Ground State and Transition State Effects in the Acylation of α-Chymotrypsin in Organic Solvent–Water Mixtures

By **Ronald P. Bell,* John E. Critchlow,** and **Michael I. Page,** Chemistry Department, University of Stirling, Stirling, Scotland

The kinetics of the α-chymotrypsin catalysed hydrolysis of *p*-nitrophenyl acetate and *N*-acetyl-L-tryptophan methyl ester have been investigated at pH 7·85 and ionic strength 0·2M in solutions containing from 1 to 20% v/v dioxan or propan-2-ol. The solubilities of the two esters in these media were also measured. The ratio of the activity coefficients of the two transition states shows a much smaller variation with the organic solvent content of the medium than does the ratio of the activity coefficients of the substrates in their ground states, from which it is concluded that the substrate undergoes significant desolvation in the activation process. After allowing for the increased stability of the ester brought about by the addition of organic solvent, the inhibitory effect of dioxan can be represented by the simple formation of an inactive 1 : 1 complex of dioxan with the enzyme.

In an enzymic reaction taking place according to the scheme (1) the observed rate of formation of the product

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} ES' + P \qquad (1)$$

is given by the familiar Michaelis–Menten equation (2)

$$d[P]/dt = k_2[S][E]_0/([S] + K_m) \qquad (2)$$

where $[E]_0 = [E] + [ES]$ and $K_m = (k_{-1} + k_2)/k_1$. (If $k_{-1} \gg k_2$, K_m represents the equilibrium constant for the dissociation of ES into E + S, but this condition will not always be fulfilled, and the relative values of k_{-1} and k_2 do not affect the form of the kinetic equation.) Equation (2) can equally well be written as (3) where

$$d[P]/dt = kK_m[S][E]_0/([S] + K_m) \qquad (3)$$

the parameters k_2, K_m, and k are related by $k = k_2/K_m$. k Has the dimensions of a second-order velocity constant, and has a definite physical meaning, independent of how it is split up into k_2 and K_m, being related to the free energy difference between the well defined initial state E + S and the transition state ES‡. Moreover, k is the most logical quantity to use in comparing the efficiency of two enzymes, or of an enzyme and a non-enzymatic catalyst, and is in general use for this purpose. In order to measure k as accurately as possible it is desirable that [S] should be considerably smaller than K_m, when the process approximates to simple second-order kinetics. This condition is readily fulfilled for the hydrolysis of *p*-nitrophenyl acetate (PNPA) and *N*-acetyl-L-tryptophan methyl ester (ATME) by α-chymotrypsin, since enzyme–substrate binding is weak in both these systems.

The present paper reports kinetic and solubility measurements for the above systems in dioxan–water and propan-2-ol–water mixtures, designed to obtain information about the solvation in solution of the reactants and of the transition state ES‡. Recent *X*-ray diffraction studies on crystalline materials have elucidated the structure of the adduct formed between dioxan and α-chymotrypsin,[1] and have shown that the formation of the solid complex ES involves the desolvation of the substrate.[2-4] However, these findings are not necessarily of direct relevance to the problem in hand, partly because conditions in the solid are not always a good guide to behaviour in solution, and also, more significantly, because the complex ES represents the most stable way in which the two species can combine, while the transition state ES‡ demands a particular orientation of reacting groups. It is of course the solvation of E, S, and ES‡ in solution which has a direct bearing upon the efficiency of enzyme action.

If γ_S, γ_E, and $\gamma^‡$ represent the activity coefficients of

[1] T. A. Steitz, R. Henderson, and D. M. Blow, *J. Mol. Biol.*, 1969, **46**, 337.
[2] R. Henderson, *J. Mol. Biol.*, 1970, **54**, 341.
[3] D. M. Blow, C. S. Wright, D. Kukla, A. Ruhlmann, W. Steigemann, and R. Huber, *J. Mol. Biol.*, 1972, **69**, 137.
[4] J. D. Robertus, J. Kraut, and R. A. Alden, *Biochemistry*, 1972, **11**, 4293.

