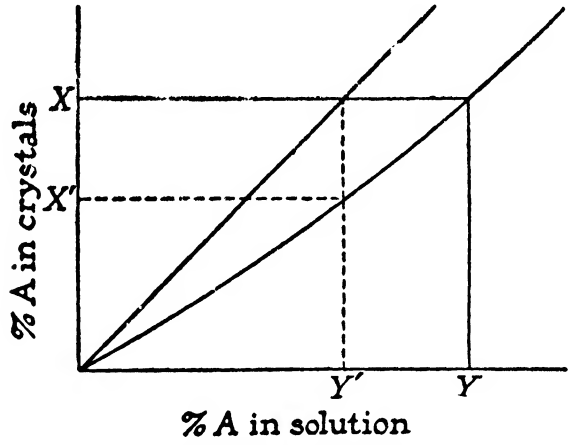

FIG. 97. %A in solution.

either one protein, or a solid solution of the two proteins. In either case, since there are now five components in two phases, the number of degrees of freedom under the stated conditions is one. Thus if the solid phase is one of the proteins, the concentration of the other in the solution is variable, while if the solid phase is a solid solution of both proteins the composition of the solution will depend on the composition of the solid solution, but if the composition of the solid solution is fixed, the composition of the aqueous solution in equilibrium with it is also fixed. Lastly, if there are two solid phases the system again becomes invariant and the composition of the solution, when the temperature, pressure, and concentrations of acid and salt are fixed, is definite.

If additional components such as salts are present, each new component

introduces a new degree of freedom; but if the concentrations of the new components are fixed, the degrees of freedom are unchanged.

These conclusions apply only to the phases actually present in the system analyzed and not necessarily to the original sample. It would be possible, for instance, for a sample to contain impurities which were removed during the preliminary washing and hence were not present in the system analyzed.

The case of a solid solution of two proteins may be discussed in greater detail. Such a solution will be in equilibrium with an aqueous solution of *fixed composition*, but the ratio of the proteins in the aqueous solution may be either the same or different from that in the solid solution. In the first case an equilibrium solution can be formed by simple dissolution of the solid phase and therefore without any change in its composition. Therefore the solubility will be independent of the amount of the solid. In the second case the formation of the equilibrium solution will result in change in the composition of the solid, and as shown by Northrop and Kunitz (1930) the effective solubility will then vary with the amount of the solid phase. The following is a simplified derivation of this conclusion, which does not depend on the particular assumptions previously made.[1]

Suppose that the relation between the composition of the solid and that of the solution in equilibrium with it is as represented in Figure 97, where X is the composition of the solid phase in equilibrium with a solution in which the ratio of the two proteins is represented by Y. In this illustration the proportion of A entering the solution is greater than that in the solid and the solid is therefore left with a smaller proportion of A. We will now consider the following cases:

1. If the quantity of solid is large compared with the amount dissolved, a solution of composition Y is obtained with a very small change in the composition of the solid and the solution obtained from the original crystals will effectively have the composition Y.

2. If the solid is nearly all dissolved, the composition of the solution must approximate to that of the original crystals and will be represented by Y'. The solid which is in equilibrium with this solution has the composition X'. If increasing amounts of the solid phase X are added to a definite quantity of the solvent, on the assumption that the solid phase can adjust itself to equilibrium with the solution, when excess of solid first appears it has the composition X' and a solubility which corresponds to the point Y'. As the excess of solid increases the solid phase remaining will change in composition from X' to X and the solubility will change correspondingly. It follows that the point at which the solid ceases to give a clear solution is not neces-

[1] The composition of either mixtures or solid solution may be calculated from solubility curves (Northrop and Kunitz 1930; Kunitz and Northrop 1938b). The analysis of solid solution curves, however, includes the assumption of Raoult's law which is open to question, and also requires knowledge of the molecular weights and solubilities of the pure components. Mixtures, on the other hand, are rarely encountered, as they are readily separable and so do not appear as a rule in purified samples.

sarily the solubility of the original solid phase, but it will be the solubility of a solid in equilibrium with the solution which has the same composition as the original solid phase. The amount of protein dissolved will be equal to the amount of protein added, up to the point at which solid first appears, but under these circumstances the solubility may then change as the excess of solid is increased until with a great excess of solid a constant value is obtained.

It is possible that the whole of the solid phase may not readily come to equilibrium with the solution and that the adjustments of composition may be confined to the surface layers of the crystals. In that case we may get the solubility which may correspond to a small residue of the solid while an appreciable amount of the solid remains.

The amount of protein present in a solution of definite acidity and salt concentration will therefore be independent of the amount of solid in contact with the solution, from the first appearance of turbidity, in the following cases:

1. The solid is a single phase: (a) a single pure protein, and (b) a solid solution of two proteins, which dissolve in the same proportion as they are present in the solid.

2. The solid consists of two distinct phases: a mixture of two proteins, present in amounts proportional to their solubilities.

To distinguish cases 1 b and 2 from 1 a, it may be observed that it is unlikely that the solubilities of two proteins would be equally affected by a change of acidity and/or salt concentration. If the solvent is changed, it is therefore probable that in the case of the solid solution the two proteins will no longer dissolve in the same proportions and the solubility will then depend on the amount of the solid phase, as described above; while in the case of two distinct solid phases, increasing the amount of solvent will give rise to a point at which one solid has completely dissolved, and here the protein concentration becomes variable. Independence of the solubility of the amount of the solid phase in several solvents can therefore be taken as strong evidence that the material is a pure protein.

On the other hand, a single protein which is present in two distinct solid forms, e.g., amorphous and crystalline, may also give the solubility curve of a mixture. If equilibrium between the two solids and the solution were established, this would be a rare occurrence, since it can only be found in particular solvents in which it happens that the two forms have the same solubility. But the approach to equilibrium may be very slow and cases may be encountered in which the two solids persist for long periods, although not in equilibrium, and so give rise to the solubility curve of a mixture. Such cases may be distinguished by adding sufficient solvent to dissolve one phase and examining the residue. If it gives identical solutions to the original solid, or can be changed into the other form, we are dealing with a case of polymorphism. To decide the nature of the material it is therefore necessary to examine not only the solubility curves in a number of solvents,

but in case the curves of a mixture are obtained, to separate the solids and determine their nature.

Equilibrium of the Protein with the Solvent

It is necessary to consider how far the conditions of constant acidity and constant salt concentration can be satisfied with varying amounts of the solid protein. In the previous experiments of Kunitz and Northrop, it has been considered that this condition is satisfied by previously equilibrating the solid by washing with successive portions of the solvent in which the solubility is to be measured, until the solubility of the protein is constant. Then it is taken that the addition of varying amounts of the equilibrated solid to the solvent will not produce variations of the acidity and salt concentration in the latter. A careful examination of the validity of this process is desirable. The following cases can be distinguished:

1. Suppose that the whole of the solid protein can come into equilibrium with the buffer. It is known that protein crystals contain considerable quantities of water and ions derived from the mother solution, and it has been found that appreciable differences of density (Norris and Elam 1940), and of cell dimensions (Northrop 1941b, 1942) occur according to the nature of the solution in which the crystals are suspended. It follows that the penetration of the salt solution in which the crystals are suspended occurs at least in some cases. In such a case the protein in the interior of the crystal may be expected to come into equilibrium with the solvent both in respect to acidity and salt concentration. On mixing a quantity of the solid with the buffer solution, the equilibrium finally reached will correspond with a pH intermediate between that of the buffer and the original protein. If the solution is poured away and a fresh quantity of the buffer solution added, a second equilibrium will be reached, and it seems evident that after successive treatments in this way the solid will approach a state in which further washings produce no change in the solid. The solubility will then be constant and will be independent of the ratio of the solid to liquid phases.

This procedure would fail only when the solubility of the protein is such that it dissolves completely before equilibrium is reached. This might be overcome by increasing the concentration of the buffer in such a way that the buffering power of a given quantity of solvent is increased, without increasing the solubility of the protein; or by using as the solvent a more concentrated salt solution in which the protein solubility is less.

