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The preparation of pure enzymes and virus proteins*

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The problem of the chemical nature of the substances which control the reactions occurring in living cells has been a subject of research, and also of controversy, for nearly two hundred years. Before the eighteenth century these reactions were considered as "vital processes", outside the realm of experimental science. The work of Spallanzani, Payen and Persoz, Schwann, Kühne, and finally Buchner proved that many of these reactions could take place without living cells and were probably caused by the presence of small amounts of unstable and active substances, which Kühne called "enzymes".

Berzelius, a century ago, pointed out that these enzymes were similar to the catalysts of the chemist and suggested that they be considered as special catalysts formed by the cells. This hypothesis was far ahead of its time and met with great opposition, since many workers considered that enzyme reactions differed qualitatively from ordinary chemical reactions. The work of Tamman, Arrhenius, Henri, Michaelis, Nelson, von Euler, Willstätter, Warburg, and other chemists, however, has shown that Berzelius' viewpoint was correct and enzyme reactions are now considered a special kind of catalysis which does not differ qualitatively from other catalytic reactions.

While the study of enzyme reactions made rapid progress all attempts to isolate an enzyme and so determine its chemical nature were unsuccessful until recently.

The early workers were of the opinion that enzymes were probably proteins and in 1896 Pikelharing isolated a protein from gastric juice which he considered to be the enzyme pepsin. He was not able to crystallize the protein and his conclusion as to the identity of the enzyme and the protein was never accepted. I repeated these experiments about 1920 but was unable to carry them further at that time. In the meantime a large amount of work,

* A detailed description of the results described in this paper may be found in *Crystalline Enzymes*, by J. H. Northrop, M. Kunitz, and R. M. Herriott, Columbia University Press, New York, 2nd ed., 1948. This book also contains complete references to the literature.



Fig. 1. Crystalline pepsin (x 90).
Northrop, 1930.



Fig. 2. Crystalline pepsin from 15 per cent alcohol (x 225). Northrop, 1946.

principally by Willstätter and his students, led to the conclusion that enzymes were a special class of unknown compounds, and certainly not proteins.

However, in 1926 Sumner isolated a crystalline protein from beans which he considered to be the enzyme urease. This conclusion was also received with skepticism but, as you know, it has turned out to be perfectly correct. Professor Sumner, himself, has reported this work to you.

Sumner's results encouraged me to take up the pepsin problem again and in 1930 I isolated a crystalline protein from a commercial pepsin preparation which appeared to be the enzyme pepsin. Since then five more enzymes, as well as some of their precursors, have been isolated in my laboratory. Trypsin, and its precursor trypsinogen, a polypeptide which inhibits trypsin, and a compound of this substance with trypsin, chymotrypsinogen, and three forms of chymotrypsin were isolated and crystallized by Kunitz and myself. Kunitz and McDonald isolated and crystallized ribonuclease and hexokinase. Anson crystallized carboxypeptidase, and Herriott isolated and crystallized pepsinogen. These experiments required a great deal of the most painstaking and difficult work and could not have been successfully carried out without my collaborators, Herriott, Anson, Desreux, McDonald, Holter, Krueger, Butler, and, especially, Dr. Kunitz, who possesses a real genius for handling these unstable and elusive substances.

In the meantime some twenty enzymes have been crystallized in all by other workers; all of these enzymes are proteins. The respiratory ferments of Warburg and other oxidative enzymes contain special groups other than

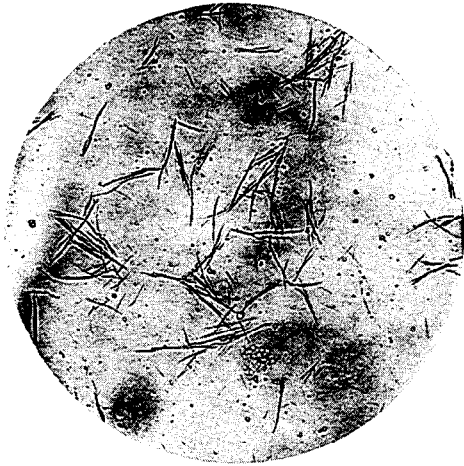


Fig. 3. Crystalline 60 per cent active acetyl pepsin (x 28). Herriott & Northrop, 1933.



Fig. 4. Crystalline pepsinogen (x 340). Herriott, 1936.

amino acids, but the purely hydrolytic enzymes, so far as is known, do not.

