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The Effect of Aprotic Dipolar Organic Solvents on the Kinetics of α-Chymotrypsin-Catalyzed Hydrolyses*

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The kinetics of several α-chymotrypsin-catalyzed hydrolyses have been determined in mixtures of water and three dipolar aprotic solvents, dioxane, acetone, and acetonitrile. The effect of the organic solvent on k_2 (the acylation constant), k_3 (the deacylation constant), k_{cat} (the catalytic or turnover constant), K_m (the Michaelis constant) and k_2/K_m was determined. The major effect of the organic solvent is to increase K_m (or decrease k_2/K_m) with only a small (lowering) effect on the rate constants, k_2, k_3, and k_{cat}. Correlations of the kinetic data with simple competitive inhibition by the organic solvent or with the dielectric constant of the medium are only qualitatively successful. However, a treatment involving the combination of these two factors affords a quantitative correlation of the kinetic data up to 15% organic solvent. The order of competitive inhibitors for α-chymotrypsin is: dioxane > acetonitrile > acetone > methanol. The concentration of active sites of an α-chymotrypsin solution is not affected by 32% dioxane-water, as measured by spectrophotometric titration with *N-trans*-cinnamoylimidazole.

In our continuing studies on the kinetics and mechanism of hydrolyses catalyzed by proteolytic enzymes, it was of interest to investigate the effect of dipolar aprotic solvents on the kinetics of such reactions. Investigations of the effect of such solvents on two groups of kinetic constants of α-chymotrypsin-catalyzed hydrolyses were carried out: (1) the effect on the turnover constant k_{cat} and the Michaelis constant K_m(app) (defined by equation 1); and (2) the effect on the acylation rate constant k_2/K_m (defined by equation 2).

$$E + S \underset{}{\overset{K_m(app)}{\rightleftharpoons}} ES \xrightarrow{k_{cat}} E + P \qquad (1)[1]$$

$$E + S \overset{K_m}{\rightleftharpoons} ES \xrightarrow{k_2} ES' \xrightarrow{k_3} E + P_2 \qquad (2)$$

$$ES' + P_1$$

The effect of organic solvents on the kinetics of chymotrypsin reactions has been noted in the literature previously. Kaufman *et al.*, (1949) reported a linear decrease in the "proteolytic coefficient" (k_{cat}/K_m) with increasing methanol concentration for the α-chymotrypsin-catalyzed hydrolysis of glycyl-L-tyrosinamide and benzoyl-L-tyrosine ethyl ester. Further, Kaufman and Neurath (1949) studied the α-chymotrypsin-catalyzed hydrolysis of acetyl-L-tyrosinamide at three methanol concentrations. From water to 5.15 M methanol, K_m was found to exhibit a 2.5-fold increase while k_{cat} remained constant. A plot of $1/K_m$ vs. methanol concentration was found to be linear. In the chymotrypsin-catalyzed hydrolysis of methyl hydrocinnamate in three aqueous methanol solutions up to 25% methanol by weight, it was found that K_m increases while k_{cat} decreases with increasing methanol concentration (Barnard and Laidler, 1952). A dielectric constant effect was suggested as a partial explanation of the results. More recently a marked inhibition of the same reaction by methanol was rationalized on the basis of a specific interaction between the enzyme and methanol (Stein and Laidler, 1959).

An extensive study of the effect of several organic solvents on the kinetics of the α-chymotrypsin-catalyzed hydrolysis of methyl hippurate was reported by Applewhite, *et al.* (1958). In acetone-water and dioxane-water solutions, it was found that the value of k_{cat} remains constant whereas the value of K_m increases with increasing solvent concentration. These data could not be explained on the basis of simple competitive inhibition or of a dielectric constant effect. A model to explain the data was suggested consisting of the partitioning of the substrate between two phases, the hydrated enzyme and the aqueous-aprotic solvent. In this study, it was also found that methanol increases K_m while it decreases k_{cat}.

In methanol-water solutions, ambiguous results can be obtained from the fact that the protic organic solvent, methanol, can participate directly in the enzymatic reaction, as well as exert a generalized solvent effect.[2] The direct participation of methanol and other alcohols in enzymatic processes has been well documented (McDonald and Balls, 1956; Koshland and Herr, 1957; Bender and Glasson, 1960). The participation of methanol in the enzymatic process results in the conversion of the hydrolysis reaction to a

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[1] K_m(app) of eq. (1) is related to K_m of equation (2) by the relationship: $K_m(app) = [k_3/(k_2 + k_3)]K_m$ (Gutfreund and Sturtevant, 1956). K_m (app) and K_m will not be further differentiated in this paper.

[2] The results cited are probably not ambiguous, because in those reactions cited acylation is probably largely or solely rate-controlling. Under these circumstances, the direct participation of methanol is of no consequence.

methanolysis reaction. This change may result in an enhancement of the deacylation rate constant (Bender and Gunter, 1962) or in an enhancement or a diminution in the turnover rate constant, depending on whether the expelled group or the carboxylic acid portion of the product, respectively, is measured.

Although the effect of methanol on chymotrypsin kinetics is of considerable interest,[3] in this paper we wish to consider the effect of organic solvents not participating in the enzymatic reaction on the kinetics of α-chymotrypsin-catalyzed reactions. We shall therefore concentrate on dipolar aprotic organic solvents. While this work was in progress a report appeared on the effect of diethyl ether and *t*-amyl alcohol on the chymotrypsin-catalyzed hydrolysis of methyl hippurate and methyl hydrocinnamate. (Miles, Robinson, and Canady, 1962; Miles, Morey *et al.*, 1962.) Low concentrations of organic solvent were used to avoid a "material alteration" of the solvent. Under these conditions, the authors were able to analyze the effects of organic solvent in terms of competitive inhibition. The effect of dioxane, methanol, and acetonitrile on the kinetics of the turnover and acylation reactions of *p*-nitrophenyl acetate with chymotrypsin was considered from two points of view: a correlation of the kinetic effect with the dielectric constant of the medium, and a correlation of the kinetic effect with competitive inhibition by organic solvent molecules. The first of these correlations was unsatisfactory. The second was found satisfactory over a limited range (Awad, 1959).

