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A CHEMICAL STUDY OF ENZYME ACTION[1]

IN making up the list of papers to be presented at this meeting to-day, it was stated that the intention was to "get at the fundamental things in enzyme activity." Since the chemical nature of an enzyme is as fundamental for the understanding of an enzyme action as any other factor, I shall present some results obtained during the last six years bearing on this question.[2] It will not be necessary to give a definition of enzymes here or to present a classification of enzyme actions. This has been done repeatedly and it would appear that at present nothing essential can be added in this respect. The question will be taken up as a chemical problem. Certain definite chemical changes may be accelerated under definite conditions; certain products obtained from living organisms have the property of accelerating these changes; these accelerations can be controlled within limits by altering the conditions. The problem in its simplest terms is the study of the chemical nature of these products of animal or plant origin which accelerate the changes. At the same time, influences physical in nature, such as the solvent and the colloidal properties of the materials must not be lost sight of, as they undoubtedly play a part in modifying the velocities of the reactions.

[1] Presented at the meeting on "Enzymes and their Behavior" before the Division of Biological Chemistry, American Chemical Society, Boston, September 12, 1917.

[2] The work was published in a series of papers in *J. Am. Chem. Soc.*, 1912–15, and in *Proc. Nat. Acad. Sci.*, 1, 136 (1915), 2, 557 (1916); *J. Biol. Chem.*, 31, 97 (1917).

Since enzymes manifest their actions by increasing the velocities of chemical reactions, a large amount of work has been done in studying the kinetics of such reactions. The actual results obtained from such studies in so far as light has been thrown on the chemical nature of enzymes has been disappointingly meager. In fact the results which might be expected from such studies have been in large measure unsatisfactory. This may be shown by a brief survey of some of the work on the kinetics of invertase action, to which, from this point of view, more attention has been paid than to any other enzyme action. Invertase, as is well known, hydrolyzes cane sugar to form glucose and levulose. O'Sullivan and Tompson[3] in 1890, as a result of the study of the kinetics of this reaction, concluded that the reaction was of the first order, the velocity being proportional to the concentration of the cane sugar. Duclaux[4] in 1898, Brown[5] and also Henri[6] in 1902, found that the velocity of this reaction was not of the first order as shown by the lack of constancy of the velocity coefficients. Henri[7] suggested in 1905 that because of the colloidal nature of enzymes, the reaction belongs to a two-phase system to which the simple mass law is not applicable in the given manner. Hudson[8] in 1908 as a result of some new work in which the mutarotation of the invert sugar was taken into account, found that the hydrolysis of cane sugar in the presence of invertase gave velocity coefficients that were constant when calculated by the unimolecular formula. He therefore claimed to have confirmed the conclusions of O'Sullivan and Tompson. Michaelis and Menton[9] in 1913 disagreed with Hudson in attempting to express the velocity of the reaction as a simple logarithmic function of the sugar concentration and elaborated the view of Henri of the two-phase system and formation of an intermediate compound. Bayliss[10] in 1911 developed the view of such intermediate compounds as adsorption compounds and concluded that the rate of enzyme action was a function of the amount of adsorption compound in existence at any particular time. Nelson and Griffin[11] in 1916 developed the two-phase system view of invertase action and in 1917, as a result of an extended series of experiments, Nelson and Vosburgh[12] summarized and stated clearly the present status of the problem of the kinetics of invertase action. Their conclusions may be stated briefly as follows:

I. The velocity of inversion is directly proportional to the concentration of the invertase.

II. The velocity is nearly independent of the concentration of the cane sugar in the more concentrated sugar solutions, while in very dilute sugar solutions the velocity increases with increase in concentration of the substrate and finally reaches a maximum.

III. The results obtained agree with the heterogeneous reaction view and contradict the claim that the kinetics of invertase action conform to the unimolecular law for homogeneous reactions.

[3] O'Sullivan and Tompson, *J. Chem. Soc.*, 57, 834 (1890).

[4] Duclaux, *Ann. Inst. Pasteur*, 12, 96 (1898).

[5] Brown, *J. Chem. Soc.*, 81, 375 (1902).

[6] Henri, *Z. Physik. Chem.*, 39, 215 (1902).

[7] Henri, *Z. Physik. Chem.*, 51, 19 (1905).

[8] Hudson, *J. Am. Chem. Soc.*, 30, 1160, 1564 (1908).

[9] Michaelis and Menton, *Biochem. Z.*, 49, 333 (1913).

[10] Bayliss, *Proc. Roy. Soc. London* (*B*), 84, 90 (1911).

[11] Nelson and Griffin, *J. Am. Chem. Soc.*, 38, 1109 (1916).