the substrate, enzyme, and transition state, respectively, defined relative to a common value of unity in purely aqueous solution, the second-order rate constant k for the acylation reaction in a given solvent mixture is related to its value k^0 in the absence of organic solvent by equation (4). For sparingly soluble substrates γ_S

$$k = k^0\gamma_S\gamma_E/\gamma^\ddagger \quad (4)$$

may be obtained separately from the solubilities, s and s^0, measured respectively in the presence and absence of organic solvent [equation (5)]. Further analysis is

$$s = s^0/\gamma_S \quad (5)$$

prevented by the lack of any method for measuring γ_E, but if solubility and kinetic data are obtained for two substrates in a given solvent system it becomes possible to study the effect of the organic solvent on the ratio of the two transition state activity coefficients [equation (6)]. The right hand side of equation (6)

$$\frac{\gamma^\ddagger_{PNPA}}{\gamma^\ddagger_{ATME}} = \frac{k_{ATME}s_{ATME}(k^0s^0)_{PNPA}}{k_{PNPA}s_{PNPA}(k^0s^0)_{ATME}} \quad (6)$$

contains only measurable quantities, and $\gamma^\ddagger_{PNPA}:\gamma^\ddagger_{ATME}$ can therefore be compared with the ground state ratio $\gamma_{PNPA}:\gamma_{ATME}$, obtained from equation (5). It was hoped that this comparison would throw some light on the availability of the substrate system for solvation in the transition states formed in the two solvent systems.

EXPERIMENTAL

Materials.—*p*-Nitrophenyl acetate was recrystallised from ethanol–water, m.p. 77·5° (lit.,[5] 79°). L-Tryptophan (B.D.H.) was treated with methanol–hydrogen chloride and the methyl ester was then acetylated with acetic anhydride in aqueous potassium acetate. After three recrystallisations from ethyl acetate, the product *N*-acetyl-L-tryptophan methyl ester had m.p. 153·5° (lit., 152·5, 154·5°), and $[\alpha]_D^{25}$ +13·2° (*c* 2%, methanol) {lit. $[\alpha]_D^{23}$ +11·5° (*c* 2%, methanol); $[\alpha]_D^{24}$ +13·8° (*c* 5·6%, methanol)}. Three-times recrystallised α-chymotrypsin was obtained from Miles Seravac, and was found to be 82% pure, based on a molecular weight of 24,800.

Spectroscopic grade dioxan was distilled over sodium, b.p. 101—102° (lit., 101·3°), and AnalaR grade propan-2-ol was dried ($MgSO_4$) and distilled, b.p. 82·2—82·6° (lit.,[6] 82·4°). The purified dioxan was kept in the dark and used within a few days of distillation, and under these conditions no variation in the kinetic results with the age of the sample could be detected. Water was distilled from potassium permanganate, and all other materials were of AnalaR or equivalent purity. The compositions of the solvent mixtures are expressed as volume per cent of the organic component.

Solubilities.—Saturated solutions were prepared by stirring magnetically a mixture of a 2—4 fold excess of the ester and 10 cm³ of the appropriate solvent mixture containing 0·2M-sodium chloride in a flask sealed with Parafilm and maintained at 25·0 ± 0·1°. After a period ranging from 2 to 20 h, the mixture was rapidly filtered with minimum vacuum through a sintered glass funnel into a flask held in the thermostat bath.

In the case of ATME 1 cm³ of the filtrate was withdrawn using a previously warmed pipette and diluted in such a way that the final solution was 20% in the organic component and 0·1M in sodium chloride. These solutions were analysed spectrophotometrically at 280 nm using a Unicam SP 500 spectrophotometer. The molar absorptivity of ATME under these conditions was found to be $5{\cdot}66 \times 10^3$ in 20% dioxan and $5{\cdot}61 \times 10^3$ dm³ mol⁻¹ cm⁻¹ in 20% propan-2-ol, and the absorbance readings were stable over a period equal to the duration of each solubility experiment, indicating negligible hydrolysis of the ester.

Because of the faster spontaneous hydrolysis of PNPA, a somewhat different analytical technique was required to avoid interference from *p*-nitrophenol. Two 1 cm³ portions of the filtrate were transferred simultaneously to 10 cm³ of a phosphate buffer solution of pH 7·55 and to 100 cm³ of a 0·01M solution of sodium hydroxide. The former was rapidly analysed at 400 nm, and the slowly increasing absorbance extrapolated back to the time of mixing, giving a measure of the concentration of *p*-nitrophenol in the filtrate. The ester added to the 0·01M-sodium hydroxide solution quickly underwent hydrolysis, and the total concentration of *p*-nitrophenol could then be measured from the absorbance at 400 nm. The molar absorptivity of *p*-nitrophenol was found to be $1{\cdot}38 \times 10^4$ in the buffer solution and $1{\cdot}84 \times 10^4$ dm³ mol⁻¹ cm⁻¹ (lit.,[5] $1{\cdot}83 \times 10^4$ dm³ mol⁻¹ cm⁻¹) in 0·01M-sodium hydroxide. The correction for hydrolysis varied from 0·5 to 5·0%, depending on the time allowed for the saturated solution to reach equilibrium.

