[CONTRIBUTION FROM THE LABORATORY OF PHYSIOLOGICAL CHEMISTRY, UNIVERSITY OF IOWA]

THE MECHANISM OF THE ACTION OF AMINO PROMOTERS UPON ENZYMES

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It has been shown in this Laboratory, as well as elsewhere,[1] that α-amino-carboxylic acids act with ptyalin and urease as auxo-amylases and auxo-ureases. Sherman and Naylor[2] have criticized the conclusion drawn that the effect of ptyalin is a specific one between the enzyme and the auxo-amylase. They prefer to regard it as "due to the influence of the added substance upon hydrogen ion or electrolyte concentration rather than to any specific effect of the organic groups."[3] They suggest that the action of the amino acid may be "due to the conservation of the enzyme through diminution of its hydrolytic destruction." "The results can all be explained on the basis of the conservation hypothesis alone."[4] Sherman and Walker repeat this opinion.[5]

The experiments here reported were planned for the purpose of analyzing the activities of urease and ptyalin into their several factors, and determining, as far as possible, the extent to which each of these factors is responsible for the increased hydrolysis.

The promoters were exactly neutralized before mixing with the other solutions. The hydrogen-ion concentration was determined colorimetrically. In the ptyalin fermentation it remained constant. Under these conditions of fermentation with urease, which are essentially those of urinary fermentation, there can naturally be no constancy of hydrogen-ion concentration.

A freshly prepared solution of urease from the Jack bean was made by Van Slyke and Cullen's method[6] and divided into three parts, A, B and C. To A no promoter was added. To B the promoter was added immediately. C was allowed to stand without a promoter until the beginning of the fermentation period, when the promoter and urease solution were mixed. The length of the fermentation period for C was one hour at room temperature. The three solutions were kept at the same temperature.

Calling the decrease in activity which takes place when the urease

[1] Rockwood, THIS JOURNAL, **39**, 2745 (1917). Jacoby and Umeda, *Biochem. Z.*, **68**, 23 (1915). Sherman and co-workers, THIS JOURNAL, **41**, 1867 (1919) and later papers. Rockwood and Husa, *ibid.*, **45**, 2678 (1923).

[2] Sherman and Naylor, *ibid.*, **44**, 2957 (1922).

[3] Ref. 2, p. 2962.

[4] Ref. 2, p. 2966.

[5] Sherman and Walker, THIS JOURNAL, **45**, 1960 (1923).

[6] Van Slyke and Cullen, *J. Biol. Chem.*, **19**, 211 (1914).

solution stands, its decay, the action of a promoter may be due to its preventing the decay, to its stimulation of the enzyme through some specific interaction of the two, or to both factors. A consideration of the relationship of the results from the three fermentations leads to the following analysis of the problem.

Using the abbreviations, D = decay before fermentation period, d = decay during fermentation period, P = prevention of decay before fermentation period, p = prevention of decay during fermentation period; fermentation A = activity − (D + d); fermentation B = activity − (D + d) + stimulation + P + p; fermentation C = activity − (D + d) + stimulation + p; then B − C = P = prevention of decay before fermentation; C − A = stimulation + prevention of decay during fermentation hour (p); $A_1 - A_2$ = D + d = decay.

Experiments with Urease

A mixture of 0.1 g. of the dry urease with 100 cc. of water was filtered to give a uniform solution. The buffer was a mixture of mono- and disodium phosphates; its Sörensen value (P_H) was 7.0. The mixture most commonly employed was composed of 5 cc. of urease solution, 10 cc. of buffer, 5 cc. of amino acid solution, 15 cc. of 3% urea solution and water to make 50 cc. The concentration of amino acid given in the tables is that of the whole mixture. The urea was added last, at the beginning of the fermentation period, and an excess was always present. At the end of the fermentation period an equal volume of a saturated solution of potassium carbonate was added and the ammonia was aspirated into an excess of 0.1 *N* hydrochloric acid. The time of aspiration was not less than 15 hours, the air being first drawn through a sodium bicarbonate solution. The excess acid was titrated with 0.05 *N* ammonium hydroxide, with methyl red as indicator. The numerical results given represent cubic centimeters of 0.05 *N* ammonia formed by the fermentation, in other words the activity of the urease under the conditions given.

