Kinetics of α-chymotrypsin action. I. pH, solvent, and temperature effects

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An investigation has been made of the influence of pH on the kinetics of the α-chymotrypsin-catalyzed hydrolysis of *N*-acetyl-L-tyrosine ethyl ester, *p*-nitrophenyl acetate, and *N*-benzoyl-D- and -L-alanine methyl esters. From the pH variations of $\tilde{k}_c$, $\tilde{K}_m$, and $\tilde{k}_c/\tilde{K}_m$, it is deduced that in the free enzyme there are ionizing groups of pK 6.9 and 9.2. From the variation of these pK values with dielectric constant, it is concluded that, when protonated, one group is cationic and the other neutral. A temperature-dependence study was carried out on *N*-benzoyl-D- and -L-alanine methyl esters. The sharp break in the plot of log $\tilde{k}_c$ against $1/T$ is attributed to a rapid reversible denaturation of the enzyme at the higher temperatures. In the low-temperature region, the activation energies are $\Delta E_L = 16.2 \pm 0.3$ kcal/mole and $\Delta E_D = 16.5 \pm 0.6$ kcal/mole.

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INTRODUCTION

Numerous studies have been made of the influence of pH on chymotrypsin kinetics, with a variety of substrates, and the results have been summarized and interpreted by Bender and co-workers (1). The previous work has been mainly concerned with the behavior at higher substrate concentrations, under which circumstances either acylation or deacylation is rate controlling.

Relatively little work has been done at lower substrate concentrations and at higher pH values. In the present investigation such a study has been made with four substrates, namely, *N*-acetyl-L-tyrosine ethyl ester, *p*-nitrophenyl acetate, *N*-benzoyl-D-alanine methyl ester, and *N*-benzoyl-L-alanine methyl ester. The work has been done with water as solvent, and also with various dioxane–water mixtures. The object of the work in mixed solvents was to deduce, from the variations of the pK value with dielectric constant, the nature of the ionizing groups.

The present paper also describes a study of the temperature dependence of the kinetic parameters for the hydrolysis of *N*-benzoyl-D-alanine methyl ester and *N*-benzoyl-L-alanine methyl ester, the object being to determine whether the marked difference in rate of hydrolysis is associated with a difference in energy or entropy of activation.

EXPERIMENTAL

Materials

All water used was doubly distilled, deionized, and free of carbon dioxide. The dioxane was purified by the method of Fieser (2) and was stored under nitrogen. Precautions were taken to prevent the solvent from coming into contact with the atmosphere, to avoid the formation of peroxide impurities.

N-Benzoyl-L-alanine methyl ester was prepared essentially by the method used by Hein and Niemann (3), with a modification to the benzoylation procedure which doubled the previously reported yield. Esterification of 5.0 g of L-alanine was carried out with methanol and thionyl chloride according to the procedure of Brenner and Huber (4). Benzoylation of the unpurified ester hydrochloride was carried out with the use of a pH-stat according to the following procedure. The ester hydrochloride was dissolved in water and the pH adjusted to 8.0 with base. One equivalent of benzoyl chloride was slowly added. The pH was kept constant at 8.0 with sodium hydroxide solution delivered by the pH-stat, and a milky white liquid settled to the bottom of the flask. The liquid was separated, and after two recrystallizations from high-boiling petroleum ether, it yielded 7.4 g (60%) of *N*-benzoyl-L-alanine methyl ester, m.p. 57–58 °C, $[\alpha]_D$ +30.7° (*c*, 5 in *sym*-tetrachloroethane).

N-Benzoyl-D-alanine methyl ester was prepared as described for the L-enantiomer; m.p. 57–58 °C, $[\alpha]_D$ −30.6° (*c*, 5 in *sym*-tetrachloroethane).

N-Acetyl-L-tyrosine ethyl ester was purchased in pure form from Mann Biochemicals and used without further purification. *p*-Nitrophenyl acetate was prepared by the method of Chattaway (5) and was purified by three crystallizations from an alcohol–water mixture; m.p. 82–84 °C.

The titrant used to follow the course of the reaction was 0.02 *N* sodium hydroxide, prepared from the Fisher certified reagent.

Kinetic Procedure

The reactions were followed with a pH-stat, 0.02 *N* sodium hydroxide solution being continuously and automatically added to maintain a constant pH. The pH-stat equipment was that supplied by the Radiometer Company of Copenhagen (TTT 1 titrator, Ole Dich recorder, G202C glass electrode, and saturated calomel reference electrode).

The reactions were carried out in 15.0 ml of solution containing 15 meq of sodium chloride and maintained at 20.0 ± 0.05 °C in a thermostatically controlled bath. Stock solutions of enzyme and substrate were made up in water.

