Trypsin–Organic Solvent Interaction. The Simultaneous Operation of Competitive Inhibition and Dielectric Effect†

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ABSTRACT: The influence of dioxane and isopropyl alcohol on the trypsin-catalyzed ester hydrolyses was investigated with *N*-benzoyl-L-arginine ethyl ester in the presence and in the absence of benzamidine. It was found that k_{cat} did not change significantly with decrease of the dielectric constant, whereas K_m for Bz-Arg-OEt hydrolysis and K_i for benzamidine inhibition did considerably increase. The results were interpreted as being due to the cooperation of two effects, a dielectric one and a competitive inhibition of trypsin by the organic solvents used. These effects could be incorporated into a set of equations which described the behavior of K_m or K_i as a function of the decrease in dielectric constant and solvent concentration. The nature of the effects is such that they preclude the estimation of the distance of approach of the charges on the enzyme and substrate in the complex by the approximations derived from the theory of ionic association equilibrium, through variation of the bulk dielectric constant of the medium. However, using the free energies of binding of inhibitors with no side chain, such as formamidinium and guanidinium, it was estimated that the distance of closest approach of the ions in the complex is close to 4 Å, that is, the ionic groups of enzyme and inhibitor establish van der Waals contact. This was found by reference to a calculated curve expressing change of electrostatic free energy of interaction as a function of the equilibrium distance of the ions in the complex.

The requirement of a positively charged group in the side chain of α-acylamino acid amides for optimum hydrolysis by trypsin was demonstrated by Bergmann *et al.* (1939; Bergman and Fruton, 1941) and Hofmann and Bergmann (1939, 1941). Later on these findings were extended to esters of α-*N*-acyl-L-arginine by Schwert *et al.* (1948; Schwert and Eisenberg, 1949). Thus, the specificity of trypsin was proven to be directed to the carboxyl end of L-lysine and L-arginine derivatives. Further work demonstrated that cysteine could be conveniently modified chemically so as to bear a side-chain positive charge at a distance approximately equal to that between the ammonium and the carbonyl groups in L-lysine (Lindley, 1956). Other analogs of lysine were prepared by Tesser and Nivard, (1964) that contained S and O in the place of the CH_2 group of α-*N*-acyl-L-lysine methyl ester. These compounds were hydrolyzed by trypsin, although changes were noticed in the values of the kinetic constants.

On the other hand, it could be demonstrated that trypsin would be able to catalyze the hydrolysis of compounds that did not carry a positive group in the side chain, but at much lower rates. Hofstee (1957) investigated the tryptic hydrolysis of fatty acid esters of *m*-hydroxybenzoic acid. However, these esters are much more reactive than the ethyl esters of benzoylnorleucine and -norvaline, which resist trypsin-catalyzed hydrolysis (Neurath and Schwert, 1950). More recently, Gorecki and Shalitin (1967) showed that α-*N*-acyl-L-amino acid esters can be efficiently split by trypsin if they carry, instead of the positively charged group, a group that is able to form hydrogen bond with the enzyme. Along the same line, Sanborn and Hein (1967) have shown that trypsin catalyzes the hydrolysis of benzoyl-L-citrulline methyl ester. Mares-Guia and Shaw (1965) used positively charged amidines and guanidines and found them to be efficient competitive inhibitors of trypsin. From previous work of this laboratory (Mares-Guia and Figueiredo, 1970) it is clear that about 40% of the free energy of binding of benzamidinium to trypsin, that is, about 2.7 kcal/mol, derives from electrostatic interaction. In the present work we report the results of experiments done with the goal of investigating the effect of changes in dielectric constant on the trypsin–inhibitor interaction. Through these experiments we hoped to be able to estimate the distance of closest approach of the ionic group on the enzyme surface (−) and in the inhibitor molecule (+) from plots of log K_i *vs.* $1/D$. This result could then be compared to that obtained from ΔG_u for binding of small ionic inhibitors to the enzyme.

Some of the earlier work on the effect of dielectric constant on enzyme-catalyzed reactions has been reviewed by Laidler (1958). Works on the dielectric effects on the catalyses by α-chymotrypsin, myosin, adenosine triphosphatase, papain, pancreatic carboxypeptidase, and yeast enolase have been discussed by Webb (1963). In what concerns trypsin, Schwert and Eisenberg (1949) observed an increase in the rate of Bz-Arg-OEt hydrolysis with increase in ethanol concentration which passed through a maximum and then decreased. At the 16 vol % level methanol, ethanol, 1-propanol, and *tert*-butyl alcohol increased the rate of Bz-Arg-OMe and Bz-Arg-OEt hydrolysis. A detailed study of the changes in dielectric constant upon trypsin kinetics was carried out by Castañeda-Agulló and Del Castillo (1959). These authors found a correlation between the rate of Bz-Arg-OEt hydrolysis and $1/D$ values for 18 different mixtures of water and organic compounds, ranging from 70.2 to 82.5 in the value of dielectric constant. Inagami and Sturtevant (1960) investigated the effect of dioxane on the trypsin-catalyzed hydrolysis of Bz-Arg-OEt, and observed that whereas the k_{cat} changes were not pronounced, the K_m values increased very much with decreasing dielectric constant.

More recently, Kallen-Trummer *et al.* (1970) observed

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that a mixture of *tert*-butyl alcohol and acetonitrile activates the tryptic hydrolysis of tosyl-L-arginine ethyl ester.