[12] Nelson and Vosburgh, *J. Am. Chem. Soc.*, 39, 790 (1917).

IV. Adsorption is one of the controlling factors in the kinetics of invertase action, and the velocity of inversion curve has the same general shape as adsorption curves, as suggested by Henri.

This brief review will show the uncertainty of the conclusions from the results obtained in the study of the kinetics of one of the most carefully measured of enzyme actions. The factors controlling the velocity of this reaction are just beginning to be cleared up, the simple earlier views being incomplete.

An unsuccessful attempt to formulate the kinetics of enzyme action in a comparatively simple way may be mentioned. The hydrolysis of urea to form ammonia and carbon dioxide was used by D. D. van Slyke[13] to develop a general theory of the kinetics of such enzyme actions based upon the assumption of an intermediate compound between enzyme and substrate. Unfortunately, in the development of the equations a further assumption was introduced which limits their validity and applicability to definite conditions which are realized only in special cases.[14]

The study of the kinetics of enzyme action has not, therefore, led to any results with regard to the chemical nature of enzymes, even in the simplest cases of chemical changes. Practically all enzymes are colloids, and when the substrate also is a colloid, as in the action of a protease on a protein, it is obvious that the conditions are complicated to such an extent that a quantitative study of the kinetics of such a reaction appears to be almost hopeless, although valuable qualitative results may be obtained.

The study of the chemical nature of enzymes is complicated in most cases by reason of the complexity of the substances whose changes they accelerate. This difficulty can be obviated for a few of the enzymes. For example, the lipases and esterases accelerate the hydrolysis of fats and esters. While the mechanism of the hydrolysis of an ester to form acid and alcohol in the absence of lipase is not known definitely, still the compositions and properties of the initial and final products undergoing the enzymatic change are known. This eliminates, partly at any rate, one of the unknown factors of the enzyme problem, and is the main reason for studying lipase in connection with the question of the chemical nature of the active catalyst, the enzyme.

Practically all enzymes are colloids or are intimately associated with substances having colloidal properties. Furthermore, in a large number of cases, it seems that the enzyme is associated with protein matter, either as an essential part of the protein molecule, or accompanying it in such a way that separation has not yet been effected. Among the enzymes which chemically show the characteristics of proteins may be mentioned the amylase obtained by Sherman,[15] proteases and lipases. On the other hand, the invertase described by Nelson[16] is a carbohydrate phosphoric acid complex containing about one per cent. of nitrogen in the form of protein.

These facts make it evident that for the case of lipase, to use a specific example, the isolation of the enzyme in a pure state is a phase or part of the problem of the isolation of a pure protein, since in the separation of the active lipase from inactive material present with it, the resulting bodies approach more and more nearly in proper-

[13] D. D. Van Slyke and G. E. Cullen, *J. Biol. Chem.*, 19, 146 (1914).

[14] *J. Biol. Chem.*, 28, 389 (1917).

[15] Sherman and coworkers, *J. Am. Chem. Soc.*, 1912–1917.

[16] Nelson and Born, *J. Am. Chem. Soc.*, 36, 393 (1914).

ties and composition those which are generally taken to typify proteins. In the problem of isolating pure proteins, it has been possible by careful treatment to obtain bodies having the same properties at different times. This is somewhat different from obtaining a pure protein possessing the same properties as when present in living matter. The operations involved in such isolations are always sufficient to change the properties of the protein to some extent. The problem of isolating a pure lipase, for example, must wait therefore for the solution of the problem of the isolation of proteins possessing the properties which they exhibit in living matter, using the term living to include also matter showing the actions of enzymes.

If, therefore, there is little hope at present of isolating a pure enzyme, considering also the colloidal nature of the material with which it is necessary to work, there is a possibility of attacking the problem in a somewhat different way. An enzyme, as a rule, accelerates a more or less specific reaction or group of reactions. Considering the very complex nature of the protein or other molecule which includes the enzyme, or with which the enzyme may be associated, and the more or less specific reaction which it accelerates, it would appear as if some definite grouping in the complex enzyme molecule were responsible for a given enzyme action. Although this is an assumption there is considerable ground for making it, considering the views held at present with regard to the probable mechanism of the reaction of ester hydrolysis, and the complex nature of the protein enzyme molecule compared with the comparatively simple ester, acid, alcohol molecules involved in the catalyzed reaction. At any rate, this is the view upon which the work on the chemical nature of enzymes was based; namely, that part of the complex molecular grouping of the protein material is responsible for a given action. The problem therefore resolves itself into a study of the chemical nature of this grouping.