After making the above correction for PNPA hydrolysis, the solubility measurements for both esters were reproducible within 1—2%, whether or not the ester was first dissolved in the organic component, and for equilibration times ranging from 1 to 20 h. The filtrates were kept for *ca.* 2 h to check for subsequent precipitation, particularly in cases in which the mixture had been prepared by adding aqueous salt solution to a concentrated solution of the ester in the organic component. Attainment of equilibrium was found to be relatively slow, especially in solutions of lower organic content, and equilibration times of <30 min or experiments allowing up to 20 h for equilibration with only occasional shaking were found to give spurious results.

Kinetics.—Absolute enzyme concentrations were determined by spectrophotometric titration against PNPA at 400 nm, using the principle of Schonbaum *et al.*[7] 1% of a 0·1M solution of PNPA in dioxan was rapidly mixed with a solution of the enzyme in phosphate buffer at pH 7·55. The absorbance was extrapolated back to the time of mixing, and the process repeated in the absence of enzyme to correct for small impurities of *p*-nitrophenol in the ester. Under these conditions the half-life for acylation should be at least 1000 times smaller than that for deacylation,[5] and the initial burst should therefore provide a measure of the true concentration of active sites. This was confirmed in the present work by showing the size of the burst to be changed by $<1\%$ on making a two-fold change in the ester concentration.

Stock solutions of the enzyme were made up in 10^{-3}M-hydrochloric acid, stored at 5°, and titrated before and after each set of experiments. Kinetic measurements

[5] F. J. Kezdy and M. L. Bender, *Biochemistry*, 1962, **1**, 1097.
[6] A. Weissberger, 'Technique of Organic Chemistry,' Interscience, New York, vol. VII, 2nd edn., 1965.
[7] G. R. Schonbaum, B. Zerner, and M. L. Bender, *J. Biol. Chem.*, 1961, **236**, 2930.

were made with a Gilford 2400 recording spectrophotometer, with the cell compartment held at 25·0°. All reaction solutions were maintained at pH 7·85 ± 0·02 by means of a phosphate buffer of ionic strength 0·20M and their pH measured after each run.

The kinetics of PNPA hydrolysis were measured by following the release of *p*-nitrophenol anion at 400 nm. The reaction was initiated by adding 1% of a 10^{-4}M solution of the ester in the organic component from a microsyringe to a buffered solution of the enzyme. Since the enzyme was always in at least a ten-fold excess over the substrate, the free enzyme concentration remained effectively constant throughout the run, and the observed change was attributable solely to the acylation reaction.[5] The reaction was accordingly found to be first order in substrate, and the corresponding first-order rate constant was found by non-linear least squares analysis of the trace.

In a previous study of this system in the presence of excess of enzyme Kezdy and Bender [5] reported that the reaction was not first order in enzyme, an observation subsequently attributed to the formation of oligomers of chymotrypsin.[8] At the lower enzyme concentrations and higher pH employed in the present work the degree of polymerisation should be <1%, at least in the more aqueous solutions for which sedimentation data are available.[9,10] Experiments conducted in 20% dioxan–water and 20% propan-2-ol–water with enzyme concentrations ranging from 2×10^{-5} to 2×10^{-4}M confirmed that in these solutions the reaction is also first order in enzyme, and kinetic runs in solutions of lower organic solvent content could therefore be carried out using a single enzyme concentration lying between 10^{-5} and 2×10^{-5}M. These experiments also confirmed the conclusion based on the recent work of Hardman *et al.*[11] that enzyme–substrate binding can be neglected under these conditions.

Separate measurements were made of the spontaneous rate of hydrolysis of PNPA in the absence of enzyme. At 20% dioxan, where the enzyme-catalysed reaction is at its slowest, the spontaneous rate still makes only a 1% contribution, and so has been neglected in calculating the second-order rate constants. A number of duplicate runs were carried out at each dioxan and propan-2-ol concentration, particularly in the more aqueous solutions where the rates are highest, and these gave standard deviations ranging from 2 to 10% depending on the rate.