Due to the complex nature of k_{cat} and K_m(app) in the trypsin catalysis, their dependence on the dielectric constant allows no simple interpretation in terms of the structures of the enzyme–substrate complex. The use of K_i as a dependent variable in dielectric effect studies should be much more convenient than the use of K_m(app), because K_i is an equilibrium constant. We observed, however, that the change of the dielectric constant of the medium affects K_i for the trypsin inhibition by benzamidine in a complex manner, which involves both electrostatic and inhibitory effects by the organic solvents employed.

Experimental Section

Reagents. The substrate used was α-*N*-benzoyl-L-arginine ethyl ester (Sigma, lot 36B-1410), from which 0.010 M stock solutions were prepared in the appropriate solvent system. Bovine trypsin (Sigma, lot 117B-8030) was dissolved in HCl at pH 3.0, at 0°, to give a final active-center molarity of the order of 20 μM. Isopropyl alcohol was reagent grade from Merck (Darmstadt, Germany). Dioxane was a high-purity product from Carlo Erba (Milan, Italy), containing a stated maximum value of 0.0045% peroxide and 0.007% acetic acid. It was stored in black bottles tightly stoppered. The dioxane–electrolyte mixtures were prepared immediately before each experiment. It was purified according to Fieser (1955) and stored in dark bottles. Benzamidine-HCl was an Aldrich product. An approximately 1 M stock solution of the compound in water was stored frozen. The exact molarity of dilutions was determined by absorbance measurements, as in the previous paper (Mares-Guia and Figueiredo, 1970). The dilutions were 1:10 in the appropriate solvent system.

Equipment and Kinetic Technique. The assays of enzyme activity were carried out with a Radiometer Titrator Model TTT-1c, equipped with the SBU-1 syringe unit, a SBR-2c recorder, a plug-in temperature compensator, and G-202B (glass) and K-401 (reference) electrodes. The reagents were contained in 250-ml flasks in a water bath maintained at 37 ± 0.05°. During the titrations a steady flow of water-saturated, CO_2-free nitrogen was blown over the surface of the liquid, and constant stirring was maintained.

The reaction mixture consisted of 200 ml of 0.10 M NaCl solution, 2 mM in $CaCl_2$, containing varying proportions of organic solvent, to which was added according to the case, 0.40–1.20 ml of Bz-Arg-OEt solution, 0.20 ml of benzamidine solution, all prepared in the same solvent, and 25 μl of trypsin dissolved in HCl (pH 3.0).

The final concentrations of Bz-Arg-OEt, benzamidine, and trypsin were corrected for these small differences in volume. The molarities of NaCl and $CaCl_2$ given above, 0.10 M and 2 mM, respectively, are final molarities in the solvent system, so that the ionic strength was kept as close to constant as possible throughout the whole set of experiments.

The proportions of dioxane and 2-propanol are by weight. The weight of water was calculated from the exact volumes and the density at the temperature of preparation. The dielectric constants of the solutions containing isopropyl alcohol and dioxane at 37° were obtained from plots of log *D* *vs.* weight per cent of organic compound, as calculated from data published by Åkerlof (1932) for isopropyl alcohol, and by Åkerlof and Short (1936) for dioxane.

The sodium hydroxide solution used in the titrations was prepared by dilution (to 0.0100 M) with 0.10 M NaCl of a stock 1.00 M solution. The diluted solution was prepared and standardized daily against a primary standard potassium acid phthalate.

The hydrolysis of Bz-Arg-OEt under the conditions used (Bz-Arg-OEt concentrations in the range 5–40 μM) followed zero-order kinetics during the first 1 or 2 min at the low substrate concentrations, corresponding to about 25% hydrolysis of the substrate at the lowest concentration and to about 50% hydrolysis at the highest concentration used. To obtain initial velocities we drew tangents to the initial parts of the tracings, and determined their slopes. With help of appropriate factors the data were converted to rates in moles of substrate per liter per second, per mole of enzyme per liter, or sec^{-1}.

All absorbance measurements were carried out in a Hitachi-Perkin-Elmer Model 139 spectrophotometer equipped with photomultiplier.

Methods of Computation. It has been demonstrated by the works of Stewart and Ouellet (1959), Bender *et al.* (1965a), Bender and Kezdy (1965), and Bender *et al.* (1965b) that the trypsin-catalyzed hydrolysis of esters obeys eq 1.

$$E + S \overset{K_S}{\rightleftharpoons} ES \xrightarrow{k_2} \underset{\underset{P_1}{+}}{E\text{-}S} \xrightarrow{k_3} E + P_2 \qquad (1)$$

$$K_m(\text{app}) = K_S/[1 + (k_2/k_3)] \qquad (2)$$

$$k_{cat} = k_2k_3/(k_2 + k_3) \qquad (3)$$

The presence of a competitive inhibitor adds the equilibrium equation

$$E + I \overset{K_i}{\rightleftharpoons} E\cdot I \qquad (4)$$

$$K_i = (E)(I)/(E\cdot I) \qquad (5)$$

The values of K_m(app), from now on referred to as K_m, together with those of k_{cat} and K_i, were calculated by the double-reciprocal plot of Lineweaver and Burk (1934) in the form

$$\frac{1}{v} = \frac{1}{V} + \frac{K'_m}{V}\frac{1}{(S)} \qquad (6)$$

$$K'_m = K_m[1 + (I)/K_i] \qquad (7)$$

and V stands for the maximum velocity. The values of K_m and V were calculated by the least-squares technique proposed by Wilkinson (1961).

