THE INFLUENCE OF THE MEDIUM DIELECTRIC STRENGTH UPON TRYPSIN KINETICS

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ABSTRACT

The use of aqueous alkali for the titration of esterolytic activity when the esters are dissolved in alcoholic solutions, results in an error due to changes in the ionization of the buffer. This is corrected by titrating with alkali in the same solvent as the substrate.

Alcohols and other substances which change the dielectric strength of water modify the rate of hydrolysis of BAEE[1] and TSAME by trypsin to an extent proportionate to their effect on the dielectric strength. The reaction rate increases with diminished dielectric strength and *vice versa*. At low concentrations of substance there seems to be no specific effect other than that derived of the variation in dielectric strength. At higher concentrations, the enzyme might be denatured. In addition, it is probable that specific effects of each substance might intervene. The Coulombic and thermic energies of activation were calculated for the two esters in various solvents. The plot of the logarithm of rate constant *vs.* reciprocal of dielectric constant yields a straight line with positive slope. This behavior is similar to that of a non-enzymatic positive ion-dipole reaction. Trypsin reacts like a positive ion. The possible influence of the dielectric strength on the regulation of the equilibria involved in the interconversion of the various forms of trypsin in solution (active, inactive, denatured) is discussed.

INTRODUCTION

Schwert and Eisenberg (19) hydrolyzed BAME and BAEE in different alcohols at various concentrations with the hope that by varying the solvent it might be possible to differentiate the two supposed steps of the enzymatic reaction (formation and activation of Michaelis complex). Although these investigators did not realize their objective, they did find that hydrolyses of these esters failed to follow zero order kinetics in the presence of alcohols. These solvents gave rise to an increase in the initial rate of hydrolysis to an extent

[1] Throughout this paper the following abbreviations will be used:
BAEE, benzoyl-L-arginine ethyl ester.
TSAME, *p*-toluenesulfonyl-L-arginine methyl ester.
BTEE, benzoyl-L-tyrosine ethyl ester.
BAME, benzoyl-L-arginine methyl ester.
ATEE, acetyl-L-tyrosine ethyl ester.

varying with the kind and concentration of alcohol. This observation agreed with that formerly made by Risley *et al.* (17) that the hydrolysis of bovine serum by trypsin proceeds faster in the presence of a suitable concentration of alcohol. No explanation of the possible mechanism of this activation by alcohols has so far been offered and it appeared of interest to the present authors to attempt to elucidate this point. Accordingly a study was undertaken of ester hydrolysis by trypsin in the presence of several members of a homologous series of alcohols.

In the course of preliminary experiments in which the reaction rate in an alcoholic medium was determined by the titration procedure employed by Schwert and coworkers (20) the values obtained were found to vary with the concentration of phosphate buffer, alcohol, or alkaline titrating solution. Under these circumstances it was difficult to reach valid conclusions with respect to the mechanism of activation. On the other hand it was possible to obtain consistent values of rate constants when the composition of the solvent was maintained constant during the entire course of the reaction, so that the only variable concentrations were those of solutes present in relatively dilute solution. Under these conditions an apparent relationship was observed between the activation by alcohols and the dielectric strength of the medium. To determine whether the activation was in reality due only to diminished dielectric strength of the medium and not to some specific characteristic of the alcohol molecule (which might also vary in the same order within a homologous series) subsequent experiments were performed in which the dielectric strength of the medium was varied with substances chemically unrelated to alcohols. The substances selected for this first investigation were those which fulfilled the following requirements: (1) solubility in water to an extent which permitted a noticeable change in the dielectric strength; (2) values of dielectrical constant in existence in the literature for aqueous solutions of various concentrations of the compound; (3) no detectable specific effect of the compound on the reaction other than to modify the dielectric strength of the medium in the concentration used; and (4) non-interference with the method of determination of the activity; *e.g.*, by buffering action.

Materials and Methods

The characteristics of the enzymes and substrates employed here were the same as those described in a previous paper (8). The alcohols and other chemicals used for modifying the dielectric strength of the medium were of the best grade commercially available and fulfilled the conditions of purity that Åkerlöf (1) considered necessary in his determinations of dielectric constants.

The titrimetric determination of esterase activity was that described by Schwert *et al.* (20), modified as indicated below.

EXPERIMENTAL

Fig. 1 A shows the curves of titration of BAEE hydrolysis in 30 per cent methanol carried out with the same amount of trypsin and in the presence of

two concentrations of phosphate buffer. The apparent rate of hydrolysis and the alkali consumption were greater with the more concentrated phosphate. Furthermore, the total volume used in either case was higher than the theoretical value, reaching an excess of nearly 40 per cent when 0.15 M phosphate

