THE TRYPSIN-CATALYZED HYDROLYSIS OF BENZOYL-L-ARGININE ETHYL ESTER

I. THE KINETICS IN DIOXANE–WATER MIXTURES*

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(Received May 5th, 1959)

SUMMARY

The rate of hydrolysis of benzoyl-L-arginine ethyl ester catalyzed by trypsin has been measured in dioxane–water mixtures containing up to 88 volume percent dioxane. The initial rate of the reaction follows the requirements of MICHAELIS-MENTEN kinetics under all experimental conditions employed. The pH-variation of the rate at enzyme saturation in the mixed solvents is similar to that in water. At pH 8 the rate first increases as dioxane is added and then decreases at higher dioxane concentrations. The apparent MICHAELIS-MENTEN constant is a minimum at approximately pH 8, and increases with increasing dioxane concentration. The value at pH 8.6 in 88 % dioxane is 5500 times the value in water at this pH. Interpretations of various of the observed effects are suggested.

INTRODUCTION

In view of the fact that variations of solvent have led to important results in many kinetic studies of small molecules of known structure, it is of interest to apply this attack to an enzyme system. A hydrolytic enzyme was desired for a first study because solvent variation would alter the concentration of the essential reactant water. The trypsin-catalyzed hydrolysis of benzoyl-L-arginine ethyl ester (BAEE) has been shown[1] to be very convenient for kinetic measurements, and preliminary experiments indicated that it would be possible to obtain kinetic data with this system in dioxane–water mixtures up to high dioxane concentrations.

The effect of temperature and pH on the maximum rate of hydrolysis of BAEE in aqueous solution was investigated by GUTFREUND[2]. In the present paper we report the effect of pH, temperature and substrate concentration on the rate of the hydrolysis in dioxane–water mixtures.

A few experiments with hydrolytic enzymes in organic solvent–water mixtures have been previously reported. SCHWERT AND EISENBERG[3] found that the rate of the trypsin-catalayzed hydrolysis of benzoyl-L-arginine methyl ester is increased by the addition of ethanol up to a concentration of 32 volume percent, and that further

* Contribution No. 1545 from the Sterling Chemistry Laboratory of Yale University.
** Adapted from Ph.D. thesis of T. INAGAMI, June 1958.

additions of ethanol cause a decrease in rate. The maximum rate observed was 50 % higher than the rate in water. Similar effects were observed to result from the addition of various other alcohols. On the other hand, KAUFMAN AND NEURATH[4] found that the rate, at enzyme saturation, of the chymotrypsin-catalyzed hydrolysis of acetyl-L-tyrosine amide was unaffected by methanol up to a concentration of 21 volume percent, although the apparent MICHAELIS-MENTEN constant for the system was nearly tripled. BARNARD AND LAIDLER[5] reported that the saturation rate of hydrolysis of methyl hydrocinnamate by chymotrypsin decreased by 40 % in going from 15 to 25 weight percent of methanol, while LAIDLER AND ETHIER[6] found that the enzymic hydrolysis of adenosine triphosphate was 20 % higher in 40 volume percent methanol than in water. These latter authors found more complicated variations of rate in dioxane–water mixtures. KIMMEL AND SMITH[7] studied the papain-benzoyl-L-arginine amide system in various alcohol–water mixtures. Ethanol had little effect on the maximum rate, while methanol and propanol-2 decreased it.

NELSON AND SCHUBERT[8] studied the enzymic hydrolysis of sucrose at high concentrations of the substrate in water, and in 10 % and 20 % ethanol. In this system the data indicated that the rate is the same function of water concentration whether this is lowered by sucrose or by ethanol.

LAIDLER[5,6,9] has discussed the influence of solvent on the kinetics of enzymic reactions in terms of the effect of dielectric constant on electrostatic interactions, and has shown that the behavior observed in various systems can be reasonably interpreted on this basis. It will appear below that the dependence of the kinetics of the trypsin-catalyzed hydrolysis of BAEE on solvent composition is too complicated to permit any such simple treatment, although it will be of interest to invoke electrostatic interactions in connection with some of the observed phenomena.

All trypsin-catalyzed reactions which have been subjected to kinetic analysis appear to follow the simple MICHAELIS-MENTEN mechanism under usual experimental conditions:

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

As pointed out in earlier papers[10,11] measurements of the over-all rate of product formation by classical (as contrasted with fast-reaction) techniques can only lead to values for the maximum rate, $k_{2(app)}$, and the apparent MICHAELIS-MENTEN constant, $K_{m(app)} = (k_{-1} + k_{2(app)}/k_1$. It should be kept in mind that in cases where the mechanism is more complicated than indicated by eqn. (1), the kinetic constants will include several individual rate constants.

We shall assume in subsequent discussion that $K_{m(app)}$ is the dissociation constant for the enzyme–substrate complex. The only justification for this assumption, aside from convenience, is that the variation of $K_{m(app)}$ with experimental conditions appears to be quite independent of that of $k_{2(app)}$.

EXPERIMENTAL

Materials

Dioxane was purified by a modification of the method of KRAUS AND VINGEE[12]. Commercial "purified dioxane" was stored over NaOH pellets for several days, refluxed over sodium wire for 2–3 h, and then distilled over sodium at atmospheric

pressure. A central cut of the distillate was stored over sodium under an atmosphere of nitrogen; exposure to atmospheric oxygen during use was kept to a minimum. Dioxane stored in this way does not contain sufficient sodium to give a flame test, and the pH of a solution in water is identical with that of a similar solution of freshly distilled dioxane. Dioxane concentrations are given in volume percent, a 30 % solution being prepared by diluting 3 volumes of dioxane to 10 volumes with water.

