cinnamoylimidazole is complicated by the protonation of the substrate as well as the protonation of the enzyme, and by the apparent reaction of both protonated and unprotonated substrate species. Although the correspondence of this scheme (eq. 13) to the experimental data is not completely satisfying quantitatively, it is the simplest one which at least qualitatively fits the data.

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Kinetic Isotope Effects of Deuterium Oxide on Several α-Chymotrypsin-catalyzed Reactions[1,2]

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The kinetics of several reactions catalyzed by α-chymotrypsin have been determined in D_2O as solvent, and the results have been compared with the kinetics of the same reactions in H_2O as solvent. By a suitable choice of substrates it has been possible to estimate the effect of D_2O on the binding of the substrate to the enzyme, on the acylation of the enzyme and on the deacylation of the acyl-enzyme intermediate. The substrate *p*-nitrophenyl trimethylacetate was used for studying the acylation reaction, and *trans*-cinnamoyl-α-chymotrypsin and trimethylacetyl-α-chymotrypsin were used for studying the deacylation reaction. A sizable isotope effect was observed for both the acylation and deacylation reactions (k^H/k^D = 2 to 3). The kinetics of the hydrolysis of N-acetyl-L-tryptophan methyl ester were also compared in D_2O and H_2O and it was observed that at high substrate concentrations the limiting rate constant is greater in H_2O than in D_2O by a factor of 2.83. The results indicate a rate-determining proton transfer in the catalytic steps of the enzymatic reaction.

Introduction

Several mechanisms for the chymotrypsin-catalyzed hydrolysis of carboxylic acid derivatives have been proposed; in general these mechanisms involve a catalytic group on the enzyme acting as a general base or as a nucleophile or, in some cases, as both.[4] Usually it has been proposed that this group is an imidazole group of a histidine residue. Evidence in the literature suggests that the rate of a general base-catalyzed reaction is decreased in deuterium oxide relative to that in water by approximately 2- to 3-fold, whereas the rate of a reaction subject to nucleophilic catalysis may be affected to only a small extent by deuterium oxide.[5] Therefore, from the effect of deuterium oxide on the rate of the chymotrypsin-catalyzed reactions, one should be able to distinguish between general base and nucleophilic catalysis by the enzyme. The mechanisms proposed for the enzyme-catalyzed reactions are probably oversimplified; however, from the effect of deuterium oxide on each step of the reaction it should at least be possible to determine whether a proton is transferred in the rate-determining part of each step. Consequently, the effects of deuterium oxide on the rate of each of the various steps of the enzyme reaction were studied and are reported in this part.

From previous work[4] on the mechanism of chymotrypsin catalysis it appears that the reaction involves initially a reversible adsorption of the substrate (S) on the enzyme (E) to give an enzyme-substrate complex (ES). For many substrates the complex then reacts to give an acyl-enzyme (ES′) with concomitant release of the alcohol or amine portion (P_1) of the substrate, and the final step of the sequence involves deacylation of the acyl-enzyme to give the free enzyme (E) and carboxylic acid (P_2). By a suitable choice of substrates it is possible to study the effect of deuterium oxide on each of these steps.

$$\begin{aligned} &E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES && \text{adsorption} \\ &ES \xrightarrow{k_2} ES' + P_1 && \text{acylation} \\ &ES' \xrightarrow{k_3} E + P_2 && \text{deacylation} \end{aligned} \qquad (1)$$

Various workers[1,4,6] using several different acyl-enzymes have studied the kinetics of the deacylation step in water. In the present research the kinetics of the deacylation of *trans*-cinnamoyl-α-chymotrypsin was studied thoroughly using deuterium oxide as solvent; this acyl-enzyme was used because the kinetics of its deacylation in water had been studied extensively and the reaction can be followed conveniently.[1]

Gutfreund and Sturtevant,[7] by using fast reaction techniques, were able to study the kinetics of the acylation of chymotrypsin by *p*-nitrophenyl acetate and 2,4-dinitrophenyl acetate. Since the ester *p*-nitrophenyl trimethylacetate reacts more slowly than the acetate esters with the enzyme,[1] its kinetics are more easily studied and thus this ester was used in the present research. The kinetics of the acylation reaction in both water and deuterium oxide were determined. Gutfreund

(1) This research was supported by grants from the National Institutes of Health and the U. S. Atomic Energy Commission. Paper XIV in the series, The Mechanism of Action of Proteolytic Enzymes; previous paper M. L. Bender, G. R. Schonbaum and B. Zerner, *J. Am. Chem. Soc.*, **84**, 2562 (1962).

(2) Some of the present results have been presented in preliminary form: M. L. Bender, G. R. Schonbaum, G. A. Hamilton and B. Zerner, *ibid.*, **83**, 1255 (1961).

(3) Alfred P. Sloan Foundation Research Fellow; present address: Northwestern Univ., Evanston, Ill.

(4) For recent reviews see (a) M. L. Bender, *Chem. Revs.*, **60**, 53 (1960); (b) M. L. Bender, G. R. Schonbaum and G. A. Hamilton, *J. Polymer Sci.*, **49**, 75 (1961).

(5) M. L. Bender, E. J. Pollock and M. C. Neveu, *J. Am. Chem. Soc.*, **84**, 595 (1962).

(6) M. L. Bender, G. R. Schonbaum and B. Zerner, *ibid.*, **84**, 2540 (1962).

(7) H. Gutfreund and J. M. Sturtevant, *Biochem. J.*, **63**, 656 (1956); *Proc. Natl. Acad. Sci., U. S.*, **42**, 719 (1956).

and Sturtevant[7] were able to separate the rate constant for the formation of the acyl-enzyme intermediate into two parts, k_2 and K_m, where $K_m = (k_2 + k_{-1})/k_1$. For acylation with *p*-nitrophenyl trimethylacetate the same separation can be made and thus one can estimate the effect of deuterium oxide on both k_2 and K_m. From the above investigations it was therefore possible to determine the effect of deuterium oxide on each step of scheme 1.