Our interest has centered on observing the effects of organic solvents over as large a concentration range as possible. Such an investigation was carried out by Inagami and Sturtevant (1960) who measured the effect of dioxane-water mixtures on the kinetics of trypsin-catalyzed hydrolysis. Since trypsin has been shown to catalyze reactions in a manner mechanistically similar to that of α-chymotrypsin (Bender and Kaiser, 1962), it is reasonable to expect that it should exhibit the same type of solvent effects as α-chymotrypsin. The rate constant for the trypsin-catalyzed hydrolysis of benzoyl-L-arginine ethyl ester increases slightly and then decreases as the concentration of dioxane increases while K_m steadily increases (over a 5500-fold range from water to 88% dioxane-water).

α-Chymotrypsin is precipitated by dioxane (or other dipolar aprotic solvents) above 30% organic solvent in water under our conditions. However, it is still possible to use an appreciable concentration of organic solvent. Those used were dioxane, acetone, and acetonitrile. The effect of all three organic solvents on the kinetics of the acylation of α-chymotrypsin by *p*-nitrophenyl acetate and *N-trans*-cinnamoylimidazole was investigated under first- and/or second-order conditions. The effect of dioxane on the kinetics of the deacylation of cinnamoyl-α-chymotrypsin was also investigated. Finally the effect of acetonitrile on the α-chymotrypsin-catalyzed hydrolysis of *N*-acetyl-L-tryptophan methyl ester, a specific substrate, was investigated. These data and pertinent data in the literature will be analyzed in order to develop a general framework within which the effect of dipolar aprotic organic solvents can be quantitatively treated and predictions made.

Experimental

Materials.—*N-trans*-Cinnamoylimidazole was prepared by the reaction of *trans*-cinnamoyl chloride with imidazole in benzene solution (Schonbaum *et al.*, 1961). This material was recrystallized from dry distilled hexane and melted to a completely colorless liquid, mp 134–134.5°. *p*-Nitrophenyl acetate (Aldrich Chemical Co.) was dissolved in the minimum amount of hot absolute ethanol, charcoal was added, and the solution was stirred for 5 minutes, filtered, and the filtrate cooled in an ice bath. The ester was recrystallized from dry chloroform-hexane solution, mp 79.5–80.0° (Kézdy and Bender, 1962). *N*-Acetyl-L-tryptophan methyl ester was obtained from the H. M. Chemical Co., Ltd., Santa Monica, Calif., mp 154–155°, lit. mp 152.5° (Huang and Niemann, 1951); $[\alpha]_D^{25}$, + 13.8° [*c* = 2 (methanol)]. The saponification equivalent of this compound was within 3% of the theoretical value. α-Chymotrypsin (3 × crystallized, salt free, Worthington Biochemical Corporation) was used without further purification. In some kinetic runs the enzyme solution was prepared by dilution with the appropriate buffer from a $1 - 3.5 \times 10^{-3}$ M centrifuged stock solution and in others it was prepared directly by addition of the buffer to a weighed amount of enzyme. The normality of the enzyme solutions was determined by spectrophotometric titration (Schonbaum *et al.*, 1961) at the beginning and end of each series of runs. The water used in the enzymatic experiments was distilled water, redistilled in an all-glass apparatus from alkaline permanganate. Acetonitrile was an Eastman Kodak Co. practical grade chemical, purified by multiple distillation from phosphorus pentoxide followed by distillation from anhydrous potassium carbonate (Steinhardt, 1938). The dioxane was a Fisher reagent grade chemical purified according to the method of Fieser (1955). Immediately before a kinetic experiment, this purified dioxane was distilled from sodium, bp 101°. The acetone, an Eastman Kodak Spectrograde chemical, was used directly. Buffer components including sodium acetate, potassium dihydrogen phosphate, disodium hydrogen phosphate, acetic acid, hydrochloric acid, imidazole, and Tris were of reagent grade quality. The phosphate and acetate buffers were prepared according to Kolthoff and Rosenblum (1937) and the Tris buffers were prepared according to Bates and Bower (1956).

A Radiometer *p*H meter Model 4c (precision ±0.002 *p*H unit) was used to determine the *p*H of the solutions immediately after reaction. An organic solvent increases the *p*H, as measured by the glass electrode, of acetate and phosphate buffers, but has no effect on Tris or imidazole buffers (Wheland, 1960). For example, a *p*H 5.05 acetate buffer increases 0.27 *p*H unit from pure water to 12.8% dioxane-water. The increases in *p*H of acetate and phosphate buffers in acetone and acetonitrile solutions are smaller for the same volume per cent of organic solvent. It has been noted previously that glass electrode measurements of the hydrogen ion activity in dioxane-water mixtures are the same as those measured by the hydrogen electrode (Marshall and Grunwald, 1953), indicating that the *p*H changes noted above are real. However, the changes are small enough not to preclude the kinetic grouping of all kinetic measurements of a given substrate-solvent system with one parent buffer.

Kinetics.—The kinetics of most reactions were followed spectrophotometrically using a Cary Model 14 PM recording spectrophotometer with a cell compartment thermostatted at 25.0 ± 0.1°.

Acylation of α-Chymotrypsin by p-Nitrophenyl Acetate.—The buffer solution, which sometimes contained the organic solvent, was thermostatted in the cell compartment for *ca.* 15 minutes. An aliquot (100 μl) of the enzyme solution was added and the reaction

[3] We shall discuss the effect of methanol on the kinetics of α-chymotrypsin-catalyzed hydrolysis in a future publication.