The kinetics of ATME hydrolysis were followed at 300 nm by the method of Zerner *et al.*[12] The solutions contained 10^{-7}M-enzyme and 10^{-4}M-substrate, and the reaction was initiated by adding 1% of a 10^{-2}M solution of the ester in the organic component. The traces were recorded to completion, and were analysed by means of equation (7)

$$\frac{2{\cdot}303}{t}\log_{10}\frac{A_\infty - A_0}{A_\infty - A} = -\frac{A - A_0}{tK_m\varepsilon} + k[E]_0 \quad (7)$$

where A is the absorbance and ε the molar absorptivity difference between reactant and product. As expected from previous studies,[12] the reaction traces obtained in the more highly aqueous solutions showed significant enzyme–substrate binding in the early stages of the reaction, and extrapolations of up to 100% were required to obtain a value of k. However, K_m increases on adding organic solvent, and in solutions containing 20% of either organic component the kinetics were found to be strictly first order in ester.

The results of Huang and Niemann [13] indicate that inhibition by *N*-acetyl-L-tryptophan can be neglected in the more highly aqueous solutions. That this is also justified at the other extreme of solvent composition was confirmed by experiments in 20% dioxan, which showed that changing the ester concentration from 10^{-4} to 10^{-3}M produced no significant decrease in k. Further experiments in the same solvent mixture using enzyme concentrations ranging from 10^{-7} to 10^{-6}M established that neither

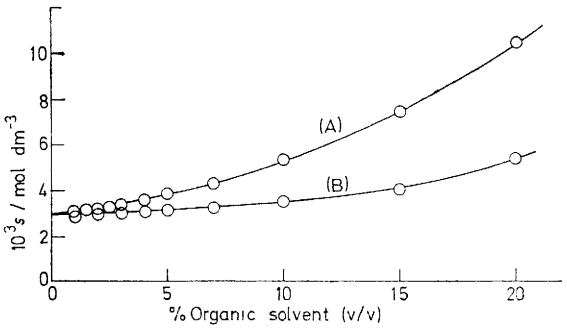

FIGURE 1 Solubility of *p*-nitrophenyl acetate in mixtures of water with dioxan (A) and propan-2-ol (B)

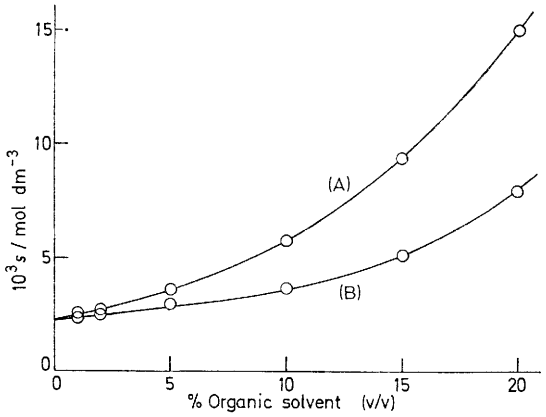

FIGURE 2 Solubility of *N*-acetyl-L-tryptophan methyl ester in mixtures of water with dioxan (A) and propan-2-ol (B)

adsorption of the enzyme on the sides of the cuvette nor inhibition of the enzyme by impurities in the dioxan were important under these conditions. While the rate constants in solutions containing 20% of organic solvent could be defined as closely as those for PNPA hydrolysis, the results in highly aqueous solutions showed larger random deviations, primarily because of the low values of K_m.

RESULTS AND DISCUSSION

$\gamma^\dagger$ and γ Ratios.—The solubilities of the two esters in each of the solvent systems are shown in Figures 1 and 2. The values reported by Hardman *et al.*[10] for PNPA

[8] A. Himoe, P. C. Parks, and G. P. Hess, *J. Biol. Chem.*, 1967, **242**, 919.
[9] M. S. N. Rao and G. Kegeles, *J. Amer. Chem. Soc.*, 1958, **80**, 5724.
[10] R. Egan, H. O. Michel, R. Schlueter, and B. J. Jandorf, *Arch. Biochem. Biophys.*, 1957, **66**, 366.
[11] M. J. Hardman, P. Valenzuela, and M. L. Bender, *J. Biol. Chem.*, 1971, **246**, 5907.
[12] B. Zerner, R. P. M. Bond, and M. L. Bender, *J. Amer. Chem. Soc.*, 1964, **86**, 3674.
[13] H. T. Huang and C. Niemann, *J. Amer. Chem. Soc.*, 1951, **73**, 1541.