2. The solid may not equilibrate at all with the buffer. The only function of successive washings would then be to remove the mother liquor adhering to the crystals. A given quantity of solvent would then always dissolve the same amount of protein; which will produce a constant change of pH. The change of pH produced by the dissolution of the protein would, however, depend on the pH of the protein preparation and the solubility will therefore probably depend on the nature of the solution in which the protein crystals were formed.

3. Suppose that the buffer solution reacts with the protein to form a new phase, e.g., a protein salt. It has been shown that two solid phases of a single protein can only coexist with a solution in which one variable concentration, e.g., acidity, is fixed. The buffer solution will therefore be changed by the reaction to a composition which corresponds to the pH fixed by the protein solids. The addition of more buffer will produce more of the protein salt, and only when the first solid phase has been completely converted into the second can the pH change and approach the value defined by the buffer solution. In this case it is evident that a stoichiometrical excess of the buffering substances is required to bring the protein into a state in which it can exist unchanged in contact with fresh portions of the buffer solution.

Experimental Methods for Determination of Solubility Curves of Proteins

The determination is easily carried out provided an analytical method is available. The quantity of sample required depends very largely on this analytical method. If one gamma protein may be determined with sufficient accuracy, then a rough curve could be established with 10 gamma and a complete one with 50 gamma. Ordinarily 10–100 milligrams are used since even micro-Kjeldahl analyses require several milligrams of protein.

A good idea of the purity of the sample may be obtained from two points, one corresponding to the total concentration where the first turbidity appears, and one corresponding to a total concentration of about ten times this amount.

Choice of solvents.—The protein must be stable for several hours at least, in the solvent used, as otherwise decomposition will occur during the test and the result will show the presence of impurities which may not have been present in the original solution. Pepsin crystals in dilute acid are an example of such a system.

It is also necessary to choose a solvent in which the solubility is sufficiently high so that the analyses may be carried out accurately. If the solubility is too high, on the other hand, large quantities of sample may be lost during the preliminary washing. As a rule a solubility of from 0.1–1 milligrams protein per ml. is satisfactory.

Time for equilibrium.—It is absolutely essential that the system be at equilibrium since otherwise the phase rule cannot be applied. This condition can only be established with certainty by approaching equilibrium from both the super- and undersaturated sides. This was actually done in Butler's study of chymo-trypsinogen. Practically, however, it is usually sufficient to determine the solubility at two different times. If no drift is evident, the system may be considered to be in equilibrium. The rate of solution depends on the size of the particles of solid, the rate of diffusion and the rate of stirring. With amorphous preparations, equilibrium is reached in a few minutes, even with very gentle stirring, owing to the enormous surface of the small particles. With large crystals, however, several hours may be required, even with continuous stirring.

Stirring.—The method of stirring is of great importance since proteins are very rapidly denatured at air-water interfaces and most methods of stirring cause the formation of air bubbles. In our experience the best method is as follows:

A small test tube, containing a glass marble, or stainless steel ball bearing, is completely filled with the suspension and allowed to stand for a few minutes so that all air bubbles rise to the top. A rubber stopper which fits rather tightly is then pressed into the tube and also pressed from the side with the thumb nail. This process results in a small amount of solution being forced out at one side and allows the stopper to be pushed into the tube far enough to remain in place. A one-hole rubber stopper may be used and the hole plugged with a glass rod after the stopper has been pushed into the tube. If properly done, no air whatever is left in the tube and the stopper will remain in place without further fastening. The tube is then attached to the side of a wheel which rotates 10 to 20 times a minute, so that it is inverted at each revolution.

Temperature.—The tests should be carried out at 0–5°C., owing to the greater stability of protein at this temperature.

Method of analysis.—A small portion of the suspension is removed for analysis for total protein. A sample of the solution only must then be obtained. This must be done without change of temperature and is best accomplished by centrifugation. If small tubes which fit an angle centrifuge are used, they may be unstoppered and placed directly in a centrifuge which is in the same cold room or ice box, in which the equilibration of the suspension was carried out. In case the suspension must be filtered, care must be taken to see that the filter paper does not absorb some of the protein.

Time required.—An entire solubility curve of amorphous preparations may be obtained in an hour or less, provided the proper solvent is at hand. Crystalline preparations require more time for equilibrium to be reached.

The following table is an example of such a determination as actually carried out on a sample of partially purified amorphous diphtheria antitoxin (Table 48).

The solubility evidently increases rapidly as the total concentration of protein increases. Practically, analyses of the most dilute suspension and the most concentrated one would be sufficient to show that the preparation was very inhomogeneous.

Method for the Determination of the Diffusion Coefficient by Means of a Porous Diaphragm (Northrop and Anson 1929; Anson and Northrop 1937)

An approximate knowledge of the molecular weight of a newly discovered, little known compound is frequently a very valuable indication of the chemical nature of the compound. Such information is of special value in attempts to isolate biologically active material. Once the substance has

TABLE 48

DETERMINATION OF SOLUBILITY OF PARTIALLY PURIFIED AMORPHOUS DIPHTHERIA ANTITOXIN IN {0.5 SATURATED AMMONIUM SULFATE / 0.05 M pH 7.4 PHOSPHATE BUFFER

		PN/Ml. Mgm.
2 ml. solution containing about 20 mgm. antibody protein N in 0.1 M pH 7.4 phosphate, added to 2 ml. saturated ammonium sulfate. Centrifuge.		
	Supernatant	1.1
Precipitate, stir gently with 2 ml. solvent.	Centrifuge	
	Supernatant	0.62
Repeat.	Supernatant	0.51
Repeat.	Supernatant	0.50
Repeat.	Supernatant	0.51
The precipitate is now in equilibrium with the solvent, since no change in solubility can be detected on repeated suspension.		
Precipitate, stir with 2 ml. solvent, and add the following amounts of this suspension to 2 ml. solvent. Stand 20 minutes with occasional gentle stirring and centrifuge and analyze.		

Ml. suspension	0.05	0.1	0.2	0.5	1.0
PN/Ml. Total	0.08 (slightly cloudy)	0.16	0.3	0.6	1.2
In solution	0.07	0.12	0.2	0.25	0.3

been isolated, many methods are available for the determination of its molecular weight but the diffusion method is still the only method of general application which may be applied to any substance for which a method of analysis exists, no matter how impure the preparation may be. The value of this method was pointed out by Arrhenius many years ago. Arrhenius (1907) studied the diffusion of toxins and antitoxins into gels. Other properties of a substance in addition to the molecular weight may be determined from its diffusion rate. For instance if the rate of diffusion in low salt concentrations is independent of pH then the substance is probably neither a weak acid nor a weak base. If the diffusion is minimum at a certain pH, the substance is amphoteric with an isoelectric point at the pH of minimum diffusion. If the rate of diffusion is affected little or not at all by the addition of neutral salts, it is probably not ionized, whereas if the diffusion is decreased markedly by the addition of neutral salts the substance is probably an acid or base. These effects follow from the theory of the diffusion of ions (Nernst 1888; Arrhenius 1892).

This method may also be used to test the homogeneity of the preparation. The first part of the substance which diffuses through the membrane is placed in another cell and the diffusion measurement repeated. If the measurements agree, the substance is homogeneous in size.

The relation between biological activity and other properties of a substance may also be determined by diffusion measurements. If, for instance, a protein has been isolated which carries with it some biological activity, then the diffusion coefficient, as determined by tests of the biological activity and by protein determinations, must agree. This method has been applied to pepsin (Northrop 1930) and trypsin (Northrop and Kunitz 1932b; Scherp 1933).

The older methods of determining diffusion coefficients were very laborious and uncertain especially where large molecules were concerned so that the method was very seldom used.

The diaphragm method (Northrop and Anson 1929; Anson and Northrop 1937) was designed to obviate these experimental difficulties and to increase the speed and accuracy of the determination.

The method contains several assumptions of somewhat doubtful validity (cf. Anson and Northrop 1937) and it was originally intended for use as a means of obtaining an approximate value, especially in terms of molecular weight. Recent work has shown, however, that the method is capable of yielding very accurate figures for the value of the diffusion coefficients and also that the assumptions involved in the Einstein-Sutherland equation are applicable to molecules at least as small as cane sugar.