Enzyme concentrations, calculated on the basis of a molecular weight of 24 800, were as follows, for the various substrates studied: *N*-acetyl-L-tyrosine ethyl ester, 10^{-8} *M*; *p*-nitrophenyl acetate, 10^{-5} *M*; *N*-benzoyl-L-alanine methyl ester, 10^{-6} *M*; *N*-benzoyl-D-alanine methyl ester, 10^{-5} *M*.

In a typical run, substrate and sodium chloride solutions were mixed to make 10.0 ml of solution. Then dioxane or water or both were added to a total volume of 4.0 ml. The pH was adjusted to the desired value with 2 *N* base delivered by a micrometer screw gauge. The reaction was started by the addition of 1.0 ml of enzyme. Reactions were followed to less than 5% completion.

Since reactions were carried out up to high pH values, considerable base hydrolysis was encountered. It was found that the best method for correction was to find the first-order constant ($k = v/[S]$) at various pH values, and then to plot k against $1/[H^+]$. These plots, of which an example is shown in Fig. 1, were linear. The correction can then be directly calculated.

In the case of *p*-nitrophenyl acetate, a blank correction was made for each rate determination, since the rate of basic hydrolysis is not linear with hydroxide ion concentration (6).

RESULTS AND DISCUSSION

Figures 2, 3, 4, and 5 show the pH dependencies of $\tilde{k}_c$, $\tilde{K}_m$, and $\tilde{k}_c/\tilde{K}_m$ for the four substrates. The parameter $\tilde{k}_c$ is in all cases independent of pH on the basic side of the pH optimum and dependent on pH on the acid side. $\tilde{K}_m$ shows an inverse behavior to that of $\tilde{k}_c$, being independent of pH on the acid side of the pH optimum and dependent on pH on the basic side. The ratio $\tilde{k}_c/\tilde{K}_m$ shows a pH dependence on both sides of the pH optimum.

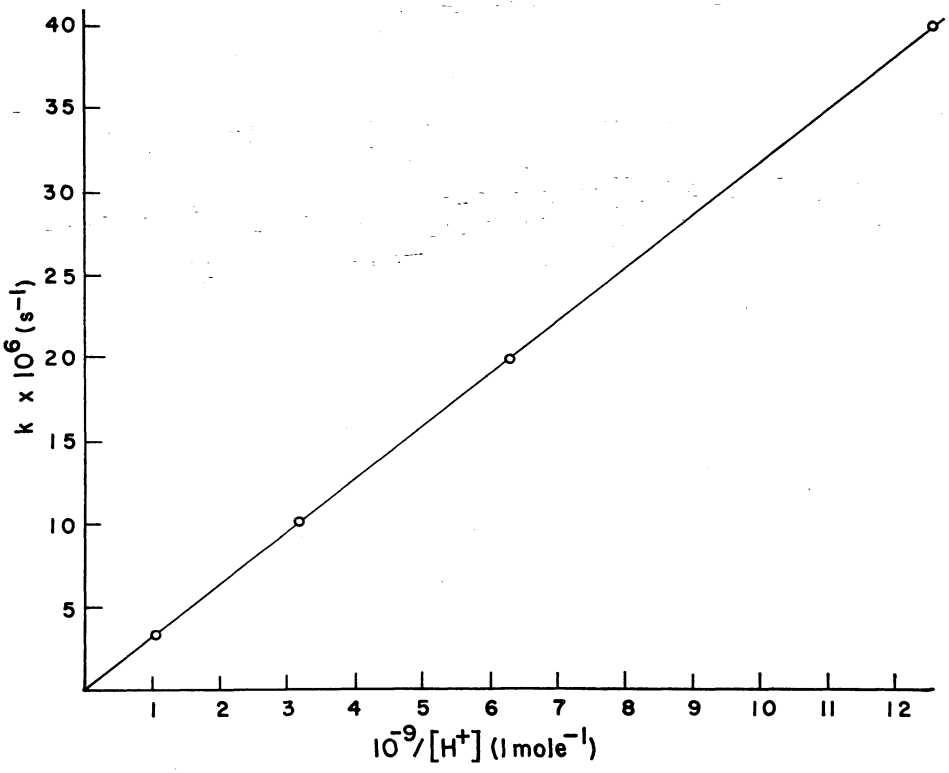


FIG. 1. The first-order rate constant ($k = v/[S]$) for the basic hydrolysis of *N*-benzoyl-L-alanine methyl ester in dioxane (13.3%) – water (v/v) at 20.0 °C plotted against the reciprocal of the hydrogen ion concentration.