In addition to investigating the effect of deuterium oxide on the reactions of labile substrates which have been shown to proceed through an acyl-enzyme intermediate, it was thought desirable to perform a comparable investigation with a specific substrate of α-chymotrypsin. To this end the kinetics of the α-chymotrypsin-catalyzed hydrolysis of N-acetyl-L-tryptophan methyl ester in H_2O and D_2O were determined. With this substrate the kinetics were analyzed according to scheme 2. Values for k_{cat} and K_m' (where $K_m' =$

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \qquad (2)$$
$$ES \xrightarrow{k_{cat}} E + P$$

$(k_{cat} + k_{-1})/k_1$) were obtained using both water and deuterium oxide as solvent.

Experimental

Materials.—Worthington three times recrystallized α-chymotrypsin was used without further purification. When the concentration of the enzyme solutions was required, it was determined by titration with N-*trans*-cinnamoylimidazole.[8] The deuterium oxide used as solvent was purchased from General Dynamics Corporation and contained greater than 99.5% D_2O. The *p*-nitrophenyl trimethylacetate (m.p. 95–96.5°) was prepared as previously described.[9a] N-Acetyl-L-tryptophan methyl ester was purchased from H. M. Chemical Co., Ltd., Santa Monica, Calif. The compound, which melted at 154–155.5°, was used without further purification.[9b] In each of the enzymatic runs with this compound one can calculate its equivalent weight assuming complete hydrolysis to N-acetyl-L-tryptophan. This was always within 3% of the theoretical value. The buffers were prepared from standard reagent grade chemicals; those in D_2O were prepared from chemicals with a minimum of hydrogen ions; for example, phosphate buffers were prepared from Na_2HPO_4 and KH_2PO_4, and Tris buffers from Tris and standard DCl solution (prepared by bubbling dry HCl into D_2O). For the acylation of chymotrypsin with *p*-nitrophenyl trimethylacetate and the enzymatic hydrolysis of N-acetyl-L-tryptophan methyl ester the buffers in H_2O and D_2O were prepared with the same acid to base ratio in each solvent.

The *p*H's of the solutions were determined using a Radiometer *p*H meter, model 4b. For solutions in D_2O, the *p*D of the solution was calculated from the measured *p*H by adding 0.40 pH unit.[10]

The Deacylation of *trans*-Cinnamoyl-α-chymotrypsin in D_2O.—The procedure for measuring the kinetics was similar to that outlined previously for the same reaction in H_2O.[6] The formation of cinnamic acid from *trans*-cinnamoyl-α-chymotrypsin was followed at 260 mμ using a Beckman DK-2 spectrophotometer. In a typical run 3 ml. of the buffer (in D_2O) plus 25 μl. of a stock enzyme solution (2.37×10^{-3} *M* enzyme in D_2O) were equilibrated at 25.6 ± 0.1°. At zero time 25 μl. of a stock solution of N-*trans*-cinnamoylimidazole in acetonitrile (2.38×10^{-3} *M* N-*trans*-cinnamoylimidazole) was added and the reaction was followed spectrophotometrically. The acylation is complete within a few seconds; the rate of the deacylation reaction was calculated from a first-order plot using the observed absorption at infinity. The *p*D of the solutions was calculated from the measured *p*H after the reaction was complete. In all these runs the solvent was approximately 0.8% in acetonitrile.

The Acylation of Chymotrypsin by *p*-Nitrophenyl Trimethylacetate.—The procedure for treating the kinetic data was similar to that used by Gutfreund and Sturtevant[7] for the acylation of α-chymotrypsin by *p*-nitrophenyl acetate, but it differs enough in detail to warrant a full description here. The reaction of α-chymotrypsin with *p*-nitrophenyl trimethylacetate, like that with *p*-nitrophenyl acetate or 2,4-dinitrophenyl acetate, follows scheme 1, but the trimethylacetate ester reacts more slowly than the acetate esters. However, for all these esters the acylation step is more rapid than deacylation and thus (using stoichiometric amounts of enzyme) one can study the acylation step separately.

In scheme 1 no allowance is made for the possibility that the enzyme (E) or the enzyme-substrate complexes (ES or ES′) can add or lose a proton to give an inactive species. Experiments using *p*-nitrophenyl trimethylacetate,[1] and also results with many other substrates,[4] indicated that such ionizations occur below *p*H 8 and that above this *p*H the rate of the enzyme-catalyzed hydrolysis is at a maximum. Therefore in this study the reaction was studied at a *p*H greater than 8 in water and a *p*D greater than 8.5 in deuterium oxide; presumably at these *p*H's no complications arise due to ionization of the enzyme or enzyme-substrate complexes.

Gutfreund and Sturtevant[7] integrated the kinetic expression for scheme 1 and found that if the substrate is always present in excess, the concentration of P_1 at any time t is given by an expression of the form

$$(P_1) = At + B(1 - e^{-kt}) \qquad (3)$$

where A and B are constants and $k = (k_2 + k_3)(S) + k_3K_m/(K_m + (S))$ where k_2 and k_3 are those of scheme 1 and $K_m = (k_2 + k_{-1})/k_1$. It will be shown later that for the reaction of *p*-nitrophenyl trimethylacetate with chymotrypsin $k_2 >> k_3$ and $k_2(S) >> k_3K_m$. Therefore

$$k = k_2(S)/(K_m + (S)) \qquad (4)$$

and k_2 and K_m can be determined by a Dixon–Webb[11] plot if k is known for various values of (S).