We have programmed Wilkinson's equations in Fortran IV and carried out all calculations with an IBM-1130 digital computer. The computer output includes a table containing primary data followed by values of rate and substrate concentration; then, the calculated values of K_m and V are printed, together with the respective standard deviations.

When a competitive inhibitor was present K_i was evaluated from eq 7 in the form

$$K_i = \frac{(I)}{\alpha - 1} \qquad (8)$$

$$\alpha = K'_m/K_m \qquad (9)$$

The variance of K_i was obtained from eq 10.

$$V(K) = V(\alpha)\left(\frac{\partial K_i}{\partial \alpha}\right)^2 = V(\alpha)\left[\frac{(I)^2}{(\alpha - 1)^4}\right] \qquad (10)$$

The variance of α was estimated through the use of eq 9 in Wilkinson's (1961) paper.

The calculations were carried out in the computer with the help of a program called EPKI.

Results

Theoretical

The association of trypsin with amidinium ions was treated as an ionic association equilibrium, where the active center contained the negative charge and the amidinium group housed the positive charge.

Although trypsin at pH 8.0 might carry a maximum positive charge of 11–12, as judged by its amino acid composition (Domont *et al.*, 1964; Walsh and Neurath, 1964), only the negative charge at the anionic site is taken into account, since the extent of interaction was determined by competitive inhibition. The process is represented by the following equation

$$E^-I^+ \overset{K_i}{\rightleftharpoons} E^- + I^+,\ K_i^0 = (E^-)(I^+)/(E^-I^+) \qquad (11)$$

where K_i^0 is obtained by extrapolation to infinite dilution.

The effect of the dielectric constant on the association equilibrium is given by

$$K_i = K_i^0 \exp\left(\frac{\epsilon^2}{DakT}\right) \qquad (12)$$

as deduced by Denison and Ramsey (1955). In eq 12, K_i^0 is the dissociation constant for a pair of uncharged particles, ϵ is the electronic charge, a is the distance of separation of charges in the ion pair, D is the dielectric constant of the medium, and k is the Boltzmann constant. The simplification in the theory of ionic association introduced by Denison and Ramsey (1955) was to assume that the oppositely charged ions existed either in contact (a is the sum of the van der Waals radii), thus constituting the associated ion pair, or at such a large distance apart that the coulombic force between them becomes negligible.

At any other condition different from infinite dilution the equilibrium constant becomes

$$K_i = \frac{C_{E^-}{}^0 C_{I^+}{}^0}{C_{E^-I^+}{}^0} \cdot \frac{f_{E^-} f_{I^+}}{f_{E^-I^+}} \qquad (13)$$

from which one obtains

$$\ln K_i = \ln K_i^0 - \frac{Z_E Z_I \epsilon^2 \exp[k(a - r)]}{DkT(1 + ka)r} \qquad (14)$$

where a has the same meaning as above, r represents the distance of separation of the ions at which the potential is ψ, k is the Debye κ, which gives $1/k$ as the Debye–Hückel radius of the ionic atmosphere, equal to 9.53 Å under our conditions (see Amis, 1966, for further details).

The value for the distance of closest approximation, 3.75 Å, was estimated from the head-on van der Waals radius of the carboxylate ion, 2.15 Å, and the head-on radius of the $=N^+H_2$ component of the amidinium group, 1.60 Å (values of Table 6-9 in Webb, 1963). Substituting the known quantities in eq 15, changing to decimal logarithms and allowing $a = r$ one obtains

$$\log K_i = \log K_i^0 + 167.9\frac{Z_E Z_I}{a}\frac{1}{D} \qquad (15)$$

According to eq 15 a plot of log K_i as a function of $1/D$ yields a straight line whose slope gives the equilibrium distance of the ions in the complex.

Equation 15 implies that the trypsin molecule is spherical in shape, rigid, and impenetrable to solvent. The adequacy of these assumptions for certains kinds of proteins has been discussed by Tanford and Kirkwood (1957).[1] In the present case, the straight lines obtained in Figure 1 allow for the additional simplifications of using the bulk dielectric constant and the linearized form of the Debye–Hückel theory.

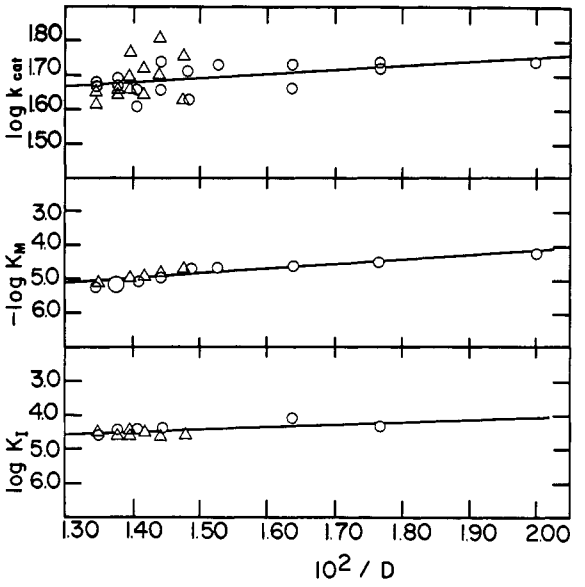

FIGURE 1: The linear dependence of log k_{cat}, log K_m, and log K_i on $1/D$. At each value of $1/D$ one point was obtained with added inhibitor, the other is the control without inhibitor. Open circles: dioxane; open triangles: isopropyl alcohol. All experiments were carried out at pH 8.0, 37.0°, with Bz-Arg-OEt as substrate, in 0.10 M NaCl–2 mM $CaCl_2$.

