

in which it was only sparingly soluble, and the purified material melted at 120°.

Anal. Calcd. for C₁₆H₁₂O₈: C, 75.0; H, 5.0. Found: C, 75.0; H, 5.06.

The rearrangement product was shown to be the benzoate of benzoylcarbinol (IV) by a mixed melting point with an authentic sample of that carbinol prepared by the method of Rather and Reid.¹²

(12) Rather and Reid, *THIS JOURNAL*, **41**, 75 (1919).

Summary

Dibenzoylcarbinol has been prepared and its chemical behavior described. The carbinol, which does not exist as an ene-diol, is very readily oxidized. It is cleaved by alkali and rearranges on heating to furnish the benzoate of benzoylcarbinol.

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[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, COLUMBIA UNIVERSITY]

A Study of the Influence of Heavy Water upon the Activity and upon the Stability of Pancreatic Amylase

BY M. L. CALDWELL, S. E. DOEBBELING AND S. H. MANIAN

Since the discovery of heavy water there have been conflicting reports as to its influence upon biological processes including enzyme action. The data so far obtained are too meager to be properly evaluated but sufficient to indicate the need for further carefully controlled work with typical enzymes.¹

A quantitative study is reported briefly here of the influence of heavy water upon the activity and upon the stability of pancreatic amylase. Such studies are of special interest because the amylases, of which this one is typical, catalyze hydrolytic reactions in which water is an important factor. Moreover, there were available for this work, preparations of pancreatic amylase of exceedingly high purity and activity.² This makes the systems more sensitive and the interpretation of the results in terms of the influence of heavy water upon the enzyme more convincing than would be the case if less highly purified enzyme preparations had been used.

The heavy water was kindly supplied by Professor H. C. Urey. The methods for its concentration, purification, and analysis have been described previously.³ It was further purified for the enzyme work by three redistillations: the first, from permanganate made alkaline by the addition of anhydrous sodium carbonate; the second, after the addition of a few milligrams of phosphorus pentoxide; and the last, without fur-

ther treatment. The distillations were carried out slowly, under atmospheric pressure, in all Pyrex glass apparatus equipped with suitable traps. The first and last few cubic centimeters of the distillate were not used for the enzyme work. The heavy water was found to be free from oxidizing substances (ozone and peroxides) as shown by the potassium-iodide-starch test, which was found to be sensitive to one part of hydrogen peroxide in 500,000. The ordinary distilled water used was treated in the same way in the same apparatus.

Influence of Heavy Water upon the Activity of Pancreatic Amylase.—The activity of the enzyme was studied both by determinations of its so-called amyloclastic and of its saccharogenic action. For the former, a modification of the Wohlgemuth method⁴ was used. In the latter, the reducing sugar formed in the reaction mixtures was determined iodimetrically⁵ and calculated to maltose. In all cases, the conditions were those which have been found most favorable to the activity and stability of the enzyme.⁶ Every effort was made to keep the measurements in the presence of heavy and of ordinary water strictly comparable and to eliminate as far as possible the influence of any other variable factors.

Briefly, equal weights of the enzyme preparation were dissolved under the same conditions at 0° in purified 100% (99.9 +)³ heavy water and in 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

(1) (a) Barnes and Larson, *THIS JOURNAL*, **55**, 5059 (1933); (b) *Protoplasma*, **23**, 431 (1934); (c) Hughes, Yudkin, Kemp and Rideal, *J. Chem. Soc.*, 1105 (1934); (d) Steacie, *Z. physik. Chem.*, **27B**, 6 (1934); **28B**, 236 (1935); (e) Pacsu, *THIS JOURNAL*, **56**, 245 (1934); (f) Fox, *J. Cell. and Comp. Physiol.*, **6**, 405 (1935).

(2) Sherman, Caldwell and Adams, *J. Biol. Chem.*, **88**, 295 (1930).

(3) (a) Brown and Dagget, *J. Chem. Phys.*, **3**, 216 (1935); (b) Crist, Murphy and Urey, *ibid.*, **2**, 112 (1934).

(4) (a) Wohlgemuth, *Biochem. Z.*, **9**, 1 (1908); (b) Sherman and Thomas, *THIS JOURNAL*, **37**, 823 (1915).

(5) Caldwell, Doebbeling and Manian, unpublished data.

(6) Sherman, Caldwell and Adams, *THIS JOURNAL*, **50**, 2529, 2535, 2538 (1928).

of two substrates, made up in the same way from the same starch and buffer salts but in one case in purified 100% heavy water and in the other in purified ordinary water. The maltose formed in the reaction mixtures was determined^{5,6} in aliquots removed immediately after mixing the enzyme with the substrate and at frequent intervals thereafter.