To determine k, use is made of the integrated expression 3. This accounts for the amount of P_1 formed by the enzymatic reaction. Some P_1 will also be formed by the spontaneous hydrolysis of the substrate. The rate of this reaction is pseudo zero-order at high substrate concentrations and thus the amount of P_1 obtained from the spontaneous hydrolysis is given by $(P_1) = C(S)t$ where C is a first-order rate constant and (S) is the substrate concentration. Therefore the total amount of P_1 formed is given by

$$(P_1) = At + C(S)t + B(1 - e^{-kt}) \qquad (5)$$

When t is large (equal to t'), then $e^{-kt'}$ becomes negligible and thus the amount of P_1 present is given by

$$(P_1') = At' + C(S)t' + B \qquad (6)$$

where (P_1') is the concentration of P_1 at time t'.

Subtracting 5 from 6 one obtains

$$[(P_1') - t'(A + C(S))] - [(P_1) - t(A + C(S))] = Be^{-kt} \qquad (7)$$

Thus, k can be obtained from a plot of the logarithm of the left side of the above equation *versus* t. This method was used in the present research to obtain the values of k for various values of (S).[12]

In eq. 7, C is the first-order rate constant for the spontaneous hydrolysis of the substrate and can be obtained from the rate of formation of P_1 in the particular buffer

(8) G. R. Schonbaum, B. Zerner and M. L. Bender, *J. Biol. Chem.*, **236**, 2930 (1961).

(9) (a) M. L. Bender and K. Nakamura, *J. Am. Chem. Soc.*, **84**, 2577 (1962); (b) H. T. Huang and C. Niemann, *ibid.*, **73**, 1541 (1951), report a melting point of 152.5° for this compound.

(10) B. Zerner and M. L. Bender, *ibid.*, **83**, 2267 (1961), and also ref. 12 and 13 of their paper.

(11) M. Dixon and E. C. Webb, "Enzymes," Academic Press, Inc., New York, N. Y., 1958, p. 21.

(12) This method for treating the data was suggested by Prof. J. M. Sturtevant.

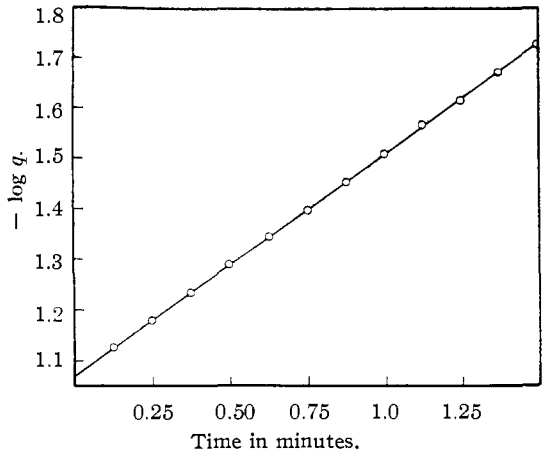


Fig. 1.—The acylation of α-chymotrypsin by *p*-nitrophenyl trimethylacetate in water, *p*H 8.17, 0.01 *M* Tris buffer, ionic strength 0.06; 25.6 ± 0.1°, [E] = 0.515 × 10^{-5} *M*, [S] = 7.67 × 10^{-5} *M*, $q = [(P_1') - t'(A + C(S))] - [(P_1) - t(A + C(S))]$.

with no enzyme present. From Gutfreund and Sturtevant's[7] derivation it can be shown that $A = k_3 e$ where e is the initial concentration of the enzyme. The quantity $[A + C(S)]$ can be easily determined from the zero-order rate obtained after the enzyme system has come to the steady state. In the present study t' was always equal to approximately 8 to 10 half-lives of the acylation reaction.

For the above treatment of the data it is necessary to have the substrate always present in excess. However the substrate, *p*-nitrophenyl trimethylacetate, is only soluble in water to a concentration of approximately 10^{-4} *M*. Therefore it was necessary to use enzyme concentrations of approximately 5 × 10^{-6} *M*. The substrate concentration was varied from 3.76 × 10^{-5} to 15.5 × 10^{-5} *M*. The substrate concentrations quoted in the following tables were the average substrate concentrations to approximately 60% acylation of the enzyme.

The reaction was followed by measuring the absorption at 400 mμ of the *p*-nitrophenolate anion which is produced. A Beckman DK-2 recording spectrophotometer was used. With an enzyme concentration of approximately 5 × 10^{-6} *M*, the total absorbance change during the acylation reaction was 0.08 to 0.10 absorbance unit. This change is conveniently followed on the 75 to 125% transmission scale of the DK-2 instrument.

In a typical run, 3 ml. of the buffer solution was equilibrated at the temperature of the cell compartment (25.6 ± 0.1°). Approximately 2 minutes after the addition of 25 μl. of a stock enzyme solution the reaction was initiated by adding 50 μl. of the substrate in acetonitrile. (Substrate solutions of differing concentrations in acetonitrile were prepared from a common stock solution by quantitative dilution.) Usually 5 sec. was required for stirring the solution and then the formation of *p*-nitrophenolate ion was followed to at least 10 half-lives of the acylation reaction. In Fig. 1 a plot of the data according to eq. 7 is shown for an individual run. Usually such plots gave good straight lines to about 70% of the acylation reaction. The *p*H (or *p*D) of the solution was determined after the run was completed. For all of these runs the solvent was 1.6% in acetonitrile.

