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.

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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.

in this laboratory as previously described.[6] As substrate we used a commercial[7] "Soluble Starch according to Lintner" after purification by washing 9 times with distilled, and 6 times with thrice distilled, water. In weighing out portions of starch for test, allowance was made for its moisture content. Both the soluble starch to be used as substrate, and each amino acid to be tested, was titrated with 0.01 *N* sodium hydroxide solution or hydrochloric acid using rosolic acid as indicator. From the results of these titrations the amounts of 0.01 *N* alkali needed to neutralize the soluble starch, and of acid or alkali needed to neutralize each of the amino acids used, were calculated and these amounts were added to the starch dispersions and the amino acid solutions in preparing them for the experiments to be described. The hydrogen-ion concentrations of the starch pastes with and without the addition of the neutralized amino acid solutions were then verified by electrometric determinations and by the indicator method as explained below.

In the purification of all materials, and the cleaning and protection of all apparatus used in the experiments we have observed the precautions described in previous papers from this laboratory.

Method.

Preliminary experiments showed that the gravimetric method worked out in this laboratory for determining the saccharogenic power of the enzyme[8] could not be used in the presence of arginine and histidine because of their interference with the determination of reducing sugar by Fehling solution. This had also been found to be true with cystine. For this reason the amyloclastic power of the amylase was measured instead.

The procedure followed was based on the method of Wohlgemuth[9] and has been previously used and described in this laboratory.[10] Enough starch to make 600 cc. of a 1% starch paste was weighed out, mixed with 50 cc. of thrice distilled water and poured into 100 cc. of boiling water. The paste was boiled for 3 minutes, cooled, and 25 cc. poured into each of six 100cc. cylinders. To each of these were added the proper activating agents (5 cc. of *M* sodium chloride solution and 2.5 cc. of 0.02 *M* disodium phosphate), enough 0.01 *N* sodium hydroxide solution to neutralize the acidity of the starch, and the amino-acid to be tested, which was also properly neutralized. The mixture in each cylinder was then made up to 100 cc. with thrice distilled water and carefully stirred.

Forty-two clean, dry test-tubes were placed in a special wire frame basket in a bath of ice-water, and carefully measured portions of enzyme solution introduced into each by means of a 1cc. pipet which was accurately standardized and graduated to 0.01 cc. Portions of 5 cc. of one of the starch pastes prepared above were then introduced into each of 7 test-tubes. This was done carefully by means of a buret with a very long delivery tip reaching to the bottom of the test-tube. In this way the lodging of any

[6] THIS JOURNAL, **41**, 1855 (1919).

[7] From Merck and Company.

[8] THIS JOURNAL, **32**, 1082 (1910); **37**, 628 (1915).

[9] Wohlgemuth, *Biochem. Z.*, **9**, 1 (1908).

[10] THIS JOURNAL, **37**, 634 (1915).

of the starch paste on the sides of the tube was avoided. Since the tubes were kept in ice-water no measurable reaction took place.

The basket of tubes so prepared was shaken and placed in a Freas thermostat in which the temperature varied only about $\pm 0.01°$. The tubes were thus kept at an average temperature of 40° for 30 minutes, when the basket was taken out and placed in ice-water to stop the action of the enzyme. After a few minutes' cooling 0.1 cc. of 0.1 *N* iodine in potassium iodide solution was added to each tube. This was done very carefully by means of a dropping bottle which delivered drops of 0.1 cc. About 20 cc. of distilled water was then poured into each tube and the contents thoroughly mixed. Each set of tubes containing the same amount of the same starch paste with various amounts of enzyme was then observed for the end-point; that is, the tube of lowest enzyme concentration which is definitely red and shows no blue or violet color due to starch.[11] To obtain the value of the amyloclastic power of the enzyme, the weight of the 1% starch paste, (5000 mg.) is divided by the weight in mg. of enzyme present in the tube showing "the Wohlgemuth end-point" *i. e.*, the first red color not tinged by violet. Blank determinations were always made in each set; that is, starch pastes neutralized and containing the proper activators but not amino acids were used as the standard each time, since results vary slightly from day to day due to deterioration of the enzyme in solution and possibly other factors.

Data of Typical Experiments.

Table I shows the influence of 50 mg. of arginine and 50 mg. of histidine on the amyloclastic action of the amylase. It will be seen that the arginine has a distinct activating influence, while the histidine has not. These results were confirmed by other experiments.

