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INFLUENCE OF TRYPTOPHAN AND OTHER AMINO ACIDS UPON THE STABILITY AND ENZYMIC ACTIVITY OF PANCREATIC AMYLASE

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In previous investigations of the starch-splitting enzymes, and particularly of pancreatic amylase, we have found that the enzymic activity is influenced by amino acids in such a manner as to indicate that the enzyme is of protein nature or contains protein as an essential constituent, and that the favorable influence of added amino acids consists, in part at least, in retarding the hydrolytic destruction of the enzyme.[1]

To guard against the possibility of confusion it should perhaps be explicitly stated here that the favorable influence of amino acids upon enzymic hydrolysis of starch which we have reported and discussed in this and in previous papers cannot be attributed to diastatic action exerted by the amino acids themselves. This possibility was considered by us at the outset and has been definitely eliminated in all of our work by frequent "blank" experiments in which starch has been heated with various amino acids under the conditions of our experiments but in the absence of enzyme. Since all of our work has been controlled by such blank experiments, observations such as those of Biedermann[2] (who, working under conditions quite different from ours and employing enormously greater concentrations of amino acid, believes that he has found some evidence of a diastatic effect of the amino acid itself) plainly have no application to the interpretation of our work. If any amino acid exerts a diastatic action under the conditions of our experiments, such influence is negligible as compared with that which we are studying and is entirely excluded by our "blank" tests from playing any part in the explanation of the effects with which we are concerned.

A large proportion of the amino acids known to result from the hydrolysis of typical proteins have been tested, and without exception these have shown a favorable influence upon the saccharogenic activity of the amylase, that is, its activity as measured by the rate of production of reducing sugar from starch.[1] In tests of the amyloclastic activity of the enzyme, that is, its activity as measured by the rate at which it changes starch into substances which do not give a blue or violet color with iodine, added amino

[1] (a) Sherman and Walker, THIS JOURNAL, **43**, 2461 (1921); **45**, 1960 (1923). (b) Sherman and Caldwell, *ibid.*, **43**, 2469 (1921); **44**, 2926 (1922). (c) Sherman and Naylor, *ibid.*, **44**, 2957 (1922).

[2] Biedermann, *Arch. Néerland. Physiol.*, **7**, 151 (1922).

acids in most cases showed similarly favorable effects; but a few, of which tryptophan is typical, did not appreciably influence the amyloclastic action as shown in our usual method of testing which involves an experimental period of 30 minutes at 40°.[1b]

That tryptophan (and lysine) should aid the saccharogenic but apparently not the amyloclastic action of the enzyme was of distinct theoretical interest. If these observations stood alone they would suggest the presence of two different enzymes in the amylase preparations, one splitting starch to dextrin and the other splitting dextrin to maltose; but, as we have shown in previous papers,[3] the ratio of amyloclastic to saccharogenic activities of the purified amylase preparations is practically the same as in the original pancreatin from which they are made, and it is highly improbable that two distinct enzymes would pass through all the operations of the purification process and both remain in the small fraction which constitutes the final product, and in unaltered quantitative relations to each other. The experiments described below give additional evidence that we are here dealing not with two enzymes but with one enzyme acting through two (or more) stages. It is the first (amyloclastic) stage of the enzyme's activity which did not appear to be influenced by lysine and tryptophan,[1b] while the later (saccharogenic) stage was favorably affected.[1c] According to our theory that the favorable influence of the amino acid upon the enzyme is at least largely due to retarding the hydrolysis of the enzyme by the water in which it is dispersed, it is evident that the amino acid will be effective only if it checks the hydrolysis of the enzyme protein at a point earlier than that at which the enzymic activity is lost. If certain amino acids begin to be liberated early in the series of hydrolytic changes through which the enzyme is gradually inactivated, while other amino acids are liberated only at later stages, then the former and not the latter may be expected to conserve the enzyme in the earliest stages of its activity while both would favorably influence the later stages. Hence, our results with lysine and tryptophan indicated that these two amino acids are so bound in the enzyme molecule as not to be liberated by hydrolysis until (under the conditions of our previously published experiments) the enzyme has largely completed the amyloclastic stage of its action, whereas the saccharogenic activity is chiefly exerted later and it is in this later stage of the activity of the enzyme that the favorable influence of the added lysine and tryptophan first appeared. This view suggested the further possibility that a favorable effect of tryptophan (or of lysine) upon amyloclastic action might be found if tests were made under conditions such as to induce a more extensive hydrolysis of the enzyme, either by exposing the enzyme to the action of water for a longer time, or at a higher temperature, or both. The present paper summarizes

[3] Sherman and Schlesinger, THIS JOURNAL, **35**, 1784 (1913); **37**, 1305 (1915).

the results of new experiments planned in both of these ways, which show that under such conditions the favorable influence of tryptophan upon amyloclastic action becomes clearly demonstrable, thus verifying and developing further our view of the chemical nature of the enzyme,[4] and the relation of added amino acids to its action.