Before use, the starch, Lintner's soluble potato starch,⁷ and the buffer salts⁶ were purified and dried in vacuum (1-mm. pressure) at room temperature to constant weight over phosphorus pentoxide. All manipulations were carried out in glass-stoppered Pyrex glass containers. It was found necessary to reflux the starch for thirty minutes to obtain satisfactory dispersion in heavy water. The starch made up in ordinary distilled water was therefore also refluxed for thirty minutes in the same apparatus.

Results, typical of the saccharogenic action of pancreatic amylase in the presence of heavy water and of ordinary water, are summarized in Table I and in Fig. 1. Table I gives a representative series of measurements while the curves in Fig. 1 are average values. Here, the maltose formed in the reaction mixtures is plotted (mg. per cc.) against time in minutes. As has already been pointed out, the data for the different curves are strictly comparable. It is evident that even 100% heavy water has no marked influence upon the saccharogenic activity of pancreatic amylase provided the conditions of the experiments are such as to minimize the deterioration of the enzyme.

TABLE I
HYDROLYSIS OF STARCH BY PANCREATIC AMYLASE IN THE PRESENCE OF HEAVY WATER OR OF ORDINARY WATER

Time, min.	Enzyme dissolved in H ₂ O Starch dispersed in H ₂ O		Enzyme dissolved in D ₂ O Starch dispersed in D ₂ O	
	maltose, ^a mg.	maltose, ^a mg.	maltose, ^a mg.	maltose, ^a mg.
Immediately	1.3	1.1	1.4	1.3
17	5.6	4.8	5.8	
22				7.2
31			8.7	8.7
34	8.5	7.6		
48			11.1	10.5
53		9.7		
56	11.1			
76.5	11.9	11.4	12.5	12.5
100.5	12.5	11.9	12.8	12.8
131	13.2	12.5	13.2	13.2
161.5	13.6	13.3	13.7	13.8
280	14.7	14.0	14.6	14.7

^a Maltose per 2 × 10⁻³ g. of enzyme preparation.

(7) Lintner, *J. prakt. Chem.*, **34**, 378 (1886).

The same conclusion is reached from a study of the results of an extended series of measurements dealing with the so-called amyloclastic action⁴ of the amylase. The data for this phase of the work are omitted for the sake of brevity but also show no marked differences in the hydrolysis of the starch in the presence of heavy and of ordinary water as evidenced by the speed with which products which give a blue color with iodine disappear from the reaction mixtures made up in purified 100% heavy water or in similarly purified ordinary water.

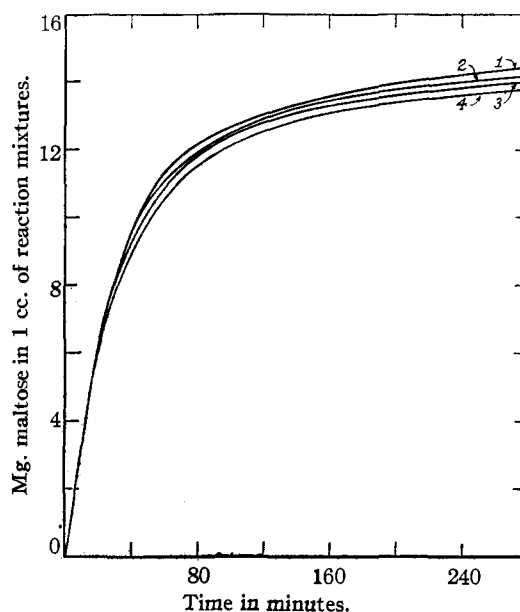


Fig. 1.—A comparison of the hydrolysis of starch by pancreatic amylase in the presence of heavy and of ordinary water. Starch: 2% soluble potato; 0.025 *M* NaCl; 0.01 *M* phosphate; pH 7.1. Each curve represents the average of two sets of values. The numbers in parentheses represent the average deviation of all the experimental points from the curves as drawn. Curve 1. Amylase dissolved in ordinary water reacting with starch made up in ordinary water (0.1 mg. maltose). Curve 2. Amylase dissolved in heavy water reacting with starch made up in ordinary water (0.3 mg. maltose). Curve 3. Amylase dissolved in heavy water reacting with starch made up in heavy water (0.5 mg. maltose). Curve 4. Amylase dissolved in ordinary water reacting with starch made up in heavy water (0.3 mg. maltose).