While this work was in progress a new controversy, very similar to the old controversy over the nature of enzymes, arose concerning the chemical nature of viruses. Dr. Stanley succeeded in crystallizing the virus of tobacco mosaic and has isolated a number of other viruses. Bawden and Pirie have crystallized the bushy stunt virus of tomatoes. I have isolated a nucleoprotein which appears to be one of the bacterial viruses, or bacteriophages. This is Dr. Stanley's field and he will report to you concerning it.

As a result of these experiments, it appears probable that all enzymes and at least some viruses are proteins. The mere fact that the preparations are crystalline proteins is not, of course, sufficient to warrant this conclusion and we have spent a great deal of time in establishing the purity of our preparations and in testing by every method available the relation between the activity and the protein.

Before discussing these results, I will describe briefly the experimental methods we have used in the isolation and crystallization of these active proteins. No one general method has been found which will lead to the isolation and crystallization of an enzyme, but certain general principles have been found to be of great assistance. In the first place large quantities of material are used, so that actual solid material is handled and not merely dilute solutions. The failure of so many early attempts to isolate enzymes was due, I believe, largely to the fact that nearly all of the work was carried out with

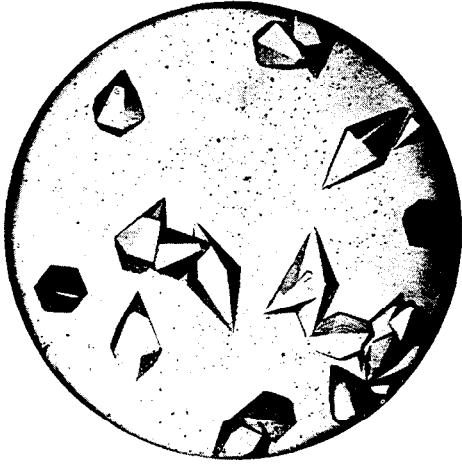


Fig. 5. Crystalline active acetyl pepsin (x 34). Herriott & Northrop, 1934.

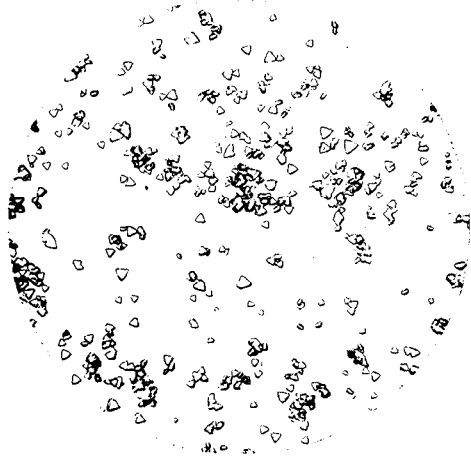


Fig. 6. Crystalline trypsinogen (x 320). Kunitz & Northrop, 1935.

dilute solutions. In the second place filtration by suction was used wherever possible since this method results in far better separation of the precipitate from the mother liquor than does the use of the centrifuge. Had Pekelharig filtered his pepsin preparation and then dissolved it in a very small amount of water, instead of centrifuging the precipitate and dissolving it in a large amount of water, I am quite sure he would have crystallized the enzyme nearly fifty years ago.

In the third place fractionation was carried out largely by the use of concentrated neutral salts in the presence of which proteins are much more stable than in dilute salt solutions.

Pepsin, for instance, decomposes at the rate of about 3 per cent a day in hydrochloric acid solution at pH 2.7 and 0° C. Even in nearly saturated magnesium sulfate solutions at 0° C, the most stable condition known, the rate is 1 per cent a day.

Trypsin at pH 8.0 and 30° C loses 90 per cent or more of its activity in a day. This reaction is exceptional since it is bimolecular so that in this case dilute solutions are more stable than concentrated ones.

Tests of purity

Solubility. There is no doubt that the crystalline materials isolated by Sum-

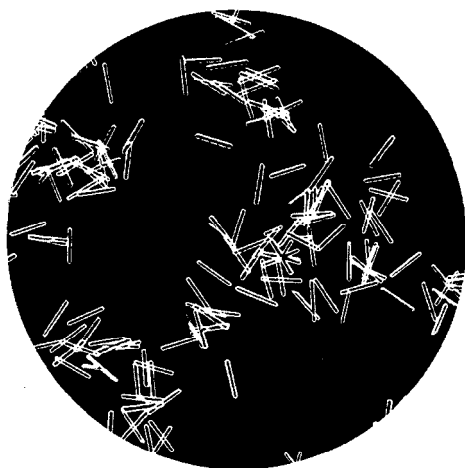


Fig. 7. Crydine trypsin ($\times 200$).
Kunitz & Northrop, 1935.