Experimental

Table I contains the values of K_m, k_{cat} and K_i for the trypsin–Bz-Arg-OEt–benzamidine system, measured in NaCl–$CaCl_2$ and containing isopropyl alcohol; similar data in the presence of dioxane are shown in Table II. Plots of log k_{cat}, log K_m, and log K_i as function of $1/D$ are reproduced in Figure 1. The slope of log k_{cat} *vs.* $1/D$ is indistinguishable from zero within P = 0.05:11.6, with SD = 5.7. The slope of log K_m *vs.* $1/D$ is 139, with SD = 13, whereas the slope of log K_i *vs.* $1/D$ is 64.7, with SD = 24. A Student t test shows that this slope is significantly different from zero at P = 0.05.

[1] One of the reviewers criticized the assumption of the rigid structure of trypsin which would, as a consequence, be totally unaffected by drastic changes in surrounding medium. Our data in Figure 1 (upper) and in Tables I and II bear out the assumption, for no major change in k_{cat} was detected within the concentration ranges of solvents used. If changes in tertiary structure did take place, they did not modify the functioning of the catalytic site of the enzyme.

TABLE I: The Values of K_m, k_{cat}, and K_i for the System Bz-Arg-OEt–Trypsin–Benzamidine Measured in Different Isopropyl Alcohol Concentrations.

Line	Isopropyl Alcohol % w/w	Isopropyl Alcohol M	Dielectric Constant	K_m (μM)[a]	SE (μM)	k_{cat}[b] (sec^{-1})		$10^6 \times k_{cat}/K_m$	K_i (μM)	SE (μM)
1	0	0	74.20	8.17	1.0[c]	44.9	42.0	5.50	42.2	5.4
2	1.97	0.325	72.61	7.60	0.46	44.4	45.6	5.85	25.4	1.6
				9.04	0.98	46.9	50.2	5.19	27.3	2.6
3	3.86	0.634	71.62	9.04	0.98	46.9	59.4	5.19	31.8	3.1
4	5.68	0.925	70.47	14.7	0.49	52.3	74.2	4.11	43.9	7.5
5	7.41	1.206	69.18	14.7	1.2	65.2	50.2	4.43	41.8	3.3
6	9.09	1.477	67.61	19.6	1.6	57.4	42.4	2.93	71.2	18

[a] The substrate concentration range was 5–40 μM. [b] The value at left was determined with K_m, that at right was determined with K_i; the standard deviation was *ca.* 2.0 in most cases. [c] Twelve assays were run for each K_m or K_i determination.

TABLE II: The Values of K_m, k_{cat}, and K_i for the System Bz-Arg-OEt–Trypsin–Benzamidine Measured in Different Dioxane Concentrations.

Line	Dioxane % w/w	Dioxane M	Dielectric Constant	K_m (μM)[a]	SE (μM)	k_{cat}[b] (sec^{-1})		$10^6 \times k_{cat}/K_m$	K_i (μM)	SE (μM)
1	0	0	74.20	7.01	0.63[c]	47.8	36.7	6.83	32.6	2.7
2	2.13	0.242	72.44	8.56	1.1	47.0	49.4	5.49	32.4	3.3
3	4.21	0.483	80.80	9.30	0.41	46.2	41.0	4.97	40.6	8.9
4	6.38	0.724	69.18	11.9	0.53	54.7	46.0	4.61	43.3	20
5	8.46	0.961	67.30	18.4	1.30	51.4	42.7	2.79	104.3	80
6	10.61	1.205	65.31	21.1	4.3	53.8	38.8	2.55	116.9	34
7	15.89	1.804	60.81	25.1	2.4	53.6	45.9	2.13	72.4	7.9
8	21.15	2.401	56.36	31.1	2.0	52.0	54.9	1.68	47.21	18
9	28.93	2.284	49.66	68.4	13	55.5	32.9	0.81	364	199

[a] The substrate concentration range was 5–40 μM. [b] The value at left was determined with K_m, that at right was determined with K_i; the standard deviation was *ca.* 2.0 in most cases. [c] Twelve assays were run for each K_m and K_i determination.

In relation to this latter plot, it is important to note that if eq 15 were obeyed a negative slope should be obtained, since the anionic site in trypsin bears a negative charge, whereas the inhibitor is positively charged.

The slope of log (k_{cat}/K_m) *vs.* $1/D$ is -132, with SD = 9.9 (Figure 2). A t test indicates that it is highly significantly different from zero at $P = 0.01$.

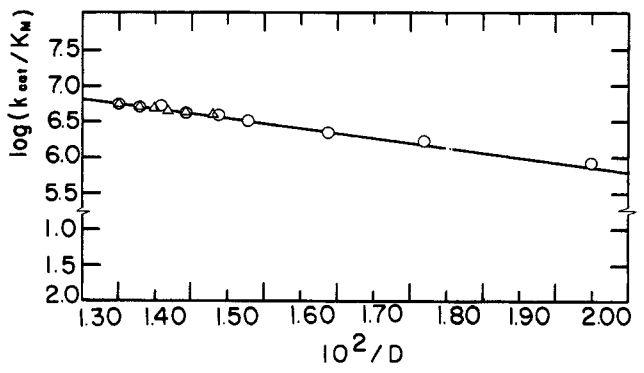

FIGURE 2: The linear dependence of log (k_{cat}/K_m) on $1/D$. Conditions as in Figure 1.