When critically examined, however, the data for both types of measurements show slightly less amylase activity in the presence of heavy than of ordinary water. The differences, although exceedingly small and often within experimental error, were consistently obtained and appeared of possible theoretical significance. They might

TABLE II
INFLUENCE OF HEAVY WATER UPON STABILITY OF DILUTE SOLUTIONS OF HIGHLY PURIFIED PANCREATIC AMYLASE
Enzyme solution diluted at 25° with

Time after dilution, min.	H ₂ O		Time after dilution, min.	50% D ₂ O		Time after dilution, min.	99% D ₂ O	
	Maltose, ^a mg.	Activity remaining, %		Maltose, ^a mg.	Activity remaining, %		Maltose, ^a mg.	Activity remaining, %
I Immediately	8.0	93.0 ^b	Immediately	7.8	90.7 ^b	Immediately	7.7	89.5 ^b
6	5.6	65.2	5	4.1	47.7	4	4.0	46.5
10.5	5.5	64.0	10	3.7	43.0	8.5	3.7	43.0
20	5.2	60.5	20	2.6	30.2	44	3.2	37.2
45	5.1	59.5	42	2.1	24.4	90.5	3.0	34.9
92	4.2	48.8	92	2.1	24.4			
II Immediately	7.4	88.0 ^c	Immediately	7.0	83.3 ^c	Immediately	7.0	83.3 ^c
6	6.1	72.5	4	4.5	53.5	3.5	4.3	51.2
10	5.9	70.2	8	3.7	44.0	7.5	4.1	48.8
13	5.4	64.3	19	3.3	39.2	11.5	4.0	47.6
20.5	5.3	63.0	25	3.2	38.0	18.5	3.5	41.6
63.5	4.3	51.1	62	2.2	26.2	25	3.4	40.5
						62	2.6	30.9

^a Maltose per 2×10^{-6} g. of enzyme preparation. ^b 100% activity is that of enzyme solution in H₂O at 0° before dilution (8.6 mg. maltose per 2×10^{-6} g. enzyme preparation). ^c 100% activity is that of enzyme solution in H₂O at 0° before dilution (8.4 mg. maltose per 2×10^{-6} g. enzyme preparation).

indicate an actual retardation of the hydrolysis of the amylose, possibly due to side or exchange reactions with heavy water, or an increased inactivation of the enzyme in the presence of heavy water, or both. While the first possibility did not lend itself to further immediate study, the

phase of the work are given in the next section of this paper.

Influence of Heavy Water upon the Stability of Pancreatic Amylase.—Experiments designed to study the influence of heavy water upon the stability as distinguished from the activity of pancreatic amylase were carried out, briefly, as follows. Portions of an enzyme solution, made up in purified ordinary water at 0°, were diluted one hundred-fold with purified heavy or with purified ordinary water which had been brought to 25°. The resulting diluted enzyme solutions were held at 25° and examined immediately and at intervals for saccharogenic activity.⁵ This was compared with that of the undiluted enzyme solution taken as 100%. In all cases, the enzyme reacted for thirty minutes at $40 \pm 0.02^\circ$ with 2% soluble potato starch made up in purified ordinary water and adjusted⁶ so as to minimize further deterioration of the enzyme and to enable it to exert its optimal activity. Thus the activity measurements were all strictly comparable. The only variable was that introduced by the changes in the kind of water used to dilute the enzyme solution and the influence of this water upon the enzyme, before the activity measurements, while it was held in solution at 25°.

The typical data summarized in Table II and in Fig. 2 show that, under the conditions of these experiments, purified 99% heavy water causes a more rapid loss of activity of pancreatic amylase than similarly purified ordinary water. This is

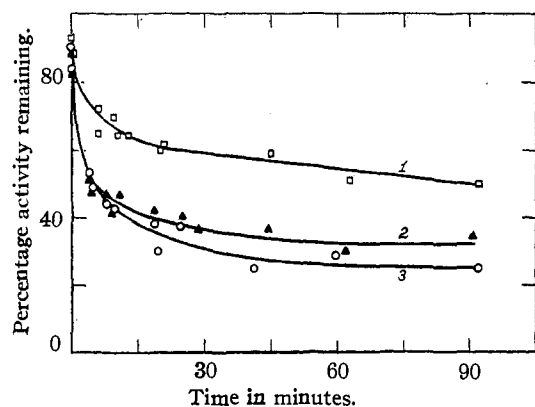


Fig. 2.—Inactivation of pancreatic amylase at 25° as influenced by heavy as compared with ordinary water. (Each curve represents the average of two sets of values.) Curve 1. Amylase in purified ordinary water. Curve 2. Amylase in purified 99% heavy water. Curve 3. Amylase in purified 50% heavy water.