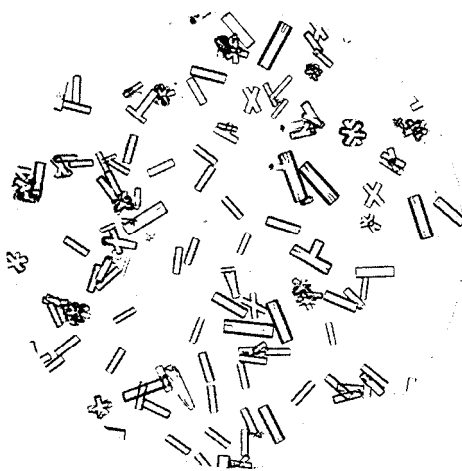


Fig. 8. Crystalline trypsin-inhibitor ($\times 225$).
Kunitz & Northrop, 1935.

ner, myself, and others are proteins but the proof that these proteins are the enzymes, themselves, is open to question owing to the difficulty of establishing the purity of a protein. In organic chemistry constant properties, composition, and melting point, through repeated fractional crystallization are considered as sufficient proof of purity. In the case of proteins, constant analysis means very little and the melting point, which is the most sensitive test, cannot be used since proteins decompose instead of melting. However, the solubility of the protein, which, from the point of view of the phase rule, is strictly analogous to the melting point, may be determined instead.

According to Willard Gibbs's phase rule, a system consisting of a single solid in equilibrium with its solution is of fixed composition. That is to say, if more of the same solid is added no change in concentration will occur. If any other substance is added, however, a change will occur. The test is exceedingly specific, even more so than the serological tests as Landsteiner and Heidelberger pointed out. It will, in fact, distinguish between optical isomers, even though the two isomers have exactly the same solubility. In addition, the concentration of the active material as well as the protein may be determined. From the experimental point of view it has the great advantage that the test may be carried out in concentrated salt solution, in which the proteins are most stable. Very few pure proteins have ever been obtained, as judged by this test. Of these, chymotrypsinogen is probably the best. Pepsin, pepsinogen, chymotrypsin, trypsin, ribonuclease, and hexokinase all give

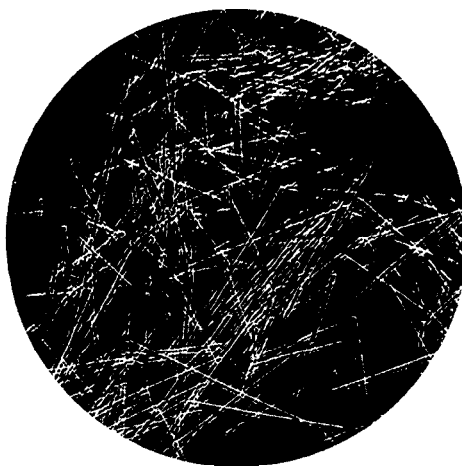


Fig. 9. Crystalline inhibitor and trypsin compound (x 370). Kunitz & Northrop, 1935. Fig. 10. Crystalline chymotrypsinogen (x 260). Kunitz & Northrop, 1934.

solubility curves closely approaching those of a single substance and as good or better, than those obtained with most other proteins.

The fact that strictly homogeneous proteins are so difficult to prepare indicates that very closely related groups of proteins, and not one particular individual, are synthesized by the organism. The recent interesting results of Pedersen and of Wyman, Rafferty, and Ingalls, who found that the proteins of young animals differ from those of old animals, confirm this explanation. It is very unlikely that an animal synthesizes the "young" protein for a certain time and then suddenly synthesizes the "old" protein and hence it is probable that the various kinds of protein are present at the same time in any one animal.

Analysis in the ultracentrifuge. The many important and striking results which Professor Svedberg has obtained with the ultracentrifuge are familiar to all of you. I need only say that it is a very valuable criterion of homogeneity in protein solution. There is an experimental difficulty in the case of the extremely unstable proteins with which we are involved in that concentrated salt solutions cannot be used owing to their high specific gravity, and some of the proteins are too unstable in dilute salt, even at 0° C, to enable the measurement to be made. Nevertheless, the enzymes pepsin (Philpot and Eriksson-Quensel), ribonuclease and hexokinase (Rothen), and the bacteriophage nucleoprotein (Wyckoff) were found to be homogeneous by this test.



Fig. 11. Crystalline chymotrypsin (x 100).
Kunitz & Northrop, 1934.



Fig. 12. Crystalline beta chymotrypsin
(x 300). Kunitz, 1937.