TABLE I

SECOND-ORDER RATE CONSTANTS FOR THE ACYLATION OF α-CHYMOTRYPSIN BY *p*-NITROPHENYL ACETATE IN ORGANIC SOLVENT–WATER MIXTURES[a]

% Organic Solvent[c]	k_2/K_m in Dioxane-Water mole^{-1} sec^{-1} Exp.	Calcd.[m]	k_2/K_m in Acetone-Water mole^{-1} sec^{-1}	k_2/K_m in Acetonitrile-Water mole^{-1} sec^{-1}
		*p*H 5.1 ± 0.1[b]		
0	120[l]			
0.80			103.0	
1.59	64.1 ± 0.5[d]	65.6	96.4 ± 0.9	79.4 ± 1.2
3.20	39.5	41.0	80.7 ± 2.0	64.9 ± 0.9
4.62	28.7 ± 0.6	30.5	64.4 ± 2.0	52.4 ± 0.1
6.35	20.0 ± 0.9	21.8	47.9 ± 0.1	41.0 ± 1.2
7.30	...	...	40.2	...
11.11	9.7 ± 0.2	9.9	27.0 ± 0.7	24.2 ± 0.6
		*p*H 7.7 ± 0.1[e]		
0	50.8[l]			
0.32				46.8[j]
1.59	22.4 ± 0.6[f]	26.6	39.9 ± 0.01	36.0
3.13	12.2 ± 0.2	16.0	29.5 ± 0.3	19.6[h,j]
4.62	8.8 ± 0.1	10.6	24.9 ± 0.8	
6.35	6.2 ± 0.03	6.8	17.0 ± 0.04	
11.11	2.7 ± 0.1[g]	2.3	9.7 ± 0.1	5.6[i,k]

[a] 25.0 ± 0.1°. [b] [Ester]$_0$ = 10.0 to 6.3 × 10^{-6} M; [enzyme]$_0$ = 140 to 119 × 10^{-6} M; 0.1 M acetate buffer (*p*H 5.05 in water). [c] Volume/volume. [d] Deviations are average deviations from the mean; ordinarily duplicate runs were used to determine the average rate constant. [e] [Ester]$_0$ = 33 to 38 × 10^{-6} M; [enzyme]$_0$ = 40 to 43 × 10^{-6} M; 0.066 M phosphate buffer (*p*H 7.60 in water). [f] *p*H 7.7. [g] *p*H 7.9. [h] 4.0% acetonitrile-water. [i] 10.0% acetonitrile-water. [j] Kézdy and Bender (1962). [k] Bender and Nakamura (1962). [l] This value was calculated using Figure 2 and equation (16). [m] Calculated using equation (16) and Table VI.

TABLE II

SECOND-ORDER ACYLATION RATE CONSTANTS AND TITRATION OF α-CHYMOTRYPSIN BY *N-trans*-CINNAMOYLIMIDAZOLE IN ORGANIC SOLVENT–WATER MIXTURES[a]

*p*H[b]		k_2/K_m[d] mole^{-1} sec^{-1} 10^{-3} Exp.	Calcd.[h]	Enzyme Concentration × 10^6 M Calcd.[g]	Found[f]
	% dioxane[c]				
5.05	0	18.25[g]			
5.06	0.32	12.40	13.1	3.67	3.79
5.09	1.56	9.44 ± 0.07	9.3	1.92	1.85
5.09	3.13	5.42 ± 0.20	5.4	3.16	3.11
5.14	4.07	4.00 ± 0.05	4.1	3.16	3.16
5.22	7.82	1.57 ± 0.10	1.6	3.85	3.80
5.32	12.8	0.74 ± 0.05	0.54	38.50	38.80
5.51	22.2	0.18 ± 0.003	0.13	38.50	38.60
5.82	32.2	0.048 ± 0.001	0.07	108.70	101.00
	% acetone[c]				
5.08	1.56	13.60 ± 0.20	13.2	3.24	3.22
5.10	3.13	10.35 ± 0.05	9.7	3.24	3.19
5.13	4.69	8.19 ± 0.10	7.6	3.24	3.21
5.21	10.0	2.99 ± 0.03	3.2	3.24	3.25
5.24	14.1	1.82 ± 0.05	1.8	3.24	3.22
	% acetonitrile[c]				
5.06	0.32	14.66	15.2	3.70	3.64
5.06	1.60	10.00 ± 0.20	12.2	4.24	
5.09	3.13	8.19 ± 0.20	8.9	4.13	4.13
5.13	7.82	3.99 ± 0.06	4.1	3.11	3.11
5.19	12.8	2.02 ± 0.03	2.1	4.76	4.71

[a] 25.0 ± 0.1°. [b] 0.1 M acetate buffer. [c] Volume/volume. Under these conditions the enzyme precipitated in 35% dioxane and 20% acetonitrile. [d] Deviations are average deviations from the mean of at least two experiments. [e] The stock enzyme solution (containing no organic solvent) was titrated before the reactions were run. The concentration in the cell was calculated using the appropriate dilution factor. S_0/E_0 = 1.1:2.1. [f] The enzyme concentration (in the cell) determined in the presence of the organic solvent. [g] This value was calculated using Figure 2 and equation (16). [h] Calculated using equation (16) and Table VI.

initiated by adding 10–100 μl of the substrate in the appropriate solvent from the tip of a stirring rod and stirring vigorously for a few seconds. Recording was usually started within 10 seconds after initiation of the reaction. The kinetics at *p*H 5.05 were followed at 330 mμ (liberation of *p*-nitrophenol) under pseudo first-order conditions with $S_0 << E_0 < K_m$. The kinetics at *p*H 7.80 were followed at 400 mμ (liberation of *p*-nitrophenoxide ion) under second-order conditions with $E_0 \cong S_0 << K_m$. The enzyme concentration

was always greater than the substrate concentration in order to eliminate a possible turnover of the enzyme. The second-order acylation rate constants were calculated according to the method of Bender, *et al.* (1962). The second-order plots were linear to greater than two half-lives. The first-order constants were calculated by either conventional first-order kinetics employing an infinity reading of the absorbance, or by a modified Guggenheim plot (Kézdy *et al.*, 1958).