BAEE was purchased from Mann Biochemical Co., New York, N.Y., and was used without further purification. M.p. 130° (lit.[3] 129–130°). SMITH AND PARKER[13] have recently reported kinetic anomalies in the hydrolysis of BAEE by papain at 37°, which they attributed to impurities in the substrate which were removed by chromatography. We have observed no such anomalies in the hydrolysis of BAEE by trypsin at 25°. Tris(hydroxymethyl)aminomethane (THAM) was recrystallized from ethanol. Other reagents were of analytical grade.

Crude trypsin, purchased from British Drug Houses, Ltd., was treated[14] as follows. The powdered material was extracted with 0.01 *N* HCl and the trypsin was precipitated with 3 % trichloroacetic acid. The precipitate was transferred to a dialysis sac and dissolved by dialysis against 0.01 *N* HCl. The solution was taken to pH 8 and a small amount of precipitate removed by centrifugation. The trichloroacetic acid precipitation and solution in 0.01 *N* HCl were repeated. Stock solutions prepared in this way contained 2.6–3.0 mg N/ml, and had tryptic activities (BAEE substrate) of $3–4 \cdot 10^{-6}$ moles sec^{-1} mg N^{-1} measured at 25° and pH 8.0 in a solution containing 0.01 *M* phosphate and 0.1 *M* NaCl, with a substrate concentration sufficient to saturate the enzyme. The maximum activity observed was $4.18 \cdot 10^{-6}$ moles/sec/mg N. From the maximum rate of hydrolysis found by GREEN AND NEURATH[15] and the molecular weight (23,800) and N content (14.4 %) reported by CUNNINGHAM[16], we can conclude that 99.0 % of the nitrogenous material in this sample was trypsin*. Some preparations had as low as 80 % of the specific activity of this sample, but were indistinguishable from it so far as relative kinetic behavior was concerned.

Some of the enzyme prepartions were found to have considerable activity towards acetyl-L-tyrosine ethyl ester (ATEE), perhaps in part because of contamination by chymotrypsin. Any such contamination was shown to have negligible effect on the rate of BAEE hydrolysis by experiments in the presence of indole, a good inhibitor for chymotrypsin. Thus in one experiment, 0.01 *M* indole reduced the rate of hydrolysis of ATEE ($2.5 \cdot 10^{-3}$ *M*) by 85 % without detectably changing the rate of hydrolysis of BAEE. Indole at a concentration of 0.1 *M* had no effect on the BAEE hydrolysis in 50 % dioxane between pH 8 and 11, and in 85 % dioxane at pH 8.

Trypsin concentrations were in all cases inferred from the observed activity under assay conditions (25° and pH 8 in aqueous solution containing 0.005 *M* THAM and 0.025 *M* $CaCl_2$) which we shall hereafter refer to as standard conditions, using the data of GREEN AND NEURATH[15] and CUNNINGHAM[16].

pH Scale

No satisfactory definition of a pH scale in dioxane–water mixtures, in terms of

* In arriving at this figure use has been made of our observation that the rate of hydrolysis under the conditions given above is 0.8% lower than under the conditions used by GREEN AND NEURATH[15], namely 25°, pH 8 in a solution containing 0.005 *M* THAM and 0.001 *M* $CaCl_2$.

standard buffers, is available. MARSHALL AND GRUNWALD[17] have shown that the glass electrode responds to changes in hydrogen ion activity in the same way as the hydrogen electrode in solutions up to 82 % (by weight) dioxane. FREDERICQ[18] found that a glass electrode and a saturated KCl bridge leading to a reference electrode can be employed to measure pH in 40 % dioxane with the same meaning as the measurement has in water.

We have found the usual pH measuring equipment including a Beckman glass electrode to function conveniently and reproducibly in solutions containing as much as 88 % dioxane, and have adopted the following simple and operationally satisfactory procedure for setting up an arbitrary "pH scale". The pH meter and electrodes were standardized in water using Beckman pH 7 standard buffer, and were then used directly in dioxane–water mixtures to measure the apparent pH. That pH values read in this way are closely related to pH values in water is indicated by the fact that the apparent pH at 25° of a 0.05 *M* THAM–0.05 *M* THAM·HCl solution remained constant at 7.99 ± 0.02 as the dioxane concentration was increased from 0 to 88 %. This behavior would be expected if the pH scale were approximately correct since the ionization of THAM is isoelectric and therefore relatively unaffected by changes in dielectric constant. In contrast, the apparent pH of a 0.05 *M* formic acid–0.05 *M* sodium formate solution increased from 3.72 in water to 6.11 in 80 % dioxane.

Kinetic measurements

The hydrolysis of BAEE at neutral pH is accompanied by the liberation of one proton per molecule hydrolyzed, so that the rate of the reaction was conveniently followed by the method of SCHWERT *et al.*[1]. The reaction solution, usually 10 ml, was immersed in a water bath controlled to 0.05° and was stirred by a magnetic stirrer. Nitrogen gas previously saturated with the solvent being used was led over the solution. At low pH values suitable corrections for incomplete ionization of benzoylarginine were applied to the rates of alkali consumption. These corrections were evaluated from titration curves for benzoylarginine which led to the apparent p*K'* values listed in Table I. The fact that these values increase less with increasing

TABLE I

APPARENT pK' VALUES FOR BENZOYLARGININE AT 25° IN AQUEOUS SOLUTIONS CONTAINING 0.025 *M* $CaCl_2$

Percent dioxane	0	50	70	80	85	88
pK'	3.34	4.59	4.87	4.69	4.75	4.57

dioxane concentration than was observed in the case of formic acid is presumably to be attributed to increasing electrostatic interaction between the guanido and carboxyl groups as the dielectric constant is decreased. At high pH values corrections for hydroxide ion catalysis were applied.

Titration of benzoylarginine showed that even at pH 11 there was no significant loss of protons from the guanido groups.

Some of the later rate measurements employed a pH-stat of the type developed at the Carlsberg Laboratory[19].