Discussion

General

Isopropyl alcohol was selected to lower the dielectric constant of the system because it does not participate as a nucleophilic agent in the deacylation of the acyl-enzyme intermediate. This has been experimentally observed by Seydoux *et al.* (1969). In addition, our experiments show that isopropyl alcohol, at 10% (w/w) in the hydrolysis system, allows the correct infinity for total hydrolysis of Bz-Arg-OEt to the acid. There is, however, evidence that other alcohols compete with water in this step (Glazer, 1965; Seydoux and Yon, 1967; Seydoux *et al.*, 1969). The positive slope of the line in the log K_i *vs.* $1/D$ plot eliminates the possibility of calculating the distance of closest approach of the ions in the enzyme–inhibitor complex. It indicates that additional factors are at play in the system if an organic solvent is present. The positive slope means that the affinity of the enzyme for the ion is decreasing as the dielectric constant decreases, the opposite of the expected results for interaction of ions of unlike charge. On the other hand, the very small slope of the line in log k_{cat} *vs.* $1/D$ plot associated to the considerable increase in K_m that accompanied a decrease in dielectric constant are coherent results. They indicate that the organic solvents used

behave as competitive inhibitors of trypsin. A similar conclusion was reached by Clement and Bender (1963) in their work on chymotrypsin.

An Estimate of the Equilibrium Distance of the Ions in the Complex via ΔG_{el}

The electrostatic contribution to the free energy of interaction may be evaluated from eq 14, and is found to be

$$\Delta G_{el}^{\circ} = 238.3\frac{Z_E Z_I}{r(6r - 7)} \exp[k(3.75 - r)] \qquad (16)$$

where $6r - 7$ approximates the dielectric constant at the site of interaction.

The value of Debye's κ was calculated for every value of r, using the approximation $D = 6r - 7$, through a computer program. The range of k values was 0.112–0.229.

At present there is no way of calculating what the dielectric constant in the neighborhood of a protein molecule would be. In eq 16 we are using an approximation, $D = 6r - 7$, suggested by Webb (1963), based in published data on the interaction of univalent ions of opposite charge.

The evaluation of D through this expression has also been used by Bechet *et al.* (1966), for estimating distances in the catalytic site of trypsin. A plot of ΔG_{el}° as a function of r is shown in Figure 3. From this graph it is possible to estimate r if ΔG° is known. This is exactly the case with two of the simplest competitive inhibitors of trypsin previously used, formamidinium and guanidinium ions. Thus, guanidinium binds to trypsin with a K_i value of 14 mM at 37° (Mares-Guia and Figueiredo, 1970). Assuming that only one of its three NH_2 groups comes close to the carboxylate group of the enzyme, one obtains for the value of the unitary free energy of binding: $-(\Delta G_u^{\circ})_{el} = RT \ln K_i + RT \ln 3 - 2.381 = 4.34$ kcal/mole.

From Figure 3 a value of 3.8 Å for the equilibrium distance of the ions in the complex is obtained. For the case of the inhibitor formamidinium (Mares-Guia, 1968), after correcting for the fact the ion has two NH_2 groups, and assuming ΔG° to have the same values at 15 and 37° one obtains: $-(\Delta G_u^{\circ})_{el} = RT \ln K_i + RT \ln 2 - 2.381 = 3.58$ kcal/mole.

This value yields 4.0 Å for the equilibrium distance of the ionic groups in the enzyme–inhibitor complex. A closest approach distance of 4 Å implies that they practically establish van der Waals contact, that is, there are no water molecules located between the ionic groups in the complex.

This conclusion is reasonable because, first of all, the interaction free energy is large, and second, because an interaction similar to that described was discovered by Lipscomb *et al.* (1968). These authors determined the three-dimensional structure of a complex of carboxypeptidase A and glycyl-L-tyrosine at 2.0-Å resolution. They demonstrated the direct ionic interaction of the carboxylate group of the substrate with the guanidinium group of arginyl residue 145. In the ionic interaction reported here, the carboxylate group belongs to the enzyme residue Asp-177 (Smith and Shaw, 1969; E. Shaw, quoted in Neurath and Bradshaw, 1970; Eyl and Inagami, 1970), whereas the positively charged group belongs to the inhibitors or to the typical substrates.

Competitive Inhibition by the Organic Solvent

The competitive inhibition by the organic solvent, indicated under the heading General, extends to the point of decreasing the binding of another competitive inhibitor, benzamidine.