TABLE I.

INFLUENCE OF ARGININE AND HISTIDINE ON AMYLOCLASTIC ACTION.

Enzyme, mg. Amino acid.	Power.	0.003.	0.004.	0.005.	0.006.	0.007.	0.008.	0.009.
None	625,000	Blue-violet	Violet	Red-violet	Violet-red	Violet-red	Red[a]	Orange-red
Arginine (gelatin-a) 50 mg.	714,000	Violet-blue	Violet	Red-violet	Violet-red	Red[a]	Red-orange	Orange
Arginine (gelatin-b) 50 mg.	714,000	Blue	Violet	Red-violet	Violet-red	Red[a]	Orange-red	Orange
Histidine (casein) 50 mg.	625,000	Violet-blue	Blue-violet	Violet	Red-violet	Violet-red	Red[a]	Orange-red
Histidine (purchased) 50 mg.	625,000	Blue	Blue-violet	Red-violet	Violet-red	Violet-red	Red[a]	Red-orange

[a] The "end-point tube."

[11] The terms used in describing the colors are those of the Milton Bradley Standard Color Chart as given by Mullikin in his "Identification of Pure Organic Compounds." John Wiley and Sons, New York, 1904.

The results of a comparison of the influence of arginine and glycine on the amyloclastic action of the amylase are given in Table II. Here it is seen that both glycine and arginine activate the hydrolysis, that the activation is about the same, that a 50mg. mixture of the two gives about the same results as 50 mg. of either alone, and that a 100mg. mixture of 50 mg. of each does not seem to increase the effect.

TABLE II.

INFLUENCE OF ARGININE AND GLYCINE ON AMYLOCLASTIC ACTION.

Enzyme, mg, Amino acid.	Power.	0.003.	0.004.	0.005.	0.006.	0.007.	0.008.	0.009.
None	625, 000	Blue	Violet-blue	Violet	Violet-red	Violet-red	Red[a]	Orange-red
Glycine 50 mg.	714, 000	Blue	Blue-violet	Red-violet	Violet-red	Red[a]	Orange-red	Red-orange
Arginine 50 mg.	714, 000	Violet-blue	Red-violet	Violet-red	Violet-red	Red[a]	Orange-red	Red-orange
Glycine Arginine 25 mg. each	714, 000	Blue-violet	Violet-red	Violet-red	Violet-red	Red[a]	Orange-red	Orange-red
Glycine Arginine 50 mg. each	714, 000	Blue	Violet	Violet-red	Violet-red	Red[a]	Orange-red	Orange-red
None	625, 000	Blue	Blue-violet	Violet	Violet-red	Violet-red	Red[a]	Orange-red

[a] The end-point tube.

A comparison of the influence of glycine, phenylalanine, cystine and tryptophane was made. The results are given in Table III. They show that activation is produced with each of these amino acids except tryptophane. Previous work[12] has shown that glycine and phenylalanine also activate the saccharogenic power of this amylase.

TABLE III.

INFLUENCE OF GLYCINE, PHENYLALANINE, CYSTINE and TRYPTOPHANE ON AMYLOCLASTIC ACTION.

Enzyme, mg. Amino acid.	Power.	0.004.	0.005.	0.006.	0.0065.	0.007.	0.0075.	0.008.
None	714, 000	Violet	Red-violet	Violet-red	Violet-red	Red[a]	Orange-red	Orange-red
Glycine 50 mg.	833, 000	Violet	Violet-red	Red[a]	Red	Orange-red	Red-orange	Orange
Phenyl-alanine 50 mg.	833, 000	Red-violet	Violet-red	Red[a]	Red	Orange-red	Red-orange	Red-orange
Cystine 50 mg.	833, 000	Violet	Violet-red	Red[a]	Red	Orange-red	Red-orange	Red-orange
Tryptophane 50 mg.	less than 625, 000	Blue-violet	Violet	Red-violet	Red-violet	Violet-red	Violet-red	Violet-red
None	714, 000	Violet	Violet-red	Violet-red	Violet-red	Red[a]	Orange-red	Orange-red

[a] The end-point tube.

[12] Sherman and Walker, THIS JOURNAL, 43, 2461 (1921).

Table IV shows that results obtained with histidine and tryptophane are similar when tested side by side; but as suggested by the foregoing experiments the activity of the enzyme was slightly lower in the presence of the tryptophane than of the histidine.