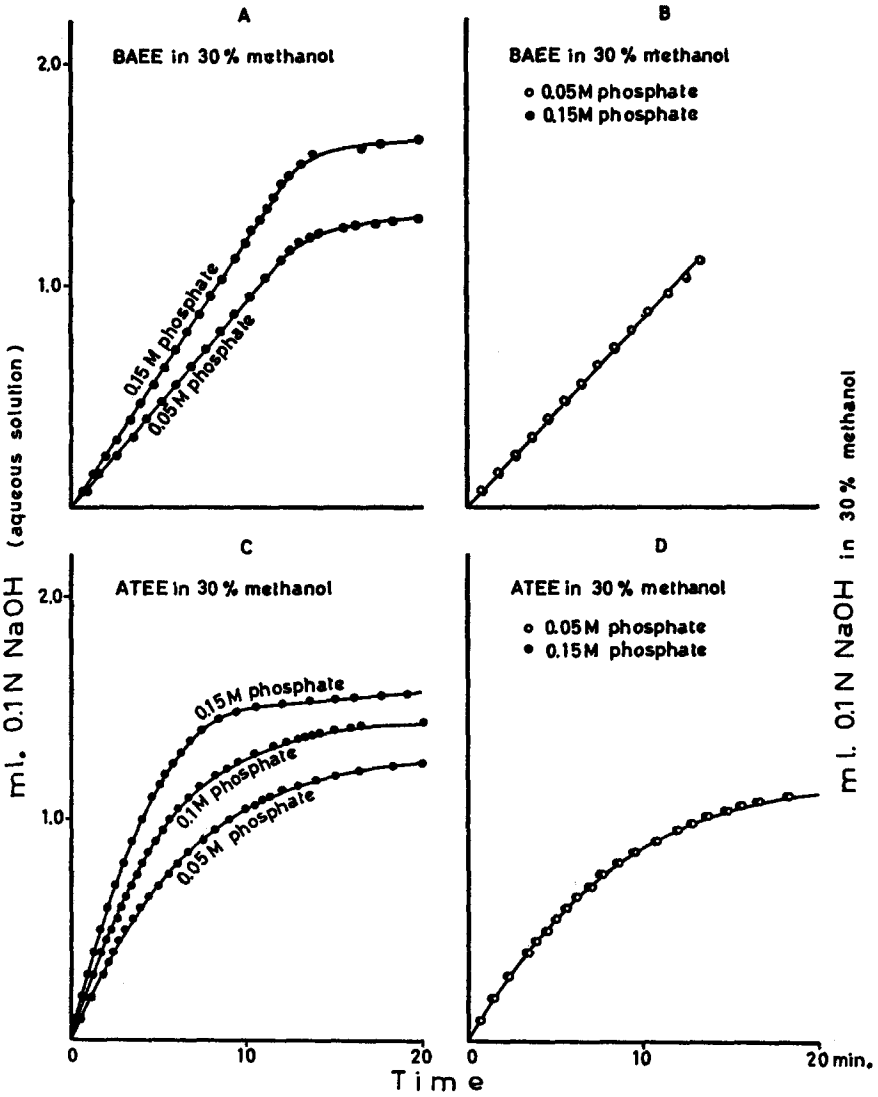

FIG. 1. Effect of the phosphate concentration on the titration values of BAEE-trypsin and ATEE-chymotrypsin, when the titration is carried out with either aqueous alkali (A and C) or alcoholic alkali (B and D). Ester concentration, 0.012 M; temperature, 25°C.; pH, 7.8.

was used. The same anomalous results were observed when the hydrolysis of ATEE by chymotrypsin in 30 per cent methanol was determined in the presence of three concentrations of phosphate, *i.e.*, the reaction rate and the alkali consumption increased with phosphate concentration, Fig. 1 C.

The close resemblance of the behavior of two unrelated esters hydrolyzed by different enzymes led to the suspicion of an error in method. It was observed that methanol added in a proportion of 30 volumes per cent to Na_2HPO_4-

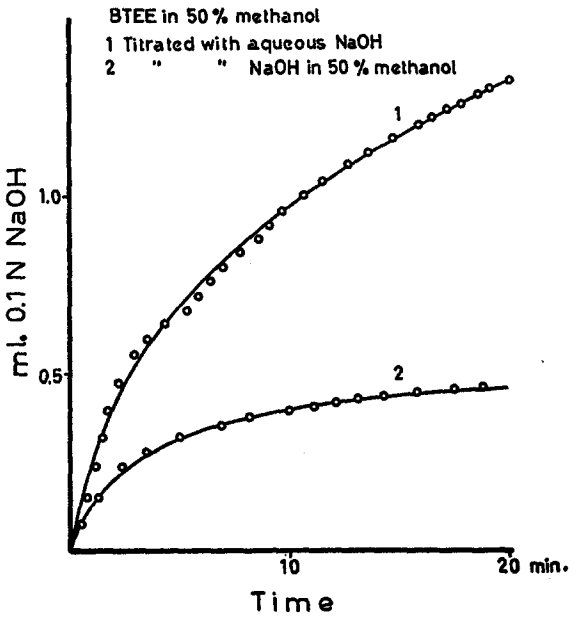

FIG. 2. Titration of BTEE hydrolysis by chymotrypsin with either aqueous or alcoholic alkali solution. Phosphate buffer concentration, 0.05 M.; pH, 7.8; temperature, 25°C.; ester concentration, 0.012 M.

