[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, COLUMBIA UNIVERSITY]

A Study of the Influence of Heavy Water upon the Activities and upon the Stabilities of the Amylases of Barley and of Malted Barley

BY M. L. CALDWELL, S. E. DOEBBELING AND F. C. VONWICKLEN

The study of the influence of heavy water upon the activity and stability of pancreatic amylase[1] has been extended to include similar quantitative measurements of its influence upon the activities and stabilities of the amylases of barley and of malted barley, representative starch-splitting enzymes of the plant kingdom, which are now also available as highly purified and active products[2] suitable for such work. The results of this extensive study with several different preparations of each of these amylases are summarized briefly here. The general methods used have already been described.[1] The precautions previously found necessary[1] were again carefully observed. The heavy water was kindly furnished by Professor H. C. Urey and was further purified[1] for the enzymic measurements.

Influence of Heavy Water upon the Activities of the Amylases of Barley (β) and of Malted Barley (α and β).—The influence of heavy water upon the activities of these amylases was studied by comparable measurements of their action in the presence of heavy water (99%) and in its absence. In each case, the conditions were those which previously[2] had been found to be most favorable to the action and stability of the enzyme concerned.

In the measurements of saccharogenic action, the reducing sugar formed from 1% soluble potato starch was determined iodometrically[3] and calculated to maltose. Briefly, equal volumes of a concentrated aqueous solution of the highly purified amylase being studied were diluted under the same conditions at 5° with purified 99.9%[1] heavy water or with similarly purified ordinary water. Equal portions of these two enzyme solutions then reacted side by side at 40 ± 0.02° with equal portions of each of two substrates, made up in the same way from the same starch and buffer salts but, in one case, in purified 99.9%[1] heavy water, and, in the other, in purified ordinary water. The maltose formed in the reaction mixtures was determined[3] in aliquots removed at frequent intervals.

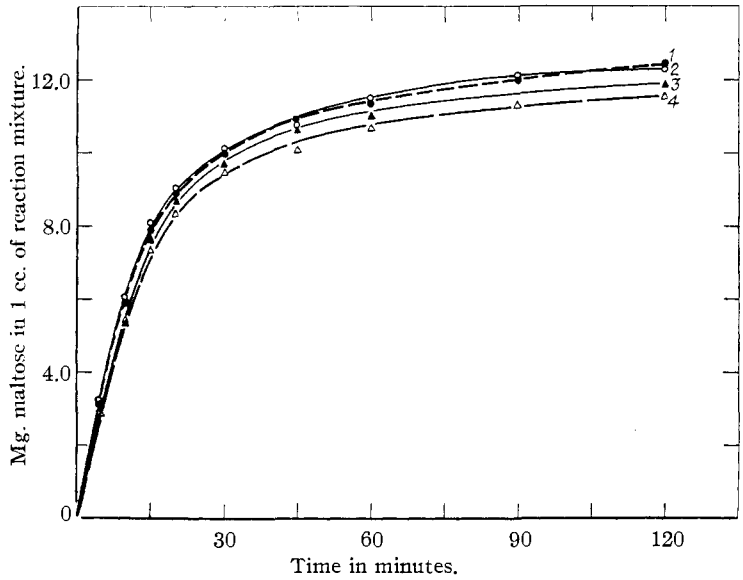

Fig. 1.—A comparison of the hydrolysis of starch by β-amylase of malted barley in the presence of heavy and of ordinary water. Starch: 1% soluble potato; 0.01 *M* acetate; *p*H 4.5. Each curve represents the average of two sets of values. Curve 1, amylase dissolved in heavy water reacting with starch made up in ordinary water. Curve 2, amylase dissolved in ordinary water reacting with starch made up in ordinary water. Curve 3, amylase dissolved in heavy water reacting with starch made up in heavy water. Curve 4, amylase dissolved in ordinary water reacting with starch made up in heavy water.

Results typical of the saccharogenic action of the β-amylase of malted barley in the presence of heavy and of ordinary water are summarized in Table I and in Fig. 1. The data for the different curves in Fig. 1 are strictly comparable and it is evident that even 99% heavy water has no appreciable influence upon the saccharogenic activity of this amylase under the conditions of these experiments.

The data on this point for the saccharogenic

(1) Caldwell, Doebbeling and Manian, THIS JOURNAL, **58**, 84 (1936).

(2) (a) Sherman, Caldwell and Doebbeling, *J. Biol. Chem.*, **104**, 501 (1934); (b) Caldwell and Doebbeling, *ibid.*, **110**, 739 (1935); (c) Caldwell, Doebbeling and VonWicklen, unpublished data.