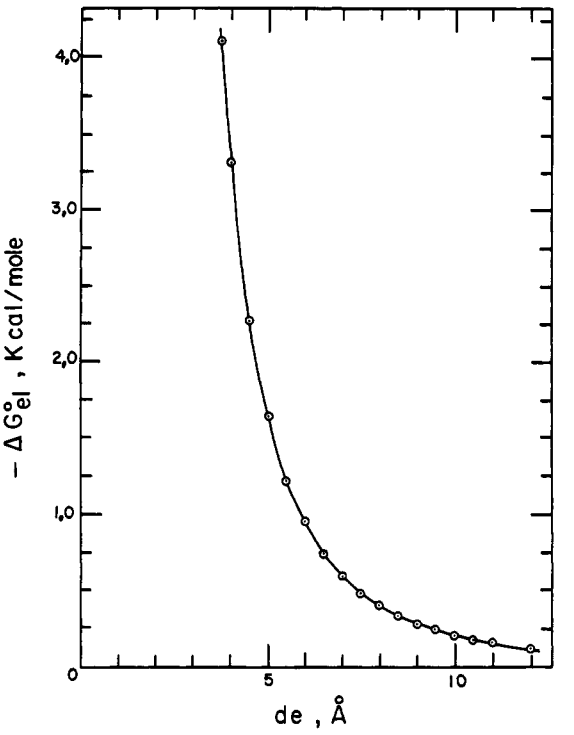

FIGURE 3: Electrostatic free energy of interaction as a function of the distance of the ions. The values were calculated from eq 16. The distance of closest approach of the ions in the complex is 3.75 Å (see text for further details).

Several simple organic compounds were shown to act as competitive inhibitors of proteolytic enzymes. The competitive inhibition of α-chymotrypsin by diethyl ether and *tert*-amyl alcohol, as well as noncompetitive inhibition by methanol, have been shown by Miles *et al.* (1962). More recently Tang (1965) studied the competitive inhibition of pepsin by aliphatic alcohols and concluded that from methanol to amyl alcohol the inhibition increased with increasing size of the side chain.

Clement and Bender (1963) discussed the possibility of better explaining the effects of organic solvents on enzyme-catalyzed reactions, in terms of simultaneous operation of a dielectric and a competitive effect. These authors applied these views to the effects of dioxane, acetone, and acetonitrile on α-chymotrypsin-catalyzed reactions, and succeeded in quantitatively accounting for the results observed. As our work contains, in addition, the effects of organic compounds on the interaction of enzyme and competitive inhibitor, we applied to it the method of Clement and Bender (1963), and succeeded in fitting the experimental data to the equations describing the simultaneous operation of dielectric and competitive effects by the solvent. As we worked in relatively low concentrations of organic solvent (see Tables I and II), we did not take into account the effect of the solvent upon its own complex formation with the enzyme.

Effect of Organic Solvent on K_m. Given the system

$$E + S \overset{K_m}{\rightleftharpoons} E\cdot S \longrightarrow E + P;\ K_m = (E)(S)/(E\cdot S) \qquad (17)$$

$$E + I_S = E\cdot I_S;\ K_{iS} = (E)(I_S)/(E\cdot I_S) \qquad (18)$$

where I_S stands for the organic solvent, one obtains, according to Clement and Bender (1963)

$$K_m(\text{obsd})/K_m(\text{org}) = 1 + (I_S)/K_{iS} \qquad (19)$$

$$K_m(\text{org})/K_m(H_2O) = e^{AX} \qquad (20)$$

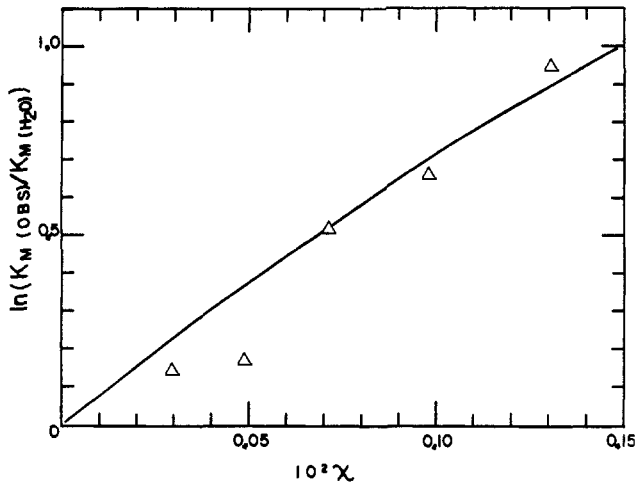

FIGURE 4: The increase in K_m as a consequence of simultaneous operation of dielectric effect and competitive inhibition of trypsin by 2-propanol. Substrate: Bz-Arg-OEt; other conditions as in Figure 1.

where K_{iS} is the enzyme–solvent dissociation constant, X is the difference of the reciprocals of the dielectric constants of solvent–water mixture and pure water, and A is a constant which includes the temperature dependence of the dielectric effect (Clement and Bender, 1963). The combination of the two effects is expressed in eq 21

$$K_m(\text{obsd})/K_m(H_2O) = e^{AX}[1 + (I_S)/K_{iS}] \quad (21)$$

Effect of Organic Solvent on K_i. In analyzing the effect of organic solvent on K_i, two alternatives should be considered: first, the organic solvent competes with substrate and competitive inhibitor for a common site in the active center; second, it is conceivable that the organic solvent, while competing with the substrate, could bind to the active center in such a way that a ternary complex would ensue. The calculations granted the disregard of the second alternative.

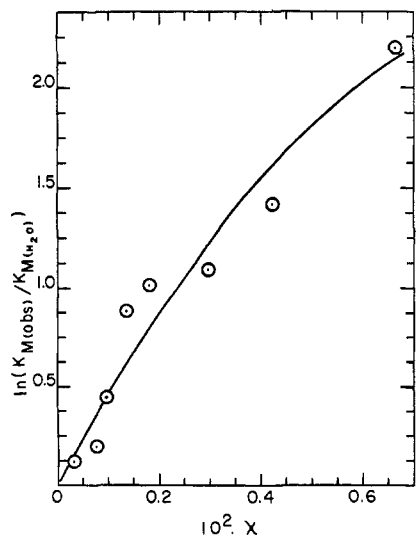

FIGURE 5: The increase in K_m as a consequence of simultaneous operation of dielectric effect and competitive inhibition of trypsin by dioxane. Substrate: Bz-Arg-OEt; other conditions as in Figure 1.