KH_2PO_4 buffer causes its pH to shift about 0.5 unit toward the alkaline region. This suggested that progressive dilution of the alcohol during the titration with aqueous solution of NaOH might shift the pH back towards the acid side with the consequence that phosphate was being titrated in addition to the acidic groups liberated in the hydrolysis. In order to ascertain whether this supposition was correct the following experiment was performed. To 10 ml. of 0.15 M phosphate buffer in 30 volume per cent methanol, 1.2 ml. of water was added (a volume of water equal to the volume of 0.1 N NaOH required to titrate the total hydrolysis of 0.012 M ester). The resultant deviation in pH was compensated for by the addition of 0.57 ml. of 0.1 NaOH. This amount of alkali corresponded quantitatively to the observed excess in the titration curve of BAEE

in the same concentration of phosphate, Fig. 1 A. The conclusion was reached that the abnormally high titration values would disappear if the concentration of alcohol was maintained constant during the whole titration. The only way to do this was to titrate with alkali in the same concentration of alcohol used for dissolving the esters. The curves resulting when 0.1 N NaOH in 30 volume per cent methanol was used for the titration of BAEE and ATEE hydrolyses respectively are reported in Figs. 1 B and 1 D. In both cases the phosphate concentration did not influence either the reaction rate or the alkali consumption. The order of the reaction kinetics also remained unaltered.

Fig. 2 represents the curves of hydrolysis of BTEE by chymotrypsin in 50 volume per cent methanol and 0.05 M phosphate. Two strikingly different titration curves are obtained when the titration is made with aqueous or alcoholic alkali.

When aqueous solutions of alkali are used to follow the hydrolysis of ester in alcohol-water media, the error in titration is greater with higher concentrations of phosphate, higher concentrations of alcohol, or more dilute titrating solutions. On the basis of this finding, the titration method was modified in that the titrating alkali was dissolved in the same solvent as the ester. In addition to this modification of the method, the following remarks must be made with regard to the experimental conditions and calculations: (1) compounds with acid or alkaline reaction were neutralized to pH 7.8 before mixing with the ester or KOH solution and the titer of this solution was checked to avoid any error due to reaction with the alkali; (2) the ionic strength of the solutions was 0.14 in all the experiments; (3) the kinetics of the reaction remained zero order in most cases, especially when the concentrations of the compounds tested was 1 molar or lower. However, with tertiary butanol, pyridine, and dioxane, it was observed that as the concentration was raised the reaction rate tended to decrease with time. With dimethylurea, the opposite phenomenon was observed. After a few minutes in which the maximum delaying action was demonstrated without altering the reaction order, the rate showed a tendency to increase. Because of these observations, the compounds tested in this work were used at concentrations which in no case affected notably the reaction order.[2] Rate

[2] The accuracy of pH measurements with a glass electrode in aqueous alcohol solutions has been questioned (Shedlovsky, T., and Kay, R. L., *J. Physic. Chem.*, 1956, **60,** 151). Purlee and Grunwald (*J. Am. Chem. Soc.*, 1957, **79,** 1366) observed that the glass electrode requires a relatively long period of time for equilibration with a given solvent medium. The error observed in a determination of E.M.F. in a solution of 70 weight per cent dioxane, without previous equilibration of the electrode in the same solvent, amounted to 1.7 mv. Recently, Bacarella, Grunwald, Marshall, and Purlee (*J. Physic. Chem.*, 1958, **62,** 856) by the comparison of acid dissociation constants obtained potentiometrically with the glass electrode and conductometrically, conclude that the two methods yield equal results within experimental error, provided

constants were calculated within the initial straight portion of the hydrolysis curve or its prolongation. In this report the symbol K is used as the zero order rate constant in a medium of dielectric strength D, while K_0 is the equivalent rate constant in water (D = 78.5 at 25°C. (1)).

Experiments with a Homologous Series of Alcohols

The hydrolysis curves of BAEE by the same trypsin solution in aqueous solution and in 16 volume per cent of methanol, ethanol, *n*-propanol, isopro-

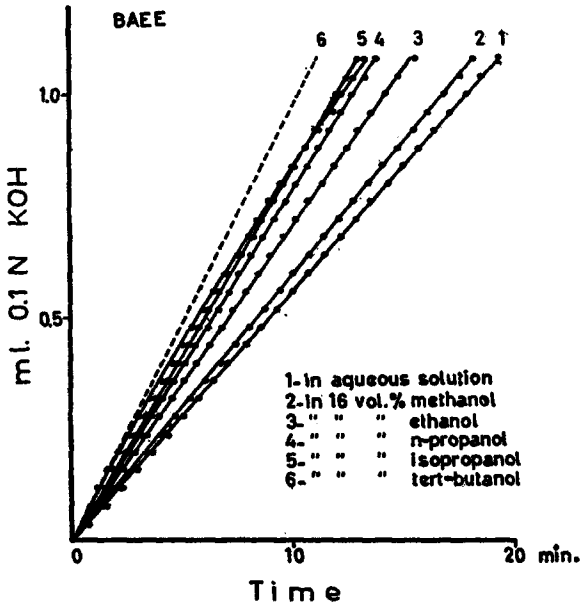

FIG. 3. Effect of a series of alcohols on the hydrolysis of BAEE by trypsin. Ester concentration, 0.008 M; temperature, 25°C.; pH, 7.8; ionic strength, 0.14.

panol, and tertiary butanol are given in Fig. 3. This experiment was a repetition of that which Schwert and Eisenberg (19) made previously, but with the modified titration technique. The results are similar to those obtained by these authors in that the rates increased in the same order with the series of alcohols, but they differ in that, except for *tert*-butanol, the order of the reaction was not altered. Such discrepancy might be explained in terms of the effects of the

that certain experimental conditions are satisfied. In the experiments reported here the maximum concentration of solvent used was 22.8 weight per cent methanol. Even though one assumes the same error as in the case of the concentrated solution of dioxane, the difference between the real and observed pH would be insufficient to account for a significant change in the rate of trypsin hydrolysis.

progressive dilution of the alcohol which results when the esterase activity is determined by titration with aqueous alkali. The two effects of the alcohol, one on the phosphate and the other on the reaction rate will diminish as the concentration decreases. This would give rise to two errors with opposite sign, the first of which makes the reaction appear to go faster, and the second of which in reality progressively slows the reaction by decreasing the concentration of the activating agent. At the beginning of the reaction the error with positive sign would predominate, and as the hydrolysis progresses, the extent of the negative error would be increasing constantly. The resultant of the two effects would be a non-linear course of reaction.