The Enzymatic Hydrolysis of N-Acetyl-L-tryptophan Methyl Ester.—The rate of the enzyme-catalyzed hydrolysis was measured by titration at constant *p*H of the acid produced during the hydrolysis. The method was similar to that developed by Schwert, *et al.*,[13] and used by Bender and Glasson.[14] For each run approximately 0.04 to 0.05 g. of the ester was dissolved (by heating to 85 to 90°) in 55 or 60 ml. of a 0.002 *M* Tris buffer. After the ester had dissolved the solution was cooled rapidly to room

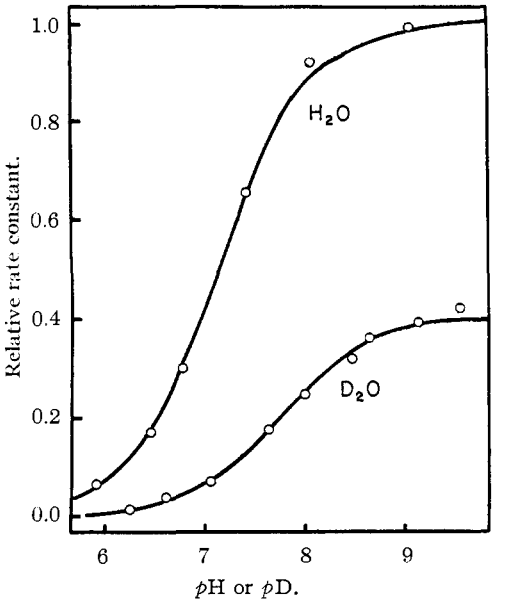


Fig. 2.—The effect of deuterium oxide on the deacylation of *trans*-cinnamoyl-α-chymotrypsin.

temperature and 50 ml. was pipetted into a 150-ml. beaker. The beaker was half-submerged in a constant temperature bath at 24.73 ± 0.05°. Nitrogen was bubbled through the solution for approximately 20 min., but just before adding the enzyme the apparatus was changed so that the nitrogen was blown gently over the surface of the solution. This procedure was adopted because it was found that if nitrogen was bubbled through the solution after the enzyme was added, then the enzyme was slowly denatured, presumably on the surface of the bubbles. The solution was stirred with a motor-driven glass stirrer and the tip of the microburet[14] (which contained 0.3861 *N* NaOH) was placed below the surface of the solution. The initial *p*H of the solution was taken and then enough base was added in 0.01-ml. quantities until the *p*H was greater then the desired zero-point *p*H for the reaction. For the reactions in water the zero-point *p*H was 8.250, and in deuterium oxide the zero-point *p*D was 8.850. The reaction in water was initiated by adding 25 μl. of a dilute aqueous enzyme solution. In deuterium oxide, 50 μl. of the same enzyme solution was used. Zero time was taken when the *p*H reached the zero-point *p*H. Shortly after this, another 0.01 ml. of base was added and the time was again taken when the *p*H equalled the zero-point *p*H. This was continued to the end of the run. Usually the *p*H was allowed to go as far below the zero-point *p*H as 0.01 ml. of base would then put it above this *p*H, and thus the zero-point *p*H is the average *p*H of the run. In this buffer, around *p*H 8.25, 0.01 ml. of the 0.3861 *N* base would change the *p*H by about 0.05 *p*H unit.

The results were calculated using the integrated form of the Michaelis–Menten equation for the case of inhibition by one of the reaction products, *i.e.*, N-acetyl-L-tryptophan.[15,16] This equation is

$$[(S_0) - (S)]/t = \left[\frac{-2.3K_m'[1 + (S)_0/K_i]}{1 - K_m'/K_i}\right]\frac{\log (S)_0/(S)}{t} + \frac{k_{cat}(E)}{1 - K_m'/K_i} \quad (8)$$

where $(S)_0$ is the initial substrate concentration, (S) is the substrate concentration at any time t, k_{cat} is the limiting rate constant, (E) is the initial enzyme concentration, K_m' is the Michaelis–Menten constant and K_i is the dissociation constant for the enzyme-inhibitor complex. Thus a plot of $[(S)_0 - (S)]/t$ *vs.* $[\log(S)_0/(S)]/t$ should yield a straight line. The intercept is approximately equal to $k_{cat}(E)$ be-

(13) G. W. Schwert, H. Neurath, S. Kaufman and J. E. Snoke, *J. Biol. Chem.*, **172**, 221 (1948).

(14) M. L. Bender and W. A. Glasson, *J. Am. Chem. Soc.*, **82**, 3336 (1960).

(15) R. S. Foster and C. Niemann, *Proc. Natl. Acad. Sci.*, **39**, 999 (1953).

(16) L. W. Cunningham and C. S. Brown, *J. Biol. Chem.*, **221**, 287 (1956).

cause the factor $(1 - K_m'/K_i)$ is approximately equal to unity for N-acetyl-L-tryptophan ethyl ester[16] and it will be shown later that this factor equals approximately 1 for the ester used in this research also. Knowing K_i,[16] one can get a value for K_m' from the slope of the above plot.

Results

The Deacylation of *trans*-Cinnamoyl-α-chymotrypsin in D_2O.—The kinetic results in deuterium oxide are shown in Table I and are plotted in Fig. 2. The kinetics of the deacylation reaction in water are also plotted in Fig. 2 for comparison.

TABLE I

THE DEACYLATION OF *trans*-CINNAMOYL-α-CHYMOTRYPSIN IN D_2O[c]

pD	$k_3' \times 10^3$, sec.$^{-1}$	pD	$k_3' \times 10^3$, sec.$^{-1}$
Buffer, 0.067 *M* phosphate[a]		Buffer, 0.067 tris[b]	
6.26	0.163	8.68	4.24
6.62	.366	9.13	4.60
7.08	.820	9.55	4.83
7.64	2.09		
8.02	2.97		
8.44	3.72		

[a] No attempt was made to keep the ionic strength constant since experiments had indicated that changes in the ionic strength had negligible effect on the rate. [b] These buffers were 0.067 *M* in Tris and sufficient KH_2PO_4 to give the desired pD. [c] 25.6 ± 0.1°, 0.8% acetonitrile.