Barnes (1934) showed that the simplifying assumption of a linear diffusion gradient across the diaphragm was very nearly correct.

J. W. McBain and his collaborators (1930, 1934, 1935) (M.E.L. McBain 1933) used a modified form of cell in a study of the diffusion of electrolytes. Orr and Butler (1935) used this method for the determination of the diffusion of deuterium.

Friedman and Carpenter (1939) obtained the correct molecular weight of sugar, provided low concentrations were used. Dean and Loring (1945) also found that the Einstein-Sutherland equation gave correct values for glycerophosphate. Mehl and Schmidt (1937) used the method for amino acids. Frieden (1945) applied the method to penicillin and arrived at a molecular weight of 490. Mouquin and Cathcart (1935) designed a cell in which the solutions were stirred, in order to be sure of uniform concentrations. Results in this laboratory have shown that stirring is not necessary provided the solution in the cell is slightly heavier than the solvent outside the cell. It is extremely difficult to stir the solution without giving rise to slight vibrations which, in turn, cause very serious errors.

Hershey, Kimura, and Bronfenbrenner (1947), have found that the diffusion of bacteriophage by this method is abnormal and gives rise to too small values for the particle size. They ascribe this to flow of solution through the membrane. Such abnormal results have not been obtained in this laboratory except when the apparatus is exposed to vibration. The diffusion of protein of molecular weights of 40–50,000 is consistently found to be a little slower than expected, and not faster as Hershey, Kimura, and Bronfenbrenner state. It is quite true, however, as pointed out by these

authors that a very minute constant source of error would entirely vitiate the results for the diffusion coefficient of very large particles, such as the bacteriophage or viruses, with molecular weights in the millions, whereas it would be entirely negligible in the case of small molecules or even proteins of 30,000 molecular weight. The use of the method with very large particles requires great care and the results can be relied upon only as regards the order of magnitude.

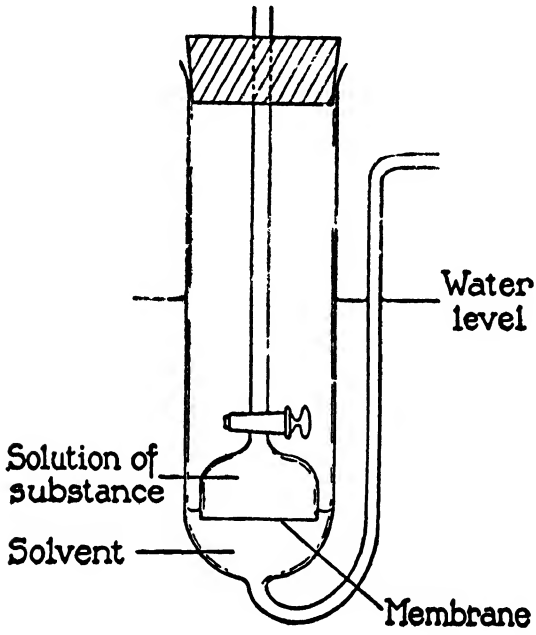

FIG. 98. Apparatus for the determination of diffusion coefficients.

The theoretical aspects, as well as the application of the method, are reviewed by Gordon (1944).

Experimental Procedure (Northrop and Anson 1929)

Diffusion cell.—Cells with scintered glass disks are made by the Corning Glass Company. These disks are thick so that diffusion is slow. They may be ground to any desired thickness by filling the pores with melted rosin and grinding on a glass plate with carborundum powder. The rosin is then dissolved out with warm chloroform. Much smaller cells may be made if desired.

The cell is held in a large tube with a drain tube at the bottom (Figure 98).

The volume of the cell may be determined by weight or by allowing the contents to drain into a graduate.

The solution in the cell must be slightly heavier than that outside. If the solution of the sample contains 1 gram or more per 100 ml. the substance itself is sufficient to produce this difference in specific gravity. If very dilute solutions are used, however, it is better to use a slightly more concentrated buffer or salt solution inside the cell than is used outside.

Assembly of apparatus.—Cf. Figure 98.

Calculation of the diffusion coefficient and determination of the cell constant (Northrop and Anson 1929).—Theory of the method. The diffusion coefficient is defined as the quantity of material that will diffuse across a plane of unit dimensions in unit time under unit concentration gradient, or

$$(1) \qquad D = \frac{dQ}{A\,dt\,\frac{dc}{dx}}$$

where D is the diffusion coefficient, dQ is the quantity which passes across the plane of area A in time dt under a concentration gradient of dc/dx. There are a number of solutions of this differential equation depending on the conditions of the experiment (cf. Mellor 1913). The present case is in many respects the simplest. Suppose a solution of concentration C_1 is separated from a more dilute solution of concentration C_2 by a porous membrane through the pores of which the solute can diffuse. Both solutions are stirred so that diffusion occurs only across the membrane. The solute will diffuse from C_1 to C_2 and if the volume of these solutions is relatively large and the experiment is carried on for a short time the concentrations will remain practically constant. Let the effective area of the pores be A and the effective length (the distance through which the solute diffuses) be h. The concentration gradient will then be constant and equal to $\frac{C_1 - C_2}{h}$ and the quantity Q diffusing in time t will be simply

$$(2) \qquad Q = D\,At\,\frac{C_1 - C_2}{h}$$

or

$$(3) \qquad D = \frac{hQ}{At(C_1 - C_2)}$$

If the experiment is so arranged that C_2 is zero when $t = 0$ and is negligible compared to C_1 the equation is still further simplified to

$$(4) \qquad D = \frac{h}{A}\frac{Q}{tc}, \text{ or } D = \frac{K\,V_2\,C_2}{C_1\,t} \text{ where } K = \frac{h}{A}$$

General equation.—If the concentrations change significantly during the experiment the equation for the diffusion coefficient is (Northrop and Anson 1929):

$$D = \frac{2.3\,K\,V_1V_2}{(V_1+V_2)t}\log\frac{V_2S-(V_2+V_1)Q_0}{V_2S-(V_2+V_1)Q} \tag{5}$$

when S = total solute present

and $Q = Q_0$ when $t = 0$

Dimensions of D. Since concentration may be expressed as quantity per unit of volume, the units used to measure the quantity cancel out provided the same unit is used to express the concentration as is used to measure the quantity diffusing. D therefore reduces to area over time, or if time is expressed in days and length and volume in centimeters, to cm.² per day.

It is evident that in order to obtain the diffusion coefficient in absolute units it is necessary to know the dimensions of the membrane through which the diffusion occurs. In the case of porous membranes this value cannot be measured directly since the effective radius and the arrangement of the pores is not known. For any one membrane, however, the effective thickness and area may be assumed constant and therefore h/A is constant and may be called K, the membrane constant. In order to obtain this value it is necessary to standardize the apparatus against some solution the diffusion constant of which is known, just as is the case with a conductivity cell. K, the cell constant, may then be found from the equation

$$K = \frac{Dt\,C_1}{C_2\,V_2} = \frac{Dt\,C_1}{Q} \tag{6}$$

When this constant has been determined for a particular membrane it may then be used to determine the diffusion coefficient for unknown substances, provided of course that the effective pore area is the same for the standard and for the unknown.

Choice of standard solutions (Anson and Northrop 1937).—Sodium chloride is the most suitable substance for standardization since the absolute diffusion coefficient is well established and does not vary much with the concentration. The diffusion coefficient of sodium chloride at 5°C. is 0.720 cm.² per day. The value at other temperatures between 0 and 25°C. may be calculated from the equation (Öholm 1905) $D = 0.588 + 0.0263\ t$ when t = degrees Centigrade.