In trying to find some relationship between the effects of these alcohols upon the rate of hydrolysis of BAEE by trypsin and their physicochemical properties, it was observed that the activating action of the series of alcohols increased in the same order as their diminishing effects on the dielectric strength of the water. This suggested that if these results were due to a relationship between dielectric strength of the medium and reaction rate, the velocities of hydrolysis in solutions of the same dielectric strength might be expected to be independent of the nature of the alcohol. The influence of six alcohols, methanol, ethanol, *n*-propanol, isopropanol, normal butanol, and tertiary butanol, was studied comparatively on the hydrolysis of BAEE by trypsin. The concentration of each alcohol was varied progressively to obtain a change of dielectric constant (dD) from 0 to -10 units with intervals of -1. The concentration of each alcohol, except *n*-butanol, was calculated in accordance with Åkerlöf's (1) data of dielectric constants in mixtures of different alcohols with water at various temperatures. Harned and Samaras (13) obtained the dielectric constant of pure *n*-butanol, but the values of mixtures with water were not reported. The dielectric constant of pure *n*-butanol is slightly lower than that of *tert*-butanol. Using this value and the supposition that values of mixtures of *n*-butanol with water probably parallel those with *tert*-butanol, a hypothetical curve of dielectric strength *vs.* concentration of *n*-butanol was constructed and the corresponding values of D calculated from this curve. The relative velocity of hydrolysis in each concentration of alcohol with respect to that in water was plotted against the dielectric increment taken with positive sign, Fig. 4. The six resulting curves had similar shapes and slopes of the initial portion (up to approximately a value of $dD = -7$). With greater values of dD more notable differences in the curves are present, especially in the case of *n*-propanol when the relative speed reached a maximum and then became constant. It is possible that at the relatively high concentrations necessary for obtaining dielectric increments from -7 onward, the alcohols exert denaturing effects on the enzyme and the extent of such effect for a particular alcohol might be related to the number of carbon atoms in the molecule, or rather to the length of the chain. Considering that the alcohol may influence the hydrolysis rate by two

different mechanisms, diminution of the dielectric strength and/or, by denaturing the enzyme, as long as the concentration of the alcohol is below the limit at which it begins to affect the enzyme at a measurable extent, the activation effect will be related to the dielectric strength and independent of the