The new experiments cover an extensive study of both the amyloclastic and the saccharogenic activities of the pancreatic amylase including (1) determinations of the rate of inactivation of the enzyme upon standing for several different time intervals at each of the temperatures 10°, 25° and 40° in the absence of substrate in aqueous solutions with and without the addition of the salts regularly used in our ordinary determinations of pancreatic amylase activity (sodium chloride 0.05 *M*, and disodium phosphate 0.0005 *M*); (2) quantitative studies of the activities of the enzyme after standing for various time intervals at 40° and 50° in the absence of substrate in aqueous solutions of P_H 6.9 containing the above-mentioned salts with and without the addition of the typical amino acids, glycine or tryptophan; (3) quantitative measurements of the activities of the enzyme in the hydrolysis of starch at 40° and at 50° when acting upon starch in the presence of optimum concentrations of chloride, phosphate and hydrogen ions, with or without the addition of glycine or of tryptophan. These two amino acids were chosen for these experiments because previous work had shown that glycine is typical of the many amino acids which influence favorably both the amyloclastic and saccharogenic activities of the enzyme when tested in the usual experiments of 30 minutes' duration at 40°, whereas tryptophan is typical of the few which, as above explained, have appeared in our earlier experiments to show favorable influence only upon the saccharogenic action.

Typical experiments of each of the three series just mentioned are next described.

Experimental Part

The general method of procedure may be divided into two main parts: (1) the preparation and treatment of the enzyme solutions to be tested for activity; (2) the measurements of the enzymic activities of these enzyme solutions.

In the present investigation the preparation and treatment of the enzyme solutions was varied in three different ways as described under the headings of the first, second and third series below.

The methods of measuring the amyloclastic and saccharogenic activities of the enzyme solutions were the same in all cases except for the use of different times and temperatures in the third series only as there

[4] Sherman and Schlesinger, THIS JOURNAL, **33**, 1195 (1911); **34**, 1104 (1912); **37**, 643 (1915). Sherman and Gettler, *ibid.*, **35**, 1790 (1913). Sherman and Wayman, *ibid.*, **43**, 2454 (1921). Sherman, *Proc. Nat. Acad. Sci.*, **9**, 81 (1923).

described. In all except the specified cases in the third series, the enzyme acted upon the substrate for 30 minutes at 40°. In all cases it acted in the presence of optimum concentrations of chloride, phosphate and hydrogen ions. In all cases the preparation of the starch dispersions used as substrates (1% for determinations of amyloclastic, and 2% for saccharogenic activities) and the technique of conducting the actual measurements of activities were the same as described in our previous papers.[1a,1b]. The starch dispersions and enzyme solutions were in all cases adjusted to P_H 6.9 at room temperature. This adjustment was effected by means of electrometric titrations of aliquot portions, and was checked by colorimetric tests of each solution used. In the presence of tryptophan, the adjustment was effected entirely by means of the colorimetric method. The colorimetric measurements were made as described by Clark,[5] using phosphate buffer mixtures which were standardized electrometrically.

First Series.—These were for the purpose of ascertaining the rate of deterioration of the enzyme when standing at different temperatures in the absence of substrate in aqueous solutions which contained optimum concentrations of sodium chloride and sodium phosphate and which had been carefully adjusted to the optimum hydrogen-ion concentration.[6] This deterioration was measured in terms of the decreasing amyloclastic and saccharogenic activities of the enzyme in solution as shown by testing the activities of portions of it withdrawn simultaneously at the beginning of each experiment and after stated intervals of time: at 10°, after two, four and 24 hours; at 25°, after two, four, eight and 24 hours; at 40°, after one, two and four hours. In this first series of experiments the enzyme was used in the form of a high grade commercial pancreatin; in the second and third series, in the form of purified pancreatic amylase.

The experiments of the first series indicate that at 10° in aqueous solution containing optimum concentrations of chloride, phosphate and hydrogen ions the hydrolysis of the enzyme proceeds but slowly, being insignificant at four hours and showing at 24 hours a loss of only about one-sixth of the original activity. The inactivation proceeds nearly 12 times as rapidly at 25° as at 10°, and nearly 12 times as rapidly at 40° as at 25°. The reaction by which the activity of the enzyme was destroyed had therefore a temperature coefficient much higher than that of most chemical reactions. Within the limits which must be allowed for experimental error in the determination of amyloclastic activity, the rates of deterioration of amyloclastic and saccharogenic activities run parallel, indicating anew that these are not the effects of two enzymes but rather two stages in the action of one enzyme, and that under these conditions

[5] Clark, "The Determination of Hydrogen Ions," Williams and Wilkins Co., 2nd ed., **1922**, p. 99.