The procedure for the determination of the inhibition constant of *N*-acetyl-L-tryptophan in 3% and 6% acetonitrile-water was exactly as described above, except that the buffer contained the *N*-acetyl-L-tryptophan and the *p*H was 7.9–8.1.

Deacylation of trans-Cinnamoyl-α-Chymotrypsin.—*trans*-Cinnamoyl-α-chymotrypsin was prepared at *p*H 4.0 as described previously (Bender *et al.*, 1962). An aliquot of the stock solution of the acyl-enzyme (100 μl) was added as described previously to 3 ml of buffer which contained the appropriate percentage of dioxane. The decrease in absorbance was followed at 310 mμ.

Acylation of α-Chymotrypsin by trans-Cinnamoylimidazole.—The kinetics of the reactions of *trans*-cinnamoylimidazole and α-chymotrypsin under second-order conditions at *p*H 5.05 were followed spectrophotometrically at 335 mμ. The decay curve at this wavelength leads directly to the second-order rate constant (Bender *et al.*, 1962). The difference in absorbance between the initial and final readings leads directly to the determination of the concentration of α-chymotrypsin-active sites in the solution (Schonbaum *et al.*, 1961). It was therefore possible to titrate the enzyme solution and observe the acylation kinetics with the same experiment. The experimental procedure was exactly that described for the *p*-nitrophenyl acetate experiments, except for three runs at high dioxane concentrations. In these experiments, the procedure was varied only in that 1.0–2.0 ml of enzyme solution was added to the cuvette and the total volume made to 3.10 ml. The reaction was then initiated by addition of the substrate. The enzyme concentration was always less than the substrate concentration, as required for the titration. The acyl-enzyme formed is completely stable under these conditions.

The pseudo first-order procedure used for the separate determination of the acylation rate constant k_2 and K_m requires a stopped-flow apparatus and has been described previously (Kézdy and Bender, 1962). Since the enzyme concentration approached the substrate concentration (the lowest limit of S_0/E_0 was 6.4) in this set of experiments, it is necessary to correct slightly the initial substrate concentration by one-half the enzyme concentration. (This correction amounts to 8% of the rate in the worst case.)

α-Chymotrypsin-Catalyzed Hydrolysis of N-Acetyl-L-Tryptophan Methyl Ester.—The kinetics of hydrolysis were measured by titration at constant *p*H of the acid produced, using a Radiometer Type TTT1C recording Titrator (*p*H-stat). Nitrogen was gently blown over the surface of the titration solution which was thermostatted. The data were treated 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 (Bender and Hamilton, 1962; Foster and Niemann, 1953; Cunningham and Brown, 1956). The inhibition constant for *N*-acetyl-L-tryptophan, needed for the above calculation, is not known in 10% and 14% acetonitrile-water solution. However, the kinetics were carried out with $S_0 << K_I$ so that the slope of the plot of $(S_0 - S)$ vs. $(\log S_0/S)/t$ is directly equal to $-2.303\ K_m$, since it was shown that K_I increases approximately as much as K_m with increasing acetonitrile (cf. Table IV, footnote *f*). The value of K_I could be estimated to meet this requirement.

The kinetics of this hydrolysis were also carried out using the Cary spectrophotometer as previously described at 300 mμ. The data were treated as above with the integrated Michaelis-Menten equation with inhibition by products, and also by the method of Lineweaver and Burk (1934).

TABLE III

THE EFFECT OF ORGANIC SOLVENT ON k_2 AND K_m IN THE ACYLATION OF α-CHYMOTRYPSIN BY *N-trans*-CINNAMOYLIMIDAZOLE AND *p*-NITROPHENYL ACETATE

*p*H	% Organic Solvent	k_2/K_m mole^{-1} sec^{-1}	k_2 sec^{-1}	$K_m \times 10^4$ M
	N-trans-cinnamoylimidazole			
5.05	2.59 acetonitrile[a]	9620 (8720)[e]	4.3	4.5
5.09	1.65 dioxane[b]	8260 (8550)[e]	3.8	4.6
5.22	7.82 dioxane[b]	2000 (1570)[e]	5.6	28
	p-Nitrophenyl Acetate			
7.82	1.61 acetonitrile[c]	3530 (3600)[e]	3.96	11.2
7.80	4.0 acetonitrile[c]	1958	3.72	19.0
7.80	20.0 2-propanol[d]		3.3	77

[a] Unpublished work of F. Kézdy. [b] This work, 25.0 ± 0.1°, E_0 = 2–4 × 10^{-4} M, S_0 = 1 to 16 × 10^{-4} M. [c] Kézdy and Bender (1962). [d] Gutfreund and Sturtevant (1956). [e] Determined from second-order kinetics.

TABLE IV

α-CHYMOTRYPSIN-CATALYZED HYDROLYSIS OF *N*-ACETYL-L-TRYPTOPHAN METHYL ESTER IN ACETONITRILE-WATER[a,b]

% Acetonitrile[c]	$K_m \times 10^5$ (M)[d] Calcd.[g]	Exp.	k_{cat} (sec^{-1})[f]
0		7.5 ± 0.5	57.5 ± 2.6[e]
3	13.1	13.9 ± 0.5	50.9 ± 1.8
6	19.1	21.1 ± 1.2	47.3 ± 0.6
10	28.3	36.9 ± 6.3	47.3 ± 3.9
14	40.0	65.5 ± 4.7	51.5 ± 0.5

[a] At 25.0 ± 0.1°. Ester concentration, 0.1–7.0 × 10^{-3} M. Enzyme concentration, *ca.* 10^{-3} M. [b] *p*H 8.20; 0.002 M Tris buffer for *p*H-Stat experiments and 0.1 M Tris buffer for spectrophotometric experiments. [c] Volume/volume. [d] Deviations are average deviations from the mean; at least two determinations were used to calculate the average constants. [e] Bender and Hamilton (1962) report K_m = 7.80 × 10^{-5} M; k_{cat} = 54.9 sec.$^{-1}$. [f] The effect of acetonitrile in water on the inhibition constant of *N*-acetyl-L-tryptophan was determined by the effect of this substance on the hydrolysis of *p*-nitrophenyl acetate in acetonitrile-water. The K_I of this compound varied from 0.87 × 10^{-2} M in water to 2.15 × 10^{-2} M in 6% acetonitrile. [g] Calculated using equation (15) and Table VI.