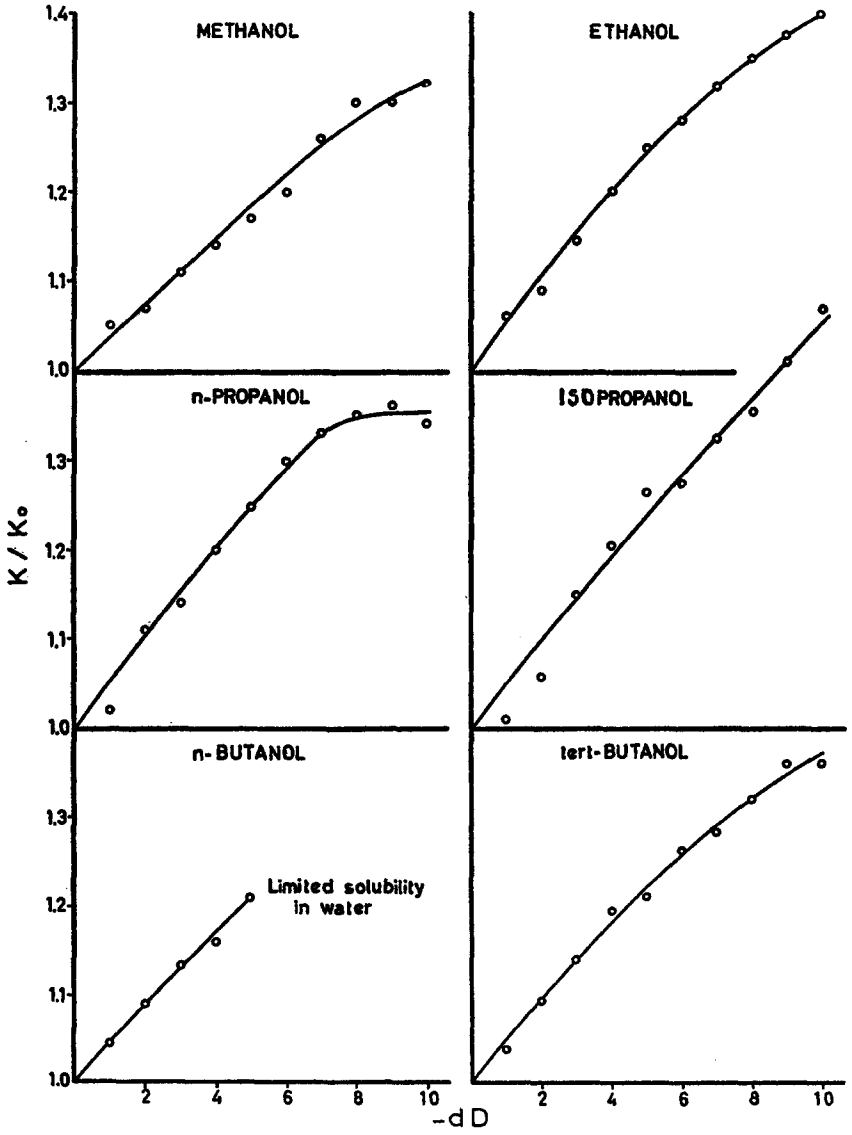

FIG. 4. Effect of the change in dielectric strength of the medium by different alcohols upon the hydrolysis rate of BAEE. Ester concentration, 0.008 M; temperature, 25°C.; pH, 7.8; ionic strength, 0.14.

alcohol molecule. When the denaturing action becomes apparent, the observed reaction rate will be the resultant of the two actions, and consequently it could be different for each alcohol. This would possibly explain the rather different courses which the curves follow after $dD -7$, and the non-linearity of the curves obtained with high concentrations of some alcohols.

Quantitative Interpretation of the Reaction Rates

Rather extensive studies have been made about the influence of the dielectric strength upon the kinetics of non-enzymatic reactions and several distinct quantitative approaches fit the experimental facts. Scatchard (18) derived an expression which relates the dielectrical constant to the rate of ionic reactions from the direct calculation of the concentration of an intermediate complex using the Debye and Hückel equations. Amis (3) arrived at the same equation from the viewpoint of the simple expression for the Coulombic force between ionic reactants. By applying Coulomb's law and the Arrhenius equation the following equation was derived by Amis to account for the dielectric effect on ionic reactions:

$$\log k'_{D_2} = \log k'_{D_1} - \frac{\Delta E_c}{2.303RT} \tag{1}$$

in which, k'_{D_2} and k'_{D_1} are the rate constants at dielectric strengths D_2 and D_1 respectively; R the gas constant; T absolute temperature and ΔE_c the Coulombic energy of activation; *i.e.*, the change in energy of activation necessary to bring the two reactant particles within the reacting distance r at two different dielectric strengths. The molar Coulombic energy of activation ΔE_c can be expressed as a function of the ionic charges as

$$\Delta E_c = \frac{N z_1 z_2 \epsilon^2}{r}\left[\frac{1}{D_2} - \frac{1}{D_1}\right] \tag{2}$$

in which z_1 and z_2 are the valences of the two ions and ϵ the electronic charge. Taking into account that N/R equals $1/k$ (k = Boltzmann constant); and considering D_2 as infinite in magnitude, so that all the electrostatic forces between reactants vanish; and D_1 as any finite value = D; the equation (1) becomes

$$\log k'_D = \log k'_\infty - \frac{z_1 z_2 \epsilon^2}{2.303krTD} \tag{3}$$

This last equation predicts that if D increases there should be an increase in k'_D for reactant ions of equal charge sign and decrease for those of unlike charge sign and conversely. A plot of log k'_D against $1/D$ should give a straight line in the region of dielectric constant at which theory is being obeyed. This line should have a negative slope for equally charged ions, or a positive one for ions of unlike charge.

Amis (4) also derived equations for reactions ion-dipolar molecule or between two dipolar molecules. In the first case, the expression

$$\ln k_D' = \ln k_\infty' + \frac{z\epsilon\mu}{DkTr^2} \tag{4}$$

includes the dipole moment of the molecule μ. According to this equation if D

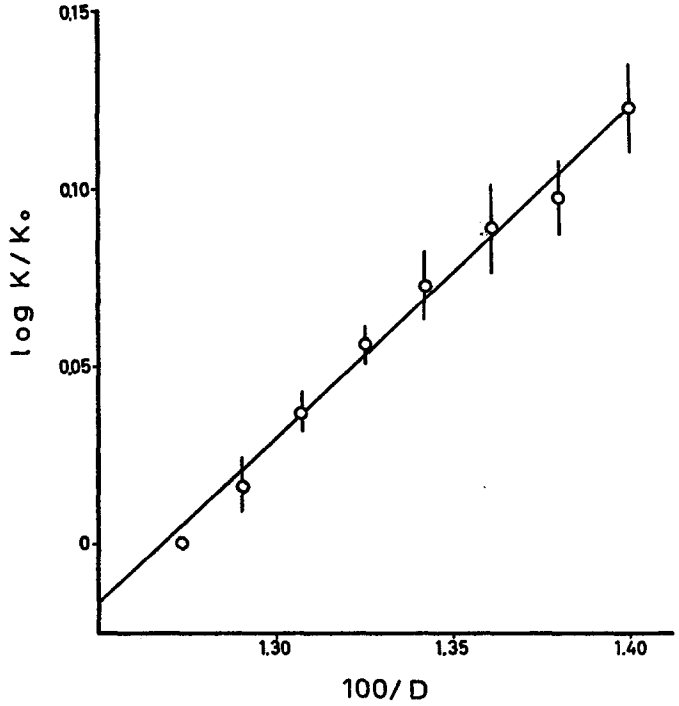

FIG. 5. Least square plot of the data obtained with alcohols. Circles represent average of six values and lines across them the standard deviations.

increases the rate should decrease for a positive ion and *vice versa* for a negative ion. If D decreases, the opposite effects will result.