The colloidal properties of the protein and other molecules as a whole also influence the rates of reaction, especially with insoluble fats in the case of lipase, for example. Emulsifying agents also play a part, but it appears from the experimental evidence available that such agents do not cause enzyme action in the absence of a definite enzyme grouping, while, on the other hand, enzyme action occurs even without the emulsifying or other agent, though perhaps not to as great extent as in their presence.

The main factor, therefore, may be taken to be the chemical grouping, the physical and other properties modifying to a greater or less extent the typical action of the active enzyme grouping though not changing its nature.

The point of view has been presented from which the study of the chemical nature of enzymes was developed. As stated before, the experimental work was done for the most part with lipase because of the better known properties of the substrate and its reaction products. The greater part of the lipase work was carried out with preparations from castor beans, although other sources were also used. There has been a general tendency in the study of enzyme actions to attempt to attain conditions under which the enzyme would show a maximum action. This method of studying the problem is likely to introduce a number of new complicating factors, so that it was considered that if the action was due to some definite grouping, a study of the factors which caused a loss of the action

might aid in throwing light on the nature of the grouping. A systematic study of the factors which caused inactivation of the esterase and lipase was therefore undertaken. The results were presented in detail elsewhere.

Inactivation of the lipase and esterase preparations was brought about by acids, bases, neutral salts, alcohols, acetone, esters and heat.

The different ways in which these preparations may be inactivated make it appear at first sight as if different reactions occur in the inactivations. If, however, a definite chemical group is responsible for a definite enzyme action, it might perhaps be more reasonable to assume that inactivation follows a definite reaction. The preparations used were essentially protein in character. There was no evidence that a dehydration, or loss of the elements of water, caused inactivation. Some of the reactions indicated that a possible hydrolysis might be a cause of inactivation. With proteins, hydrolysis is generally taken to occur with the —CO—NH— group, the peptide linking, which goes over into the carboxyl and amino groups. Experiments with all the inactivations in no case showed an increase in the formol titration such as would be expected in this reaction, and, therefore, makes the assumption of such a hydrolysis improbable. Coagulation of the material accompanied some of the inactivations. This physical change alone does not appear satisfactory as an explanation; some change in chemical structure unquestionably must accompany or produce the physical phenomenon. Furthermore, the lipase material in suspension in water showed the same activity as in 1.5 normal sodium chloride solution when tested immediately.

The explanations of the chemical changes accompanying inactivation so far suggested are not satisfactory. The reagents used are simple. It is difficult to conceive of a very deep-seated chemical reaction taking place under so many different conditions, none of a complex nature. The only chemical change which appears probable under these conditions is that involving a simple rearrangement within the molecule, such as a tautomeric change involving the change in position of a hydrogen atom. In considering the structure of proteins it is evident that such a rearrangement is possible in the peptide linking.

The hypothesis to be suggested is that the active grouping of the esterase and lipase preparations is of the enol-lactim structure, —C(OH)=N—, the specificities being dependent in part upon the groups combined with the carbon and nitrogen, and that inactivation consists primarily in a rearrangement to the keto-lactam group, —CO—NH—.

This hypothesis was tested in several different ways. It has been found that in tautomeric substances, the presence of alkali in solution favors the enol form of compounds showing such tautomerism, while acid favors the existence of the keto form. The hydrolytic actions of some simple dipeptides on esters at different hydrogen-ion concentrations would, therefore, be evidence bearing on this point, the alkaline solutions presumably favoring the enol-lactim structure. In order to find the actions exerted by the amino-carboxyl groups of the peptide, the hydrolytic actions of a number of amino acids on different esters were determined at the same hydrogen-ion concentrations. The actions of the dipeptides and amino acids were also measured with the amino-carboxyl groups actions masked by the hydrogen of the carboxyl group being replaced by the ethyl group,

and also by testing compounds such as hippuric acids, which do not contain an amino group.

In these compounds, it is possible that the equilibrium between the keto-lactam enol-lactim forms might be changed rapidly if the conditions were changed slightly. A more stable substance was therefore studied from this point of view. Imido-esters, as shown by the formula (*a*), possess the enol-lactim structure in which the hydrogen atoms may be substituted by organic radicals. The hydrolytic actions on esters of ethyl imidobenzoate (*b*) at different hydrogen-ion concentrations and various conditions were measured.

$$\underset{(a)}{R-C(OR')=NR''}, \qquad \underset{(b)}{C_6H_5-C(OC_2H_5)=NH}$$

Finally, in order to reproduce the conditions and properties of naturally occurring lipases as far as possible, a number of different proteins were treated with alkali for the purpose of producing an enol-lactim grouping in the peptide linking if this were possible, then neutralized to different hydrogen-ion concentrations and the hydrolytic actions tested on a number of different esters.