The promoters studied were one mono-amino-monocarboxylic acid, glycine; one mono-amino-dicarboxylic acid, aspartic acid; and one substituted α-amino acid, hippuric acid. The rapidity of decay was found by comparing the amount of ammonia formed in the one-hour fermentation periods, after the enzyme solution had stood for different intervals. It was rapid in the first few hours, decreasing afterwards.

Comparing these results and using the above method of analyzing them, it is shown that the promoter effects are due to two distinct factors: first, a prevention of decay of the urease: second, a much greater specific stimulating effect. The first is obtained in the difference between the amounts of hydrolysis in Fermentations B and C. This gives the total prevention of decay and the hourly average.

The difference between Fermentations C and A represents the sum of the stimulation factor and that of the prevention of decay during the

TABLE I

ACTION OF PROMOTERS ON UREASE

In the cubic centimeters of 0.05 *N* ammonia formed from the urea

Hours of standing before fermentation	No promoter A	Promoter from beginning B	Promoter for fermentation only C	Activity increased % B/A	Activity increased % C/A	Decay $A_1 - A_2$ Total	Decay $A_1 - A_2$ Per hour	Prevention of decay B − C Total	Prevention of decay B − C Per hour	Stimulation + p one hour C − A
ACTION OF 0.005 *N* GLYCINE										
0	29.0	41.5	41.5	143	143	...	..	...	...	12.5
6	14.0	26.5	17.3	189	124	15.0	2.5	9.2	1.5	3.3
24	6.4	19.2	8.5	300	133	7.6	0.42	10.7	0.6	2.1
48	4.0	13.6	6.3	340	158	2.4	.10	7.3	.3	2.3
93	1.4	8.0	3.3	571	236	2.6	.067	4.7	.1	1.9
148	0.7	5.8	1.2	828	171	0.7	.012	4.6	.1	0.5
ACTION OF 0.002 *N* ASPARTIC ACID										
0	32.0	39.4	39.4	123	123	...	...	...	..	7.4
7	12.9	20.0	16.8	155	130	19.1	2.7	3.2	0.46	3.9
25	8.2	17.6	10.6	215	129	4.7	0.26	7.0	.39	2.4
50	5.7	14.4	8.4	253	147	2.5	.10	6.0	.28	2.7
72	3.6	15.9	6.8	442	189	2.1	.10	9.1	.41	3.2
ACTION OF 0.002 *N* HIPPURIC ACID										
0	7.4	11.0	11.0	149	149	...	...	...	..	3.6
43	5.3	7.7	5.8	145	109	2.1	0.05	1.9	0.04	0.5
65	4.4	5.7	4.7	129	107	0.9	.04	1.9	.045	0.3
89	3.5	5.0	5.0	143	143	0.9	.04	0.	0.	1.5

last hour (p). The size of p can be shown to be very small by comparison with the average hourly prevention of decay before fermentation. This average per hour must be larger than p since it is decreasing, and p is for the last hour only when the rate is less than that of the hourly average. Subtraction of this small value of p from the figures of C–A shows that the stimulation factor is definite and large.

Experiments with Ptyalin.—In testing the mechanism of the action of promoters upon ptyalin the same principles were followed as with urease. Comparisons were made of the amounts of starch hydrolyzed in the absence of an auxo-amylase (promoter), (A), of that changed when the auxo-amylase stood with the ptyalin through the whole period (B), and that where the ptyalin stood alone until the period of digestion when the auxo-amylase was added (C). Washed cornstarch was used, thoroughly boiled with water. The promoter was made neutral with dil. sodium hydroxide solution, and to each 50 cc. of the digestion mixture was added 1 cc. of a concentrated buffer, P_H 7.0. The digestion continued for one hour at 32–33°. The saliva was from the same person, was diluted with 9 volumes of water and the diluted saliva was filtered before using. The same sample of saliva was used throughout each individual series of tests.

One-sixth of the mixtures described above was taken at intervals for each digestion period of one hour. In no case was the starch completely hydrolyzed.

The amount of reducing sugar formed was determined by heating with an excess of Fehling's solution and titrating the remaining soluble copper with potassium iodide and 0.1 *N* sodium thiosulfate solutions.[7]

TABLE II

ACTION OF PROMOTERS ON PTYALIN

Mixtures used		A	B	C
10% saliva, cc.	Mixed at the beginning	9	9	9
Water, cc.		141	111	111
0.1 *N* promoter, cc.		None	30	None
0.1 *N* promoter, cc.	Added at the time of digestion	None	None	30
1% starch, cc.		150	150	150

Thus the total volume of each was 300 cc. In this the concentration of the amino compound was 0.01 *N*.