The expression for dipole-dipole reactants is the following:

$$\ln k_D' = \ln k_\infty' - \frac{2\mu_1\mu_2}{DkTr^3} \tag{5}$$

This predicts an increase of k_D' as D increases.

The tryptic hydrolysis of BAEE was influenced by the changes in dielectric strength of the medium like a reaction ion-dipole. The plot of log K/K_0 (rate

constants in a medium of dielectric constant D and in water, $D = 78.5$ at 25°C. (1) respectively) against $1/D$ with the data of the six alcohols resulted in a straight line, (Fig. 5) in agreement with Equation (4). The statistical treatment (21) of these data rendered a correlation coefficient of 0.96, which indicates a high degree of interdependence between the variables dielectric strength and reaction rate. The straight line plotted through the average velocity for each datum of dielectric increment is the least square line whose slope 0.92 ± 0.05 and intersection -1.17 ± 0.06 were calculated from 40 individual data of Fig.

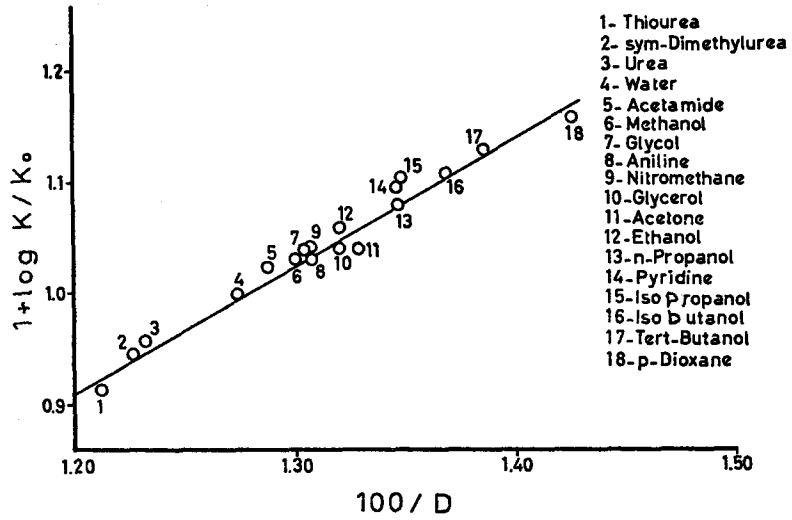

FIG. 6. Hydrolysis rate of BAEE in a series of equimolar solutions of the compounds indicated as a function of their respective dielectric strengths. Concentration of substances, 1 M, except aniline, which is 0.26 M due to its limited solubility. Ester concentration, 0.008 M; temperature, 25°C.; pH, 7.8; ionic strength, 0.14.

4 (only the values corresponding to dD from -1 to -7 were considered, because it appears that the specific solvent effects are minimum within this range). The spread of the data due to experimental variation is indicated by the standard deviation line which is drawn across each circle which represents the average data for the same dielectric constant.

Experiments with Non-Alcoholic Compounds

In spite of the high correlation index found for the change in dielectric strength with the rate of hydrolysis which indicates that the differences observed with different alcohols could be attributed to experimental variations, some doubt was left about the possibility that the alcohol effect might be re-

lated to some other property of these compounds which also varied in a parallel manner with their dielectric effect. To clear up this point, other chemicals which also modified the dielectric constant of water but are chemically unrelated to alcohols were tested. The hydrolysis rate of BAEE by trypsin with respect to that in water was measured in equimolar solutions of 17 compounds including mono-, di-, and trihydroxylic alcohols, nitromethane, aniline, pyridine, acetamide, acetone, *p*-dioxane, urea, thiourea, and dimethylurea. Of these, 14 have a lowering effect on the dielectric strength, and the 3 last mentioned when dissolved in water increase the dielectric strength. A plot of the log of the rate constant in each medium with reference to that in water against the reciprocal of the dielectric constant is given in Fig. 6. The straight line is the least square plot (21) calculated for the data. The slope is 1.15 ± 0.05 and the intersection -1.47 ± 0.06. The correlation coefficient, 0.98, was very near unity, which is the ideal value.

TABLE I

Dielectric Constants at 25°C. of Water and 1 Molar Solutions of the Studied Compounds

Compound	*D*	Reference	
Thiourea	82.5	Devoto	(10)
sym-Dimethylurea	81.5	"	(12)
Urea	81.2	Wyman	(22)
Water	78.5	Åkerlöf	(1)
Acetamide	77.7	Devoto	(11)
Methanol	77.1	Åkerlöf	(1)
Ethylene glycol	76.7	"	(1)
Aniline*	76.5	Devoto	(12)
Nitromethane	76.5	"	(12)
Ethanol	75.8	Åkerlöf	(1)
Glycerol	75.8	"	(1)
Acetone	75.0	"	(1)
n-Propanol	74.3	"	(1)
Pyridine	74.3	Devoto	(12)
Isopropanol	74.2	Åkerlöf	(1)
Isobutanol‡	73.1	"	(1)
tert-Butanol	72.2	"	(1)
p-Dioxane	70.2	Åkerlöf and Short	(2)

* The concentration was 0.26 M due to limited solubility.