In high concentrations of dioxane at high pH values the curves of alkali consumption *vs* time showed pronounced curvature. Initial rates were estimated graphically by two independent methods. In one method the observed curve was extrapolated to $t = 0$ and the slope estimated directly. In the other the mean rate of alkali consumption over short intervals of time was plotted against the time and extrapolated to $t = 0$; these plots were in most cases very nearly linear. The two methods gave values for the initial rate agreeing on the average to better than 5 %. We estimate the precision of the initial rates to be $\pm$ 5 %, except at the higher pH values in 80 to 88 % dioxane.

The initial rates obtained at varying initial substrate concentrations under each constant set of conditions were used to construct EADIE[20] plots, in which the rate is plotted against the ratio of the rate to the initial substrate concentration, for the evaluation of kinetic constants. The "best" straight line through the points in each EADIE plot was determined on the basis of a least squares criterion. Fig. 1 represents an EADIE plot, with y replacing the initial rate and x the substrate concentration.

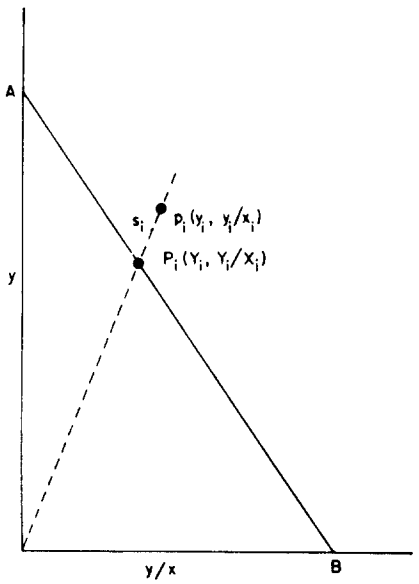

Fig. 1. Schematic EADIE plot illustrating least squares method. P_i is a point on the "true" line, and p_i is the observed point at the same substrate concentration.

Suppose p_i is an observed point and P_i is a point lying on the "true" line AB given by $Y = a + bY/X$. We wish to minimize the sum of the squares of the deviations, s_i, along lines of constant x, since this variable is considered to be essentially free from error. Since $x_i = X_i$, $s_i^2 = (y_i - Y_i)^2 \cdot (1 + 1/x_i^2) = [y_i - ax_i/(x_i - b)]^2 \cdot (1 + 1/x_1^2)$. Applying the conditions $\partial \sum s_i^2/\partial a = \partial \sum s_i^2/\partial b = 0$ gives the equations

$$\sum \left(1 + \frac{1}{x_i^2}\right) \frac{ax_i^2 + bx_iy_i - x_i^2y_i}{(x_i - b)^2} = 0 \qquad (2)$$

$$\sum \left(1 + \frac{1}{x_i^2}\right) \frac{ax_i^2 + bx_iy_i - x_i^2y_i}{(x_i - b)^3} = 0 \qquad (3)$$

In the present application the error in y at small values of x is larger than at large values of x. We have therefore least squared only the y-component of s_i; this amounts to introducing a weighting factor which gives greater weight to the deviations at large x. Eqns. (2) and (3) then become

$$\sum \frac{ax_i^2 + bx_iy_i - x_i^2y_i}{(x_i - b)^2} = 0 \tag{4}$$

$$\sum \frac{ax_i^2 + bx_iy_i - x_i^2y_i}{(x_i - b)^3} = 0 \tag{5}$$

These equations are conveniently solved by a method of successive approximations. It can be shown that if b_1 is an approximate value for b, then a better approximation is $b_1 + \Delta b_1$, where

$$\Delta b_1 = \frac{a_1a_4 - a_2a_3}{a_1a_5 - a_2a_4} \tag{6}$$

with

$$a_1 = \sum \frac{x_i^2}{(x_i - b_1)^2} \; ; a_2 = \sum \frac{x_i^2}{(x_i - b_1)^3} \; ; a_3 = \sum \frac{x_iy_i}{x_i - b_1} \; ;$$

$$a_4 = \sum \frac{x_iy_i}{(x_i - b_1)^2} \; ; a_5 = \sum \frac{x_iy_i}{(x_i - b_1)^3} \tag{7}$$

The value of a corresponding to $b_1 + \Delta b_1$ is

$$a = \frac{a_3a_5 - a_4^2}{a_1a_5 - a_2a_4} \tag{8}$$

Some typical EADIE plots are given in Fig. 2. The rates, r, are relative rates obtained by dividing the observed rates, in moles of ester hydrolyzed per ml per sec, by the rate observed in water at pH 8.0 with the same enzyme concentration but a substrate concentration large enough to insure saturation of the enzyme. The intercept of an EADIE plot at infinite substrate concentration gives the value of r_{max}, and the slope is equal to $-K_{m(app)}$.

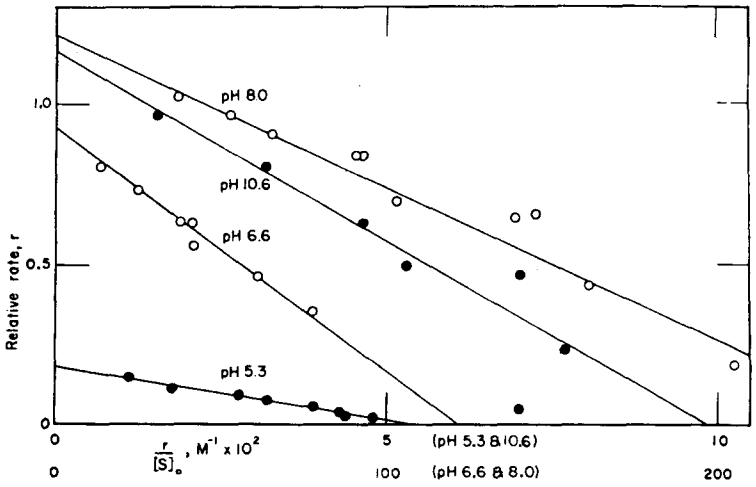

Fig. 2. The trypsin-catalyzed hydrolysis of BAEE in 50% dioxane at 25°. Typical EADIE plots at various values of the pH.