in propan-2-ol–water differ from the present values only at 10% propan-2-ol, but these authors give few details of their experimental method. The variation of the ratios $\gamma^{\ddagger}_{PNPA} : \gamma^{\ddagger}_{ATME}$ and $\gamma_{PNPA} : \gamma_{ATME}$ are listed in Tables 1 and 2, together with the experimental results from which they were derived and the overall standard deviations. The values of k_{PNPA} in dioxan–water lie 5—10% below those interpolated from the results of Clement and Bender [14] measured at pH 7·7 with [ester] $\simeq [E]_0$.

Although the accumulated standard deviations are fairly large, particularly in the more highly aqueous solutions, nevertheless there is no evidence for a systematic variation in $\gamma^{\ddagger}_{PNPA} : \gamma^{\ddagger}_{ATME}$ compared with the ground state, although they were not able to take any account of the influence of ionic strength on γ_E or $\gamma^{\ddagger}$. The substrate is also found to be desolvated in the solid complex ES,[2-4] although, as pointed out in the Introduction, this finding does not provide direct evidence about the nature of the transition *state* ES‡ in solution.

Evidence for the Binding of Organic Solvents.—Previous studies of the rates of α-chymotrypsin catalysed reactions in organic solvent–water mixtures concentrated on the variation in the rate for particular substrates. Faller and Sturtevant [16] observed empirically a linear relation between $1/\sqrt{k_{PNPA}}$ and the organic solvent content in a number of systems, while in a more

TABLE 1

$\gamma^{\ddagger}$ and γ ratios in dioxan–water mixtures

Vol. % dioxan	$10^{-3}k_{PNPA}$ / dm³ mol⁻¹ s⁻¹	$10^{-5}k_{ATME}$ / dm³ mol⁻¹ s⁻¹	$10^{3}s_{PNPA}$ / mol dm⁻³	$10^{3}s_{ATME}$ / mol dm⁻³	$\frac{\gamma^{\ddagger}_{PNPA}(k^0s^0)_{ATME}}{\gamma^{\ddagger}_{ATME}(k^0s^0)_{PNPA}}$	$\frac{\gamma_{PNPA}s^0_{ATME}}{\gamma_{ATME}s^0_{PNPA}}$
1	2·63	6·03	3·00	2·52	(1·92 ± 0·39) × 10²	0·84 ± 0·02
2	1·48	3·79	3·13	2·70	(2·21 ± 0·29) × 10²	0·86 ± 0·02
5	0·627	1·77	3·83	3·60	(2·66 ± 0·35) × 10²	0·94 ± 0·03
10	0·255	0·632	5·33	5·77	(2·70 ± 0·19) × 10²	1·09 ± 0·03
15	0·124	0·253	7·46	9·41	(2·58 ± 0·10) × 10²	1·27 ± 0·04
20	0·069	0·118	10·5	15·0	(2·44 ± 0·09) × 10²	1·43 ± 0·04

In purely aqueous solution $s_{PNPA} = 2{\cdot}83 \times 10^{-3}$ and $s_{ATME} = 2{\cdot}23 \times 10^{-3}$ mol dm⁻³.

TABLE 2

$\gamma^{\ddagger}$ and γ ratios in propan-2-ol–water mixtures

Vol. % propan-2-ol	$10^{-3}k_{PNPA}$ / dm³ mol⁻¹ s⁻¹	$10^{-5}k_{ATME}$ / dm³ mol⁻¹ s⁻¹	$10^{3}s_{PNPA}$ / mol dm⁻³	$10^{3}s_{ATME}$ / mol dm⁻³	$\frac{\gamma^{\ddagger}_{PNPA}(k^0s^0)_{ATME}}{\gamma^{\ddagger}_{ATME}(k^0s^0)_{PNPA}}$	$\frac{\gamma_{PNPA}s^0_{ATME}}{\gamma_{ATME}s^0_{PNPA}}$
1	3·23	8·08	2·86	2·38	(2·07 ± 0·45) × 10²	0·83 ± 0·02
2	2·74	6·62	2·96	2·55	(2·08 ± 0·33) × 10²	0·86 ± 0·02
5	2·06	4·73	3·14	3·00	(2·21 ± 0·29) × 10²	0·96 ± 0·03
7	1·76	3·57	3·26	3·28 [a]	(2·05 ± 0·21) × 10²	1·01 ± 0·05
10	1·38	3·00	3·52	3·71	(2·28 ± 0·20) × 10²	1·05 ± 0·03
13	1·05	2·21	3·87 [a]	4·46 [a]	(2·41 ± 0·12) × 10²	1·15 ± 0·05
15	0·84	1·47	4·08	5·15	(2·20 ± 0·16) × 10²	1·26 ± 0·03
20	0·44	0·81	5·45	8·03	(2·69 ± 0·13) × 10²	1·47 ± 0·06