(3) Caldwell, Doebbeling and Manian, *Ind. Eng. Chem., Anal. Ed.*, **8**, 181 (1936).

TABLE I

HYDROLYSIS OF STARCH BY β-AMYLASE FROM MALTED BARLEY IN THE PRESENCE OF HEAVY WATER OR OF ORDINARY WATER

Time, min.	Enzyme dissolved in H_2O; Starch dispersed in H_2O maltose[a] mg.	Enzyme dissolved in H_2O; Starch dispersed in D_2O maltose[a] mg.	Enzyme dissolved in D_2O; Starch dispersed in H_2O maltose[a] mg.	Enzyme dissolved in D_2O; Starch dispersed in D_2O maltose[a] mg.
5	3.26	2.88	3.14	3.01
10	6.15	5.55	5.91	5.40
15	8.10	7.35	7.95	7.63
20	9.03	8.38	8.97	8.70
30	10.19	9.49	10.05	9.77
45	10.78	10.03	10.93	10.65
60	11.49	10.65	11.42	11.00
90	12.10	11.30	12.09	
120	12.28	11.54	12.42	11.91

[a] Maltose per 1.17×10^{-6} g. enzyme preparation.

activities of the other amylases studied (β-amylase of barley and α-amylase of malted barley) are very similar, lead to the same conclusions, and are omitted for the sake of brevity.

Again, in corresponding measurements, the amyloclastic activity[2b] of the α-amylase of malted barley was also found not to be appreciably influenced even by 99% heavy water—no measurable α-amylase activity[2b] was observed with the β-amylase preparations from barley or from malted barley.

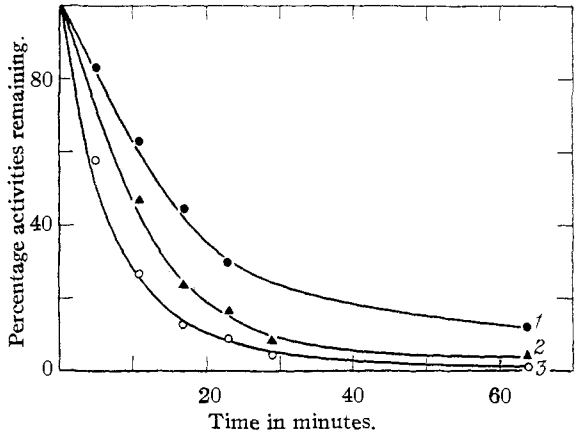

Fig. 2.—Inactivation of β-amylase from malted barley at 65° as influenced by heavy as compared with ordinary water. Curve 1, amylase in purified 99% heavy water. Curve 2, amylase in purified 50% heavy water. Curve 3, amylase in purified ordinary water.

Influence of Heavy Water upon the Stabilities of the Amylases of Barley and of Malted Barley.—Experiments designed to study the influence of heavy water upon the stability as distinguished from the activity of each of these amylases were also carried out. In these measurements, portions of concentrated purified aqueous amylase solutions at 5° were diluted 100-fold with either purified heavy or with purified ordinary water at the same temperature. The resulting diluted enzyme solutions were then held at 65° and examined at intervals for saccharogenic activity. This was compared with that of the diluted unheated enzyme solution, taken as 100%.

In the measurements of the activities of these solutions, the enzymes reacted for thirty minutes at 40 ± 0.02° with 1%-soluble potato starch made up in purified ordinary water and adjusted[2] so as to minimize further deterioration of the enzyme and to favor its action.[2]

Typical data for the β-amylase of malted barley are summarized in Table II and in Fig. 2. These show that the loss of amylase activity is less in the presence of heavy water (99 or 50%) than in that of ordinary water. Similar results, which are omitted for the sake of brevity, were also obtained consistently with the other plant amylases studied.

TABLE II

INFLUENCE OF HEAVY WATER UPON STABILITY OF DILUTE SOLUTIONS OF HIGHLY PURIFIED β-AMYLASE FROM MALTED BARLEY

Time of inactivation min.	Temperature of inactivation 65°; H_2O Maltose[a] mg.	H_2O Activity remaining %	50% D_2O Maltose[a] mg.	50% D_2O Activity remaining %	99% D_2O Maltose[a] mg.	99% D_2O Activity remaining %
0	6.83	100.0[b]	5.96	100.0[b]	6.48	100.0[b]
5	3.77	58.1			5.43	83.7
11	1.84	26.9	2.80	47.0	4.29	62.8
17	0.88	12.8	1.40	23.5	2.89	44.6
23	.61	9.0	0.96	16.2	1.93	29.9
29	.35	5.1	.53	8.8		
64	.09	1.3	.26	4.4	0.79	12.1

[a] Maltose per 3.4×10^{-6} g. of enzyme preparation. [b] 100% activity is that of enzyme solution before inactivation.