Diffusion in the presence of a standard.—Instead of calibrating the membrane with a standard solution, the standardizing compound may be added to the unknown and both determinations carried out at the same time (pro-

vided there are no experimental complications). By this method the diffusion coefficient of the unknown is found by calculation directly from the relative quantities of the two substances which diffuse. This method has the advantage over that of standardization in that any change in the membrane pores is corrected. In addition, if a standardized cell is used, any experimental errors will be detected, since then the value of D for the standard substance will be incorrect. It is preferable however to use as standard some compound which diffuses at approximately the same rate as the unknown as otherwise the appropriate time for removing a sample will be quite different. The diffusion coefficient of the unknown substance may be calculated as follows:

1. The quantity of either substance which diffuses is small compared to the total quantity originally present, and the concentration outside is 0 at the beginning.

V_1 = volume of cell.
V_2 = volume outside solution.
C_1 = concentration standard in cell.
C_2 = concentration standard outside cell at time t.
X_1 = concentration unknown in cell.
X_2 = concentration unknown outside cell at time t.
K = cell constant.
t = time of diffusion.
D_c = diffusion coefficient of standard.
D_x = diffusion coefficient of unknown.

Then $$D_c = \frac{K C_2 V_2}{C_1 t} \quad \text{and} \quad D_x = \frac{K X_2 V_2}{X_1 t}$$

or $$D_x = D_c \frac{X_2}{X_1} \cdot \frac{C_1}{C_2} = D_c \frac{Q_x}{S_x} \times \frac{S_c}{Q_c}$$

2. In case a significant fraction of the total quantity diffuses so that the concentration in the cell cannot be considered constant the complete equation (Northrop and Anson 1929, page 544) must be used.

S_c = total quantity standard present in cell at beginning
S_x = total quantity unknown present in cell at beginning
Q_x = quantity of unknown present in outside solution at time t
Q_{xo} = quantity of unknown present in outside solution when $t = 0$
Q_c = quantity of standard present in outside solution at time t
Q_{co} = quantity of standard present in outside solution when $t = 0$

then

$$D_x = D_c \frac{\log [V_2 S_x - (V_2 + V_1) Q_{xo}] - \log [V_2 S_x - (V_2 + V_1) Q_x]}{\log [V_2 S_c - (V_2 + V_1) Q_{co}] - \log [V_2 S_c - (V_2 + V_1) Q_c]}$$

If $V_1 = V_2$

$$D_x = D_c \frac{\log (S_x - 2Q_{xo}) - \log (S_x - 2Q_x)}{\log (S_c - 2Q_{co}) - \log (S_c - 2Q_c)}$$

If Q_{xo} and $Q_{co} = 0$

$$D_x = D_c \frac{\log S_x - \log (S_x - 2Q_x)}{\log S_c - \log (S_c - 2Q_c)}$$

Determination of Pepsin or Chymo-Trypsin by Rennet Action (Kunitz 1935; Herriott 1938a)

"Klim" solution.—20 gm. of Klim are worked into a paste in a mortar with water, poured into a 100 ml. graduate, and 10 ml. of M/1 pH 5.0 acetate buffer is added, finally diluting the suspension to 100 ml. with water. This solution should be kept in the ice box when not in use and for precise work it should not be used after 5 days from the time it is made up. A control tube should not clot in 24 hours at 35°C.

Procedure.—5.0 ml. of the above Klim solution is brought to 35.5°C. in a water bath and 0.5 ml. of the pepsin solution diluted in M/10 pH 5.0 acetate is added. The pipette should be held about 1 inch above the Klim solution and the last drop blown out of the pipette. The test tube containing the digestion mixture is now twirled once or twice to mix the solutions and to wash down any enzyme solution which may be on the side of the tube. A stop-watch is started as the enzyme is added. The tube is now left in the bath until a minute or two before it should clot and then the tube is tipped and slowly rotated so that the worker can examine a thin film of the solution which thickens and coagulates in small particles just before clotting. The end point is arbitrary and therefore depends upon the worker. However, the variation in the end point determined by two workers is not great.

Rennet activity units $[P.U.]^{Ren.}$.—1 rennet unit is defined as the amount of enzyme which, under the above conditions, will clot 11 ml. of the enzyme-Klim mixture in 1 minute. The rennet activity of any solution is obtained then by dividing the dilution by the time in minutes required to clot the Klim. Thus, if 1.0 ml. of an enzyme solution was diluted to 500 ml. and 1.0 ml. was added to 10.0 ml. of the 20 percent Klim (or 0.5 ml. to 5.0) with a clotting time of 5 minutes, the original solution would contain 500/5 or 100 $[P.U.]^{Ren.}_{ml.}$.

The Estimation of Pepsin, Trypsin, Papain, and Cathepsin with Hemoglobin[1]

In the hemoglobin method for the estimation of proteinase, denatured hemoglobin is digested under standard conditions, the undigested hemoglobin is precipitated with trichloracetic acid, and the amount of unpre-

[1] This section, except for the omission of several introductory paragraphs, is reprinted from the paper of M. L. Anson (1938).

cipitated protein split products, which is a measure of the amount of proteinase present, is estimated with the phenol reagent which gives a blue color with tyrosine and tryptophane.

Hemoglobin, unlike casein and gelatin, is a reproducible substrate. Different batches of hemoglobin are digested at the same rate by a given proteinase solution.

Even when peptidase is present in addition to proteinase, the formation of products not precipitable by trichloracetic acid is due, so far as is known, to proteinase alone.

The preparation of hemoglobin.—Whipped beef blood is centrifuged. The serum and the white corpuscles which form a thin layer on top of the red corpuscles are siphoned off and the red corpuscles are then mixed with an equal volume of cold 1 percent sodium chloride solution and after cen rifugation the supernatant solution is siphoned off again and the corpuscles are either stored frozen or dialyzed immediately and then stored frozen. The corpuscles are largely freed of color-producing substances not precipitable by trichloracetic acid by dialysis in DuPont cellophane tubing of 3/4 inches diameter. Cellophane deteriorates on standing, especially the outer layer of the coil, which is most exposed to the air. It is necessary to test the tubing for leaks. One end of the tubing is wetted with water and a knot is tied in the end of the tubing. The tube is then filled with water and the open end is twisted and folded over. While the folded part is pressed closed with one hand the tubing is squeezed with the other. The squeezing, in addition to showing up leaks, stretches the tubing and thus excessive stretching and dilution during dialysis are avoided. If the tubing is satisfactory the water is poured out, a marble is put in, the tube is filled with the washed corpuscles, and the end of the tubing is closed with a knot in the cellophane itself. The tubes are placed in a tall vessel. Cold tap water is run into the lower part of the vessel at a rate sufficient to cause stirring. Occasionally the tubes are inverted and the hemoglobin solution is thus stirred by the marbles. After 24 hours' dialysis the hemoglobin solutions from all the cellophane tubes are mixed and the mixed solution is stored frozen in small aluminum containers or cardboard ice-cream containers. It is easily possible to prepare enough dialyzed hemoglobin at one time for thousands of proteinase estimations.

To estimate the concentration of proteins in the dialyzed corpuscles a 3–5 gm. sample is weighed out in a porcelain evaporating dish, and dried overnight at 105°C., and the dry weight is recorded.

When it is not convenient to store the dialyzed hemoglobin frozen it can be stored at room temperature as a dry powder. If the hemoglobin solution is frozen while the drying takes place the hemoglobin remains soluble.

Bacto-Hemoglobin of the Difco brand can be used for rough work when the blank is not of importance. It consists of dried washed corpuscles. The

other commercial hemoglobins are probably prepared from unwashed corpuscles or from whole blood. Some of them give results very different from those obtained with the hemoglobin whose preparation has just been described.

Casein and edestin or egg albumin can be used instead of hemoglobin. Gelatin cannot be used since it is not precipitated by trichloracetic acid.

Phenol reagent.—To the phenol reagent prepared according to Folin and Ciocalteu (1927) twice its volume of water is added. Whenever the phenol reagent is referred to this diluted reagent is meant.

Determination of Pepsin with Hemoglobin by Means of a Quartz Spectrophotometer

Preparation of acid hemoglobin solution.—20 ml. of 0.3 M hydrochloric acid is added to 80 ml. of 2.5 percent hemoglobin solution. This acid solution is unstable and should be prepared fresh every day.