The amount of fermentation is expressed in terms of cubic centimeters of Fehling's solution reduced after a digestion period of one hour.

Hours of standing before fermentation	No promoter A	Promoter from beginning B	Promoter for fermentation only C	Activity increased % B/A	Activity increased % C/A	Decay $A_1 - A_2$ Total	Decay $A_1 - A_2$ Per hour	Prevention of decay B − C Total	Prevention of decay B − C Per hour	Stimulation + p one hour C − A
ACTION OF 0.01 *N* GLYCINE										
43	7.0	11.8	9.7	168	139	...	..	2.0	0.046	2.7
49	5.7	11.1	8.4	194	147	1.4	0.25	2.7	.45	2.7
67	2.7	9.0	4.7	337	174	3.0	.17	4.3	.24	2.0
ACTION OF 0.01 *N* GLYCINE										
0	7.6	7.7	7.7	101	101	...	...	...	...	0.1
48	4.8	7.8	7.1	170	148	2.8	0.958	0.7	0.015	2.3
55	3.4	7.3	5.6	215	165	1.4	.020	1.7	.24	2.2
ACTION OF 0.01 *N* ASPARTIC ACID										
0	14.1	21.5	21.5	152	152	...	...	...	...	7.4
16	14.5	21.5	21.1	148	145	...	...	0.4	0.025	6.6
41	13.8	21.1	20.6	153	150	0.7	0.017	.5	.020	6.8
98	12.1	19.1	18.2	159	150	1.7	.03	.9	.017	6.1
120	9.0	15.2	14.5	169	161	3.1	.14	.7	.032	5.5

Analysis of these results leads to the same conclusions as those from urease, namely, that the specific stimulating effect of these amino compounds is much greater than their action in preventing decay of the enzyme, although both effects are evident. These amino compounds are auxo-amylases.

Summary

The rate of decay of urease and ptyalin in solution has been measured. The activity of these enzymes has been determined alone and in the presence of α-amino compounds, which act as promoters.

[7] Lehmann and Maquenne, *Z. physiol. Chem.*, **88**, 207 (1913).

The mechanism of the action of the promoters has been studied and found to be due partly to the prevention of decay of the enzyme. The greater part of the action of the promoter is not due to this but to a specific stimulating action of the amino compound.

Any possible hydrogen-ion effect has been eliminated by the use of a buffer.

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ORTHO-BENZOYL-BENZOIC ACIDS CONTAINING FLUORINE, IODINE AND SULFUR[1]

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On account of their ready conversion into anthraquinone derivatives the substituted *o*-benzoyl-benzoic acids have been extensively studied. As the chloro- and bromobenzoyl-*o*-benzoic acids and a number of their methyl derivatives are well known, it seemed of interest to investigate the corresponding fluoro- and iodo-compounds. In addition the methyl ether of thiophenol has been condensed with phthalic anhydride. The *o*-benzoyl-benzoic acids obtained have been condensed to anthraquinone derivatives.

Results

All condensations were made in the usual way using two molecules of aluminum chloride to one each of phthalic anhydride and the substituted hydrocarbon.

Fluoro-benzoyl-*o*-benzoic Acid and Methyl Derivatives.—Fluorobenzene condenses readily to give the 4′-acid.[2] This constitution was assumed on account of analogy with the chloro- and bromo-acids.[3] Fluorine orients a negative group to the *para* position even more strongly than the other halogens, since the nitration of fluorobenzene yields 90% of *p*-nitrofluorobenzene. This constitution was proved by the fact that the acid formed yields *p*-hydroxybenzoyl-*o*-benzoic acid[4] when boiled with a concd. solution of sodium hydroxide. The hydroxy acid proved to be identical with that prepared by Friedländer.

From *p*-fluorotoluene two acids, I and II, are possible. The low melting point of the crude acid and of crystals from the mother liquors even after it is recrystallized, appears to indicate that both are formed; but one predominates and is easily obtained pure. Since it has been shown

[1] From the Doctor's Dissertation of Frederick C. Hahn, 1923.

[2] Ger. pat. 75,288; Meister, Lucius and Brüning.

[3] Ullmann, *Ann.*, **380**, 337 (1911).

[4] Friedländer, *Ber.*, **26**, 176 (1893).