Discussion

The data reported here indicate that the unfavorable influence previously observed[4] for heavy water upon the germination of barley and upon the generation of amylase activities during this process is not explained by its inactivation of the amylases or by its interference with their activities. The heavy water may, however, influence the stabilities of inactive combinations of these amylases in the grain or may unfavorably affect the action of other enzymes in the grain which are normally needed to set free or

(4) Caldwell and Doebbeling, *J. Biol. Chem.*, **123**, 479 (1938).

to form the amylases from some inactive combination or state.

A comparison of the influence of heavy water upon the plant amylases studied here and upon pancreatic amylase previously investigated[1] is of interest.

The activity measurements with all of these different amylases lead to the same conclusion: that even 99% heavy water has no appreciable effect upon the activities of these enzymes provided the hydrolysis of the starch is carried out under conditions which favor the action and minimize the inactivation of the amylase concerned. This finding assumes added significance when it is remembered that the different amylases were studied as highly purified products, in a condition which increases their sensitivity to their chemical environment.

The measurements of the stabilities of these amylases, on the other hand, give different results. With pancreatic amylase, there is decreased stability of the enzyme in the presence of heavy water,[1] while with the amylases of barley (β) and of malted barley (α and β) an increased stability of the enzyme in the presence of heavy water is observed. No attempt is made to explain these differences at this time.

Summary

Working with highly purified heavy water and with highly purified preparations of the amylases of barley (β) and of malted barley (α and β), it has been found that heavy water (99%) has no appreciable influence upon the hydrolysis of starch as catalyzed by any of these enzymes provided the conditions of the hydrolysis are such as to minimize the deterioration of the amylase and to favor its action. Similar results previously have been reported for highly purified pancreatic amylase.

Stability measurements show that inactivation of these plant amylases is much less rapid and less pronounced in highly purified heavy water than in similarly purified ordinary water.

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The Production of Fumaric Acid by Molds Belonging to the Genus *Rhizopus*[1]

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Fumaric acid is one of the less commonly encountered products of mold metabolism. Aside from the important biochemical fact that this acid is unsaturated, it is of particular interest because, with only two exceptions, the organisms capable of producing this acid were always found to belong to the family *Mucoraceae*. The two exceptions are the isolation by Wehmer[2] of *Aspergillus fumaricus*, which gave almost 70% conversion of the sugar to fumaric acid, and the separation by Raistrick and Simonart,[3] of an unstated quantity of this acid from the culture medium of a *Penicillium*. However, it was later found[4] that upon continued cultivation Wehmer's organism lost the ability to produce fumaric acid.

Many species of *Rhizopus* have been found capable of producing varying quantities of fumaric acid, frequently accompanied by one or more other acids. Ehrlich,[5] using a culture designated as *Mucor stolonifer* (*Rhizopus nigricans*), first established, in 1911, this property for filamentous fungi. No neutralizing agent was added to the cultures, as a result of which low yields of acid were obtained. Ehrlich considered this acid to be an intermediate product in the breakdown of the carbohydrate by the fungus, rather than an end-product of metabolism. Takahashi, *et al.*,[6] found that *Rhiz. japonicus* gave a 16% yield of fumaric acid, accompanied by a small amount of lactic acid, whereas other species of *Rhizopus* produced smaller quantities of the fumaric acid; ethyl alcohol, formic, malic and acetic acids were also detected in the culture solutions. Takahashi suggested that the lactic acid originated from the fumaric acid by decarboxylation; gluconic acid was believed[7] to be an intermediate

(1) Journal Series Paper, N. J. Agricultural Experiment Station, Department of Soil Chemistry and Microbiology.

(2) C. Wehmer, *Ber.*, **51**, 1663 (1918).

(3) H. Raistrick and P. Simonart, *Biochem. J.*, **27**, 628 (1933).

(4) W. Thies, *Centrbl. Bakt.*, II, **82**, 321 (1930).

(5) F. Ehrlich, *Ber.*, **44**, 3737 (1911); **52**, 63 (1919).

(6) T. Takahashi, K. Sakaguchi and T. Asai, *Bull. Agr. Chem. Soc. (Japan)*, **2**, 5 (1926); **3**, 59 (1927).

(7) T. Takahashi and T. Asai, *Proc. Imp. Acad.* (Japan), **3**, 86 (1927).