From Fig. 2 it is obvious that the deacylation occurs more slowly in D_2O than in H_2O at all pH's studied. At high pH's (or pD's) when the rate constant appears to reach a maximum in both solvents, the deacylation proceeds 2.5 times more rapidly in H_2O than D_2O. The curves shown are those calculated assuming that the rate of deacylation depends on the presence of the unprotonated form of a base with a pK_a of 7.15 in H_2O and 7.75 in D_2O. This shift in pK_a in D_2O is what is expected for a group which ionizes around neutral pH.[17] Since the theoretical curves follow the experimental data so closely, then at all pH's the reaction goes more rapidly in H_2O than in D_2O by a factor of 2.5 if the reaction in the two solvents is compared when the buffer is present at the same acid to base ratio, *i.e.*, if the rate constant at a given pH is compared with that at a pD which is 0.6 unit higher around neutral pH.[18] The H_2O/D_2O factor does not remain constant if the rate constants are compared when pH is equal to pD. Therefore, presumably reactions in these two solvents should be compared in buffers with the same acid to base ratio and not when pH is equal to pD.[19] It is reasonable that the comparison should be made thus, because when the same acid to base ratio is present in each solvent then the effective acidity is approximately the same, whereas if the rates are compared when pH = pD, one is comparing the rate at a purely arbitrary acidity in one solvent to the rate at a different acidity in the other. The acidities are not the same when pH = pD because the solvents have different acid and base properties.

(17) R. P. Bell, "The Proton in Chemistry," Cornell University Press, Ithaca, N. Y., 1959, p. 188.

(18) The difference in pK_a of dihydrogen phosphate ion (one of the buffers used in this research) in D_2O relative to that in H_2O is 0.56 unit.

(19) In a recent communication (P. A. Srere, G. W. Kosicki and R. Lumry, *Biochim. et Biophys. Acta*, **50**, 184 (1961)) the authors give relative rate data for the effect of pH on the kinetic isotope effect of deuterium oxide for several enzyme systems. The reason that k_H/k_D varies so greatly and unintelligibly with pH is probably because the rates were compared in the two solvents when pH = pD.

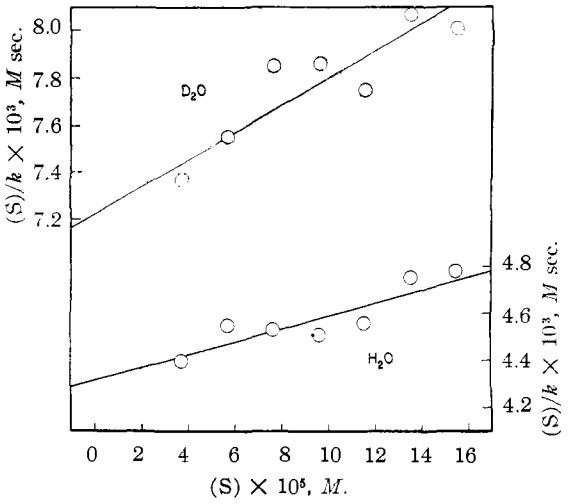


Fig. 3.—Dixon-Webb plots of the data for the acylation of α-chymotrypsin by *p*-nitrophenyl trimethylacetate in water and deuterium oxide (for the experimental conditions, see Tables II and III).

The Acylation of α-Chymotrypsin by *p*-Nitrophenyl Trimethylacetate.—The kinetic results in H_2O are shown in Table II and those in D_2O in Table III. Figure 3 shows a Dixon–Webb[11] plot of both sets of data.

TABLE II

THE ACYLATION OF α-CHYMOTRYPSIN BY *p*-NITROPHENYL TRIMETHYLACETATE IN H_2O[a]

[Enzyme] (E) × 10⁵, *M*	[Substrate][b] (S) × 10⁵, *M*	$k \times 10^2$, sec.$^{-1}$	(S)/k × 10³, *M* sec.	Av. (S)/k × 10³, *M* sec.
0.515	3.76	0.849	4.43	
.530	3.76	.860	4.37	4.40
.530	3.76	.854	4.41	
.515	5.72	1.26	4.53	
.530	5.72	1.23	4.64	4.55
.530	5.72	1.28	4.47	
.515	7.67	1.68	4.56	
.530	7.67	1.73	4.44	4.53
.530	7.67	1.67	4.59	
.515	9.63	2.20	4.39	
.515	9.63	2.10	4.59	4.51
.530	9.63	2.15	4.48	
.530	9.63	2.10	4.59	
.515	11.59	2.57	4.52	
.515	11.59	2.53	4.59	4.56
.530	11.59	2.53	4.58	
.530	11.59	2.55	4.55	
.515	13.55	2.87	4.72	4.75
.515	13.55	2.83	4.79	
.515	15.50	3.29	4.71	
.515	15.50	3.18	4.88	4.78
.530	15.50	3.27	4.75	

[a] 0.01 *M* Tris–HCl buffer, ionic strength 0.06 adjusted with NaCl, pH 8.17, temperature 25.6 ± 0.1°. [b] Average substrate concentration to approximately 60% acylation.

The lines shown in the Fig. 3 were calculated by the method of least squares from all the data of Tables II and III. The constants k_2 and K_m can be calculated from the slopes and intercepts of the

TABLE III

THE ACYLATION OF α-CHYMOTRYPSIN BY *p*-NITROPHENYL TRIMETHYLACETATE IN D_2O[a]

[Enzyme] (E) × 10^5, *M*	[Substrate][b] (S) × 10^5, *M*	$k \times 10^3$, $sec.^{-1}$	(S)/*k* × 10^3, *M* sec.	Av. (S)/*k* × 10^3, *M* sec.
0.530	3.76	0.526	7.14	
.530	3.76	.494	7.62	7.37
.518	3.76	.504	7.46	
.518	3.76	.519	7.25	
.530	5.72	.741	7.72	
.530	5.72	.745	7.68	7.55
.518	5.72	.792	7.22	
.518	5.72	.756	7.57	
.530	7.67	.983	7.80	
.530	7.67	.983	7.80	7.85
.518	7.67	.964	7.96	
.530	9.63	1.18	8.18	
.530	9.63	1.21	7.95	7.86
.518	9.63	1.30	7.44	
.530	11.59	1.49	7.78	
.530	11.59	1.52	7.65	7.75
.518	11.59	1.46	7.95	
.518	11.59	1.52	7.64	
.530	13.55	1.66	8.18	
.530	13.55	1.75	7.75	8.07
.518	13.55	1.64	8.26	
.518	13.55	1.68	8.08	
.518	15.50	1.94	8.01	8.01