‡ The value of *D* given by Åkerlöf is that of pure isobutanol. No data are reported for mixtures with water. The datum for 1 M isobutanol was interpolated from those of isopropanol and tertiary butanol taking into account their respective *D* values for pure solvent.

The dielectric constants of 1 M solutions of the substances and the source of the data are indicated in Table I.

Calculation of the Thermic and Coulombic Energies of Activation

According to Amis (3), the Coulombic energy of activation can be estimated by applying Coulomb's law to obtain the difference of the energy of activation for a reaction which takes place at two different dielectric strengths. In order to

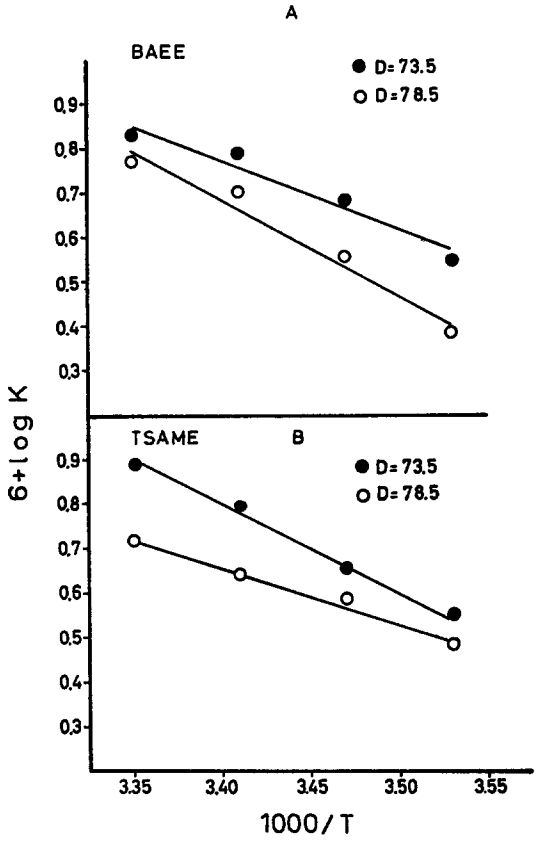

FIG. 7. Arrhenius plots for the calculation of the energies of activation at two dielectric strengths. pH, 7.8; ionic strength, 0.14.

have equal dielectric strengths at various temperatures, a pair of solvents at variable proportions must be used. Fig. 7 A shows the effect of temperature upon the hydrolysis rate of BAEE at the dielectric constants 73.5 and 78.5 respectively. The plot of log K against $1/T$ in accordance with the Arrhenius equation was made for the calculation of the energy of activation. Each point is the average of two determinations carried out with a distinct pair of solvents. These solvents were dioxane-water and isopropanol-water. The concentrations

of each substance required to produce the needed dielectric strength at a given temperature are listed in Table II.

The slope of each line was estimated by the least square method, and the energy of activation determined by equating this value to $-\Delta E/2.303R$. The Coulombic activation energy ΔE_c was determined by difference of the energies

TABLE II

Concentration in Per Cent by Weight of Dioxane or Isopropanol Required to Give the Specified Dielectric Strength at Each Temperature

Temperature °C.	D = 78.5		D =73.5	
	Dioxane	Isopropanol	Dioxane	Isopropanol
10	6.21	7.58	11.60	14.16
15	4.16	5.10	9.64	11.81
20	2.08	2.56	7.64	9.41
25	0	0	5.66	6.97

These concentrations were calculated according to Åkerlöf's data for isopropanol (1) and dioxane (2).

TABLE III

*Thermic and Coulombic Energies of Activation of BAEE and TSAME in Solutions of Dioxane or Isopropanol**

Substrate	D	Slope	ΔE (cal.)	ΔE_c ($\Delta E_{73.5} - \Delta E_{78.5}$)	$\Delta E_c/\Delta D$
BAEE	73.5	−2206	−10095	−2860	−572
BAEE	78.5	−1581	−7235		
TSAME	73.5	−1277	−5836	3332	666
TSAME	78.5	−2006	−9168		

* Calculated from data of Fig. 7.

of activation at D = 73.5 and 78.5. The values of thermic and Coulombic energies of activation are given in Table III.

The effect of temperature upon the hydrolysis of another ester, TSAME, at two dielectric strengths was studied in the same way that the experiment with BAEE was done. The results are given in Fig. 7 B. The activity corresponding to the lower dielectric constant was greater as in the case of BAEE. Nevertheless, the results differ somewhat in that the increase of rate produced by the same diminution of dielectric constant with TSAME is greater at the higher temperature and with BAEE is greater at the lower temperature.

To investigate a possible influence of solvent, the Coulombic energy per unity of dielectric increment, $\Delta E_c/\Delta D$ of BAEE were determined with other solvents, including urea and thiourea which augment the dielectric strength.

The rates were measured at two temperatures and ΔE calculated by the formula

$$\Delta E = \frac{2.303RT_1T_2\,\Delta\log K}{\Delta T}$$

The results obtained with the systems thiourea-water with ethanol-water and urea-water with glycerol-water, are similar, within the experimental error, to those obtained with dioxane or isopropanol as solvents (Table IV).