RESULTS

The effect of three dipolar, aprotic organic solvents, dioxane, acetone, and acetonitrile, on the kinetics of several α-chymotrypsin reactions are shown in Tables I, II, III, and IV. Table II indicates that the concentration of active sites in an α-chymotrypsin solution is not affected in dioxane-water solutions up to 32.2% dioxane, in acetone-water solutions up to 14.4% acetone, and in acetonitrile-water solutions up to 12.8%

TABLE V

DEACYLATION OF *trans*-CINNAMOYL-α-CHYMOTRYPSIN IN DIOXANE-WATER MIXTURES[a]

% Dioxane[b]	$k_3 \times 10^3$ sec^{-1}[c]
0.5	11.9 ± 0.4
5.3	11.2 ± 0.2
10.2	9.4 ± 0.2
15.0	9.2 ± 0.1
20.0	9.0
30.0	8.9

[a] 25.0 ± 0.1°; 0.05 M Tris buffer, pH 8.50. [b] Volume/volume; precipitation occurred when an aliquot of the acyl-enzyme in water was added to a 40% dioxane-water mixture. [c] Deviations are deviations from the mean.

acetonitrile. These conclusions are based on the spectrophotometric titration of α-chymotrypsin using *N-trans*-cinnamoylimidazole as substrate. Knowing that the enzyme concentration is not affected by organic solvents, it is then feasible to investigate the effect of these solvents on the kinetic parameters.

The qualitative observation of most interest is apparent in Tables III and IV, namely, that the introduction of a dipolar aprotic organic solvent increases the apparent K_m profoundly, but has a small or negligible effect on the various rate constants k_2, k_3, and k_{cat}. For example, in the reaction of *N-trans*-cinnamoylimidazole with chymotrypsin, k_2 changes only by 50% while K_m changes by a factor of six; further, in the reaction of *p*-nitrophenyl acetate with chymotrypsin, k_2 changes by only 20% while K_m changes by a factor of six (Table III). Finally, in the α-chymotrypsin-catalyzed hydrolysis of *N*-acetyl-L-tryptophan methyl ester, k_{cat} changes by only 20% while K_m changes by a factor of nine.

The virtual independence of α-chymotrypsin rate constants on the presence of organic solvents in the medium is seen in the effect of dioxane on the deacylation of *trans*-cinnamoyl-α-chymotrypsin. The kinetic data reported in Table V indicate that a change from 0 to 30% dioxane decreases k_3 by a factor of only 25%. Previously it was noted in the α-chymotrypsin-catalyzed hydrolysis of methyl hippurate that increasing dioxane or acetone concentration increased K_m while k_{cat} remained relatively constant (Applewhite *et al.*, 1958). The present results corroborate and extend this earlier finding.

Table III indicates that the acylation rate constant k_2 is negligibly affected by large changes in organic solvent concentration in the reactions of *p*-nitrophenyl acetate and *N-trans*-cinnamoylimidazole. It is therefore reasonable to assume that the changes in k_2/K_n for α-chymotrypsin-catalyzed reactions of these compounds in Tables I and II, respectively, are due in fact to changes in K_m with organic solvent composition. The ease of the experimental technique and the excellent reproducibility of the second-order kinetic data make the determination of k_2/K_m an attractive approach for the determination of effects of solvent on K_m in comparison to the determination by classical Michaelis-Menten kinetics. Of course, this method is suitable at best for substrates whose deacylation step is completely rate determining.

DISCUSSION

Competitive Inhibition.—The virtual independence of any of the enzymatic rate constants on the concentration of the organic solvent and the dependence of the apparent Michaelis constant on this parameter can be superficially explained on the basis of a competitive inhibition by the organic solvent molecule (I); that is, that the organic solvent molecule is acting as a competitive inhibitor for the substrate. Qualitatively competitive inhibition rationalizes the effect of organic solvents, since the apparent K_m would be expected to increase and k_{cat} should be unaffected. A satisfactory quantitative correlation was found using data on the α-chymotrypsin-catalyzed hydrolysis of methyl hippurate and methyl hydrocinnamate in low concentrations of diethyl ether and *t*-amyl alcohol (less than 3% of organic solvent). (Miles, Morey, *et al.*, 1962.) However, the change in solvent composition was quite small, and application of the simple competitive inhibition process to the data contained in the present paper failed completely: plots of v_0/v or k_0/k versus I were curved over their entire length.

Therefore an attempt was made to determine whether a modified competitive inhibition treatment would better empirically fit the data. Since high concentrations of organic solvent were used, it was then postulated that a second molecule of inhibitor (I) could bind, leading to a hypothesis of the following kind:

$$E + S \underset{}{\overset{K_m}{\rightleftharpoons}} ES \xrightarrow{k_{cat}} E + P \qquad (3)$$

$$E + I \overset{K_I}{\rightleftharpoons} EI \overset{K_{II}}{\underset{I}{\rightleftharpoons}} EI_2 \qquad (4)$$