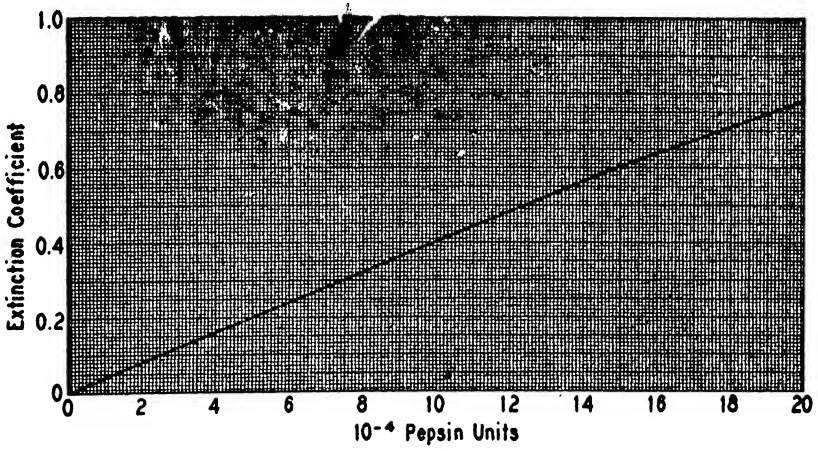

Fig. 99. Plot of pepsin units *vs.* extinction coefficients at 35.5°C.

Experimental procedure.—5 ml. acid hemoglobin solution is added to a series of 175 × 15 mm. test tubes and the tubes placed in a water bath at 35.5°C. (or 25°C.) for 5 to 10 minutes. 1.0 ml. of the pepsin samples to be tested is added to each tube and the time noted. After 10 minutes, 10 ml. of 0.3 M trichloracetic acid is added as rapidly as possible, the tubes are shaken, and then replaced in water bath for 2 to 5 minutes. The suspension is then filtered through a thick retentive paper such as Whatman No. 3. The filtrate should be perfectly clear. 5 ml. of the filtrate is added to 10 ml. of water and the absorption coefficient of this solution is determined at a

wave length of 280 mμ. This corresponds to the maximum of the absorption spectrum of tyrosine and tryptophane (Holiday 1936).

Blank determination.—The pepsin solution may contain some substance which absorbs in this region and the hemoglobin solution has a small blank. This value is obtained by adding the pepsin sample to the 10 ml. trichloracetic acid before mixing with the hemoglobin solution. The extinction coefficient of the blank is substracted from that of the corresponding test.

The pepsin units corresponding to the extinction coefficients of the tests (corrected for the blank) are then read off from the standard curve (Figure 99).

Correction for 25°C.—The curve in Figure 99 corresponds to the value found at 35.5°C. If the digestion is carried out at 25°, the value for the pepsin units must be multiplied by 1.8 (Anson 1938).

Calibration of the curve in absolute units.—One pepsin unit was originally defined as that quantity which would produce 1 milliequivalent of tyrosine (measured by Folin's phenol reagent), not precipitated by 0.3 M trichloracetic acid per minute per 6 ml. standard hemoglobin digestion mixture, at 35.5°. The tyrosine equivalent of a solution as determined by Folin's reagent differs from that obtained by ultraviolet absorption due to the fact that tyrosine and tryptophane affect the two measurements differently.

In order to obtain an absolute value for the pepsin units, therefore, it is necessary to carry out a series of determinations with some one pepsin solution using the original Folin reagent method. When the value has been obtained for this solution the units so found may be plotted against the ultraviolet absorption as in Figure 99.

Determination by means of the phenol reagent (Anson 1938).—Reagents and procedure are the same as described above until the trichloracetic acid filtrate is obtained (page 305).

10 ml. 0.5 M sodium hydroxide and then 3 ml. of diluted phenol reagent are added to 5 ml. of the trichloracetic acid filtrate.

The phenol reagent should be added as a rapid stream of drops and the tube shaken during the addition of the reagent. The color depends somewhat on the rate of addition of the reagent. The solution is allowed to stand for 2 to 10 minutes and then read in a colorimeter against a standard solution of tyrosine in 0.2 M hydrochloric acid containing 8×10^{-4} milliequivalents per 5 ml.

The color developed is not permanent and for this reason a stable solution, such as copper sulfate or chrome alum is used as a reference standard with photoelectric colorimeters. A blue glass may also be used as a reference standard in instruments of the Du Bosque type. If a glass standard is used then very low readings as in the blank cannot be obtained and it is necessary to add 1 ml. of a standard tyrosine solution containing 8×10^{-4} milliequivalents per ml. to the trichloracetic filtrate from the blank before adding the phenol reagent. In this case the value for the blank is as follows:

$$\text{Blank} = \frac{19}{18} \times 0.0008 \frac{(\text{colorimeter reading of standard})}{(\text{colorimeter reading of (blank + standard)})} - 0.0008$$

The factor 19/18 is a correction for the change in volume due to the addition of 1 ml. standard tyrosine solution.

The pepsin units corresponding to the corrected reading are then read off from the standard curve (Figure 100).[1]

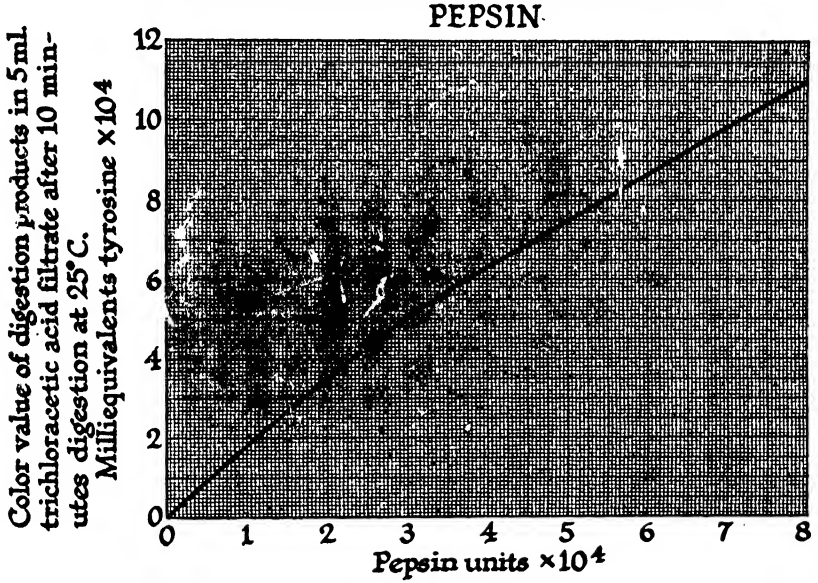

FIG. 100. Digestion of hemoglobin with increasing concentrations of pepsin[1].

THE ESTIMATION OF TRYPSIN

Hemoglobin substrate.—A solution is made up containing 8 cc. of 1 N sodium hydroxide, 72 cc. water, 36 gm. urea, and 10 cc. of 22 percent hemoglobin (22 gm. hemoglobin per 100 cc. solution). This alkaline solution is kept at 25°C. for 30–60 minutes to denature the hemoglobin and is then mixed with a solution containing 10 cc. 1 M potassium dihydrogen phosphate and 4 gm. of urea. The final pH is 7.5. One mg. Merthiolate (Lilly) is added to each 50 cc. of hemoglobin solution as a preservative and the hemoglobin solution is stored at 5°C.

The activity curve is given in Figure 101.

The procedure for the estimation of trypsin is the same as that for the

[1] The numbers on the ordinates of all four hemoglobin activity curves (Figures 100, 101, 104, 105) represent the numbers of ten thousandths of milliequivalents of tyrosine. The numbers on the abscissae represent the numbers of ten thousandths of activity units.

estimation of pepsin except that, because of the urea in the substrate solution, it is necessary, after the addition of trichloracetic acid, to wait 30 minutes before filtration, both in the preparation of the digestion filtrate and in the preparation of the blank filtrate.