Electrophoresis. The elegant electrophoresis technique of Tiselius also needs no description in Stockholm. Ribonuclease, hexokinase, and chymotrypsinogen have been examined by Rothen and found to be homogeneous. Tiselius, Henschen, and Svensson studied the electrophoresis of pepsin and found the main protein component to be strictly homogeneous under a wide variety of conditions, but some inactive nitrogenous material was separated from the protein by this method.

Ågren and Hammarsten found that the enzyme migrated to the anode at pH 3.4 and to the cathode at pH 2.7. Tiselius, Henschen, and Svensson, on the other hand, found the protein migrated always to the anode, while I had found by cataphoresis experiments that the protein was isoelectric at about pH 2.4. The electrophoresis experiments were repeated by Herriott, Desreux, Rothen, and Longworth, using a specially purified pepsin preparation, and both the Tiselius, and the Theorell apparatus. The experiments showed that the various conflicting results were due to decomposition products in the pepsin preparations. In the presence of those products the enzyme has an isoelectric point at pH 2.5 to 3.0, whereas the pure protein has no determinable isoelectric point. This marked effect of hydrolysis products on the electrophoresis of pepsin had been observed many years ago by Ringer. These non-protein decomposition products may be removed partially by electrophoresis but we could find no evidence for the presence of more than one protein by this method.

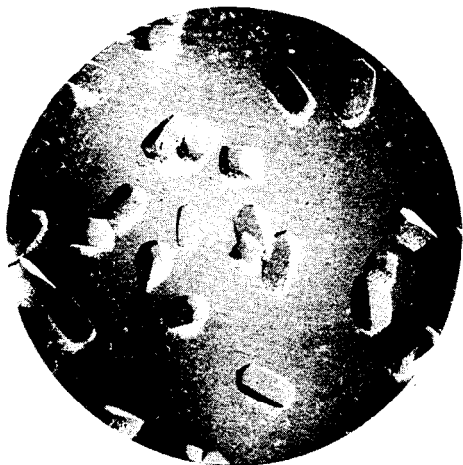


Fig. 13. Crystalline gamma chymotrypsin (x 15). Kunitz, 1937.



Fig. 14. Crystalline carboxypeptidase (x 85). Anson, 1936.

Ågren and Hammarsten were also able to separate crystalline carboxypeptidase into several components, so it appears that this enzyme has not yet been prepared in pure form.

The results of these various tests of purity warrant the conclusion, I believe, that the enzymes pepsin, trypsin, chymotrypsin, hexokinase, and ribonuclease are at least as homogeneous as any known proteins. Chymotrypsinogen is probably the purest protein so far isolated. This statement refers only to specially prepared and highly purified samples. The first crystallization usually results in a product which still contains impurities and in some cases more than one protein.

Relation of the activity to the protein by other methods

Formation of enzymes from their precursors. Pepsin, trypsin, and chymotrypsin are derived from inactive precursors. These precursors were isolated and crystallized and the formation of the active enzyme studied. The formation of pepsin from pepsinogen and trypsin from trypsinogen are autocatalytic reactions. These enzymes may therefore be "propagated", just as are bacteria. The formation of trypsin from trypsinogen may also be catalyzed by enterokinase, an enzyme of the digestive tract, or by an enzyme produced by a mold (*Penicillium*.) The formation of chymotrypsin from chymotrypsinogen

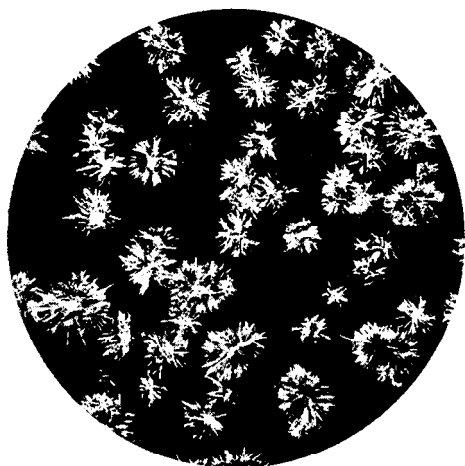


Fig. 15. Crystalline ribonuclease (x 200).
Kunitz, 1939.