RESULTS AND DISCUSSION

The effect of calcium chloride

Preliminary experiments showed that in 50% dioxane containing 0.05 *M* KCl the rate of BAEE hydrolysis decreased during the reaction, while in aqueous medium the rate remained constant until very near to completion. Since the possibility existed that this was due to denaturation of the enzyme and since Ca^{++} has been reported[21,22] to have a stabilizing influence on trypsin, the effect of $CaCl_2$ was investigated. As seen in Fig. 3, curves A and B, 0.01 *M* $CaCl_2$ in the absence of KCl is quite effective in decreasing the curvature of the reaction plot. Curves C and D indicate that the presence of KCl counteracts the effect of $CaCl_2$; it is quite unlikely that the difference between curves C and D is due to an increase in $K_{m(app)}$ caused by the KCl, since the substrate concentration employed was approximately 100 times the $K_{m(app)}$ in the absence of KCl.

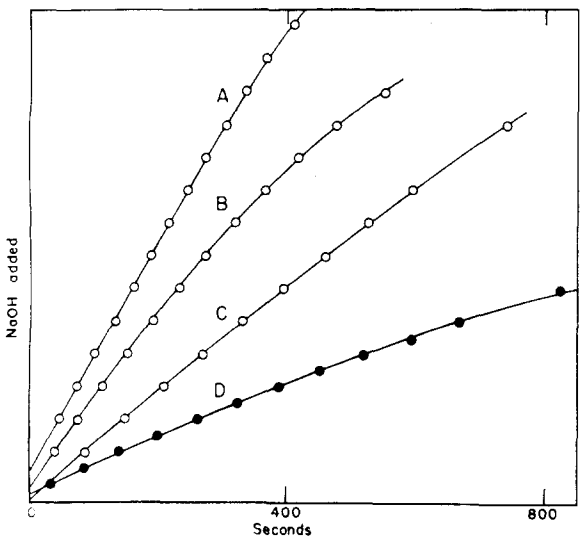

Fig. 3. The effect of $CaCl_2$ and KCl on the rate of the trypsin-catalyzed hydrolysis of BAEE at 25°. Curves A and B, 50% dioxane, pH 8; A, 0.01 *M* $CaCl_2$; B, 0.05 *M* KCl. Curves C and D, 70% dioxane, pH 8; C, 0.025 *M* $CaCl_2$; D, 0.025 *M* $CaCl_2$ and 0.05 *M* KCl.

GREEN AND NEURATH[15] reported that the esterase activity of trypsin is increased by increasing concentration of Ca^{++} to a maximum extent of 28%, this maximum being reached in approximately $5 \cdot 10^{-4}$ *M* Ca^{++}. We have observed similar increases, with subsequent decreases taking place at Ca^{++} concentrations higher than 0.1 *M*; these decreases are much accentuated in solutions containing dioxane, as seen in Fig. 4.

The experiment illustrated in Fig. 5 shows that trypsin is sufficiently stable in dioxane solutions in the presence of 0.025 *M* $CaCl_2$ so that initial rates can be evaluated. This experiment was run at pH 9.2 in 85% dioxane at a substrate concentration of about one-twentieth of $K_{m(app)}$. A small volume of concentrated substrate solution was added to the enzyme solution, and after the reaction was nearly complete a second volume of substrate solution was added. This process was repeated two additional times. The approximate constancy of the initial rates indicates that no significant loss of activity took place during a period of 40 min. Thus our usual experimental

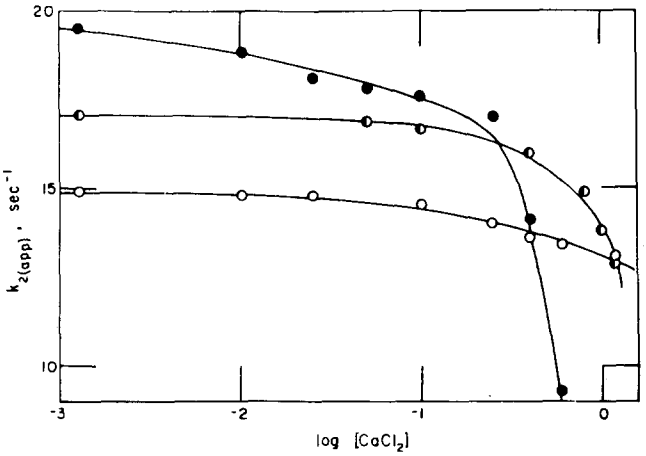

Fig. 4. The effect of $CaCl_2$ on the rate of the trypsin-catalyzed hydrolysis of BAEE at 25°. Open circles, water; half-filled circles, 30% dioxane; filled circles, 50% dioxane.

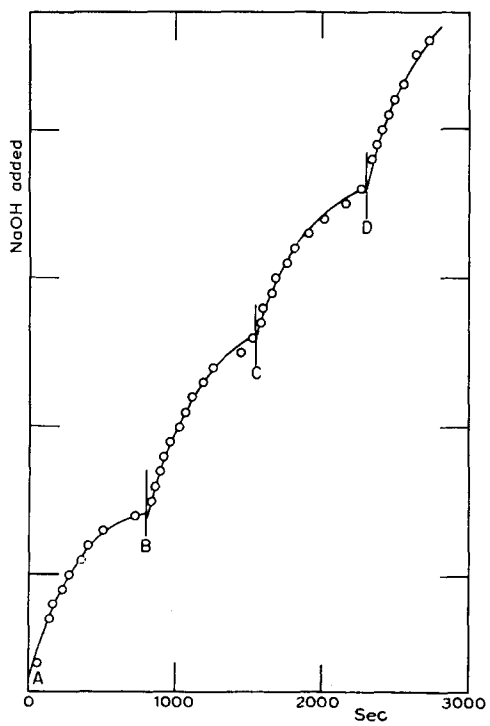

Fig. 5. Test of the stability of trypsin under reaction conditions in 85% dioxane. Substrate was added at A, B, C and D. Initial substrate concentrations and approximate relative initial rates were respectively: A, $1.30 \cdot 10^{-4}$ *M*, (1.00); B, $1.40 \cdot 10^{-4}$ *M*, 1.05; C, $1.51 \cdot 10^{-4}$ *M*, 1.08; D, $1.78 \cdot 10^{-4}$ *M*, 1.18.

procedure, in which a small volume of an aqueous solution of the enzyme was added to the substrate in the appropriate solvent, should be quite adequate. We have not investigated the long term stability of the enzyme in dioxane solutions.