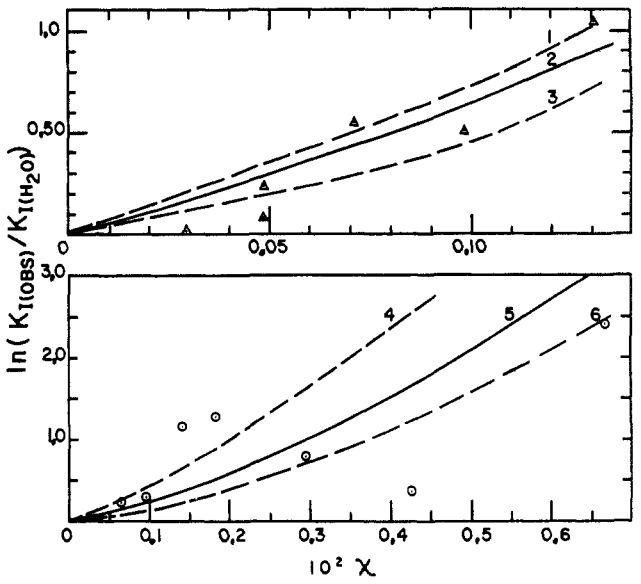

FIGURE 6: The variation of K_i for benzamidine as caused by the simultaneous operation of dielectric effect and competitive inhibition of trypsin by isopropyl alcohol and dioxane. Upper: isopropyl alcohol; lower: dioxane. The dashed lines indicate ranges of A values: (1) 1050; (2) 950; (3) 750, for isopropyl alcohol; (4) 800; (5) 600; and (6) 500, for dioxane. Other conditions as in Figure 1.

FIRST ALTERNATIVE. COMPETITION. Equations 17, 18, 4, and 5 describe the system. One obtains for K_i

$$K_i(\text{obsd})/K_i(\text{org}) = 1/\{1 + [(I_S)K_i(H_2O)/(I)K_{iS}]\} \quad (22)$$

$$K_i(\text{org})/K_i(H_2O) = e^{AX} \quad (23)$$

and finally

$$K_i(\text{obsd})/K_i(H_2O) = e^{AX}\{1/[1 + (I_S)K_i(H_2O)/(I)K_{iS}]\} \quad (24)$$

Equations 21 and 24 were solved by adjusting the values of K_{iS} and A so as to provide the best possible agreement between the experimentally observed and the calculated values of ln $(K(\text{obsd})/K(H_2O))$. The equations were programmed in Fortran IV and processed in an IBM-1130 digital computer.

In Figures 4 and 5 the experimental K_m values obtained in the presence of dioxane or isopropyl alcohol, respectively, are seen to agree well with the lines calculated through eq 21.

The values of the constants K_{is} and A which yielded the best lines are reproduced in Table III. Although there is some

TABLE III: The Dielectric and Inhibitory Effects of Isopropyl Alcohol and Dioxane on the Trypsin-Catalyzed Hydrolysis of Bz-Arg-OEt, in the Presence and in the Absence of Benzamidine: Best Values of A and K_{is}.

	Effect on K_m		Effect on K_i for Benzamidine Inhibn	
Solvent	A	K_{iS} (M)	A	K_{iS} (M)
Isopropyl alcohol	370	3.0	950	1.2
Dioxane	225	2.5	600	2.0

scattering of points, eq 24 seems to represent well the real situation, as can be seen in Figure 6 for isopropyl alcohol and dioxane. In this figure we added curves calculated with K_{iS} values corresponding to the best range of fitting. Judging from the values of A and K_{iS} (Table III) that gave the best fit to eq 24, it is possible to conclude that, concerning the effect of solvent on K_i, the dielectric contribution is still more important than the competitive inhibition. The values of A and K_{iS} in Table III are of the same order of magnitude as those found by Clement and Bender (1963) in their work with α-chymotrypsin.

The competitive inhibition by the solvent allows an attempt at interpreting the log (k_{cat}/K_m) *vs.* $1/D$ plot. In the trypsin-catalyzed Bz-Arg-OEt hydrolysis, $K_m = K_s k_3/(k_2 + k_3)$, and $k_2 \gg k_3$ (Schwert and Eisenberg, 1949; Bender and Kézdy, 1965). This reduces K_m to $K_m = K_s(k_3/k_2)$, and $k_{cat} = k_3$ (*cf.* eq 3). Under these circumstances $k_{cat}/K_m = k_2/K_s$. As we do not know how k_2 or K_s vary with D, independent of each other, it seems reasonable to suppose that K_s should vary with D approximately like K_i did. In that case, the negative slope observed in the plot (Figure 2) is accounted for by an increase in K_s as D decreases, with k_2 remaining constant or decreasing.[2]

The competitive inhibition of substrate hydrolysis, together with a decreased affinity for the competitive inhibitor benzamidine, caused by the organic solvents used, indicates that binding of the solvent to the enzyme affects or involves the specificity site of the enzyme active center, that is, the anionic and/or the hydrophobic sites.

There is, however, the possibility that the solvents are affecting the enzyme through binding to the secondary hydrophobic site indicated by Heidberg *et al.* (1967) and by Seydoux *et al.* (1969).