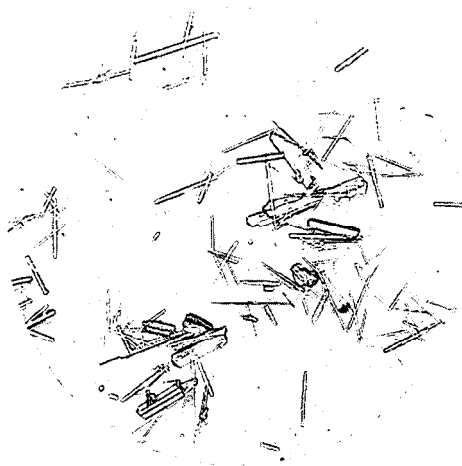


Fig. 16. Crystalline hexokinase (x 100).
Kunitz & McDonald, 1942.

is catalyzed only by trypsin, so far as is known. In all these reactions the increase in enzymatic activity is accompanied quantitatively by the appearance of the new enzyme protein which is quite different in all its properties from the original precursor. It seems to me that these results are perhaps the most convincing evidence that the enzymatic activity is actually a property of the protein molecule.

Rate of diffusion of the protein and the active substance. The relationship of the protein and the enzyme may be tested further by measuring the rate of diffusion of the preparation by means of protein determinations and also by enzyme activity measurements. The rate of diffusion was suggested by Arrhenius years ago as a means of determining the size of unknown substances. The method has been developed and used by von Euler.

Theoretically it is simple but experimentally it was difficult. These difficulties were overcome by Anson and myself, who developed a diffusion cell made of a porous disk across which diffusion takes place. This greatly accelerates and simplifies the experiment. The method is the only one, I believe, which permits the estimation of the molecular weight of unknown and impure substances.

The diffusion of pepsin, trypsin, chymotrypsin, chymotrypsinogen, and bacteriophage has been followed by this method. The results show that the protein and the active substance diffuse at exactly the same rate.

Effect of hydrolysis of the protein on the enzymatic activity. Digestion of trypt-

sin or ribonuclease with pepsin results in loss of activity which is almost exactly parallel to the loss of protein. There is no indication that the protein may be hydrolyzed without also destroying the enzymatic activity, nor that any of the hydrolysis products possess measurable activity. Similar results were obtained during the autolysis of pepsin and the digestion of bacteriophage with chymotrypsin.

Denaturation of the protein and loss of activity. The denaturation of proteins is a characteristic property and has an extremely high temperature coefficient. The rate of formation of denatured protein and the loss in enzymatic activity has been compared in the case of pepsin, trypsin, chymotrypsin, ribonuclease and hexokinase. In every case the loss of native protein is accompanied by a concomitant loss of enzymatic activity.

An equilibrium exists between native and denatured trypsin which is accurately predicted by Van 't Hoff's equation.

Reversal of the reaction results in recovery of the enzymatic activity in proportion to the recovery of native protein.

Effect of chemical changes in the protein molecule on the activity. Ketene reacts with pepsin to form a series of acetylated products. Herriott has isolated and crystallized three of these derivatives. One contains three or four acetyl groups, probably attached to the primary amino groups. This derivative has the same activity as the original pepsin. A second derivative has about ten acetyl groups and 60 per cent of the original activity, while the third preparation contains twenty to thirty acetyl groups and is inactive. There is evidence that the acetyl groups which are attached to the hydroxyl group of tyrosine are responsible for the loss of activity. This conclusion is borne out by the fact (Herriott) that iodination of pepsin results in the addition of iodine to the tyrosine groups, and also results in loss of activity.

Pepsin, as well as other enzymes, is also inactivated by mustard gas (dichlor ethyl sulfide). This substance is an excellent protein reagent. It reacts in slightly acid solution with carboxyl groups and probably with the hydroxyl group of tyrosine.

Summary

The enzymes, pepsin, trypsin, chymotrypsin, carboxypeptidase, ribonuclease, and hexokinase have been isolated and crystallized. The precursors of pepsin, trypsin, and chymotrypsin have also been isolated and crystallized. A

nucleoprotein which appears to be bacteriophage has been isolated but not crystallized.

The purity of the enzyme preparations, with the exception of carboxypeptidase, has been tested by means of solubility measurements, ultracentrifuge analysis (pepsin, ribonuclease, hexokinase, and bacteriophage), and electrophoresis (pepsin, ribonuclease, hexokinase, chymotrypsinogen). They appear to be pure proteins by all these methods.

The relation of the enzymatic activity to the protein has been tested by diffusion measurements (pepsin, trypsin, chymotrypsin, chymotrypsinogen, bacteriophage), denaturation of the protein (pepsin, trypsin, chymotrypsin, ribonuclease, hexokinase), hydrolysis of the protein (trypsin, ribonuclease, pepsin, bacteriophage), formation of the active enzyme from an inactive precursor (pepsin, trypsin, chymotrypsin), and by the formation and isolation of definite chemical derivatives of pepsin. These experiments confirm the conclusion that the enzymatic activity is a property of the protein molecule itself, and is not due to a non-protein impurity.