Commercial pancreatin, purified pancreatic amylase, saliva, malt extract, purified malt amylase, commercial takadiastase, and the purified amylase of *Aspergillus oryzae* were all injured by formaldehyde even in small amounts. Takadiastase was the least, and purified pancreatic amylase the most, affected. The percentage of loss of the enzyme action increased in all cases with increasing concentration of formaldehyde.

A very low concentration of formaldehyde (0.0000116 *M*) gave a 3% destruction of the activity of commercial pancreatin.

All of the enzymes studied were very sensitive to copper sulfate. Pancreatic amylase was much more sensitive than any of the others. Most of the enzymes were injured by as low a concentration of copper sulfate as 0.000006 *M*. Almost complete destruction of the activity of purified pancreatic amylase was caused by 0.00054 *M* copper sulfate. The inhibiting effect of copper sulfate on the activity of amylases increased with increasing concentration of copper sulfate.

The percentage loss of enzyme action due to formaldehyde and to copper sulfate solution did not depend upon the ratio of antiseptic to enzyme or of antiseptic to substrate, but upon the ratio of antiseptic to water, or the concentration of the antiseptic in the system.

The results demonstrate the need of attention to the possible effects of antiseptic upon enzyme in cases in which antiseptics are used to suppress microörganisms in studies of enzyme activity.

The much greater sensitiveness of the amylases to formaldehyde and copper sulfate than to toluene is of further interest in connection with the problem of the protein nature of these enzymes.

We are indebted to the Carnegie Institution of Washington for the use of enzyme preparations which had been purified in connection with work done under the auspices of the Institution.

NEW YORK CITY.

[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY OF COLUMBIA UNIVERSITY, No. 375.]

THE INFLUENCE OF CERTAIN AMINO ACIDS UPON THE ENZYMIC HYDROLYSIS OF STARCH.

BY H. C. SHERMAN AND FLORENCE WALKER.

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Our experiments on the influence of amino acids upon the rate of hydrolysis of starch by different enzymes, begun with the study of asparagine and aspartic acid,[1] have been extended to glycine, alanine, tyrosine and phenylalanine. Essentially the same experimental methods have been employed as in our work with asparagine and aspartic acid. In the experiments described below, however, "soluble" starch, prepared by the Lintner

[1] Sherman and Walker, THIS JOURNAL, **41**, 1867 (1919).

method and further purified by washing 9 times with ordinary distilled and 6 times with specially purified thrice distilled water, has in all cases been used as substrate. The amino acids here used were imported. The amylase preparations and other enzyme-containing materials tested were (1) pancreatic amylase preparations Nos. 58, 59, 60, 77B and 81B, (2) commercial pancreatin No. 8, (3) malt amylase preparation No. 155, (4) malt extract, (5) aspergillus amylase preparations Nos. 22, 22b, and 23, (6) commercial takadiastase, and (7) fresh saliva. Thrice distilled water was used for making starch dispersions, solutions of activators, enzymes, etc., and for rinsing all glassware.

Method.—The method[2] of testing the influence of the amino acids is briefly as follows. An amount of air-dry starch equivalent to the required amount of anhydrous material is weighed out, mixed with a little cold water, dispersed by pouring into boiling water (about 80 cc. per g. of starch) and boiled for about 3 minutes. This is transferred to 100cc. cylinders, neutralized with 0.01 *N* sodium hydroxide solution and the salts most favorable for the action of the amylase[3] added. The dispersions are then made up to 100 cc. so that the concentration of starch is exactly 1%, mixed thoroughly by stirring and placed in the 40° bath to reach the desired temperature. In the meantime, the enzyme solution is prepared and the required amount pipetted into dry flasks. The starch dispersions are then poured into the flasks containing the enzyme at intervals of 15 seconds and the flasks placed in the 40° bath. At the end of 30 minutes, enzymic action is stopped by pouring 50 cc. of Fehling solution into the digestion mixtures, at intervals of 15 seconds and in the same order in which the starch was poured on the enzyme. The amount of reducing sugar formed is determined by immersing the flasks in a boiling water bath for 15 minutes. The cuprous oxide is filtered into weighed Gooch crucibles, washed with hot water, alcohol and ether, dried at 100°, and weighed. Glycine and alanine being quite soluble were dissolved in a small volume of water and added to the starch paste, after it was poured into the cylinders and before being made up to volume. Since tyrosine and phenylalanine are difficultly soluble, the amount of each used was added to the water in which the starch was dispersed and boiled with it. To show whether this variation in procedure affected the action of the amino acid on the enzyme, digestions were carried out in which equal amounts of asparagine were added before boiling in some cases and after cooling in others. Activation due to asparagine was the same in both cases. The same test was made with aspartic acid with the same result.