The pH dependence of the hydrolysis of *p*-nitrophenyl acetate has previously been investigated at least four times (1, 7–9). In all but one study, the pH dependence was not studied above pH 7.8. Bender and co-workers (1) have studied the reaction up to pH 9.98 by the stopped-flow technique in the second-order kinetic region, in which $[E]_0 \simeq [S]_0 \ll K_s$. The results leave little doubt that *p*-nitrophenyl acetate is hydrolyzed by a three-step mechanism, the first step being the binding of the substrate, the second acylation, and the third deacylation. In the present work, we have studied to pH 10.0 the overall steady-state kinetics. It should be noted that, although the four substrates studied have considerable structural differences, and different kinetic specificities for the enzyme, they all show almost identical pH dependencies for the kinetic parameters $\tilde{k}_c$, $\tilde{K}_m$, and $\tilde{k}_c/\tilde{K}_m$. This observation suggests that there is a similar mechanism of action of α-chymotrypsin on all these substrates.

Nature of the Ionizing Groups

The study in dioxane–water was carried out with *N*-benzoyl-L-alanine methyl ester. The $\tilde{K}_m$ value for this compound is relatively large in water and it increases on the addition of dioxane (10). The substrate concentrations used were much lower than the $\tilde{K}_m$ values; the rates therefore relate to the second-order constant $\tilde{k}_c/\tilde{K}_m$, and the pK values obtained are those for the free enzyme. If the condition of low substrate concentration is satisfied, a plot of rate against [S] should be a straight line passing through the origin. Figure 6 shows that this is the case.

Figure 7 shows the pH profiles for dioxane (6.67, 13.3, and 26.6%) – water (v/v) mixtures. It also shows a superposition of the curves, with the maximum rates normalized to the same value.

Ionizing groups on an enzyme may be

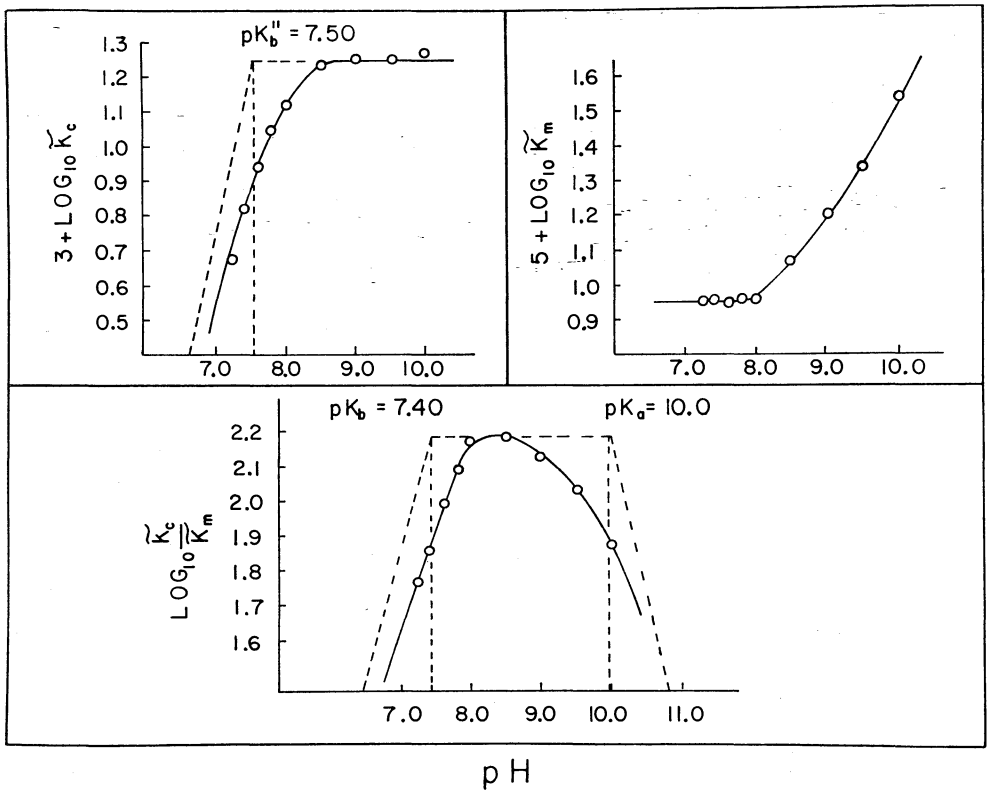


FIG. 2. Plot of $\log_{10} \tilde{k}_c$, $\log_{10} \tilde{K}_m$, and $\log_{10} \tilde{k}_c/\tilde{K}_m$ for the α-chymotrypsin-catalyzed hydrolysis of *p*-nitrophenyl acetate in isopropyl alcohol (20%) – water (v/v). T = 20.0 °C; 0.10 N NaCl.