[6] Sherman, Thomas and Baldwin, THIS JOURNAL, **41**, 231 (1919).

the inactivation of this enzyme is probably a hydrolytic reaction (at least in part) as previously explained.

In the absence of the chloride and phosphate ordinarily used in our work with this enzyme, its rate of deterioration was much more rapid. That deterioration under these conditions could not be measured with equal accuracy, may be largely due to unfavorable and variable hydrogen-ion concentrations in the unbuffered solutions.

Second Series.—As the experiments of the first series had shown that even in the presence of optimum concentrations of chloride, phosphate and hydrogen ions, the inactivation of the enzyme in aqueous solution is sufficiently rapid (at 40°) to permit quantitative investigation by our methods, a series of experiments was carried out to test the influence of glycine and of tryptophan (present in the enzyme solution in the concentration of 0.01 *M*) upon the inactivation of the enzyme when held in such solutions at P_H 6.9 at 40° and at 50° in the absence of substrate. Control experiments were made in all cases with solutions prepared and treated in exactly the same way except that no amino acid was added. The influence of the small amount of the amino acid added with the enzyme solution to the digestion mixture was found by blank experiments to be negligible.

It was found that the favorable influence of the glycine in the enzyme solutions is apparent in all cases and that tryptophan exerts a favorable influence upon the amyloclastic activity as well as upon the saccharogenic activity of those enzyme solutions which have stood for two hours or longer at 40°, and in all of the experiments in which the temperature was increased to 50°. It is noteworthy that the favorable influence of glycine upon both the amyloclastic and saccharogenic activities of the enzyme solutions which have stood at both temperatures, and of tryptophan upon the amyloclastic activity of the solution which has stood at 50° and upon the saccharogenic activity of the enzyme solutions which have been held at both temperatures, appears even in the initial determinations, indicating that in the absence of the added amino acid an appreciable inactivation (probably due to hydrolysis) occurs immediately when the enzyme is dissolved in warm water.

The most important result here from the standpoint of the present paper is that in these experiments tryptophan showed a protective effect upon the enzyme similar to that of the typical mono-amino acid glycine.

Third Series.—Having found, as just shown, that tryptophan in experiments of sufficient duration or at a sufficiently high temperature exerts a favorable influence similar to that of other amino acids in protecting the enzyme from inactivation on standing in aqueous solution in the absence of substrate, a new series of experiments was planned to determine whether, in experiments in which the enzyme acts upon the substrate at

50°, tryptophan would also resemble the other amino acids in causing an apparent increase of the amyloclastic as well as saccharogenic activity of the enzyme. This proved to be the case.

Discussion

The results of experiments upon the inactivation of the enzyme (pancreatic amylase) both when standing in solution in the absence of substrate (first and second series) and when acting upon substrate (third series) show that in both cases the tryptophan exerts a favorable influence, as do the other α-amino acids tested, upon the conservation of both the amyloclastic and the saccharogenic activities. To make this apparent for tryptophan in the case of amyloclastic action requires simply the use of a higher temperature or of a longer time than had been employed in our previous experiments. There is no reason to doubt that this is also true of the two other amino acids (histidine and lysine) which we have found in previous experiments to act similarly to tryptophan.[1b] As previously explained, we believe that the quantitative difference in behavior toward amyloclastic action which distinguishes these from all of the other α-amino acids that we have studied is probably due, at least in part, to the position or mode of linkage of the corresponding amino acid radicals in the enzyme molecule, and not simply to differences in structure among the amino acids and the influence of these structural differences upon the actual interaction of enzyme with substrate. We do not, however, seek to exclude this latter factor. Certainly, many substances have unfavorable effects upon enzymes, especially when the latter are highly purified as are our preparations of pancreatic and malt amylases, and it seems quite probable that such inhibitory effects may be correlated with the chemical structures of the inhibitory substances. As we have previously recorded,[1c] indole shows such an inhibitory influence and it therefore seems probable that in the case of tryptophan there is a very slight initial inhibitory effect upon the amyloclastic activity of the enzyme exerted by the indole radical of the tryptophan and attributable to its inherent structure, which slight inhibitory effect is soon more than compensated by the favorable influence of the tryptophan as a protein cleavage product in protecting the enzyme from hydrolytic cleavage in the same way that it is protected by other amino acids. The view that the influence of tryptophan may be explained by the position of the tryptophan radical in the enzyme molecule is in accordance with observations such as those of Fürth and Lieben[7] in which it has been found that in the gradual hydrolysis of protein certain amino acids, for example, tyrosine, are liberated more rapidly and apparently earlier than is tryptophan.

[7] Fürth and Lieben, *Biochem. Z.*, **109**, 153 (1920).