Added in Proof

Circular dichroism spectra of β-trypsin in 0.1 M Tris (pH 7.5), containing 21.4% (v/v) dioxane, or at pH 8.0, containing 10% (v/v) isopropyl alcohol, failed to show any major changes in the β-trypsin spectrum, both in the near- and far-uv regions. These findings, which will be reported in detail in a future paper, give a direct support to our contention that the effects reported in this paper are not a consequence of a major conformational change in the enzyme (see footnote 1). Circular dichroism was measured with a highly sensitive, modified Jasco CD-SP dichrograph, at the Department of Biochemistry, the University of Texas, M. D. Anderson Hospital and Tumor Institute, Houston, Texas. M. M. G. expresses his appreciation to Professor Bruno Jirgensons for his hospitality.

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[2] While this manuscript was being reviewed we took notice of the work by Kassera and Laidler (1970), in which the authors have shown an increase in K_m and a decrease in k_2 studying trypsin catalysis in the transient phase.

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Purification and Properties of a Diketo Acid Hydrolase from Beef Liver†

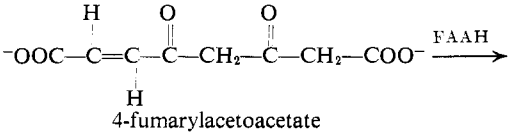
H. H. Hsiang, S. S. Sim, D. J. Mahuran, and Donald E. Schmidt, Jr.*

ABSTRACT: A diketo acid hydrolase which is probably fumarylacetoacetate fumarylhydrolase has been isolated from beef liver. The enzyme is homogeneous on Bio-Gel P-200 chromatography and on disc gel electrophoresis at two pH values. Acetopyruvic acid is cleaved by the enzyme into pyruvic acid and acetic acid. Substrate inhibition kinetics are found with acetopyruvic acid. The diketo acid hydrolase is sensitive to sulfhydryl-specific reagents but is insensitive to serine-specific reagents.

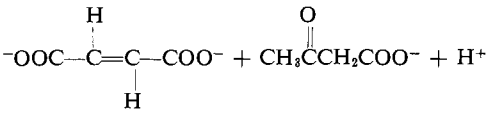
A few examples in which enzymes bring about the catalytic hydrolysis of carbon–carbon bonds are known. Oxaloacetase from *Aspergillus niger* catalyzes the hydrolysis of oxaloacetate to oxalic acid and acetic acid (Hayaishi *et al.*, 1956). Chymotrypsin is known to hydrolyze ethyl 5-(*p*-hydroxyphenyl)-3-ketovalerate to 3-(*p*-hydroxyphenyl)propionate and ethyl acetate (Doherty, 1955) and, in an analogous manner, trypsin cleaves ethyl 5-(*p*-aminophenyl)-3-ketovalerate (Roget and Calvet, 1962). L-Kynurenine hydrolase catalyzes the formation of anthranilate and alanine from L-kynurenine (Longenecka and Snell, 1955). In a bacterial system the pathway for the degradation of gentisic acid includes the enzyme fumarylpyruvate hydrolase, which hydrolyzes fumarylpyruvic acid to fumarate and pyruvate (Lack, 1961). The metabolic pathway for the degradation of tyrosine in mammalian systems utilizes the enzyme fumarylacetoacetate fumarylhydrolase (EC 3.7.1.2) to hydrolyze fumarylacetoacetate to fumaric acid and acetoacetic acid (Ravdin and Crandall, 1951). The mechanisms of these reactions have received little attention.

The study presented here describes the purification from beef liver and some properties of a diketo acid hydrolase which is probably fumarylacetoacetate fumarylhydrolase. Using this enzyme, a study of the mechanism of carbon–carbon bond hydrolysis will be undertaken.

Diketo acid hydrolases from several sources have been studied. A 2,4-diketo acid hydrolase had been isolated from rat liver (Meister and Greenstein, 1948). Other workers partially purified from beef liver a protein that hydrolyzed triacetic acid to acetoacetic acid and acetic acid (Connors and Stotz, 1949). Subsequent investigation showed that these two enzymes were probably the fumarylhydrolase (Ravdin and Crandall, 1951) which catalyzes the following reaction in rat and beef liver. Recently another diketo acid hydrolase



4-fumarylacetoacetate



fumarate acetoacetate

from rat liver has been purified 100-fold (Brock and Williamson, 1968). This enzyme hydrolyzed both triacetic acid and fumarylacetoacetic acid.

Experimental Section

Synthesis of Acetopyruvic Acid. Ethyl acetopyruvate was synthesized according to the method of Marvel and Dreger (1958). The ester was then hydrolyzed with 4 N sodium hydroxide to give the free acid (Lehninger and Witzemann, 1942). Three vacuum sublimations followed by recrystallization from carbon tetrachloride yielded colorless crystals that melted at 97.5–98.5° (uncorrected) (reported 98° (Lehninger and Witzemann, 1942)).

Enzyme Assay. A stock solution of acetopyruvic acid (1.22×10^{-3} M) was prepared by dissolving a weighed amount of the acid in 100 ml of 0.025 M sodium phosphate buffer at pH 7.2. This solution could be stored at 4° for a period of at least 1 week without significant decomposition.

Into a 3-ml cuvet was pipeted 2.6 ml of 0.025 M sodium phosphate buffer at pH 7.2 and 0.3 ml of the stock acetopyruvic acid solution. To this solution was added a measured

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