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CHEMICAL NATURE AND MODE OF FORMATION OF PEPSIN, TRYPSIN AND BACTERIOPHAGE[1]

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THE field of enzymes has always been a sort of no-men's land between the fields of chemistry and biology. In the early days of science reactions occurring in, or caused by, living organisms were grouped together as fermentations and were supposed to be qualitatively different from the reactions of inorganic material. In the course of the nineteenth century the work of Payen and Persoz, Schwann, Kühne and Buchner and many others showed that most of these reactions were caused by the presence of unknown substances formed by the living cells, but which were not living. These substances were called "enzymes" by Kühne. Berzelius early pointed out that these reactions were very similar to the catalytic reactions of inorganic chemistry, and the work of Tamman, Arrhenius, Henri, Michaelis, Nelson, Euler, Willstätter, Warburg and other chemists has shown that this view was correct. It is only recently, however, that enzymes have been accepted as a part of chemistry and it is very gratifying that research on enzymes should be selected for a chemical award.

The chemical nature of the enzymes themselves remained quite unknown until a few years ago. In the last eleven years a number of enzymes have been isolated and crystallized and have been found to be proteins. The hydrolytic enzymes, urease (Sumner), pepsin[2] (Northrop), trypsin,[2] chymo-trypsin[2] (Kunitz and Northrop), carboxypeptidase[2] (Anson), amylase

[1] Lecture delivered on the occasion of the presentation of the Charles Frederick Chandler Medal of Columbia University on October 27, 1937. The lecture was illustrated by lantern slides.

[2] Photographs of these preparations are reproduced in Harvey Lectures, 1934–35, p. 229.

(Caldwell, Booker and Sherman), a proteolytic enzyme from Ficis (Walti) and from papain (Balls, Lineweaver and Thompson) appear to be simple proteins, since no evidence of a prosthetic group (a group other than an amino acid) has been found, while Warburg's respiratory ferment, which is an oxidative catalyst, is a conjugated protein and contains a prosthetic group. Sumner and Dounee have recently crystallized catalase, and this enzyme also contains a prosthetic group.

It appears, therefore, that the characteristic chemical reactions occurring in living cells are caused by the presence of minute amounts of certain peculiar proteins, so that the nature and mode of action of enzymes is now fairly well established.

In the meantime, however, a new controversy over the nature of "viruses" has arisen, quite similar to the early controversy over the nature of enzyme reactions. Iwanowski and Beijerinck described the first of these active agents at about the time of Buchner's experiments. A large number of such filterable viruses were soon described, among them the bacteriophage of Twort and d'Herelle which possesses the remarkable property of dissolving bacteria. These filterable viruses increase in quantity in the presence of living cells and for this reason have been considered by many workers to be themselves living organisms.

The biological properties of bacteriophage have been determined by d'Herelle, who has shown that they are closely analogous to those of living cells.

Bordet, on the other hand, has shown that the facts may be more simply explained by assuming the autocatalytic production of phage from a normal cell constituent. It has been found recently that pepsin and trypsin, under the proper conditions, also increase, and there are, of course, many other known autocatalytic reactions so that the property of increasing in quantity or growth can not be considered a definite criterion for the presence of living cells. Stanley has isolated and crystallized a protein which appears to be the active agent of the tobacco mosaic, and the writer has obtained a nucleo protein which appears to be the bacteriophage. Some filterable viruses, therefore, are probably enzymes which possess the property of forming themselves under proper conditions.

Chemical Nature of the Enzymes

The isolation of enzymes in the form of crystalline proteins shows that they are essentially protein in character. 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 certain 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 has determined the general nature of the group so that the most important part of the structural chemistry of this enzyme is now known.

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. 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.

Isolation and Crystallization of Pepsin, Trypsin, Chymo-Trypsin and Their Precursors

Unfortunately experience has shown that no one method can be relied upon to lead to the isolation and crystallization of enzymes. The most that can be said is that they are best handled by the technique of protein chemistry and that large quantities 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 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.

Pepsin

Pepsin was isolated and crystallized from commercial pepsin preparations by precipitation with mag-

nesium sulfate in dilute acid. It has also been obtained from bovine gastric juice. Active pepsin exists only in the gastric juice and is present in the gastric mucosa as an inert protein, pepsinogen. Pepsinogen has also been obtained in crystalline form by extraction from the mucosa in slightly alkaline solution and fractionation by means of ammonium sulfate.