second could be investigated by changing the conditions of the experiments so as to cause increased and easily measurable inactivation of the enzyme.⁸ This was accomplished by dilution of the amylase solution and by raising the temperature at which it was held. The results of this

(8) Day, Dissertation, Columbia University, 1934.

evident not only in the greater velocity of inactivation of the amylase upon standing in heavy water at 25° but also in greater immediate losses of activity of the amylase upon dilution with heavy as compared with ordinary water.

The results obtained in comparable measurements with 50% heavy water, however, seem out of line. These are more variable but indicate greater inactivation of the enzyme in 50% heavy water than is obtained with either 99% heavy water or with ordinary water. In this connection it is of interest to note that Plantefol and Champetier⁹ also report inconsistent results with the germination of *Narcissus papyraceus* in the

(9) Plantefol and Champetier, *Compt. rend.*, **200**, 423 (1935).

presence of different concentrations of heavy water.

Summary

Working with highly purified heavy water and with highly purified preparations of pancreatic amylase, it has been found that (1) 100% heavy water has no marked influence upon the hydrolysis of starch by pancreatic amylase provided the conditions of the experiments are such as to minimize the deterioration of the enzyme and (2) that the inactivation of pancreatic amylase is more rapid and more pronounced when the amylase is held at 25° in highly purified heavy water than in similarly purified ordinary water.

NEW YORK, N. Y.

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[CONTRIBUTION FROM THE CHEMISTRY LABORATORY OF THE UNIVERSITY OF NORTH CAROLINA]

The Activity Coefficients of Lead Chloride in Aqueous Solutions of Barium Nitrate¹

BY H. D. CROCKFORD AND H. O. FARR, JR.

Crockford and Thomas² derived from theoretical considerations the following expression for the mean distance of closest approach of the ions in dilute solutions of strong electrolytes.

$$\bar{a} = \frac{A_1 + A_2}{f_1 + f_2} \quad (1)$$

wherein $A_1/f_1 = a_1$ and $A_2/f_2 = a_2$. In this expression a_1 and a_2 are the individual ion size parameter values for the two electrolytes and the f values are the frequencies of collisions. Hence the mean distance of closest approach in the solution containing the two electrolytes is a function of quantities depending upon the valence types of the electrolytes and the sizes of the individual ions concerned. It was shown by Crockford and Thomas² that the frequency of collision between the ions of a single electrolyte in dilute solutions is proportional to the square of the concentration. For two electrolytes the proportionality constants will be different but if they have small a values Crockford and Thomas have predicted that, due to compensating effects in the calculation of the individual frequencies, the following equation will be true

$$f_1 : f_2 :: C_1^2 : C_2^2 \quad (2)$$

wherein C is the concentration of the electrolyte concerned. Consequently by means of equations

(1) From a thesis presented by Mr. Farr to the faculty of the University of North Carolina as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

(2) Crockford and Thomas, *THIS JOURNAL*, **55**, 568 (1933).

(1) and (2) one can solve for the mean distance of closest approach if the two individual a values are known. Furthermore, if the ratio of the two concentrations is kept constant a constant value for the mean distance of closest approach should be obtained. Of course these considerations apply only to dilute solutions of strong electrolytes.

As a test of the above equations and in order to add to the general data concerning the a parameter of the Debye-Hückel theory a series of studies has been made with the cell.

Pb-Hg(2 phase) | PbCl₂(m_1) Ba(NO₃)₂(m_2) | AgCl-Ag
Three series of solutions have been studied: (I) a constant ratio of lead chloride and barium nitrate; (II) a constant molality of lead chloride and varying concentrations of barium nitrate; and (III) a constant molality of barium nitrate and varying concentrations of lead chloride. From the results have been calculated the activity coefficients of the lead chloride and the \bar{a} parameter. The \bar{a} values have also been calculated from existing data on the individual a values by means of equations (1) and (2).

Experimental Part

Purification of Materials.—Kahlbaum lead chloride was purified in a manner similar to that used by Crockford and Thomas.² C. P. grade of barium nitrate was purified as recommended by Hovorka and Rodebush.³ The salt was crystallized three times from conductivity

(3) Hovorka and Rodebush, *ibid.*, **47**, 1614 (1925).