The effect of substrate concentration

The variation of the initial rate of hydrolysis with BAEE concentration was found to adhere to the requirements of the MICHAELIS-MENTEN mechanism under all experimental conditions employed. A number of the EADIE[20] plots obtained in 50 % dioxane are given in Fig. 2. The standard deviations of the observed relative rates from the least squared lines averages 0.02. Since enzyme concentrations were inferred from rate measurements at the standard assay conditions (see above), it is convenient to express all rates relative to the rate under these conditions, using the symbol r for such relative rates. The value of $k_{2(app)}$ is obtained by multiplying r_{max} by 14.8 sec^{-1}.

The variation of r_{max} *with pH and temperature*

The maximum rate of hydrolysis of BAEE in water varies[2] with pH in a manner consistent with the hypothesis that a group in the catalytic site with an apparent pK' appropriate for an imidazolyl group must be unprotonated for the enzyme to be active. We have found similar behavior in dioxane–water mixtures. According to this hypothesis, a plot of rate *vs* the product of rate times hydrogen ion activity should be a straight line of slope $-1/K_i$, where K_i is the apparent ionization constant. Plots of this type for observations made in 50, 85 and 88 % dioxane are given in Fig. 6. A least squares method similar to that outlined above was employed to calculate the relative maximum rates and apparent pK'_i values, determined under

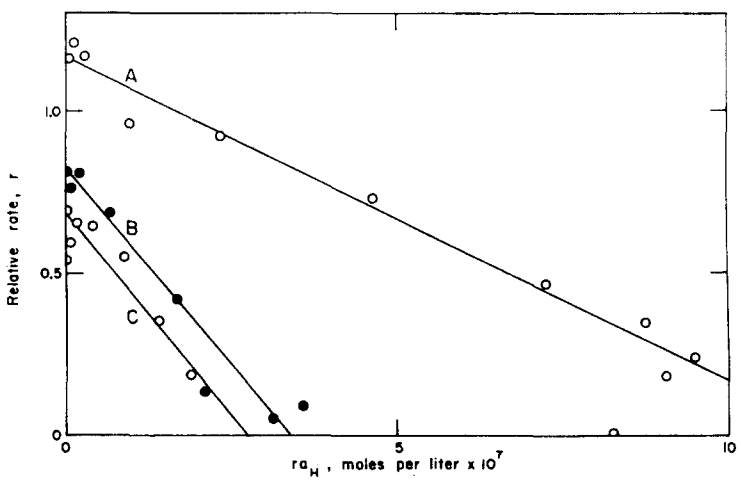

Fig. 6. The influence of pH on the maximum rate of the trypsin-catalyzed hydrolysis of BAEE in various media. A, 50% dioxane; B, 85% dioxane; C, 88% dioxane.

conditions of enzyme saturation, which are given in Table II, together with apparent heats of ionization and apparent heats of activation. Not only are the pK'_i values and apparent heats of ionization in Table II entirely reasonable for an imidazolyl group, but the fact that they are so little affected by large changes in dielectric constant indicates that the ionization involved is probably isoelectric.

The pK'_i values at 25° and 35° given in the second line of Table II differ slightly from those reported by GUTFREUND[2], and the apparent heat of ionization is approx-

TABLE II

THE EFFECT OF pH AND TEMPERATURE ON THE RATE OF TRYPTIC HYDROLYSIS OF BAEE UNDER CONDITIONS OF ENZYME SATURATION

Percent dioxane	*Added salt*	*Maximum relative rate*		pK'_i		*Apparent heat of ionization, cal/mole*	*Apparent heat of activation, cal/mole*
		25°	*35°*	*25°*	*35°*		
0	0	0.97	1.67	6.06	5.88	7600	9300
0	0.05 *M* KCl	0.99	1.68	6.22	6.01	8800	9100
50	0	1.19	2.04	5.80	5.63	7200	9200
50	0.05 *M* KCl	1.04	1.67	6.19	6.01	7600	8100
0	0.025 *M* $CaCl_2$	1.01	—	6.02	—	—	—
50	0.025 *M* $CaCl_2$	1.17	—	6.00	—	—	—
85	0.025 *M* $CaCl_2$	0.82	—	6.38	—	—	—
88	0.025 *M* $CaCl_2$	0.68	—	6.40	—	—	—

imately 2000 cal/mole larger and the apparent heat of activation 2000 cal/mole smaller than the quantities found by GUTFREUND. Some of these differences appear to be due to the fact that the ionic strength in our experiments was less than half that used by GUTFREUND.

It should be noted that all of the observed parameters pertaining to the ionization of the active site can also be accounted for on the hypothesis that the rate of the rate-controlling step is proportional to the product of the activity of hydroxide ion times the fraction of an imidazolyl group which is protonated. The interpretation of the heat values given in Table II is quite uncertain in the absence of detailed knowledge of the mechanism of the reaction. Thus the rate constants from which the heats of activation are derived may actually be composites of several individual rate constants.

The dependence of r_{max} *on dioxane concentration at pH 8.0*

At all dioxane concentrations the rate has its maximum value over a range of pH values which includes pH 8.0. This value of the pH was therefore selected for a more extensive investigation of the effect of dioxane concentration, the results of which are summarized in Fig. 7.