From this equation the relationship between the observed K_m in an organic solvent–water mixture and the real K_m in water can be derived:

$$\{[(K_m)_{obs}/(K_m)_{H_2O}] - 1\}/I = 1/K_I + I/K_IK_{II} \qquad (5)$$

Two assumptions were used in the derivation of equation (5), namely, that $(K_m + E) \cong K_m$ and $S_0 >> E_0$. The data on the second-order acylation constants can be treated in a similar fashion, leading to an equation similar to equation (5), except that the K_m's are replaced by $1/(k_2/K_m)$'s which means that the bracketed quantity is inverted (assuming $(K_m + E) \cong K_m$ and $K_m >> S_0$). A plot of the left-hand side of equation (5) vs. I (or its counterpart for second-order acylations) should give a straight line with a slope equal to $1/K_IK_{II}$ and an intercept equal to $1/K_I$. The present data can be correlated to a reasonable extent by such hypothesis involving multiple binding of the organic solvent as inhibitor. Such a correlation in Figure 1 is reasonably encouraging, for a linear relationship is obtained for the dioxane data with three substrates. However, the hypothesis embodied in equations (3) and (4) predicts that K_I, the inhibitor constant for the solvent (the intercept of Fig. 1) should be independent of the substrate. This prediction is met only approximately. Similar plots for acetonitrile-water and acetone-water solutions gave straight lines whose intercepts were generally in better agreement. However, the K_{II}'s determined from the slopes of plots such as Figure 1 have a large and unpredictable scatter, making it impossible even qualitatively to correlate them.

Competitive Inhibition and the Dielectric Constant.—The correlation of the effect of dipolar aprotic organic solvents on the kinetics of α-chymotrypsin action with simple competitive inhibition forms a self-consistent picture which makes possible the qualitative prediction of the apparent K_m (or k_2/K_m). As described above, however, the quantitative picture is not encouraging. Laidler (1958) and Barnard and Laidler (1952) have suggested that the interaction between enzyme and substrate may be correlated with the dielectric constant of the medium. From absolute reaction rate theory, Laidler proposes that

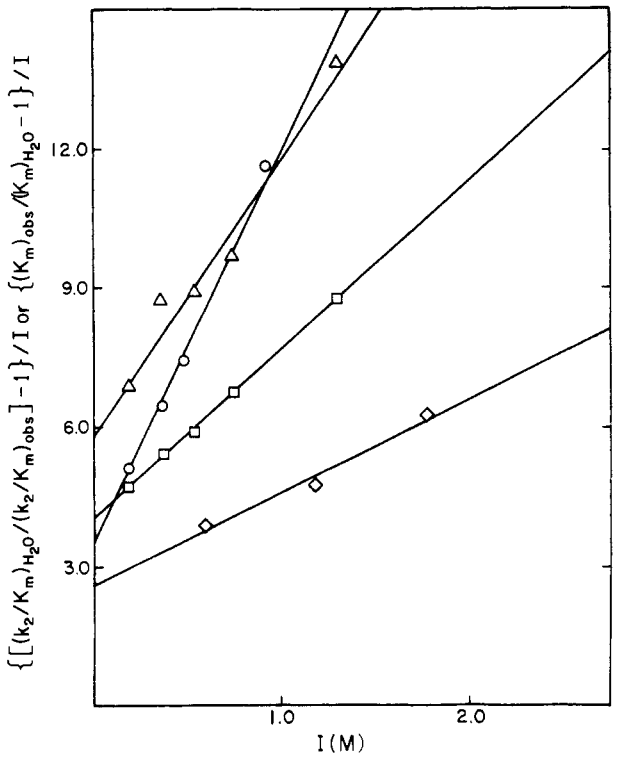

FIG. 1.—Test of equation (5) for the correlation of the effect of dioxane on α-chymotrypsin-catalyzed reactions at 25.0°: ⊡ k_2/K_m's of p-nitrophenyl acetate at pH 5.1; ⊙ k_2/K_m's of *N-trans*-cinnamoylimidazole at pH 5.1; △ k_2/K_m's of p-nitrophenyl acetate at pH 7.8; ◇ K_m's of methyl hippurate hydrolysis at pH 7.8; (Applewhite, Martin, and Niemann, 1958).

$$\ln k - \ln (k)_0 = A/DT \qquad (6)$$

where k is either a rate constant such as k_{cat} or k_2/K_m, or an equilibrium constant such as K_m or K_I; D is the dielectric constant of the medium; T is the absolute temperature; and A is a constant. Laidler and Ethier (1953) successfully applied this relationship to the myosin-catalyzed hydrolysis of ATP, and Gutfreund and Hammond (1959) did so to the ficin-catalyzed hydrolysis of *N*-α-benzoyl-arginine ethyl ester. However, Laidler (1958) has analyzed several failures of this treatment by saying that a "considerable source of uncertainty arises from the simplification of regarding the solvent as a continuous dielectric. A second difficulty is that specific effects may result from the use of mixed solvents; [solvent] molecules, may, for example, undergo some interaction with part of the enzyme molecule."

We have therefore taken the two hypotheses of competitive inhibition and dielectric constant effects, which in themselves have proved to be inadequate, to explain the effects of organic solvents, and combined them to produce a new interpretation of organic solvent effects. The simplest competitive inhibition is first postulated.

$$\mathrm{E} + \mathrm{S} \underset{}{\overset{K_m}{\rightleftharpoons}} \mathrm{ES} \longrightarrow \mathrm{E} + \mathrm{P}$$

$$\mathrm{E} + \mathrm{I} \overset{K_\mathrm{I}}{\rightleftharpoons} \mathrm{EI} \qquad (7)$$