[a] 0.01 *M* Tris–DCl buffer, ionic strength 0.06 adjusted with NaCl, *p*D 8.63, temperature 25.6 ± 0.1°. [b] Average substrate concentration to approximately 60% acylation.

lines and these are shown in Table IV along with the probable error in each quantity. One can see that the probable errors in both k_2 and K_m are quite large. This arises because both these quantities depend on the slope of the line in Fig. 3 and since the lines are almost flat the slope is not known very accurately. The ratio of constants K_m/k_2 is merely the intercept of the lines of Fig. 3 and consequently is known quite accurately. Although the probable error in k_2 is quite large, the value in H_2O appears to be greater than that in D_2O by a factor of 2.2. Quantitatively this value is not very significant but qualitatively k_2 appears to be greater in H_2O than in D_2O. However, the ratio of the K_m's in the two solvents is not significantly different from unity. The ratio $k_2^H K_m^D/k_2^D K_m^H$ is known quite accurately and is 1.68 ± 0.04.

TABLE IV

KINETIC CONSTANTS FOR THE ACYLATION OF α-CHYMOTRYPSIN WITH *p*-NITROPHENYL TRIMETHYLACETATE

Solvent	k_2, $sec.^{-1}$	$K_m \times 10^3$, *M*	$K_m/k_2 \times 10^3$, *M* sec.
H_2O	0.37 ± 0.11	1.6 ± 0.5	4.31 ± 0.06
D_2O	0.17 ± 0.07	1.2 ± 0.5	7.22 ± 0.15

It is likely that K_m is a dissociation constant for the enzyme-substrate complex ($k_{-1} >> k_2$) and one might expect, therefore, that K_m^D would be less than K_m^H; in other words, that the substrate would be more tightly bound to the enzyme in D_2O than in H_2O. The substrate *p*-nitrophenyl trimethylacetate has large hydrocarbon-like parts and it might be expected that the substrate would tend to be "squeezed out" of solution and onto the hydrocarbon parts of the enzyme more in D_2O than in H_2O. D_2O forms stronger hydrogen bonds with itself than H_2O does and most compounds are less soluble in D_2O than in H_2O.[20] If K_m^D, K_m^H is less than one then from the above ratio k_2^H/k_2^D is greater than 1.68. This discussion is very qualitative and depends on certain assumptions but it substantiates the earlier result that k_2^H/k_2^D is of the order of 2 or more.

In order to calculate k for each run, values for A (see eq. 3) were obtained in both H_2O and D_2O. It can be shown from Gutfreund and Sturtevant's[7] derivation that $A = k_3(E)$ and thus, since the initial enzyme concentration (E) is known, one can obtain the kinetic constant k_3 in both H_2O and D_2O. The average value of this constant in H_2O is 1.33 ± 0.03 × 10^{-4} $sec.^{-1}$ and in D_2O is 0.443 ± 0.005 × 10^{-4} $sec.^{-1}$.[21] These were obtained under the same conditions of temperature and *p*H as the runs of Tables II and III. The deviations in the constants are the average deviations of 5 values in H_2O and 2 values in D_2O. Therefore the ratio k_3^H/k_3^D for the deacylation of trimethylacetyl-α-chymotrypsin is 3.0. This is similar to the value given earlier (2.5) for the deacylation of *trans*-cinnamoyl-α-chymotrypsin. The ratio for the deacylation of trimethylacetyl-α-chymotrypsin is less accurate since in the calculation of k_3 in each solvent it was necessary to know the enzyme concentration and the molar absorptivity of *p*-nitrophenolate ion. This was not determined accurately in D_2O. For the deacylation of *trans*-cinnamoyl-α-chymotrypsin it was not necessary to know the enzyme concentration or any molar absorptivities accurately, since k_3 was determined from a first-order plot of the data rather than a zero-order plot. Nevertheless the result for the deacylation of trimethylacetyl-α-chymotrypsin is in essential agreement with the result for the deacylation of *trans*-cinnamoyl-α-chymotrypsin and substantiates the conclusion that a sizable isotope effect is connected with the enzymatic deacylation reaction.

The α-Chymotrypsin-catalyzed Hydrolysis of N-Acetyl-L-tryptophan Methyl Ester in H_2O and D_2O. —The kinetic results are shown in Table V.

TABLE V

THE α-CHYMOTRYPSIN-CATALYZED HYDROLYSIS OF N-ACETYL-L-TRYPTOPHAN METHYL ESTER IN H_2O AND D_2O[a]

Solvent	[Enzyme] (E) × 10^6, *M*	[Substrate] $(S)_0$ × 10^5, *M*	*p*H or *p*D[b]	k_{cat}, $sec.^{-1}$	K_m' × 10^5, *M*
H_2O	2.79	3.09	8.250	54.8	8.8
H_2O	3.25	2.73	8.250	55.0	6.7
			Av.	54.9	7.8
D_2O	6.50	2.70	8.850	19.30–19.43	1.3–3.4
D_2O	6.50	3.21	8.850	19.38–19.50	1.3–3.6
			Av.	19.4	1.3–3.6

[a] Temperature, 24.73 ± 0.05°, 0.002 *M* Tris HCl (or DCl) buffer adjusted to ionic strength 0.05 with $CaCl_2$. [b] Zero-point.

The constants k_{cat} and K_m' were determined by a least squares fit of the experimental data to the

(20) I. Kirshenbaum, "Physical Properties and Analysis of Heavy Water," McGraw-Hill Book Co., Inc., New York, N. Y., 1951, p. 39.