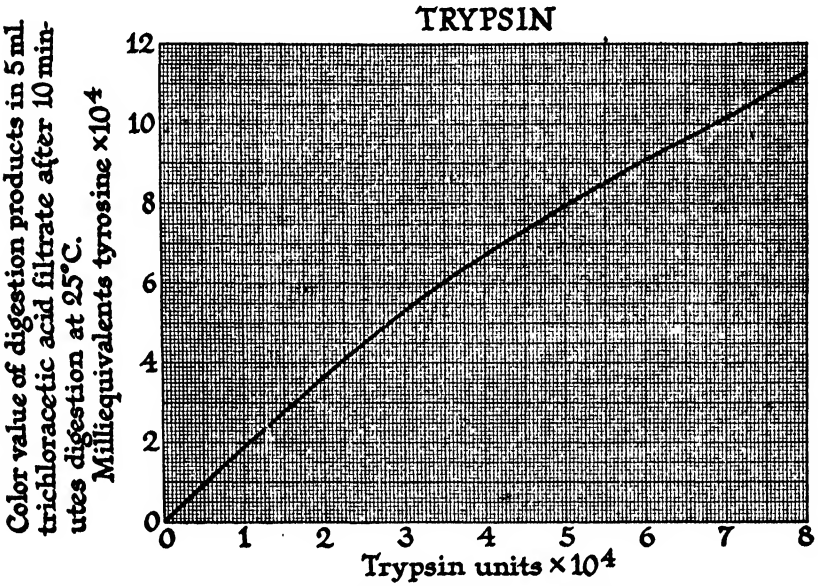

FIG. 101. Digestion of hemoglobin with increasing concentrations of trypsin. (See footnote on page 307.)

CASEIN DIGESTION METHOD (Kunitz 1947a)

Experimental procedure.—A stock solution of casein is made by suspending one gram of casein (preferably "Hammarsten") in 100 ml. 0.1 M Sørensen's phosphate buffer pH 7.6. The suspension is heated for 15 minutes in boiling water, thus bringing about complete solution of the casein. The solution designated as 1 percent casein is stored in refrigerator and is stable for about a week or longer. Samples of 1 ml. of 1 percent casein are pipetted into 15 ml. pyrex test tubes and placed in a water bath at 35°C. for about 5 minutes before being used.

The trypsin standard curve.—One ml. samples of crystalline trypsin dissolved in 0.0025 M hydrochloric acid, or in a suitable buffer solution, are added to samples of 1 ml. casein at intervals of about one minute, mixed well, and left at 35°C. for 20 minutes. The solutions are then poured back and forth into tubes containing 3 ml. of 5 percent trichloracetic acid. The precipitates formed are centrifuged after standing one hour or longer at about 25°C. The concentration of split products in the supernatant solu-

tions is determined by measuring the optical density of the solutions at 280 mμ or by Herriott's method (1941c) as follows:

1 ml. supernatant + 1 ml. 0.0025 M copper sulfate + 8 ml. 0.5 M sodium hydroxide + 3 ml. dilute Folin-Ciocalteu's phenol reagent (1 part + 2 parts of water). The reagent is added drop by drop at a rapid rate. The color developed is compared with a similar mixture containing 0.205 mg. of tyrosine.

The optical density method is simpler and has been used throughout the present studies. The readings are corrected for blank solutions which are prepared by mixing first 1 ml. of 1 percent casein solution with 3 ml. of 5 percent trichloracetic acid and then adding 1 ml. of the highest concentration of trypsin used, or 1 ml. of the buffer solution used in making up the trypsin dilutions. The corrections for blanks for the intermediate concentrations of trypsin are calculated by intrapolation. The readings (corrected for blanks) are plotted as shown in Figure 102. The plotted curve can be used for the

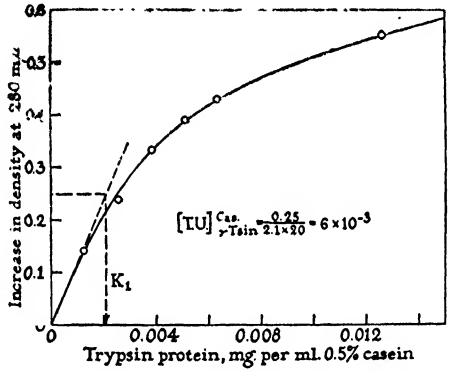

FIG. 102. Standard curve for digestion of casein by trypsin. Optical density at 280 mμ plotted *vs.* mg. trypsin protein.

determination of tryptic activity of any sample of material by reading off the mg. trypsin, corresponding to the corrected optical density reading of the sample. The activity is then expressed in terms of the sample of crystalline trypsin used for drawing the standard curve. A more general way is to calculate the specific activity of the trypsin and then replot the curve in terms of density *vs.* tryptic units. The tryptic unit is defined as the activity which gives rise, under the conditions described, to an increase of one unit of optical density at 280 mμ per minute digestion, and is designated as $[T.U.]^{cas.}$. The specific activity of the sample of trypsin used is obtained by drawing a straight line tangent to the first part of the curve. In Figure 102, the slope $\frac{0.25}{2.1}$ (indicated by the dotted lines) divided by 20 minutes is

the specific activity of the given material, i.e., the activity per microgram trypsin protein, i.e. $[T.U.]_{\gamma}^{cas.} = \frac{0.25}{2.1 \times 20} = 6 \times 10^{-3}$.

A new curve is then plotted (Figure 103), the ordinates of which are identical with those in Figure 102 while the abscissae are expressed in tryptic units, one γ being equal to 6×10^{-3} $[T.U.]^{cas.}$. The data for Figure 103 are conveniently obtained by reading off the smooth curve in Figure 102 the densities corresponding to 1γ, 2γ, 4γ, 6γ, etc., and then plotting these values as ordinates against $6, 12, 24, 36 \times 10^{-3}$ $[T.U.]^{cas.}$ as abscissae. The data on the new curve are independent of the purity of the sample of trypsin used and hence can be used as a general standard curve for determination of tryptic activity, provided the same stock of casein is used and under the same experimental conditions of pH and temperature, etc.

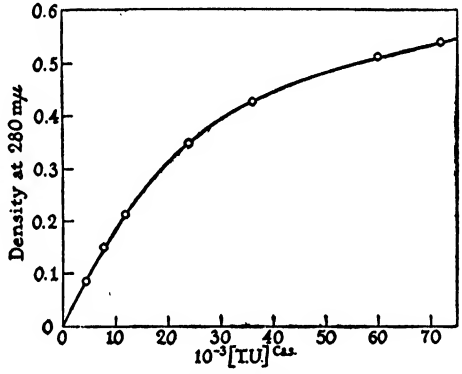

FIG. 103. Standard curve for digestion of casein by trypsin. Optical density at 280 mμ plotted *vs.* tryptic units.

The proteolytic activity of chymo-trypsin is determined in the same way.

Trypsin inhibitor activity measurements.—Inhibitor activity is expressed in terms of units of trypsin inhibited, and the measurement consists simply in comparing the tryptic activity of two samples of trypsin, one containing a definite amount of inhibitor and the other sample being free of inhibitor. The difference in the tryptic activity of the two samples of trypsin, provided the inhibitor is not in excess, expressed in [T.U.] or in weight of pure trypsin divided by the weight of the inhibitor used is a measure of its specific activity.

Experimental procedure.—Samples of 1 ml. containing 50γ trypsin dissolved in 0.0025 M hydrochloric acid were mixed with 1 ml. containing various amounts of soy inhibitor dissolved in 0.0025 M hydrochloric acid. 1 ml. of each mixture was added to 1 ml. of 1 percent casein, pH 7.6, digested 20 minutes at 35°C., then mixed with 3 ml. 5 percent trichloracetic

acid and treated as described before. The measurements and the calculations are given in Table 49. The average specific activity of the inhibitor is

TABLE 49

TRYPSIN INHIBITING ACTIVITY OF SOY INHIBITOR

Microgram soy inhibitor per ml. 0.5 percent casein	*0*	*2.5*	*5.0*	*7.5*	*10.0*	*12.5*
Optical density at 280 mμ (corrected for blank)	0.550	0.515	0.455	0.348	0.185	0.008
10^{-3} [T.U.]$^{cas.}$ read on curve, Fig. 103	75.5	61.5	43.7	25.5	10.5	0
10^{-3} [T.U.]$^{cas.}$ inhibited (by difference)	0	14.0	31.8	50.0	65.0	75.5
Specific activity 10^{-4} [T.U.]$^{cas.}$ per γ inhibitor		5.6	6.4	6.7	6.5	6.0

Average 1 γ inhibitor = 6.3×10^{-4} [T.U.]$^{cas.}$ = 1.03 γ trypsin

about 1.0 when expressed in terms of weight of pure trypsin inhibited.