It is easily shown that simple competitive inhibition of this type leads to

$$K_m(\mathrm{obs})/K_m(\mathrm{org}) = 1 + \mathrm{I}/K_\mathrm{I} \qquad (8)$$

where K_m(obs) is the observed K_m in the organic solvent–water mixture, K_m(org) is the real K_m in that mixture, and K_I is the K_I in that mixture. It is then convenient to use the exponential form of equation (6). For the effect of the dielectric constant of the medium on K_m, one can write

$$K_m(\mathrm{org})/K_m(\mathrm{H_2O}) = e^{A\chi} \qquad (9)$$

and for the effect of the dielectric constant of the medium on K_I, one can write

$$K_\mathrm{I}(\mathrm{org})/K_\mathrm{I}(\mathrm{H_2O}) = e^{B\chi} \qquad (10)$$

where the constants A and B include the temperature dependence of the dielectric effect and χ is the difference of the reciprocals of the dielectric constants of the organic solvent–water mixture and of pure water. A combination of equations (8), (9), and (10) leads directly to

$$K_m(\mathrm{obs})/K_m(\mathrm{H_2O}) = e^{A\chi}[1 + \mathrm{I}/K_\mathrm{I}(\mathrm{H_2O})e^{B\chi}] \qquad (11)$$

A similar equation can readily be derived for the effect of organic solvents on the observed second-order rate constant, k_2/K_m.

$$[k_2/K_m(\mathrm{H_2O})]/[k_2/K_m(\mathrm{obs})] = e^{A\chi}[1 + \mathrm{I}/K_\mathrm{I}(\mathrm{H_2O})e^{B\chi}] \qquad (12)$$

The parameters A and B are measures of the electrostatic interaction of the enzyme with the substrate (or inhibitor). A decrease in the dielectric constant increases the electrostatic repulsion between the enzyme and substrate (or inhibitor) and therefore decreases the binding.

The solutions of equations (11) and (12) are not straightforward and it is therefore necessary to use an indirect method to proceed further. If one considers data in the identical solvent mixture for two different substrates R and S, it can be shown that equations (13) and (14) hold, for a combination of mixed second-order and turnover reactions and for a combination of two second-order reactions, respectively. If the effect of solvent on

$$\log k^\mathrm{S}(\mathrm{obs})K_m{}^\mathrm{R}(\mathrm{obs}) = (A_\mathrm{R} - A_\mathrm{S})\chi/2.303 + \log k^\mathrm{S}(\mathrm{H_2O})K_m{}^\mathrm{R}(\mathrm{H_2O}) \qquad (13)$$

$$\log [k^\mathrm{S}(\mathrm{obs})/k^\mathrm{R}(\mathrm{obs})] = (A_\mathrm{R} - A_\mathrm{S})\chi/2.303 + \log [k^\mathrm{S}(\mathrm{H_2O})/k^\mathrm{R}(\mathrm{H_2O})] \qquad (14)$$

α-chymotrypsin-catalyzed hydrolyses can indeed be described by a combination of competitive inhibition and dielectric constant effects, a test can be applied by using equations (13) and (14). A plot of the left-hand portions of equations (13) or (14), which are experimental quantities, versus χ[4] should give a straight line with a slope of $(A_\mathrm{R} - A_\mathrm{S})/2.303$ and an intercept which is characteristic of the second-order rate constants and/or Michaelis constants in water. Figure 2 shows such plots which are in general excellent straight lines. Line C is a plot of equation (14) for two substrates, *N-trans*-cinnamoylimidazole and p-nitrophenyl acetate, in three solvent mixtures, acetone-water, dioxane-water, and acetonitrile-water at pH 5.1. The result of the same straight line for all this data is an excellent verification of the hypothesis put forward. The other straight lines are equally good.

It is possible to solve equations (11) and (12) directly if one assumption is made, namely, that B is small in comparison to A, which means that the organic solvent-enzyme interaction is much less susceptible to a dielec-

[4] The dielectric constant used for pure water at 25.0° was 78.39 which was an average of the values reported by Timmermans (1960).

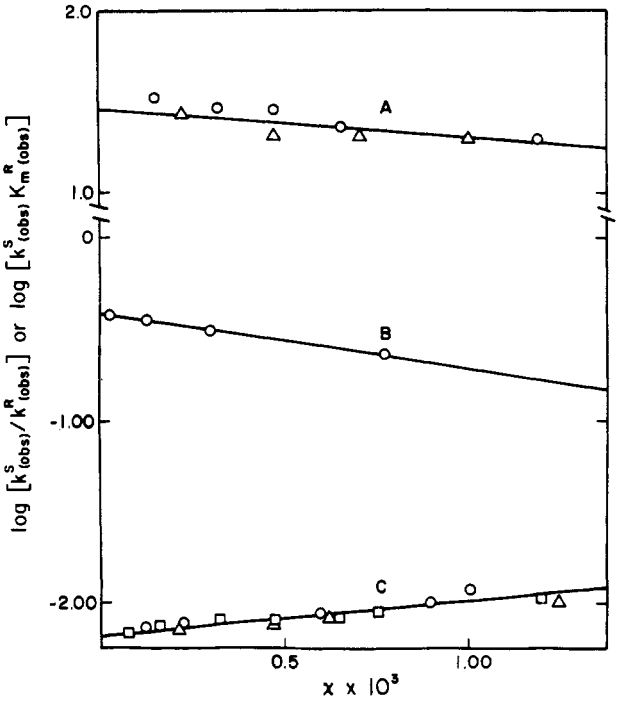

FIG. 2.—Test of equations (13) and (14) for the effect of organic solvents on α-chymotrypsin-catalyzed reactions at 25.0°: (A) log k(*p*-nitrophenyl acetate)·K_m(methyl hippurate) vs. χ at *p*H 7.8 in water-acetone ○ and water-dioxane △ solutions; (B) log k(*p*-nitrophenyl acetate at *p*H 7.7)·K_m(*N*-acetyl-L-tryptophan methyl ester at *p*H 8.2) vs. χ in water-acetonitrile solution; (C) log k(*p*-nitrophenyl acetate)/k(*trans*-cinnamoylimidazole) vs. χ at *p*H 5.1 in water-acetone □, water-acetonitrile ○, and water-dioxane △ solutions.

tric effect of the solvent than is the substrate-enzyme interaction. With this assumption equations (11) and (12) take the form

$$K_m(\text{obs})/K_m(\text{H}_2\text{O}) = e^{A\chi}(1 + \text{I}/K_\text{I}) \quad (15)$$

$$[k_2/K_m(\text{H}_2\text{O})]/[k_2/K_m(\text{obs})] = e^{A\chi}(1 + \text{I}/K_\text{I}) \quad (16)$$

These equations are equations in two unknowns, A and K_I. By employing data at different values of χ, it is possible to solve for both A, which is a function of the substrate, and K_I, which is a function of the solvent. Once an A value is determined for one substrate it is possible to use the slopes of Figure 2 [which are equal to $(A_\text{R} - A_\text{S})/2.303$] to determine the values of A for other compounds. Then knowing A values of the various substrates, it is possible to use equations (15) and (16) to determine K_I values of the various solvents. These are listed in Table VI.