As has been previously reported[3,6] for other solvent mixtures, the maximum rate at first increases as the water concentration is decreased. In the present case, the flat maximum in the vicinity of 50 % dioxane is followed by a decrease at higher dioxane concentrations, though even in 88 % dioxane the rate is still 68 % of its value in water. As explained earlier, it would be very difficult to carry the measurements to still higher dioxane concentrations; this is unfortunate, since in 88 % dioxane the water concentration has only been reduced to 6.65 *M*.

Such relatively minor changes in rate can be accounted for in a variety of ways. Thus, it might be argued that the changes in the dielectric constant of the solvent are sufficient to alter electrostatic interactions within the enzyme molecule and thus to produce slight changes in some critical spacing at the catalytic site. It is, of course, quite impossible at present to prove or disprove suggestions of this sort.

The maximum rate under a given set of conditions involves the interaction of an enzyme derivative with either free or bound water, or possibly with hydroxide ions. If we neglect the latter possibility, and secondary effects such as the one mentioned

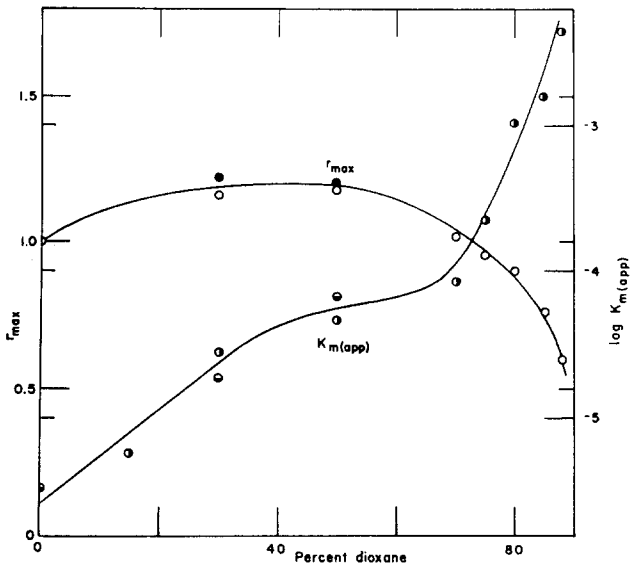

Fig. 7. The effect of dioxane concentration on the maximum rate and $K_{m(app)}$ of the tryptic hydrolysis of BAEE at 25° and pH 8.0 in the presence of 0.025 *M* $CaCl_2$. Maximum rates: ○ 0.005 *M* THAM; ●, no THAM. $K_{m(app)}$: ◑, 0.005 THAM; ◒, no THAM.

in the preceding paragraph, we should expect the rate to be essentially independent of dielectric constant and mainly dependent on water concentration. In the case that the overall reaction follows simple MICHAELIS-MENTEN kinetics and water enters into a bimolecular reaction with the enzyme–substrate complex, the dependence of r_{max} on water concentration should follow an equation of the form

$$r_{max} = k_{2(app)}\,[E]_0\,[H_2O] \tag{9}$$

It is evident from Fig. 7 that this equation does not hold. If, on the other hand, the reaction adheres to a 3-step mechanism[10]

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} ES' \overset{k_2}{\rightleftarrows} ES'' + P' \tag{10}$$

$$ES'' + H_2O \overset{k_3}{\rightleftarrows} E + P'' \tag{11}$$

"initial" rates observed after the formation of the steady-state concentration of ES'' should follow the expression

$$r_{max} = \frac{k_{2(app)}\,[E]_0\,[S]_0}{K_{m(app)} + [S]_0} \tag{12}$$

with

$$k_{2(app)} = k_2 \frac{[H_2O]}{\frac{k_2}{k_3} + [H_2O]} \tag{13}$$

and

$$K_{m(app)} = \frac{k_{-1} + k_2}{k_1} \cdot \frac{[H_2O]}{\frac{k_2}{k_3} + [H_2O]} \tag{14}$$

Consideration must also be given to the possibility that water must be bound by the enzyme before it can enter into the hydrolysis. For example, if the scheme

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} ES \tag{15}$$

$$E(H_2O) + S \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} E(H_2O)S \tag{16}$$

$$ES + H_2O \rightleftarrows E(H_2O)S \tag{17}$$

$$E(H_2O)S \xrightarrow{k_2} E + P \tag{18}$$

applies, then the steady-state approximation gives eqn. (12) with

$$k_{2(app)} = k_2 \frac{[H_2O]}{K_h + [H_2O]} \tag{19}$$

$$K_{m(app)} = \frac{k_{-1} + k_2 \dfrac{[H_2O]}{K_h + [H_2O]}}{k_1} \tag{20}$$

on the assumption that the binding of water attains an equilibrium which is independent of the binding of substrate. K_h is the dissociation constant of the enzyme–water complex. We thus have at least two cases giving a variation of maximum rate with $[H_2O]$ of the form

$$k_{2(app)} = k' \frac{[H_2O]}{k'' + [H_2O]} \tag{21}$$

according to which an EADIE plot of r_{max} *vs* r_{max}/H_2O should be a straight line.

It is evident that the 3-step mechanism with bound water required in the last step also leads to eqn. (12), but with

$$k_{2(app)} = \frac{k_2 k_3}{k_2 + k_3 \dfrac{[H_2O]}{K_h + [H_2O]}} \cdot \frac{[H_2O]}{K_h + [H_2O]} \tag{22}$$

and

$$K_{m(app)} = K_m \frac{k_3}{k_2 + k_3 \dfrac{[H_2O]}{K_h + [H_2O]}} \cdot \frac{[H_2O]}{K_h + [H_2O]} \tag{23}$$

According to eqn. (22) the dependence of $k_{2(app)}$ on $[H_2O]$ will be of the form of eqn. (21) if the reaction is rate-limited at the final step, whereas if the reaction is rate-limited at the second step $k_{2(app)}$ will be independent of $[H_2O]$. (The dependence of $K_{m(app)}$ on $[H_2O]$ is discussed in a later section.)