[a] Interpolated.

80% change in $\gamma_{PNPA} : \gamma_{ATME}$. In other words a large part of the difference between the two esters as regards the variation of k with solvent composition must be attributed to their ground state activity coefficients.

Since the changes in activity coefficients arise mainly from short-range forces, the near constancy of the $\gamma^{\ddagger}$ ratios implies that the two transition states present a similar surface to the solvent, and that the enzyme therefore largely shields the substrate portions from solvation. It is interesting that this should occur even when one of the substrates is non-specific, suggesting that the degree of desolvation is not necessarily related to the number of catalytically favourable enzyme–substrate interactions. The postulate of a largely shielded substrate portion is also supported by the results of Miles *et al.*,[15] who found that the effect of ionic strength variation on the rate of hydrolysis of a number of hippurate esters could be accounted for by changes in the activity coefficient of the substrate in detailed study Clement and Bender [14] found that their results could be accounted for by a combination of competitive inhibition by the organic solvent and an electrostatic repulsion between substrate and enzyme. None of these workers took any account of the changing activity of the substrate. In our experiments this effect is responsible for only a small part of the overall rate variation, and the fact that $\gamma^{\ddagger}_{PNPA} : \gamma^{\ddagger}_{ATME}$ is virtually solvent independent suggests that the remainder of this variation may be attributable to a simple interaction between the enzyme and organic solvent. More precisely, if γ_E is expressed as a product of $\gamma_{E'}$ representing complex formation with the organic solvent and $\gamma_{E''}$ representing weaker solvation interactions, the postulate of a largely shielded substrate portion suggests that $\gamma^{\ddagger} : \gamma_{E''}$ will be constant, leaving equation (8).

$$ks = k^0s^0\gamma_{E'} \qquad (8)$$

If $\gamma_{E'}$ can be attributed to partial formation of a 1 : 1

[14] G. E. Clement and M. L. Bender, *Biochemistry*, 1963, **2**, 836.
[15] J. L. Miles, D. A. Robinson, and W. J. Canady, *J. Biol. Chem.*, 1963, **238**, 2932.
[16] L. Faller and J. M. Sturtevant, *J. Biol. Chem.*, 1966, **241**, 4825.

complex between enzyme and organic solvent, application of the law of mass action leads to equation (9)

$$\frac{1}{ks} = \frac{1}{k^0s^0} + \frac{c}{K_ik^0s^0} \qquad (9)$$

where c is the concentration of organic solvent and K_i the dissociation of the complex. A plot of $1/ks$ against c is shown for PNPA hydrolysis in dioxan–water in Figure 3, and is linear within experimental error, with $K_i = 0{\cdot}10$ dm^3 mol^{-1}. A similar plot is obtained for ATME hydrolysis in the range 5—20% dioxan. Inhibition plots constructed without correction of the rate data for solubility changes showed significant curvature above *ca.* 7% dioxan, in accordance with the similar behaviour found previously for other substrates and solvent systems.[14,17] It therefore seems probable that the retarding effect of dioxan on the rates of acylation of α-chymotrypsin is attributable to the formation of a

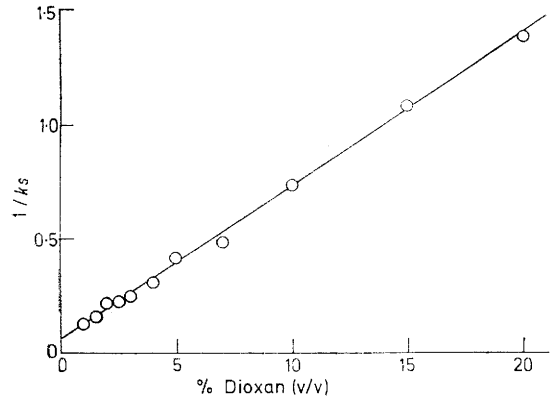

FIGURE 3 Plot of $1/ks$ against % dioxan for *p*-nitrophenyl acetate [equation (8)]