Summary and Conclusions

When the enzyme (pancreatic amylase) was dissolved in pure water and held at a temperature of 10°, it lost about one-sixth of its activity in four hours and about one-half in 24 hours. At 25° it lost nearly one-half of its activity in two hours and about two-thirds in four hours. At 40° the activity was entirely lost within one hour.

When dissolved in water containing optimum concentrations of sodium chloride and disodium phosphate and which had been brought to optimum hydrogen-ion concentration, the rate of inactivation of the enzyme was reduced to about one-sixth of that observed in the pure water solution.

Both in the presence and in the absence of salts the temperature coefficient of the inactivation of the enzyme is much higher than that of most chemical reactions.

All of the above statements apply to both the amyloclastic and saccharogenic activities of the enzyme. These and other observations give added weight to the view that the inactivation of the enzyme in solution is, at least in part, due to hydrolysis, and certainly is greatly accelerated by heat.

The addition of amino acids to the water-salt solution of the enzyme protects the enzyme from inactivation both in the absence and in the presence of its substrate.

The favorable influence of the amino acids may be exerted in more than one way. The data of our experiments are chiefly significant for the new evidence which they furnish in support of the view that the enzyme is itself a protein compound which in aqueous solution is gradually destroyed by the hydrolytic action of the water in which it is dissolved. Since such an hydrolysis must yield amino acids, the addition of amino acid to the solution tends to check the hydrolysis of the enzyme and thus conserve its activity.

This view is supported by all of the data obtained in an extended investigation of the effects of the different amino acids, the methods and results of which have been reported in this and in previous papers. Certain differences in the effects of different amino acids as reported in earlier papers are now shown to be quantitative rather than qualitative and are probably due to differences in the position or modes of linkage of the corresponding amino acid radicals in the enzyme molecule. Thus, the tryptophan radical is apparently so bound in the enzyme molecule as not to be split off in the first stages of its hydrolysis so that tryptophan did not show its full effects in experimental periods of 30 minutes at 40°; but in sufficiently prolonged experiments at 40°, or in experiments of 30 minutes' duration at 50°, its favorable influence upon the amyloclastic as well as upon the saccharogenic activity of the enzyme was clearly demonstrable.

The work recorded in this and our previous papers has, therefore, thrown light upon the chemical nature of this enzyme from three different angles:

(1) the preparation of the enzyme in the form of a highly purified product of maximum activity and uniform properties and the analysis of these purified preparations with reference to their qualitative reactions, quantitative elementary composition, and the kinds and amounts of amino acid radicals which they contain as determined by the Van Slyke method; (2) quantitative study of the comparative effects of different antiseptics, those characterized by their chemical action upon proteins being found very much more destructive than those of the lipoid-dissolving type; (3) investigation of the inactivating action of warm water upon the enzyme and the effects of amino acids in retarding this inactivation. The data of these three lines of evidence are entirely consistent and all point to the protein nature of the enzyme.

That malt amylase is also of a protein nature has been rendered in the highest degree probable by similar studies and by the further observation that it resembles typical proteins in showing an iso-electric point in electrophoresis experiments and that the iso-electric point thus established bears a definite relation to the activity, as it coincides with the hydrogen-ion concentration at which optimum activity occurs.[8] Studies of the iso-electric point of pancreatic amylase, while much more difficult because of its greater sensitiveness to deterioration, are being undertaken.

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[CONTRIBUTION FROM THE BUREAU OF CHEMISTRY, UNITED STATES DEPARTMENT OF AGRICULTURE]

TOLUIDINE DERIVATIVES. I. QUANTITATIVE PREPARATION OF 5-IODO-2-AMINOTOLUENE AND SOME OF ITS DERIVATIVES

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Iodo-*o*-toluidine (5-iodo-2-aminotoluene) has been prepared by Artmann[1] and by Wheeler and Liddle.[2] After several unsuccessful attempts to obtain satisfactory yields by this method,[3] we modified their general procedure as follows.

[8] Sherman, Thomas and Caldwell, THIS JOURNAL, **46**, 1711 (1924).

[1] Artmann, *Monatsh.*, **26**, 1097 (1905).

[2] Wheeler and Liddle, *Am. Chem. J.*, **42**, 501 (1909). See also Fichter and Philipp, *J. prakt. Chem.*, **74**, 312 (1906); Willgerodt and Heusner, *Ber.*, **40**, 4077 (1907). The complete series of possible iodine nuclear derivatives of toluene are described by Wheeler, *Am. Chem. J.*, **44**, 493 (1910).

[3] Dains, Malleis, and Meyers [THIS JOURNAL, **35**, 970 (1913)] report "good results" from preparing 5-iodo-toluidine by Wheeler and Liddle's method. The yield is not stated, however.