[2] Sherman, Kendall and Clark, THIS JOURNAL, **32**, 1082 (1910).

[3] Sherman, Thomas and Baldwin, *ibid.*, **41**, 231 (1919). Pending further investigation the substrate is prepared in the same manner for the action of saliva as for pancreatic amylase.

In these experiments the amino acids were made neutral to rosolic acid with 0.01 *N* sodium hydroxide solution.

Measurement of the Influence of Different Amino Acids.

Tables I–IV show the influence of carefully neutralized glycine, alanine, tyrosine and phenylalanine, added separately and in combination with a second amino acid, upon the rate of hydrolysis of "soluble starch" by different enzymes. The reducing sugar formed by enzymic hydrolysis is chiefly maltose, but since small amounts of glucose may also be present the results are stated in terms of the weight of cuprous oxide resulting from the reduction of the Fehling solution by the sugar or sugars present. The amounts of enzyme used in the experiments were so regulated as to result in the transformation of about 1/5 of the starch into sugar.

TABLE I.

EFFECT OF GLYCINE AND GLYCINE PLUS ASPARTIC ACID ON THE ENZYMIC HYDROLYSIS OF LINTNER SOLUBLE STARCH.

Amino acid.		Cuprous oxide.						
Glycine. Mg.	Aspartic acid. Mg.	Purified pancreatic amylase (No. 59). Mg.	Commercial pancreatin (No. 8). Mg.	Saliva. Mg.	Purified malt amylase (No. 155). Mg.	Malt extract. Mg.	Aspergillus amylase (No. 23). Mg.	Commercial takadiastase (No. 7). Mg.
None	None	246	227	316	260	277	222	292
50	None	280	243	334	270	286	226	292
100	None	284	245	341	273	283	224	292
None	50	279	242	344	269	280	223	289
50	50	279	247	334	268	281	224	292
Activation due to glycine =		38	18	25	13	9	4	0

TABLE II.

EFFECT OF ALANINE AND ALANINE PLUS GLYCINE ON THE ENZYMIC HYDROLYSIS OF LINTNER SOLUBLE STARCH.

Amino acid.		Cuprous oxide.						
Alanine. Mg.	Glycine. Mg.	Purified pancreatic amylase (No. 58). Mg.	Commercial pancreatin (No. 8). Mg.	Saliva. Mg.	Purified malt amylase (No. 155). Mg.	Malt extract. Mg.	Aspergillus amylase (No. 22b). Mg.	Commercial takadiastase (No. 7). Mg.
None	None	273	281	319	285	248	272	279
50	None	310	293	339	295	251	279	282
100	None	318	301	352	301	257	287	290
None	50	320	300	355	299	249	277	280
50	50	318	296	360	305	256	284	286
25	25	317	298	351	299	250	273	283
Activation due to alanine		42	20	33	16	9	15	11

TABLE III.

EFFECT OF TYROSINE AND TYROSINE PLUS ASPARAGINE ON THE ENZYMIC HYDROLYSIS OF LINTNER SOLUBLE STARCH.

Amino acid.		Cuprous oxide.						
Tyrosine. Mg.	Asparagine. Mg.	Purified pancreatic amylase (No. 60). Mg.	Commercial pancreatin (No. 8). Mg.	Saliva. Mg.	Purified malt amylase (No. 155). Mg.	Malt extract. Mg.	Aspergillus amylase (No. 23). Mg.	Commercial takadiastase (No. 7). Mg.
None	None	282	294	323	248	252	261	287
50	None	318	317	353	262	266	273	299
None	50	316	312	352	256	255	263	291
25	25	317	317	355	263	259	269	296
50	50	322	319	362	263	263	270	297
100	None	—	—	356	—	—	—	—
None	100	—	—	364	—	—	—	—
Activation due to tyrosine		36	23	30	14	14	12	12

TABLE IV.

EFFECT OF PHENYLALANINE AND PHENYLALANINE PLUS ASPARAGINE ON THE ENZYMIC HYDROLYSIS OF LINTNER SOLUBLE STARCH.