1 : 1 complex between the enzyme and organic solvent, coupled with an increase in the stability of the ester in its ground state. This is supported by the structure of the solid enzyme–dioxan complex in which one molecule is held at the aromatic binding site.[1]

Dioxan is a particularly favourable solvent for the demonstration of a specific complex-forming interaction since its activity in aqueous solution follows closely its volume fraction[18] and because acid–base equilibria involving no net change in charge are virtually unaffected in these media.[19] It is not therefore surprising that plots of $1/ks$ against c are not strictly linear for propan-2-ol–water mixtures, although considerably more so than when no allowance is made for the solubility changes. In general a complete demonstration of inhibition by binding in other systems would require the derivation of pH-independent kinetic parameters at each composition.

Implications of Substrate Desolvation in the Transition State.—Since the transition state requires a particular orientation of reacting groups, its formation will demand at least partial desolvation of the substrate even when ground state solvation is strong. Unless it is compensated by favourable interactions between substrate and enzyme in the transition state, such desolvation will lead to a net loss of reactivity, and should therefore affect relative specificities. In fact Lumry and Rajender[20] have concluded from enthalpy–entropy compensation effects that solvation changes are largely responsible for the different specificities exhibited by chymotrypsin.

In particular, desolvation effects may be responsible for the fact that specific substrates bearing an acylamino-side-chain are more reactive by several powers of ten than their analogues containing a free amino-group.[21] This has sometimes been explained in terms of hydrogen bonding effects, but it has been recently pointed out[22] that on any reasonable estimate the differences between the hydrogen bond energies of the amino- and acylamino-groups with the carbonyl oxygen atom of the enzyme, or with the solvent, are quite inadequate to explain the large rate differences. It seems more likely that the increase in reactivity observed when the amino-group is converted into acylamino arises more from the relatively weak solvation of the latter group in the ground state than from favourable transition state interactions, though of course the net result will depend upon the sum of these two effects. This explanation could be partially tested by measuring distribution coefficients between water and less polar solvents for a series of substrates along the lines adopted by Knowles.[23]

It should be emphasised that structural specificity attributed to the desolvation of the substrate in enzyme catalysed reactions results from a retardation effect on the less reactive substrates. Cohen and his co-workers[24] have attributed a part of the rate enhancement associated with enzyme catalysed reactions to the preliminary formation of a complex in which the site of reaction is made susceptible to attack by the removal of solvating water molecules. However, since the overall rate of reaction depends on the free energy difference between ground and transition states, any desolvation of the substrate, other than that occurring in comparable non-enzymic reactions and necessary for approach of the catalytic groups, will raise the transition state energy and lower the rate. The effect described by these workers will of course increase the first-order rate constant for the decomposition of the Michaelis complex, but only at the expense of a compensating or larger increase in K_m.

We thank the S.R.C. for fellowships awarded to J. E. C. and M. I. P. and for a grant for equipment.

[2/2596 *Received*, 16*th November*, 1972]

[17] T. H. Applewhite, R. B. Martin, and C. Niemann, *J. Amer. Chem. Soc.*, 1958, **80**, 1457.
[18] J. R. Goates and R. J. Sullivan, *J. Phys. Chem.*, 1958, **62**, 188.
[19] R. A. Robinson and R. H. Stokes, ' Electrolyte Solutions,' Butterworths, 1959, London, 2nd edn., Appendix 12.1.
[20] R. Lumry and S. Rajender, *J. Phys. Chem.*, 1971, **75**, 1387.
[21] S. Kaufman and H. Neurath, *Arch. Biochem.*, 1949, **21**, 437.
[22] M. S. Silver, M. Stoddard, T. Sone, and M. S. Matts, *J. Amer. Chem. Soc.*, 1970, **92**, 3151.
[23] J. R. Knowles, *J. Theor. Biol.*, 1965, **9**, 213.
[24] S. G. Cohen, V. M. Vaidja, and R. M. Schulz, *Proc. Nat. Acad. Sci. U.S.A.*, 1970, **66**, 249.