TABLE IV.

INFLUENCE OF HISTIDINE AND TRYPTOPHANE ON AMYLOCLASTIC ACTION.

Enzyme, mg. Amino acid.	Power.	0.004.	0.005.	0.006.	0.0065.	0.007.	0.0075.	0.008.
None	714, 000	Violet	Red-violet	Violet-red	Violet-red	Red[a]	Orange-red	Red-orange
Histidine (casein) 50 mg.	714, 000	Blue-violet	Violet-red	Violet-red	Violet-red	Red[a]	Orange-red	Red-orange
Histidine (purchased) 50 mg.	714, 000	Blue-violet	Violet	Violet-red	Violet-red	Red[a]	Orange-red	Orange-red
Tryptophane 50 mg.	666,000	Violet-blue	Blue-violet	Violet-red	Violet-red	Violet-red	Red[a]	Red
None	714, 000	Violet	Violet-red	Violet-red	Violet-red	Red[a]	Orange-red	Orange-red

[a] The end-point tube.

In Table V the results of a further comparison of the influence of the different preparations of histidine are given. As before, no activation is seen. (Duplicate determinations are omitted.)

TABLE V.

INFLUENCE OF HISTIDINE ON AMYLOCLASTIC ACTION.

Enzyme, mg. Amino acid.	Power.	0.004.	0.005.	0.006.	0.0065.	0.007.	0.0075.	0.008.
None	714, 000	Violet	Violet-red	Violet-red	Violet-red	Red[a]	Orange-red	Orange-red
Histidine (casein) 50 mg.	666, 000	Violet	Red-violet	Red-violet	Violet-red	Violet-red	Red[a]	Red
Histidine (purchased) 50 mg.	714, 000	Blue-violet	Red-violet	Violet-red	Violet-red	Red[a]	Red	Orange-red
Histidine (gelatin) 50 mg.	666, 000	Violet-blue	Violet	Violet-red	Violet-red	Violet-red	Red[a]	Orange-red
None	714, 000	Blue-violet	Red-violet	Violet-red	Violet-red	Red[a]	Orange-red	Red-orange

[a] The end-point tube.

It is interesting to note that the same result was obtained with different samples of histidine prepared from three different sources. (The purchased histidine dichloride was prepared from blood corpuscles by the method of Hanke and Koessler.)[13]

Discussion.

In order to show whether the results obtained were due to some specific action of the amino acids themselves or to a change in the hydrogen-ion

[13] Hanke and Koessler, *J. Biol. Chem.*, **43**, 521 (1920).

concentration of the hydrolysis mixtures due to the addition of the amino acids, careful measurements were made of the hydrogen-ion concentrations in the starch pastes as prepared for the experiments. The results are given in Table VI and show that a practically uniform hydrogen-ion concentration of about 10^{-7} was maintained in the different hydrolysis mixtures. This concentration had previously been found to give the optimum saccharogenic activity[14] of this amylase. In a few cases, determinations of the hydrogen-ion concentrations were made in the starch pastes both before and after digestion but no difference was found.

TABLE VI.

HYDROGEN-ION CONCENTRATION IN MOLES PER LITER AS FOUND IN THE DIGESTION MIXTURES USED.

50 mg. of amino acid used in each case.

Starch paste plus.	C_{H^+} (electrometric) $\times 10^7$	C_{H^+} (colorimetric) $\times 10^7$
No amino acid	1.1	1.1
Arginine, (gelatin-a)	1.2	1.1
Arginine, (gelatin-b)	....	1.1
Glycine	1.1	1.1
Histidine, (casein)	1.0	1.6to 1.1
Histidine, (gelatin)	...	1.1
Histidine, (purchased)	1.1	1.6 to 1.1
Tryptophane	1.1	1.1
Cystine	...	1.6 to 1.1
Phenylalanine	...	1.1

The colorimetric determinations of the hydrogen-ion concentrations were made with Sorensen's phosphate buffer mixtures, as described by Clark,[15] as standards. The electrometric determinations were made with the Clark[16] rocking electrode.

Since sodium chloride and disodium phosphate were present in optimum concentration in all cases, the effects of the added amino acids cannot be due to mere change in the concentration of electrolytes in the hydrolysis mixtures.

The possible influence of a reaction between iodine and tryptophane or histidine was considered and by experiments with different measured amounts of the iodine test solution, controlled by suitable blank tests, it was shown that this was not the reason for the difference in behavior of the tryptophane and histidine compared with the other amino acids.