(21) Using these values for k_3, one can easily show that $k_3 \gg k_2$ and $k_3(S) \gg k_2K_m$ as was assumed earlier (see Experimental section).

integrated expression of the Michaelis–Menten equation for inhibition by one product, in this case N-acetyl-L-tryptophan.[15] In order to calculate these constants one must know the value of K_i, the inhibition constant for N-acetyl-L-tryptophan. Cunningham and Brown[16] give a value of $8.7 \pm 2 \times 10^{-3}$ for this constant at 25° and *p*H 8.0 in water and this value was used for determining k_{cat} and K_m' with water as solvent. Cunningham and Brown observed that K_i did not vary greatly with *p*H above *p*H 8. The constant k_{cat} depends to only a very slight extent on the value chosen for K_i since it depends on the factor $(1 - K_m'/K_i)$ which is very close to unity. However, the calculated value of K_m' depends on the factor $[1 + (S)_0/K_I]$ (where $(S)_0$ is the initial ester concentration) and, since $(S)_0/K_i$ is of the order of 0.3 to 0.4, a large change in K_i would affect the value of K_m' significantly. In addition, a plot of the data for this ester according to the integrated expression gives a very accurate value for the intercept (from which k_{cat} is calculated) but the slope of the line (from which K_m' is calculated) is very small and difficult to determine accurately. Thus the value for k_{cat} is probably accurate within 1%, but the value for K_m' is likely good to only 10 or 20%.[22]

The value of K_i for N-acetyl-L-tryptophan in D_2O is not known and thus to calculate k_{cat} and K_m' for the experiments in D_2O one must assume some value for K_i. To obtain the values given in Table V, K_i in D_2O was assumed to be either five times greater than in H_2O or one-fifth of its value in H_2O (*i.e.*, 43.5×10^{-3} or 1.75×10^{-3} *M*, respectively). Thus a spread of values for k_{cat} and K_m' in this solvent is obtained as indicated in the table. The higher values for both these constants are obtained assuming the higher value of K_i. Again, one can see that k_{cat} depends very little on K_i and the value 19.4 sec.$^{-1}$ is probably accurate within 1%, whereas at the present time one can only say that K_m in D_2O is probably in the range $1.3\text{–}3.6 \times 10^{-5}$ *M*. One might expect that K_m' and K_i would change in the same direction in D_2O and this would thus favor the smaller values for K_m'.

From the values for k_{cat} and K_m' given in Table V one obtains $k_{cat}^H/k_{cat}^D = 2.83$ and $K_m'^H/K_m'^D = 2.2$ to 6.0.

For the runs in D_2O, 50 μl. of enzyme solution in H_2O was added and also the base used for the titration was a water solution. Since in this research the relative values of the constants in the two solvents were desired, the above procedure was followed in order to minimize any errors. The D_2O was thus diluted by approximately 1% water by the end of the run. It is not likely that this will have any large effect on the results.

Discussion

A summary of the results obtained in this research is shown in Table VI.

A possibility which must be kept in mind when interpreting kinetic results of enzyme reactions in D_2O is that the D_2O may change the hydrogen-bonded structure of the enzyme thus causing partial or full inactivation. The α-chymotrypsin used in these experiments was at least not irreversibly inactivated since enzyme solutions in D_2O could be titrated using N-*trans*-cinnamoylimidazole and the concentrations obtained were approximately equal to what was expected from the weight of the crystalline enzyme which was used to prepare the solution. Also, in the kinetic runs, enzyme solutions which had remained in D_2O for only minutes or for days gave the same results. In a few cases aqueous solutions of the enzyme were used for the D_2O runs (since such a small amount of the enzyme solution was used, the dilution by water would have a negligible effect) and the same result was obtained as when an enzyme solution in D_2O was used. Similarly, when an enzyme solution in D_2O was added to a reaction in water, a rate characteristic of the H_2O reaction was obtained. Therefore, if the enzyme is partially inactivated by D_2O, not only the deactivation but also its reactivation in H_2O must be a very rapid reaction. The inherent reasonableness of the ratios in Table VI is one argument that the enzyme is not being inactivated due to a structural change when dissolved in D_2O. All of these ratios can be rationalized in terms of what is known about non-enzymatic reactions in D_2O. This could be coincidence, although such a possibility is unlikely. Where the kinetics of other enzymes have been studied in D_2O, the effect of the D_2O is usually small which indicates that the structure of the enzymes has not been changed noticeably.[19,23–27] Also, in cases where the helix and random coil equilibrium in hydrogen and deuterium solvents have been studied,[28] it has been found that the transition

TABLE VI

THE KINETIC ISOTOPE EFFECTS OF D_2O ON SEVERAL α-CHYMOTRYPSIN-CATALYZED REACTIONS

Reaction	Constant[a]	k^{H_2O}/k^{D_2O}[a]
Acyln. with *p*-nitrophenyl trimethylacetate	k_2	2.2[b]
Acyln. with *p*-nitrophenyl trimethylacetate	K_m	1.3[b]
Deacyln. of trimethylacetyl-α-chymotrypsin	k_3	3.0
Deacyln. of *trans*-cinnamoyl-α-chymotrypsin	k_3	2.5
Hydrol. of N-acetyl-L-tryptophan methyl ester	k_{cat}	2.83
Hydrol. of N-acetyl-L-tryptophan methyl ester	K_m'	2.2–6.0

[a] k^{H_2O}/k^{D_2O} is equal to the ratio of the value of the particular constant in H_2O to that in D_2O. [b] These values are only approximate.

(22) The values for k_{cat} and K_m' obtained in this research using N-acetyl-L-tryptophan methyl ester compare favorably with those found by Cunningham and Brown[16] using N-acetyl-L-trytophan ethyl ester under comparable conditions. Their value for k_{cat} is approximately 40 sec.$^{-1}$ and for K_m' is $8.9 \pm 2 \times 10^{-5}$ *M*.

(23) K. B. Wiberg, *Chem. Revs.*, **55**, 719 (1955).

(24) S. Seltzer, G. A. Hamilton and F. H. Westheimer, *J. Am. Chem. Soc.*, **81**, 4018 (1959).