Pancreatic Enzymes

Digestion of proteins in the small intestine is caused by the pancreatic juice, and it was thought at first that this juice contained only one proteolytic enzyme called by Kühne "trypsin." It is now known, however, from the work of Willstätter, Waldschmidt-Leitz and others that pancreatic juice contains a series of proteolytic enzymes, erepsin, aminopolypeptidase, carboxypolypeptidase, protaminease, chymo-trypsin and probably others. Like pepsin, the enzymes in the pancreatic juice became active only after secretion and are present in the gland itself in the form of inactive precursors. Trypsin was originally isolated and crystallized from activated pancreatic extract by repeated fractionation with ammonium sulfate. The procedure was difficult, however, and a much more efficient method was worked out by which the inactive precursor of trypsin (trypsinogen)[2] was isolated from inactive acid extracts of fresh pancreas. The inactive precursor of chymo-trypsin was also isolated from such extracts, as well as a substance which powerfully inhibits trypsin activity. Anson isolated and crystallized carboxypeptidase from activated pancreatic extract and was able to show that this enzyme also exists in fresh pancreas in an inactive form. Conditions for the transformation of the inactive precursors to the active enzymes have beeen worked out and the active enzymes themselves crystallized.

Purification of Bacteriophage

A nucleo protein has been isolated from lysed staphylococci culture which has the properties of bacteriophage, *i.e.*, it increases when inoculated into a growing culture of staphylococci and eventually causes complete dissolution of the bacteria. The protein is practically homogeneous in the ultra-centrifuge and has a sedimentation constant of about 650×10^{-13} cm dym^{-1}, sec^{-1} corresponding to a molecular weight of about 200 million. The minimum quantity of the nucleo protein which can be detected is about 5×10^{-16} gm. If this quantity is assumed to be one molecule then the gram molecular weight of the substance is about 300 million gm. This is about the same order of magnitude as the molecular weight by the sedimentation velocity method. Diffusion measurements in concentrated solution show that the protein and the active agent diffuse at the same rate and give a value for the molecular volume which agrees approximately with that found by the centrifuge method. In extremely dilute solution the phage diffuses more rapidly, indicating that the phage particle may dissociate into smaller units as do the hemocyanins. Similar results have been previously reported by Bronfenbrenner.

Relation of Activity to the Protein

The preparations obtained in this way (with the exception of bacteriophage) are all crystalline proteins and may be recrystallized many times without change in properties. The most rigorous test of purity of a protein is solubility measurements, which take the place of the ordinary melting point. Such solubility determinations have been carried out on pepsin, pepsinogen, trypsin, chymo-trypsin and chymo-trypsinogen. The results show that the solubility is independent of the quantity of solid phase present and hence that the solid phase consists of only one component. This furnishes strong evidence that the activity is really a property of the protein molecule. The solubility of bacteriophage is constant in the presence of a large excess of solid but varies somewhat with smaller quantities of solid. The preparation, therefore, still contains two or more components one of which is probably an inactivated form of the other.

It may be shown, in addition, that loss in activity is accompanied by changes in the chemical properties of the protein. Thus, when phage or the enzymes are heated the activity is lost and at the same time denatured protein is formed. In the case of trypsin and chymo-trypsin the reaction is rapidly and completely reversible so that these enzymes exist in equilibrium with an inactive, denatured form. This equilibrium is accurately expressed by van't Hoff's equation connecting the heat of reaction and the effect of temperature on the equilibrium constant and gives a value for the latter of 67,000 calories per mol. In the case of pepsin the denatured pepsin may also be reconverted to native active pepsin. The equilibrium between native and denatured trypsin is also affected by the pH of the solution, and this accounts in part for the effect of the pH on the activity of the enzyme.

The relation between protein and activity may be further tested by digesting the protein with another enzyme. If trypsin or chymo-trypsin is hydrolyzed by pepsin there is a loss in activity corresponding to the destruction of the protein. Digestion of phage with chymo-trypsin leads to the formation of insoluble inactive protein. If the rate of diffusion of the protein and the activity is determined it is found that the diffusion proceeds at the same rate when followed by either method. The active molecule and the protein molecule therefore diffuse at the same rate.