TABLE IV

Thermic and Coulombic Energies of Activation of BAEE in Solutions of Ethanol, Thiourea, Glycerol, and Urea

D	Temperature	Solvent	Per cent by weight	$K \times 10^6$	ΔE (cal.)	ΔE_c (cal.)	$\Delta E_c/\Delta D$
	°C.			(*mols/min.*)			
82.5	10	Ethanol	2.89	1.90	−9886	−3077	−615
82.5	25	Thiourea	7.61	4.60			
77.5	10	Ethanol	11.14	3.10	−6809		
77.5	25	"	1.74	5.70			
82.5	10	Glycerol	5.99	1.80	−9338	−2580	−645
82.5	25	Urea	8.90	4.15			
78.5	10	Glycerol	16.69	2.65	−6758		
78.5	25	Water		4.85			

DISCUSSION

In the study reported here it was demonstrated that substances which modify the dielectric strength of the medium also have a pronounced effect on the rate of tryptic hydrolysis of BAEE and TSAME. With low concentrations of added substance, the changes in reaction rate observed were due only to changes in dielectric strength of the medium and not to any specific property of the molecule. This is indicated by the fact that substances as dissimilar as alcohols, acetone, aniline, nitromethane, pyridine, and dioxane increase the rate to an extent directly proportionate to the diminution of the dielectric strength of the medium they produce, while substances such as urea, thiourea, and dimethylurea, which increase the dielectric strength, decrease the reaction rate proportionately. At higher concentrations this proportionality was not obtained and this probably was due to some interaction between substance and substrate or enzyme.

The plot of the logarithm of the rate against the reciprocal of the dielectric constant gave a straight line with positive slope. Theoretically the slope of the line which passes through the experimental points which express the velocity

constant at a given value of dielectric constant must be the same whichever solvent was employed. A difference was observed in the values of the slopes when the dielectric strength was varied either by changing the concentrations of alcohol or using equimolar solutions of different compounds. However, if the two lines are plotted on the same scale, within the range of dielectric strength studied the differences noted are within the experimental error. Furthermore, some sources of error besides those involved in the experimental determination of the rate must be considered. For example, a discrepancy of 5 per cent with regard to the value of the dielectric constant given for dioxane by Åkerlöf and Short (2) has been reported more recently (9). Another error may result from the assumption that the medium is homogeneous. It is possible in mixtures of water with solvents of a low dielectric strength that the molecules of water might be preferentially oriented around the reactants, giving rise to a heterogeneous medium in which the dielectric strength in the vicinity of the enzyme or the substrate would be different from that of the bulk solution.

The dielectric strength of the medium influences the hydrolysis of BAEE or TSAME by trypsin in the same way that it affects a non-enzymatic reaction between a positive ion and a dipolar molecule. For example, the reaction of sucrose with hydronium ion (4) is activated in media of low dielectric constant and *vice versa*. For this reaction a wider range of dielectric strength was investigated (approximately 40 to 77) than in the present case (70.2 to 82.5). When enzymes are involved care must be taken to avoid high concentrations of substances like urea, alcohols, etc. which are known protein denaturants. In spite of the limited possibilities of variation in the dielectric strength, the greater sensitivity of the reaction studied here resulted in wider variations of the rates than were observed with the referred hydrolysis of sucrose. The maximum increment of rate in this latter reaction was about 20 per cent, while in the hydrolysis of BAEE a diminution of D from 82.5 to 70.2 resulted in an increase of almost 80 per cent.

The fact that an enzymatic reaction behaves like a reaction in which a non-enzymatic catalyst intervenes suggests a similar mechanism in the two reactions. In both cases the electrostatic effects arising from charges and/or moments possessed by the reactants placed in a dielectric medium play an important role. The observed behavior of trypsin, similar to that of a positive ion, is in harmony with a previous finding of Northrop (16). On the basis of the parallelism of the titration curves of several protein substrates with the activity-pH curves, and by application the Donnan theory to the distribution of trypsin between molecules of undissolved gelatin and the surrounding solution, this author reached the conclusion that this enzyme behaved like a univalent positive ion.

The classic works of Anson and Mirsky (6, 7) and Kunitz and Northrop (15)

have given the experimental ground for considering that trypsin exists in solution in four forms; inactive native protein ⇄ active native protein ⇄ reversibly denatured protein → irreversibly denatured protein. The equilibria involved are regulated by temperature, pH, and denaturing agents. In view of the observations reported here, it is possible that the dielectric strength of the medium is another factor which influences these equilibria. One might further postulate that the action of the compounds studied upon the equilibrium: inactive native protein ⇄ active native protein is due to their dielectric effect while their effect on the reaction: reversibly denatured protein → irreversibly denatured protein would be rather related to the structure of the molecule, and require higher concentrations of substance. It is possible that the two effects would be distinct for each enzyme. In support of this is the observation of Harris (14) that the reversible denaturation of trypsin and chymotrypsin by urea is directly related to the concentration of urea and to the pH, and that the effect of pH on the reversibility of the reaction is different with trypsin than it is with chymotrypsin. Furthermore, Linderstrøm-Lang and coworkers (5) reported that ribonuclease denatured by urea retains the catalytic function of the native enzyme.

Even though a diminution of the medium dielectric strength gives rise to a faster hydrolysis by trypsin of both BAEE and TSAME, the magnitude of this effect varies with temperature in a different manner with the two esters. It is difficult to find a reasonable explanation for the different trends of energies of activation. It is possible that the dielectric strength not only alters the equilibrium inactive-active enzyme, but might also exert some effect upon the substrate. An alternative possibility is that the effects result from specific solvent influences which differ for the two substrates.

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