Above 50% dioxane (for an admittedly short range of values of $r_{max}/[H_2O]$) the behavior is consistent with eqn. (21). The decrease in rate at high water concentrations is evidently not specific with respect to dioxane, having been previously observed[3] for ethanol–water mixtures. We have observed a similar effect with water–dimethylsulfoxide mixtures (see below). The non-specific nature of this effect suggests that it may result from an inhibitory effect of water, for example in the sense of substrate inhibition. If we assume the mechanism of eqns. (15) to (18), and add the assumption that the species $E(H_2O)_2$ (or $E(H_2O)_2S$) cannot react, then

$$k_{2(app)} \left(1 + \frac{[H_2O]}{K_I}\right) = k_2 - K_h \frac{k_{2(app)}}{[H_2O]} \tag{24}$$

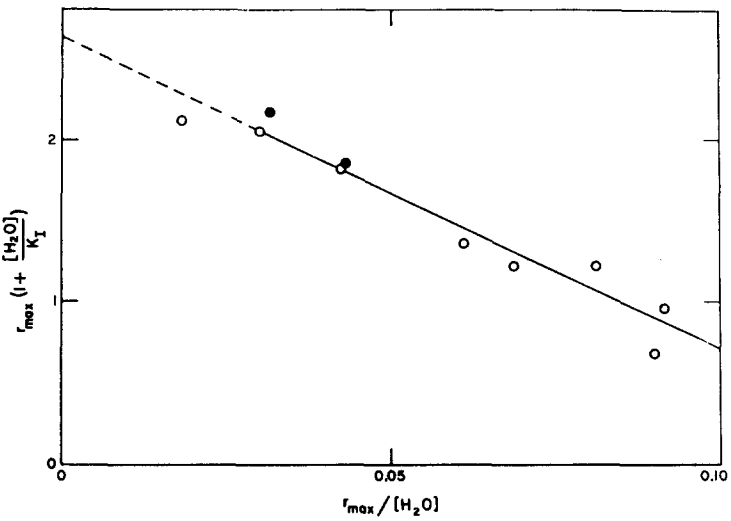

Fig. 8. The variation with water concentration of the maximum rate of the tryptic hydrolysis of BAEE, as interpreted in terms of specifically bound water and inhibition by water (see text).

where K_I is the equilibrium constant for the dissociation of the inhibiting molecule of water. It is found that the value $K_I = 50\,M$ leads to adherence to eqn. (24) within experimental error, as shown in Fig. 8. The slope of the plot gives the value $K_h = 19\,M$ and a hypothetical maximum rate 2.6 times that observed in water.

Other types of mechanism could be invoked to account for the observed variation of r_{max} with solvent composition. The above treatment is only intended to show that the experimental data can be reconciled with reasonable kinetic schemes; it cannot be proposed as a unique interpretation.

KOSHLAND AND HERR[23] have shown that water is a much more effective acceptor for the phosphate split from adenosine triphosphate by myosin than is methanol. This finding is consistent with the hypothesis that the water entering into an enzymic hydrolysis is specifically bound by the enzyme before it reacts.

The dependence of $K_{m(app)}$ *on dioxane concentration at pH 8*

The strong influence of dioxane concentration on $K_{m(app)}$ at pH 8.0 is shown in Fig. 7. Between 0* and 88 % dioxane an increase by a factor of 1300 is observed. The value in water, $3.4 \cdot 10^{-6}\,M$, is approximately one-third that reported by BERNHARD[24] for experimental conditions differing in several respects from those employed here.

The mechanisms discussed above in connection with the variation of r_{max} with dioxane concentration predict small decreases of $K_{m(app)}$ with increasing dioxane concentration. It is usually assumed that an important part of the energy of binding of BAEE to trypsin is electrostatic in origin, the positive guanidyl group interacting with a negative group on the enzyme. It would then be expected that this interaction would increase as the dielectric constant is decreased and that $K_{m(app)}$ would therefore decrease.

* In the determination[24] of $K_{m(app)}$ at low dioxane concentrations it is necessary to use large volumes of reaction solution in order to have rates of alkali consumption large enough for convenient measurement.

It is nevertheless conceivable that the large increase in $K_{m(app)}$ results from increased electrostatic repulsion. If the positive charge on the substrate comes very close to a negative charge on the enzyme, the interaction of these charges may be much less affected by changes in the dielectric constant of the medium than is the interaction of the positive charge of the substrate with the net positive charge which trypsin carries at pH 8. Application of the LINDERSTRØM-LANG[25] treatment based on the limiting DEBYE-HÜCKEL theory, which is of course a very rough approximation in media of low dielectric constant, gives for the effective charge on trypsin reasonable values in the range $+5$ to $+12$ depending on the solvent composition. In these calculations the radius of the trypsin molecule was taken as 19 Å, and $CaCl_2$ was necessarily assumed to be a strong electrolyte. Values of d log $K_{m(app)}$/d (1/D), where D is the dielectric constant[26], were read from the curve in Fig. 9.