Amino acid.		Cuprous oxide.						
Phenylalanine. Mg.	Asparagine. Mg.	Purified pancreatin amylase (No. 58). Mg.	Commercial pancreatin (No. 8). Mg.	Saliva. Mg.	Purified malt amylase (No. 155). Mg.	Malt extract. Mg.	Aspergillus amylase (No. 23). Mg.	Commercial takadiastase (No. 7). Mg.
None	None	267	293	207	244	250	252	288
50	None	300	306	222	251	253	261	290
100	None	303	307	225	256	256	263	293
None	50	309	312	225	253	254	259	291
50	50	309	312	225	257	257	264	293
25	25	308	311	225	260	255	261	292
Activation due to phenylalanine		36	14	18	12	6	11	5

The data given in the above tables show an undoubted increase in the activity of purified pancreatic amylase, pancreatin, saliva, and purified malt amylase in the presence of any one of the 4 amino acids investigated or of any 2 of them whose joint effects were tested. The apparent activation is not so marked in case of the less sensitive enzymes, malt extract, takadiastase and aspergillus amylase. It is also true that the acceleration of hydrolysis by the amino acids is somewhat greater for the purified form of the enzyme than for the natural or commercial material in which the enzyme is accompanied by other constituents of the tissue or secretion in question. It will be observed that, in general, the 4 amino acids here discussed as well as asparagine and aspartic acid previously studied[1] behave in a similar manner. The above results show no evidence that the addition of two amino acids to the same digestion mixture causes greater activation than would result from a corresponding concentration of one of them. The following combinations have been tested: aspartic acid and aspar-

agine, glycine and aspartic acid, tyrosine and asparagine, phenylalanine and asparagine, alanine and glycine.

Since some investigators have held that the activating effect of amino acids is attributable to their presence inducing a more favorable hydrogen-ion concentration in the digestion mixture, we have determined electrometrically the hydrogen-ion concentrations of our mixtures with and without neutralized amino acid, with the results shown in Table V. It is evident that the reaction of our mixtures is not changed by the addition of the neutralized amino acids to any significant degree and therefore that the favorable effect of the amino acid upon the enzyme action is due to some other cause or causes.

TABLE V.

MEASUREMENTS OF HYDROGEN-ION CONCENTRATION IN SOLUTIONS WITH AND WITHOUT NEUTRALIZED AMINO ACIDS.

50 mg. of amino acid used in the last four experiments.

Amino acid.	Solution activated as for pancreatic amylase $C_H{}^+ \times 10^7$.	Solution activated as for malt amylase $C_H{}^+ \times 10^5$.	Solution activated as for aspergillus amylase $C_H{}^+ \times 10^5$.
None	1.2	3.4	1.2
Glycine	1.3	3.1	1.0
Alanine	1.3	3.5	1.2
Tyrosine	1.3	3.4	1.2
Phenylalanine	1.3	3.3	1.2

Mode of Action of the Amino Acid.

Several possible explanations of the favorable influence of the amino acids may be suggested.

(1) **Is the Action Direct?**—It is conceivable that the amino acid may directly facilitate the interaction of the enzyme with the substrate. Until our knowledge of the mechanism of enzyme action is further developed this suggestion, while the most direct, can only be approached by somewhat speculative discussion or, experimentally, by a process of elimination of other possibilities.

(2) **Does the Effect Depend upon Some Reaction with the Products of Digestion?**—Since the activity of an enzyme is often diminished by the accumulation of the products of its action, it might be suggested that the amino acids exert their favorable effect through combining with some product or products of the hydrolysis which might otherwise combine with the enzyme itself, thus reducing its activity, or might, if remaining free in the solution, tend to bring the hydrolysis to equilibrium. To test this point, the effect of the addition of 100 mg. of pure maltose to the starch paste, with and without glycine, on hydrolysis by pancreatic amylase was determined. Similar experiments in which a certain amount of a hydrolytic mixture was substituted for pure maltose were carried out as follows. One g. of starch was digested for 1 hour at 40° by pancreatic

amylase, at the end of which time the enzyme was destroyed by boiling. The effect of 25 and 50 cc. of this digested mixture on hydrolysis with and without glycine was tested. Correction being made for the reducing power of maltose or digestion products added, and the amounts of activating salts being properly adjusted, both pure maltose and the hydrolytic products of the starch were found under the conditions of these experiments to be without measurable effect upon the activity of the enzyme used, showing that the favorable influence of amino acids cannot be explained in this way.