As the data obtained have been presented in detail in the papers referred to, they will not be repeated here. It may be stated that the assumption with regard to the active grouping has been borne out by the experimental facts with the different series of compounds. Especially interesting are the ester hydrolyzing substances obtained by the action of alkali on a number of proteins.[17]

It must be emphasized that no direct conclusive evidence is presented as to the actual chemical configuration of the active lipase grouping. The steps in the reasoning may be summarized as follows:

Inactivation (and therefore also activation) is assumed to be due to a tautomeric rearrangement whose possible nature is indicated. Simple substances possessing such structures show the actions and some other properties of naturally occurring lipases present in protein materials. Inactive proteins treated in such a way as to produce the supposedly active grouping show ester-hydrolyzing properties.

Whether it is possible to go much beyond this in the present state of the knowledge of the chemical nature of proteins and the changes they undergo with simple treatment, is an open question. However, one possible line of development bearing directly upon the present problem may be indicated.

The equilibrium in solution between the tautomeric forms of acetoacetic ester, and also of other substances, depends to a great extent upon the solvent.[18] This suggests that with the enol-lactim keto-lactam tautomerism in proteins, the colloidal properties of the protein material may well exert an influence on the grouping comparable to the effects of the solvent on the tautomerism of acetoacetic ester just mentioned. The decreased stabilities or increased rates of inactivation of enzyme preparations when separated to a greater or less extent from colloidal and other matter not connected with the actions may then parallel the actions of the solvents on the equilibria between the tautomeric forms of acetoacetic ester.

In the development of the hypothesis regarding the active grouping in lipase actions, the experimental work and discussion was limited almost entirely to the pep-

[17] F. H. Frankel, *J. Biol. Chem.*, December (1917).

[18] K. H. Meyer and F. G. Willson, *Ber.*, 47, 832 (1914).

tide linking. It is evident, however, that such tautomeric structures, enol-lactim and keto-lactam, may be present in other groupings, and the results of this investigation in no way limit the lipolytic activity to the peptide linking. In view of the complexity of the protein molecule, it is highly probable that such tautomeric groupings are present in combination with other groups and that the specificities of the actions are in part dependent upon these.

It must be admitted that the treatment of proteins with alkali to form active substances is rather drastic. Unquestionably, simpler methods, comparable to those taking place in nature, will be found to produce the same effects. The fact that dilute alkalis inactivated the castor bean globulin lipase, while a certain higher concentration of alkali produced an ester-hydrolyzing substance from the inactive globulin preparation, indicates that differently placed groups in the molecule were involved in these two changes.

In how far the conclusions reached with lipase may be applied to other enzymes is a question. It seems probable, because of the comparatively simple treatments by which most enzymes may be inactivated, that with them also a simple rearrangement or perhaps tautomeric change is connected with loss in activity. There is, however, no reason to suppose that the active grouping is the same for all enzymes. Each enzyme must be studied separately and conclusions as to the chemical nature of one active enzyme grouping can not without further evidence be applied to an enzyme grouping connected with a different action. The work described with lipase has given a definite point of view, if nothing further, from which the study of this enzyme may be continued, and it seems probable that similar systematic studies with other enzymes would yield interesting and valuable results.

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THE CONSERVATION OF WHEAT

THE U. S. Food Administrator has done, and is doing, a splendid work in the conservation of wheat, notwithstanding the many obstacles which he has had to overcome. He has met, with wonderful ability and success, one of the most difficult situations of the ages. At times he has been harassed by self-appointed experts and advisers who have often hindered when they should have helped in the conservation of food, particularly of wheat. This is a time to put aside hobbies and pet theories and look the facts squarely in the face.

One of the suggestions frequently offered to make the wheat crop go farther is to mill it so as to include with the flour a portion, or all, of the wheat by-product, and then to require universal use of such a flour. The present ruling of our Food Administrator, permitting the manufacture of whole-wheat flour and also of flour that contains approximately 75 per cent. of the wheat kernel, rests upon a sound, economic basis. The usual argument of the whole-wheat flour advocates is that the product is more nutritious, and that the wheat can be made to go farther when it is milled so as to include a part or all of the by-product.

The March 8 (1918) issue of SCIENCE contains an article: "Shall We Eat Whole Wheat Bread?" by L. A. Dutcher, in which reference is made to my work on the nutritive value of breads. This article follows the usual trend of the whole-wheat bread advocate. I would make no mention of the article if it were not for the fact that I believe attention should be called to certain omissions, a misquotation and a selective and unusual use of data from my publications that might lead to erroneous conclusions, particularly as one of the bulletins quoted, Minn. No. 54, is no longer in print, or available for distribution.