Crystalline soybean inhibitor because of its stability and purity can be used as a convenient standard for the assaying of samples of trypsin. The reaction with trypsin is independent of the method used for measuring the proteolytic activity of trypsin.

Protein determination.—Total nitrogen by Kjeldahl. The protein concentrations used in this paper were based on the total nitrogen determined by a semimicro-Kjeldahl method, one mg. of nitrogen being equivalent to 6.0 mg. of soybean protein.

Procedure.—Digestion mixture: 1 ml. sample containing 2 to 5 mg. protein + 1 ml. concentrated sulfuric acid + 1 drop selenium oxychloride + 0.25 gm. potassium sulfate + several alundum chips. Digested 5–10 minutes in 100 ml. Pyrex Kjeldahl flask, cooled, and 5 ml. water added, then steam distilled in a ground glass jointed outfit, in the presence of 5 ml. 30 percent sodium hydroxide. The distillate is received in a flask containing 5 ml. 0.02 M hydrochloric acid and is titrated with 0.02 M sodium hydroxide from a burette graduated to 0.01 ml., using methyl red as indicator.

Colorimetric method by means of copper-phenol reagent according to Herriott, as described on page 309. The color developed is measured in a colorimeter or spectrophotometer at 600 mμ. The protein concentration is read on a standard curve obtained by plotting colorimeter or density reading, *vs.* known concentrations of protein as determined by the Kjeldahl nitrogen method.

Optical density measurement at 280 mμ. A very convenient way of estimating protein in clear solutions is by measuring the ultraviolet light absorption at 280 mμ. The density readings are proportional to the con-

centration of protein up to density readings of almost 1.0. The proportionality constant varies, however, with different proteins.

The factors for calculating protein concentration from density measurement at 280 mμ are:

Soybean inhibitor 1.10
Crystalline trypsin 0.585
Crystalline chymo-trypsin 0.500.

The Estimation of Papain (Anson 1938)

The hemoglobin substrate and the estimation procedure are the same as in the estimation of trypsin. The papain must be activated before estimation.[1] This is done as follows: To 0.5 ml. papain solution are added

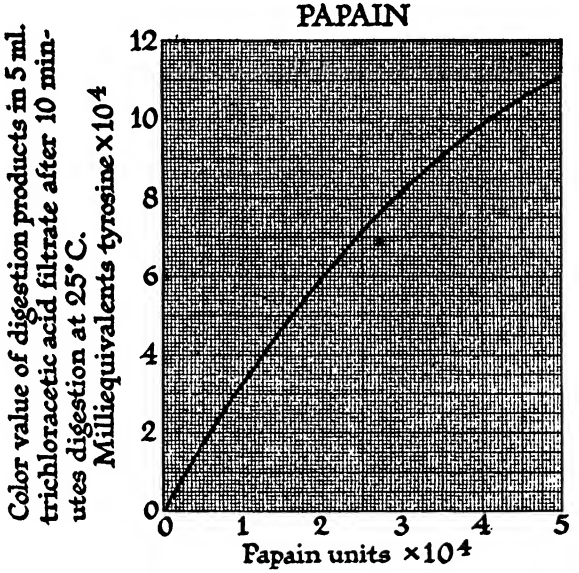

Fig. 104. Digestion of hemoglobin with increasing concentrations of papain. (See footnote on page 307.)

5 drops 2 M sodium cyanide. After 3 minutes at 25°C., 9.25 ml. of water is added. If further dilution is necessary it is carried out with a solution containing 5 drops of 2 M sodium cyanide in 10 ml. of water.

To obtain the papain activity curve (Figure 104) digestion was carried

[1] The activation procedure described gives more rapid and complete activation of crude commercial papain than the usual activation in less alkaline solution. It has not been proved that it gives complete activation. The cyanide is necessary to eliminate inhibitors in the hemoglobin solution as well as to activate the enzyme. The amount of cyanide needed for this purpose may vary with different hemoglobin preparations.

out with activated aqueous extract of Optimo Papain (S. B. Penick and Company). The amount used for digestion corresponded to 0.06–0.3 mg. of the original powder. It is not known whether or not all samples of commercial papain give the same activity curve.

The Estimation of Cathepsin

Preparation of hemoglobin substrate.—There is added from an automatic pipette to a 175 × 20 mm. test tube 4 ml. of centrifuged 2.5 percent hemoglobin solution containing 1 mg. Merthiolate per 40 cc. (the same solution used for the preparation of the pepsin substrate). From another automatic pipette 1 ml. of 1.35 M in respect to acetic acid and 0.02 M in respect to ammonium sulfate is added. The final pH is 3.5. The acid substrate solution is stored at 5°C. and used within a day or two before the blank increases.

The estimation procedure is the same as that used in the estimation of pepsin except that the digestion is carried out at 37°C. instead of 25°C. The concentration of ammonium sulfate in the enzyme solution should not be greater than 0.04 M (0.01 saturated) since ammonium sulfate in greater concentration decreases the rate of digestion.

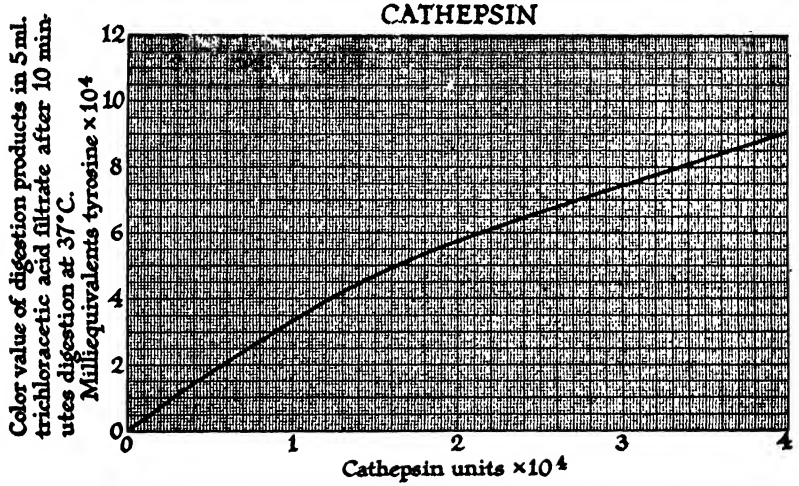

FIG. 105. Digestion of hemoglobin with increasing concentrations of cathepsin. (See footnote on page 307.)

Purified beef spleen cathepsin was used to obtain the activity curve (Figure 105).

Activity Units and the Construction of the Curves

This section defines the activity units and gives the directions for constructing a curve relating activity units to color values of digestion prod-

ucts. It is not necessary to read this section in order to use the procedures for the estimation of pepsin, trypsin, papain, and cathepsin which have been described, since the curves are already given. If the hemoglobin method is applied to other proteinases, however, a new curve must be worked out in each case, and in each case the proper substrate solution must first be found.

One unit of proteinase is defined as the amount which digests hemoglobin under the standard conditions at an initial rate such that there is liberated per minute an amount of split products not precipitated by trichloracetic acid which gives the same color with the phenol reagent as one milliequivalent of tyrosine. This unit is similar to the other proteinase units previously used in this laboratory (Northrop 1932d). The specific activity is the activity per mg. of enzyme nitrogen. The standard temperatures are taken to be 25°C. for papain and 37°C. for cathepsin, these being the temperatures used in practice. In the cases of pepsin and trypsin, the first two proteinases studied, although in practice the digestion is carried out at 25°C., the standard temperature was taken to be 35.5°C., that being the standard temperature previously used in this laboratory. The initial rate of digestion by pepsin is 1.82 times greater at 35.5°C. than at 25°C. With trypsin the rate is 1.78 times greater at 35.5°C. than at 25°C.