(3) **Does the Amino Acid Protect the Enzyme against Some Accidental or Unknown Deleterious Influence?**—Aside from the possibility of correcting an unfavorable hydrogen-ion concentration which has already been excluded as an explanation of our results, it is possible that the amino acid may act by protecting the enzyme from some active but unknown deleterious influence. This is illustrated by the following experiments with cupric sulfate.

Protective Action of Amino Acids Against Cupric Sulfate.—These experiments were designed to show whether the deleterious effect upon amylase activity of such a heavy metal salt as copper sulfate could be wholly or in part overcome by the presence of an amino acid. In the experiments the results of which are given in Table VI, the cupric sulfate and amino acid were added to the cooled starch paste and thoroughly mixed before pouring onto the enzyme solution.

TABLE VI.

ACTION OF AMINO ACIDS IN PROTECTING PURIFIED PANCREATIC AMYLASE FROM THE DELETERIOUS EFFECT OF COPPER.

Amino acid.	Mg.	Conc. $CuSO_4$ in starch paste. M.	Cuprous oxide. Mg.
None		None	274
None		0.00003	58
Alanine	10		298
Asparagine	100		293
Glycine	100		304
Glycine	50		295
Glycine	100	None	308

The above data show that a 0.00003 M concentration of cupric sulfate in the digestion mixture diminished the activity of pancreatic amylase by about 78%. This is in accordance with results recently obtained in this laboratory and reported in the preceding paper by Sherman and Wayman. However, upon the addition of 0.1% of amino acid, not only is the inhibiting influence of the cupric sulfate counteracted, but there is an increase in saccharification almost equal to that which occurs in the presence of amino acid and absence of copper. Further experiments were performed in which the cupric sulfate was added directly to the enzyme

solution in two concentrations, 0.0035 *M* and 0.00003 *M* and the efficiency of 0.1% glycine in the substrate in reactivating the enzyme was studied. Some results are given in Table VII.

TABLE VII.

REACTIVATION OF PANCREATIC AMYLASE BY GLYCINE AFTER INACTIVATION BY COPPER.

Glycine. Mg.	Conc. $CuSO_4$ in starch paste. *M*.	Conc. $CuSO_4$ in enzyme solution. *M*.	Conc. $CuSO_4$ in digestion mixture. *M*.	Cuprous oxide. Expt. 1. Mg.	Expt. 2. Mg.	Expt. 3. Mg.
None	None	None	None	280	252	264
100	None	None	None	316	—	300
100	None	0.0035	0.00003	98	—	—
None	None	0.0035	0.00003	4	—	—
None	None	0.00003	1.8×10^{-7}	—	209	—
100	None	0.00003	1.8×10^{-7}	—	260	184
None	0.00003	0.00003	0.00003	—	260	182

Numerous experiments showed that the inactivation of the enzyme by the copper and its reactivation by amino acid were considerably influenced by time and temperature. In Expt. 2 of Table VII the solution stood for about 12 minutes before testing; in Expt. 3 it stood for 55 minutes. The influence of temperature is illustrated in Table VIII.

TABLE VIII.

EFFECT OF TEMPERATURE ON REACTIVATION OF PANCREATIC AMYLASE BY GLYCINE.
After standing for 20 min. in cupric sulfate solution 0.00003 *M*.

Glycine. Mg.	Temp. of enzyme sol. after 20 min. ° C.	Cuprous oxide. Mg.
None	23	115
100	23	142
None	12	175
100	12	213

As to the bearing of these experiments with cupric sulfate upon the question whether the role of amino acids is that of a direct accelerator of the enzyme action or rather that of a protector which increases the amount of work done by the enzyme through preventing its deterioration, it cannot be doubted that they establish the possibility of a very marked protective effect without precluding the additional possibility of a more direct action upon the enzyme.

Evidently with a low concentration of copper ions in solution these react with amino acid forming copper-amino ions[4] more readily than with the enzyme; and moreover, when the copper ion has already acted upon and inactivated a part of the enzyme, it apparently may still be taken up by the amino acid, and the enzyme thus freed from the copper may become active again.