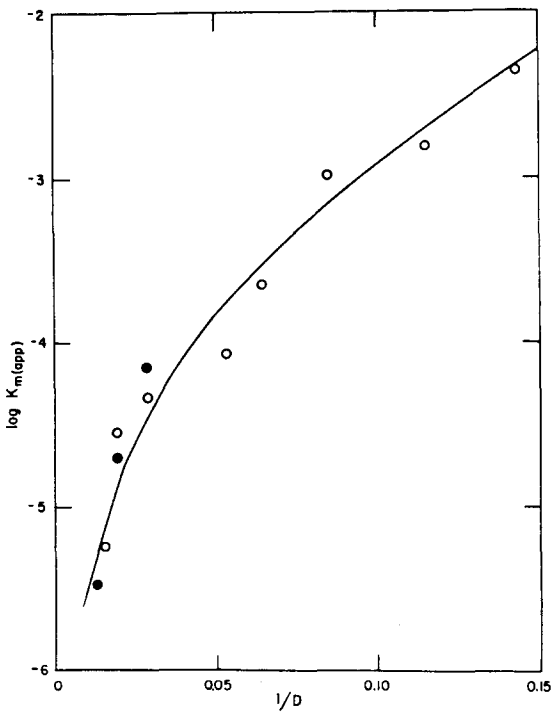

Fig. 9. The variation with dielectric constant of $K_{m(app)}$ for the trypsin-catalyzed hydrolysis of BAEE at 25° and pH 8 in the presence of 0.025 M $CaCl_2$. Open circles, 0.005 M THAM; filled circles, no THAM.

The possibility cannot be excluded that the change in $K_{m(app)}$ with solvent composition is primarily the result of configurational changes, which would not have to be very pronounced, and that any direct dielectric constant effect is of relatively minor significance.

The variation of $K_{m(app)}$ *with pH*

At a fixed dioxane concentration, $K_{m(app)}$ increases at pH values both below and above the pH range in which r_{max} has its maximum value. This behavior is shown in Fig. 10. It is difficult to devise consistent explanations for these effects. Thus the

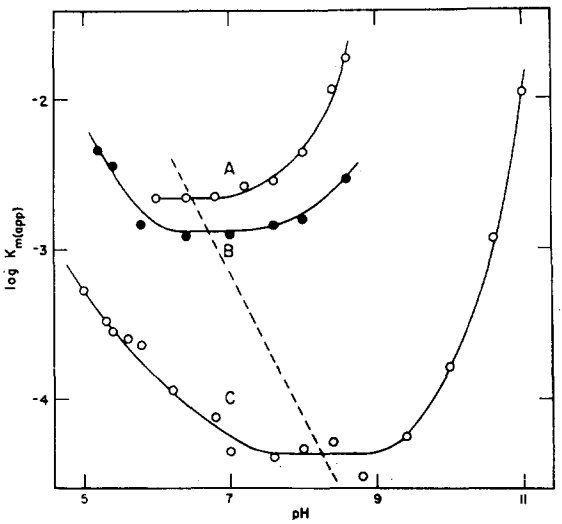

Fig. 10. The variation with pH of $K_{m(app)}$ for the trypsin-catalyzed hydrolysis of BAEE at 25° in the presence of 0.025 M $CaCl_2$ and 0.005 M THAM. Curve A, 88% dioxane; curve B, 85% dioxane; curve C, 50% dioxane.

low pH increase might be attributed to electrostatic repulsion between the guanido group of the substrate and the increasing positive charge on the enzyme. On the basis of the LINDERSTRØM-LANG[25] treatment (see the preceeding section), with $dZ/dpH = -5.0$ (Z = net charge) as read at pH 5.5 from a titration curve of trypsin in 50% dioxane, we calculate that d log $K_{m(app)}$/dpH should equal −0.60. This agrees fortuitously well with the observed value −0.57. However, this explanation is inconsistent with the observation that the rise in $K_{m(app)}$ occurs at lower pH values as the dioxane concentration is increased in spite of the increase of the pK' of the carboxyl groups with decrease of dielectric constant, and with the increase of $K_{m(app)}$ with increase of dioxane concentration discussed in the preceding section. It is evident that the increase in $K_{m(app)}$ at high pH cannot be a direct electrostatic effect.

Stability of trypsin in dimethyl sulfoxide and the tryptic hydrolysis of BAEE in dimethyl sulfoxide–water solutions

Trypsin was dissolved in 97% dimethyl sulfoxide; after storage at 12.5° or 5° for 14 h, the solution was diluted with 10 volumes of water and the activity toward BAEE determined. When the dimethyl sulfoxide solution contained 0.05 M $CaCl_2$, recovery of activity amounted to 93%; in the absence of $CaCl_2$, recovery after storage at 12.5° amounted to 79%, but only 45% after storage at 5°, at which temperature the solution was frozen.

A few determinations of the initial rate of BAEE hydrolysis in dimethyl sulfoxide solutions at 25° are summarized in Table III. These determinations were all made with $5 \cdot 10^{-3}$ M BAEE. The pH scale was defined operationally in the same manner as for dioxane–water mixtures. Deviations from zero order kinetics were evident, even at low dimethyl sulfoxide concentrations. It is not known whether this is a manifestation of large values of $K_{m(app)}$ or of other effects.

It is seen from the data in Table III that an acceleration in rate similar to that

TABLE III

RELATIVE INITIAL RATE OF HYDROLYSIS OF BAEE BY TRYPSIN IN DIMETHYL SULFOXIDE SOLUTIONS AT 25°

Rates are relative to the rate in water at 25°, pH 8.0, in the presence of 0.025 *M* CaCl, 0.005 *M* THAM. Initial substrate concentration $5 \cdot 10^{-3}$ *M*.

Dimethyl sulfoxide concn., volume %	*pH*							
	7.5	*8.0*	*8.4*	*8.5*	*8.7*	*9.0*	*9.2*	*9.5*
0		1.00				1.01		
10						1.05		
20	1.06	1.08		1.09		1.11		
30		1.21		1.30				
40		1.24		1.29				
50	1.06	1.17		1.27		1.27		
55						1.24		
60	0.79	0.88	0.98		1.08	1.06	0.85	0.59
65					0.67	0.78		
70						0.61		

found in dioxane solutions is also observed in dimethyl sulfoxide solutions, the maximum rate again occurring at a water concentration of about 50%. The non-specific character of this acceleration by organic solvents lends support to the view that a weak inhibition by water occurs in the trypsin-BAEE system.

ACKNOWLEDGEMENTS

This research was aided by grants from the National Science Foundation (G-2855) and the United States Public Health Service (RG-3996C and RG-4725).

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