To obtain an activity curve, the hemoglobin solution is digested for 10 minutes with different amounts of enzyme expressed as cc. of some stock solution. A curve is plotted, relating the color values of the digestion products, i.e., the color values of the digestion filtrates corrected for the blank, to the amounts of enzyme used. A line is drawn tangent to the first part of the curve and there is read off from this line the amount of enzyme which gives a color value of 0.001 milliequivalents of tyrosine for 5 cc. or 5/16 of the whole trichloracetic acid filtrate after 10 minutes of digestion. This amount of enzyme multiplied by $1{,}000 \times 5/16 \times 10$ is the one unit of enzymes which produces split products with a color value of 1 milliequivalent of tyrosine in the whole 16 cc. of solution in 1 minute.

The number of activity units per cc. of stock solution is now known and one can replot the curve to give activity units against color values.

LITERATURE

THE FOLLOWING abbreviations of the names of periodicals are used below.

Acta biol. belgica.—Acta biologica belgica.
Am. J. Anat.—The American Journal of Anatomy.
Am. J. Path.—The American Journal of Pathology.
Am. J. Physiol.—The American Journal of Physiology.
Am. J. Pub. Hlth.—American Journal of Public Health.
Am. Naturalist.—The American Naturalist.
Ann. Inst. Past.—Annales de l'Institut Pasteur.
Ann. N. Y. Acad. Sc.—Annals of the New York Academy of Sciences.
Ann. Rev. Biochem.—Annual Review of Biochemistry.
Ann. Soc. roy. sci. méd. et nat.—Annales et bulletin de la Société royale des sciences médicales et naturelles de Bruxelles.
Arch. Biochem.—Archives of Biochemistry.
Arch. exp. Path Pharmakol.—Archiv für experimentelle Pathologie und Pharmakologie.
Arkiv Kem. Mineral. Geol.—Arkiv för Kemi, Mineralogi och Geologi.
Australian J. Exp. Biol. and Med. Sc.—The Australian Journal of Experimental Biology and Medical Science.
Ber. chem. Ges.—Berichte der deutschen chemischen Gesellschaft.
Biochem. J.—The Biochemical Journal.
Biochem. Z.—Biochemische Zeitschrift.
Biol. Rev. Cambridge Phil. Soc.—Biological Reviews of the Cambridge Philosophical Society.
Biol. Zentr.—Biologisches Zentralblatt.
Bol. Soc. biol. Santiago Chile.—Boletín de la Sociedad de Biología de Santiago, Chile.
Brit. J. Exp. Path.—The British Journal of Experimental Pathology.
Brit. Med. J.—The Journal of the British Medical Association.
Bull. Soc. chim. biol.—Bulletin de la Société de chimie biologique.
Bull. Soc. roy. sci., Liége—Bulletin de la Société royale des sciences de Liége.
Bull. Soc. roy. sc. méd. et nat. Bruxelles—Bulletin de la Société royale des sciences médicales et naturelles de Bruxelles.
Chem. Abstr.—Chemical Abstracts.
Chem. Rev.—Chemical Reviews.
Chem. Zentr.—Chemisches Zentralblatt.
Chin. J. Physiol.—Chinese Journal of Physiology.
Cold Spring Harbor Symposia.—Cold Spring Harbor Symposia on Quantitative Biology.
C. R. Acad. Sc.—Comptes rendus hebdomadaires des séances de l'académie des sciences.
C. R. Soc. Biol. Paris.—Comptes rendus des séances de la Société de biologie et de ses filiales et associées.
C. R. Trav. Lab. Carlsberg.—Comptes rendus des travaux du laboratoire Carlsberg.
Deut. med. Wochenschr.—Deutschen medizinischen Wochenschrift.
Ergebn. Enzymforsch.—Ergebnisse der Enzymforschung.
Ergebn. Physiol.—Ergebnisse der Physiologie, biologische Chemie und experimentelle Pharmakologie.
Helv. Chim. Acta—Helvetica Chimica Acta.
Hoppe-Seyl. Z.—Zeitschrift für physiologische Chemie (Hoppe-Seyler's).

J. Amer. Chem. Soc.—Journal of the American Chemical Society.
J. Amer. Med. Assn.—Journal of the American Medical Association.
J. Bact.—Journal of Bacteriology.
J. Biol. Chem.—The Journal of Biological Chemistry.
J. Chem. Soc.—Journal of the Chemical Society (London).
J. Chem. Soc. Japan—Journal of the Chemical Society of Japan.
J. chim. phys.—Journal de chimie physique et Revue générale des colloïdes.
J. Dairy Sci.—Journal of Dairy Science.
J. Exp. Med.—The Journal of Experimental Medicine.
J. Gen. Physiol.—The Journal of General Physiology.
J. Immunol.—The Journal of Immunology.
J. Infect. Dis.—The Journal of Infectious Diseases.
J. Path. and Bact.—The Journal of Pathology and Bacteriology.
J. Phys. Chem.—The Journal of Physical Chemistry.
J. Physiol.—The Journal of Physiology.
J. Research Natl. Bur. Standards—Journal of Research of the National Bureau of Standards.
J. Roy. Statist. Soc.—Journal of the Royal Statistical Society, London.
J. Russ. Phys. Chem. Soc.—Journal of the Russian Physical-Chemical Society.
K. Danske Vidensk. Selsk. Mat-fys. Medd.—Kongelige Danske Videnskabernes Selskab, Matematisk-fysiske Meddelelser.
Kolloid-Z.—Kolloid-Zeitschrift.
Lab. Butterexportges. Valio m.b.H., Helsinki—Laboratorium Butterexportgesellschaft. Valio mit beschränkter Haftung, Helsinki.
Malys Jb.—Jahresbericht über die Fortschritte der Tier-Chemie, oder der physiologischen und pathologischen Chemie.
Med. Press & Circ.—The Medical Press and Circular.
Medd. Vetenskapsakad. Nobelinst.—Meddelanden från K. Vetenskapsakademiens Nobelinstitut.
Müllers Arch.—Müller, J. Archiv für Anatomie, Physiologie, und wissenschaftliche Medizin.
Naturwisse.—Die Naturwissenschaften.
Parasitol.—Parasitology.
Phil. Sc.—Philosophy of Science.
Physiol. Rev.—Physiological Reviews.
Phytopath.—Phytopathology.
Proc. Amer. Phil. Soc.—Proceedings of the American Philosophical Society, Philadelphia.
Proc. Indian Acad. Sci.—Proceedings of the Indian Academy of Sciences.
Proc. Nat. Ac. Sc.—Proceedings of the National Academy of Sciences of the United States of America.
Proc. Roy. Soc. London—Proceedings of the Royal Society (London).
Proc. 7th Intern. Genetical Congr. Edinburgh—Proceedings of the International Genetical Congress. 7th Congress, Edinburgh, Scotland, 1939.
Proc. Soc. Exp. Biol. N. Y.—Proceedings of the Society for Experimental Biology and Medicine.
Prod. pharm.—Produits Pharmaceutiques, Paris.
Quart. J. Exp. Physiol.—Quarterly Journal of Experimental Physiology.
Quart. Rev. Biol.—The Quarterly Review of Biology.
Sp. Rep. Ser. Med. Res. Coun., London—Medical Research Council (British) Special Report Series.
Tïb Fakültesi Mecmuasi—Bulletin de la Faculté de médecine d'Istanbul.
Tr. Faraday Soc.—Transactions of the Faraday Society.
Tr. Roy. Soc. Trop. Med. and Hyg.—Transactions of the Royal Society of Tropical Medicine and Hygiene.
Univ. Calif. Pub. Physiol.—University of California Publications in Physiology.

Virchows Arch.—Virchow's Archiv für pathologische Anatomie und Physiologie und für klinische Medizin.
Zbl. Bakt., Abt. I, Orig.—Zentralblatt für Bakteriologie, Parasitenkunde und Infektionskrankheiten, Abt. I, Originale.
Z. phys. Chem.—Zeitschrift für physikalische Chemie.

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