(4) Does the Amino Acid Act by Retarding the Hydrolytic Destruction of the Enzyme?—Another possibility is that the amino acid may act

[4] See J. T. Barker, *Trans. Faraday Soc.*, **3**, 188 (1908).

by preventing or retarding the deterioration of the enzyme in its aqueous solution. The very rapid deterioration of water solutions of pancreatic amylase, particularly when highly purified, and the influence of the sodium chloride and secondary phosphate (regularly used to "activate" this enzyme) in retarding the deterioration have been discussed in previous papers from this laboratory.[5] Since the deterioration of enzymic activity, while greatly retarded, is not entirely prevented by the presence of the salts, it is not improbable that the favorable influence of the amino acid may be due at least in part to a further protection of the enzyme from deterioration in the aqueous dispersion in which it acts.

This we have found to be the case, solutions of pancreatic amylase which had stood for 1 hour at 40° showing about 1/3 greater amylase activity when alanine had been added to the solution in advance. The conditions in this case were such as to result in greater deterioration than occurs in our ordinary tests of enzyme activity, both because of longer exposure of the enzyme to warm water and because it remained longer in water in the absence of its substrate. A series of similar experiments at different temperatures is now in progress, and the results thus far obtained go to show that the influence of the amino acid becomes more marked at the higher temperatures at which the enzyme is undergoing more rapid deterioration, thus strengthening the impression that the amino acid tends to preserve the enzyme from the destructive action of the water. Further discussion is best deferred until the completion of the projected series of experiments. Meantime, the now fully demonstrated fact that the presence of certain amino acids retards the deterioration of the enzyme constitutes an interesting addition to the evidence supporting the view that the enzyme itself is a substance of protein nature or which contains protein as an essential constituent.

Summary.

Addition of glycine, alanine, phenylalanine or tyrosine caused an undoubted increase in the rate of hydrolysis of starch by purified pancreatic amylase, commercial pancreatin, saliva, or purified malt amylase. Less marked results were obtained with the less sensitive enzyme materials, malt extract, takadiastase, and an aspergillus amylase product prepared in the laboratory from takadiastase.

Each of the 4 amino acids here studied, as well as aspartic acid and asparagine previously investigated, showed a similar favorable influence upon the enzymic hydrolysis of the starch.

The addition of a mixture of two of these amino acids, produced no greater effect than would result from the same concentration of one of them.

In these experiments the favorable effect of the added amino acid was

[5] Sherman and Schlesinger, THIS JOURNAL, and in other papers of this series.

not due to any influence upon hydrogen-ion concentration nor to combination of the amino acid with the product of the enzymic reaction.

On the other hand it is shown that the addition of one of these amino acids is a very effective means of protecting the enzyme from the deleterious effect of cupric sulfate and may even serve to restore to full activity an enzyme which has been partially inactivated by copper.

The favorable influence of the amino acid is evidently due in part at least to a protection of the enzyme from deterioration in the aqueous dispersion in which it acts.

The establishment of the importance of the last mentioned factors does not preclude the possibility of a more direct influence of the amino acid upon the activity of the enzyme.

The investigation is being continued by studying the effects of the above amino acids through a wider range of times and temperatures and by extending the study to additional amino acids.

We are greatly indebted to the Carnegie Institution of Washington for grants in aid of this investigation.

NEW YORK CITY.

[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY OF COLUMBIA UNIVERSITY, No. 376.]

A STUDY OF THE INFLUENCE OF ARGININE, HISTIDINE, TRYPTOPHANE AND CYSTINE UPON THE HYDROLYSIS OF STARCH BY PURIFIED PANCREATIC AMYLASE.

BY H. C. SHERMAN AND MARY L. CALDWELL.

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Previous work in this laboratory[1] having shown that various monoamino acids tested exhibit quite uniformly the property of increasing the enzymic hydrolysis of starch, especially by pancreatic amylase, the experiments here described were planned to extend the investigation to amino acids of different types of structure.

Arginine and histidine solutions were prepared from casein and gelatin by Kossel's method[2] with slight modifications developed in the course of this work. In the latter part of the work commercial preparations[3] of histidine dichloride and tryptophane were also used. The cystine used was prepared by another worker in this laboratory.[4] In certain of the experiments we have also made use of glycine obtained commercially[5] and an imported phenylalanine.

The enzyme used was a pancreatic amylase preparation (No. T-19-B) purified

[1] Sherman and Walker, THIS JOURNAL, **41**, 1866 (1919); and the preceding paper.

[2] Kossel, *Z. physiol. Chem.*, **31**, 165 (1900–1).

[3] Purchased from the Special Chemicals Company.

[4] Alice Thompson Merrill, *Dissertation*, Columbia University, **1921**.

[5] From Eimer and Amend.