(25) J. F. Thomson, *Arch. Biochem. Biophys.*, **90**, 1 (1960); J. F. Thomson and F. J. Klipfel, *Biochim. et Biophys. Acta*, **44**, 72 (1960).

(26) A. Stockell and E. L. Smith, *J. Biol. Chem.*, **227**, 1 (1957).

(27) R. A. Alberty, W. G. Miller and H. F. Fisher, *J. Am. Chem. Soc.*, **79**, 3973 (1957); R. Bentley and D. S. Bhate, *J. Biol. Chem.*, **235**, 1225 (1960); G. W. Kosicki and P. A. Srere, *ibid.*, **236**, 2566 (1961).

(28) M. Calvin, J. Hermans, Jr., and H. A. Scheraga, *J. Am. Chem. Soc.*, **81**, 5048 (1959); J. Hermans, Jr., and H. A. Scheraga, *Biochim. et Biophys. Acta*, **36**, 534 (1959); H. A. Scheraga in "Protein Structure and Function," Brookhaven Symposium in Biology No. 13, 1960, p. 71.

temperature for the hydrogen solvent was different from that for the deuterium solvent by only approximately 10°. Therefore, if D_2O causes some change in the structure of chymotrypsin at 25° then one should observe the same effect in H_2O at approximately 15 or 35°. This is not observed to be the case.[29]

An inspection of Table VI indicates that each of the rate constants (k_2, k_3 and k_{cat}) is decreased in D_2O relative to that in H_2O by a factor of 2 to 3. This factor is too large to be attributed only to solvation of ions which may be produced in these steps; in such cases the rate of the reaction in D_2O differs from that in H_2O usually by only a factor of 1.0 to 1.5.[30] On the other hand, the factor of 2 or 3 is what one would expect for a reaction which involved a proton transfer in the rate-controlling step.[23,31] Consequently these steps probably involve a rate-determining proton transfer. Also, these results effectively rule out any mechanism for the enzymatic reaction which has the enzyme acting only as a nucleophile. For nucleophile-catalyzed non-enzymatic reactions k^H/k^D is usually very close to unity[5]; in the few cases where this ratio is as high as 1.8 the nucleophile is a carboxylate anion and from the effect of pH on the enzyme reaction it is doubtful that a carboxylate group on the enzyme is acting as a nucleophile. However, even if a carboxylate group were involved one could not explain the fact that k^H/k^D is greater than 2 for the enzyme reactions.[32]

Several mechanisms for the enzymatic reaction have been proposed which involve a proton transfer in the rate-controlling step.[4] They include: a group on the enzyme acting as a general base (such non-enzymatic reactions do give isotope effects of the magnitude observed here[5]); two groups on the enzyme acting together, one acting as a nucleophile and the other as a general acid (if a proton was donated by the general acid in the rate-determining step then a kinetic isotope effect would be expected); two groups on the enzyme acting together, one acting as a general base and the other as a general acid; and other more complicated (and consequently more speculative) mechanisms. The deuterium isotope effects cannot distinguish between these various mechanisms.

As was indicated earlier, the effect of pD on the rate of the deacylation of *trans*-cinnamoyl-α-chymotrypsin is precisely what would be expected for dependence on a single group which ionizes around neutral pH. The pK_a of this group is 7.75 in D_2O which is 0.60 unit higher than its pK_a in H_2O (7.15). It is reasonable that this group is an imidazole group of a histidine residue as has been proposed many times previously.[4] Since the effect of D_2O on the pK_a of the active group is almost exactly what one would expect,[17] this is a further indication that the same reaction occurs in D_2O as in H_2O and that no major structural change of the enzyme occurs in D_2O.

It is difficult to conclude much from the effect of D_2O on K_m and K_m', since these ratios are not known accurately. Since K_m is most likely a dissociation constant of the initial enzyme-substrate complex, the lack of a kinetic isotope effect on this constant tends to indicate that K_m' is not merely a dissociation constant since a kinetic isotope effect appears to be associated with it. This indication is interesting when compared with the conclusion of Bernhard, Coles and Nowell[34] that K_m' is an equilibrium constant. However, it should be stressed that the effects of D_2O on K_m and K_m' are not known accurately and thus these arguments are not too conclusive.[35]

The main conclusion to be obtained from this research is that a sizable kinetic isotope effect is observed for several α-chymotrypsin-catalyzed reactions and any mechanism which is proposed for the enzymatic reaction must be able to explain this fact. Further discussion of the mechanism of the enzymatic reactions will be reserved for the final paper of this series.[33]

(29) The temperature effects on chymotrypsin-catalyzed hydrolyses are normal; for a review see K. J. Laidler, "The Chemical Kinetics of Enzyme Action," Oxford Univ. Press, London, 1958, p. 245.

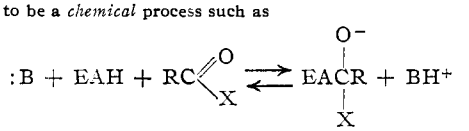
(30) R. E. Robertson and P. M. Laughton, *Can. J. Chem.*, **34**, 1714 (1956); **35**, 1319 (1957); **37**, 1491 (1959); *cf.* C. A. Bunton and V. J. Shiner, Jr., *J. Am. Chem. Soc.*, **83**, 3207, 3214 (1961).

(31) F. A. Long and J. Bigeleisen, *Trans. Faraday Soc.*, **55**, 2077 (1959).

(32) In the papain-catalyzed hydrolysis of N-α-benzoyl-L-arginine ethyl ester,[26] k_{cat} is insensitive to substitution of H_2O by D_2O indicating a different rate-determining step.

(34) S. A. Bernhard, W. C. Coles and J. F. Nowell, *ibid.*, **82**, 3043 (1960).

(35) A referee has pointed out that if the formation of ES is assumed to be a *chemical* process such as



an isotope effect on K_m would be expected, provided that E–A–H is a very weak acid.

(33) M. L. Bender, *J. Am. Chem. Soc.*, **84**, 2582 (1962).