Gates showed that the efficiency of various wave-

lengths of ultra-violet light on the inactivation of pepsin is the same as that for the relative absorption at various wave-lengths by pepsin solutions. The result indicates that each quantum absorbed by a protein molecule inactivates the molecule. Similar results were obtained with bacteriophage.

These experiments indicate strongly that the activity is a property of the protein molecule itself but unfortunately furnish no information as to the chemical structure in the molecule responsible for its activity. Owing to the extreme complexity of the protein molecule it is impossible, at present, to determine the structural configuration of the whole molecule, but some information in this connection has been obtained by studying the known changes in the molecule which affect the activity. The most definite of these reactions is the formation of acetyl derivatives of pepsin. Two crystalline[2] acetyl derivatives have been obtained by the action of ketene in aqueous solution at pH 5.0–6.0. The first compound contains 3 or 4 acetyl groups attached to the primary amino groups. It has the same activity as the original pepsin. A second derivative containing 7 acetyl groups has also been obtained. It has 60 per cent. of the activity of the original pepsin. On standing in acid this compound loses half of its acetyl groups and at the same time regains its original activity. Those acetyl groups, which decrease the activity and are removed by standing in acid, are attached to the hydroxyl group of tyrosine. Substitution of iodine for the hydrolyl groups of tyrosine also decreased the activity. These results show that the free amino groups of pepsin are not necessary for the activity, but that the phenolic groups of tyrosine are essential to the enzymatic activity.

Formation of Enzymes from Their Inactive Precursors

Further knowledge concerning the chemical structure responsible for the enzymatic activity of the molecule has been obtained by a study of the formation of the active enzymes from their inactive precursors.

Chymo-trypsin from chymo-trypsinogen[2]*:* Heidenhain and Kühne showed that freshly secreted pancreatic juice was inactive but becomes active when mixed with the secretion of the small intestine. It was assumed, therefore, that the enzymes were present in the pancreas in an inactive form. The formation of the active enzymes from inactive pancreatic juice or from inactive extracts of pancreas has been extensively studied, but the literature is surprisingly confused and contradictory. The course of the reaction has been described as catalytic, stoichiometric and autocatalytic. These contradictory results were obtained with crude preparations and under the conditions of the various experiments are probably all correct. During the course of the work on the isolation of the inactive form (chymo-trypsinogen) of chymo-trypsin and of the inactive form (trypsinogen) of trypsin we also obtained a number of apparently inexplicable, contradictory results. These varying results were eventually found to be caused by the presence in amorphous chymo-trypsinogen preparations of more or less trypsinogen and of a substance[2] which inhibits trypsin. Small amounts of these impurities influence the activation very markedly and until the protein was crystallized the activation experiments could not be repeated with certainty. When the chymo-trypsinogen has been recrystallized several times it is freed from inhibitor and trypsinogen, and its activation by trypsin becomes a perfectly definite and reproducible catalytic reaction. The rate is proportional to the trypsin concentration and also to the chymo-trypsinogen concentration. The rate of activation by trypsin is affected by the acidity of the solution in the same way as is the rate of digestion of other proteins by trypsin so that the reaction appears to be analogous to ordinary protein hydrolysis. There is one striking difference, however, in that, so far as we have been able to determine, nothing is split from the chymo-trypsinogen molecule, although there is an increase of 5 amino groups per mol. The formation of the active form from the inactive form of chymo-trypsin, therefore, may be due to the opening of a peptide ring. The isoelectric point is shifted from about pH 5.0 to about pH 5.4 and there is a slight decrease in optical rotation, but no other chemical differences have been detected so far. The elementary analysis, the tyrosine plus tryptophane content, the diffusion coefficient and molecular weight of the enzyme and of its precursor are the same within the experimental error. The x-ray defraction patterns of the two substances, according to Wyckoff, are quite different and show no more resemblance than would be expected between any two different proteins. Ten Broeck has shown that the two proteins may be distinguished by means of the anaphylactic test.

In addition to the activation by trypsin, very slow activation occurs in slightly acid solution. The activation is not accelerated by pepsin, chymo-trypsin itself, enterokinase or trypsinogen.