The inhibition constants given above are in the same range as those determined by the hypothesis based solely on competitive inhibition. The value of the second-order rate constants or Michaelis constants in pure water calculated from equations (15) or (16) using the data in Table VI are in excellent agreement with the values obtained by extrapolation of a plot of the observed constant vs. I to zero I. Equations (15) or (16) and the data in Table VI may be used to calculate observed constants for any of the substrates in any of the organic solvent–water mixtures. Such calculated values are in good agreement with the observed constants where such comparison is possible.[5]

[5] Using the data of Table VI and equations (11) or (12), it is possible to show that the value of B is indeed close to zero. This is, of course, a circular argument, but at least no inconsistency is found.

TABLE VI

DIELECTRIC PARAMETERS, A, AND INHIBITION CONSTANTS, K_I, WHICH DETERMINE THE EFFECT OF SOLVENT ON α-CHYMOTRYPSIN-CATALYZED REACTIONS

Compound	*p*H 5.1	*p*H 7.8	*p*H 8.2
	A Values[a]		
N-trans-cinnamoylimidazole	805		
p-Nitrophenyl acetate	417	814	
N-Acetyl-L-tryptophan methyl ester			168
Methyl hippurate		330–399	
	K_I Values (M)		
Acetonitrile	0.85	0.83	
Acetone	0.98	1.24	
Dioxane	0.28	0.32	

[a] The A values are a measure of the electrostatic effect between enzyme and substrate defined that a more positive A value leads to an increase in electrostatic repulsion.

Some examples of experimental and calculated rate constants are shown in Tables I, II, and IV.

In summary it can be stated that the hypothesis for the effect of dipolar aprotic organic solvents on K_m or k_2/K_m embodying the two ideas of competitive inhibition by the organic solvent and an electrostatic effect of the organic solvent on the enzyme-substrate interaction is based on a reasonable model of the enzymatic process and leads to the best empirical treatment of such data that has yet been devised. This treatment is superior to that for simple competitive inhibition in its ability to correlate data at high organic solvent concentrations. For the same reason it is superior to a treatment involving solely a dielectric constant effect. This hypothesis is general in its capability of treating all pertinent data in the literature and predicting new data.

It is of interest to compare the effect of organic solvents on enzyme-catalyzed ester hydrolyses with that on hydroxide ion–catalyzed ester hydrolyses. The effect of organic solvents such as acetone and dioxane on the alkaline hydrolyses of various esters such as ethyl acetate, methyl acetate, and ethyl formate has been investigated by Tommila and co-workers (Tommila *et al.*, 1952; Tommila and Maltamo, 1955a, 1955b). They found that the rate of the alkaline hydrolysis in 30% organic solvent–water mixtures was approximately 20–30% less than in water itself. The pure rate constants k_2, k_3, and k_cat in the enzymatic hydrolyses are depressed by roughly the same amount in comparable organic solvent–water mixtures. Thus it appears that the transition states of the hydroxide ion–catalyzed and enzyme-catalyzed hydrolysis are the same as far as this criterion is concerned.

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Substrate Activation of Trypsin*

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The initial rates of hydrolysis of D- and L-*p*-toluene-sulfonyl arginine methyl ester by trypsin were examined over a 100,000-fold range of substrate concentration. The hydrolysis of neither substrate fits simple Michaelis-Menten kinetics, but can be fitted to a scheme based on the assumption that there are complexes with both one and two molecules of substrate bound to the enzyme. In such a scheme both the binary and ternary complexes decompose to products, the latter at a much higher rate than the former. Evidence is presented which shows that the concentration dependence of the rate is not due to impure enzyme preparations. The analysis of D–L mixtures shows that both substrates compete for the same active site and further that the D isomer is capable of activating the hydrolysis of the L substrate.

The discovery that trypsin and thrombin catalyze the hydrolysis of both optical isomers of α-*N*(*p*-toluene)-sulfonyl arginine methyl ester (TAM)[1] (Laskowski *et al.*, 1958) led to a further investigation of these substrates as part of a study of the specificity of trypsin and chymotrypsin. L-TAM has been widely used as a substrate for trypsin, and several workers have made kinetic analyses of the system (Scheraga *et al.*, 1958; Ronwin, 1959; Martin *et al.*, 1959). All these workers report simple Michaelis-Menten behavior.[2] Work reported in this paper shows that trypsin/TAM does not follow simple Michaelis-Menten kinetics; the results are compatible with the formation of binary and ternary enzyme-substrate complexes, both capable of decomposing to products, the latter at a greater rate (substrate activation) than the former.

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[1] Abbreviations used in this paper are as follows: TAM, α-*N*(*p*-toluene)sulfonyl arginine methyl ester; DIP, diisopropyl phosphoryl; DIPF, diisopropyl phosphofluoridate; L-BAE, α-*N*(benzoyl)-L-arginine ethyl ester; L-ATE, N-acetyl-L-tyrosine ethyl ester.

Experimental

Materials.—Both TAM isomers were prepared from the corresponding L- and D-arginine hydrochloride

[2] Martin (1962) later published some data which show a rate-concentration relation not predicted by his constants, but which is in agreement with the analysis given in this paper.