It is plain from the evidence presented in this and previous papers that most amino acids such as result from hydrolysis of common proteins have

[14] THIS JOURNAL, **41**, 231–235 (1919).

[15] "Determination of Hydrogen Ions," by W. M. Clark, Williams and Wilkins, 1920, p. 76.

[16] Clark, *J. Biol. Chem.*, **23**, 475–86 (1915).

a favorable influence upon the hydrolysis of starch by pancreatic amylase, while tryptophane and histidine do not.

The favorable influence shown by arginine, cystine, and all of the monoamino acids which have been tested, may be attributed either to a direct or an indirect effect, or to both.

Let us consider first the hypothesis of direct action. Since all the amino acids here considered contain the α-amino group to which it would seem that any direct activating influence must be attributed (since this is the feature which they possess in common, and the only feature to which the activating influence of glycine can be ascribed) it would follow that the negative results obtained with histidine and tryptophane must be due to some inhibitory influence exerted by their respective heterocycles.

We may, however, also conceive the favorable influence of the amino acids as being exerted not directly upon the interaction of the enzyme and the starch but rather through protecting the enzyme from deterioration in the aqueous dispersion in which it acts. In previous papers from this laboratory many observations have been recorded which seem to find their best explanation in the view that the enzyme is itself a substance of protein nature or containing protein as an essential constituent. The deterioration of the enzyme in water is markedly accelerated by rise in temperature and at the heat at which the enzyme activity is rapidly and permanently lost there is evidence of the splitting of protein substance into an albumin which coagulates and a non-coagulable product or products of hydrolysis. This and observations upon loss of enzyme activity during dialysis support the view that the deterioration of the enzyme in water is due to the hydrolysis by the water of the protein material which makes up the enzyme molecule or an essential part of it. Such hydrolysis should be retarded, and the activity of the enzyme therefore conserved, by the presence of any of the amino acids which would be formed as hydrolytic products. This would account for the fact that so many amino acids have such similar effects, since they are all products of the hydrolysis of protein material of which our purified enzyme preparations are essentially composed. The extreme sensitiveness and instability of purified pancreatic amylase in water suggests that the loss of activity probably coincides with a relatively early stage of its hydrolysis. Hence if we conceive tryptophane and histidine to be so bound in the enzyme molecule that their liberation would occur only at late stages of hydrolysis when the enzyme activity had already been lost, we should have an explanation of the difference in behavior between these and other amino acids entirely consistent with the view that the favorable influence of amino acids generally is exerted through conservation of the enzyme rather than through direct activation of it, and need not necessarily be influenced by the differences of structure which exist among the amino acids themselves.

In other words according to one hypothesis the explanation of the differences observed in our experiments with different amino acids would be sought in the structure of the amino acid radical, and according to the other, in the structure of the enzyme molecule. In our opinion both of these hypotheses may be correct and experiments designed to throw further light upon them are now in progress.

Summary.

Two preparations of arginine obtained from gelatin, and two of histidine, one from gelatin and the other from casein, were tested for their influence upon the action of the enzyme in comparison with blank tests, with the mono-amino acids, glycine and phenylalanine, with each other, with purchased preparations of histidine and tryptophane, and with cystine prepared by another worker in this laboratory.

Tested by measuring the amyloclastic action of purified pancreatic amylase upon "soluble" starch, it has been shown that arginine and cystine, like glycine and phenylalanine, influence favorably the digestion of the starch and that histidine and tryptophane do not. Consistent results were obtained with the two preparations of arginine and the three of histidine.

The tests with all these amino acids were controlled as to hydrogen-ion concentration, in most cases by both the colorimetric and the electrometric methods. It is therefore established that the influence of the aminoacid upon the extent of the hydrolysis of the starch by the enzyme is not simply a matter of buffer effect or other effect upon the hydrogen-ion concentration of the digestion mixtures.

Thus it has been shown that the amino acids here studied differ among themselves in their effects upon the enzymic hydrolysis of starch, whereas the different mono-amino acids studied have shown a practically uniform behavior in this respect. Hence it appears that there are specific effects, probably though not necessarily connected with differences of structure, which may offset the favorable influence uniformly shown by the mono-amino acids studied previously. Further experiments are in progress which it is hoped will throw additional light upon the mode of action of the amino acids.

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

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