Trypsin from trypsinogen: As in the case of chymo-trypsinogen the activation of trypsinogen changes markedly after crystallization. Amorphous preparations are stable in solution and can only be activated by the addition of kinase (an extract of the small intestine), by large quantities of trypsin or by the addition of concentrated magnesium or ammonium sulfate solutions. After crystallization, however, the protein becomes transformed very rapidly into the active enzyme as soon as it is dissolved in neutral solution. This change in behavior is due to the fact

that amorphous preparations contain traces of a substance which inhibits trypsin and so prevents the autocatalytic reaction. As soon as this inhibitor is removed by crystallization the most minute trace of trypsin starts the activation reaction, which then proceeds autocatalytically. 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 chymotrypsinogen or hydrolysis of other proteins by trypsin. At pH 5.0 and 8° C. the value of the constant is 14.6, *i.e.,* with unit concentration of trypsinogen the active trypsin would increase 14 times per hour. This reaction evidently has a formal resemblance to bacteria growth curves. The addition of trypsin inhibitor to such solutions causes the production of a long lag period, and the curves obtained under these conditions are very similar to the usual bacterial growth curves. As in the case of chymo-trypsin there is no evidence for the splitting off of any part of the molecule during this activation reaction nor is there any marked change in chemical composition so that the change from the inactive protein to the enzyme is probably due to some internal rearrangement in the structure of the molecule. However, the work on this question is still in the preliminary stage. The autocatalytic nature of this reaction was correctly described by Vernon but subsequently denied by Bayliss and Starling and others.

Kunitz has recently found that trypsin is formed by trypsinogen at pH 5.0 by a proteolytic enzyme secreted by a mold (Penicillium). Trypsin is inactive at this pH, so that the activation curve is no longer autocatalytic but simply logarithmic as is the activation of chymo-trypsin. Trypsin obtained in this way is identical with that formed by autocatalytic activation. The mold enzyme, therefore, must attack the molecule at the same place as does trypsin.

Pepsin from pepsinogen: Langley showed that pepsin existed in gastric mucosa in a form which differed from the active enzyme in that it was much more resistant to alkali. Herriott has recently succeeded in isolating and crystallizing this substance. It has no proteolytic activity, but in slightly acid solution becomes converted into active pepsin. The reaction at pH 4.65 is autocatalytic and hence is caused by pepsin itself. About 15 per cent. 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. The structure responsible for the species specificity of the enzyme therefore is present in the precursor.

Kinetics of the formation of bacteriophage: Bacteriophage, in common with the other viruses, possesses the property of increasing in the presence of living cells. In the case of bacteriophage the phenomenon may be quantitatively described under certain conditions by assuming that the percentage increase in the concentration of bacteriophage is proportional to the percentage increase in the number of bacteria per cc and that when the ratio of phage to bacteria passes a certain critical value the bacteria dissolve.

Under the usual conditions no increase in phage occurs without increase in bacteria, and it was thought at first that the multiplication of bacteria was a necessary condition for the increase of phage. Krueger has recently shown that the phage does increase under certain conditions without a corresponding increase in the number of bacteria. The formation of phage is, therefore, formally at least, analogous to the autocatalytic formation of trypsin from trypsinogen. The analogy extends even to the presence of a phage inhibitor in bacterial suspensions (Burnett, Levine) corresponding to the trypsin inhibitor in crude trypsinogen solutions. This inhibitor is responsible for the difficulty encountered in producing autocatalytic activation of trypsin in crude trypsinogen solutions. Krueger has recently shown that this phage inhibitor also interferes in the production of phage in cell free extracts and that when the inhibitor is removed a small increase in phage may be noted in such cell free extracts.

The assumption that phage produces itself autocatalytically from a precursor present in normal cells appears to fit the facts at present. This assumption is far simpler than the complicated series of assumptions involved in the idea that the phage is a living organism and avoids the difficulty of accounting for the necessary energy, if it is assumed that the phage synthesizes itself from simpler compounds.

OBITUARY

EDWARD LEAMINGTON NICHOLS

EDWARD LEAMINGTON NICHOLS was born in Leamington, England, on September 14, 1854, of American parents. After his graduation from Cornell in 1875 he studied in Leipzig, Berlin and Göttingen; and from the latter university received the degree of doctor of philosophy in 1879. During the year 1879–80 he held a fellowship in the Johns Hopkins University and in the following year was one of the assistants of Edison in his famous Menlo Park Laboratory. In 1881 he became professor of physics and chemistry at Central University in Kentucky; in 1883, professor of