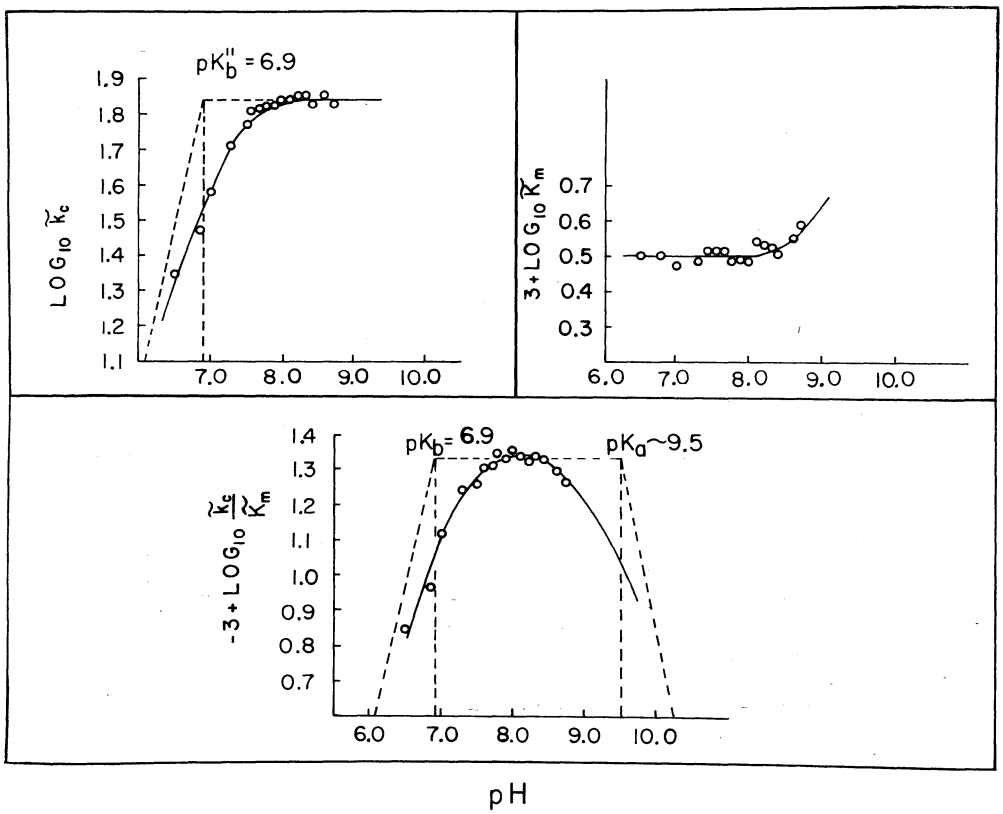


FIG. 3. Plot of $\log_{10} \tilde{k}_c$, $\log_{10} \tilde{K}_m$, and $\log_{10} \tilde{k}_c/\tilde{K}_m$ for the α-chymotrypsin-catalyzed hydrolysis of *N*-acetyl-L-tyrosine ethyl ester in dioxane (5%) – water (v/v). T = 20.0 °C; 0.10 *N* NaCl.

divided into two classes: (*a*) neutral groups, such as —COOH and —OH, which dissociate into positive and negative species on ionization, —A—H ⇌ H^+ + —A^-; (*b*) cationic groups, such as —NH_3^+, which dissociate into a proton and a neutral group, —B—H^+ ⇌ —B + H^+. The effect of changing the dielectric constant of the solvent is very different in the two cases. In group *a*, an increase in dielectric constant increases K_a (and decreases pK_a); in group *b*, it has very little effect on K_a, since cationic species are involved on both sides of the equation. This type of behavior is well established experimentally. For example, the pK of acetic acid is 4.76 in water and 10.14 in a dioxane (82%) – water (w/w) mixture (11). On the other hand, the pK values corresponding to the —NH_3^+ groups of amino acids are only very slightly affected by a change in the dielectric constant (12).

In the case of α-chymotrypsin, as with many other hydrolytic enzymes, the existence of bell-shaped rate–pH profiles at low substrate concentration reveals the presence of two kinetically significant ionizing groups in the free enzyme. Each of these may be either neutral or cationic, so that there are four possibilities: neutral–neutral, neutral–cationic, cationic–neutral, cationic–cationic. The type of behavior expected if the dielectric constant is reduced, by the addition of inert solvent, is shown in Fig. 8. These curves have been normalized to make the maximum rates the same in all cases. By observing what pattern of behavior actually occurs, it is possible to distinguish between the four possibilities.

This procedure resembles, but is simpler than, that employed by Findlay *et al.* (13) for ribonuclease. They used buffers, and had to take into account the effect of the dielectric constant on the ionization of the buffer.

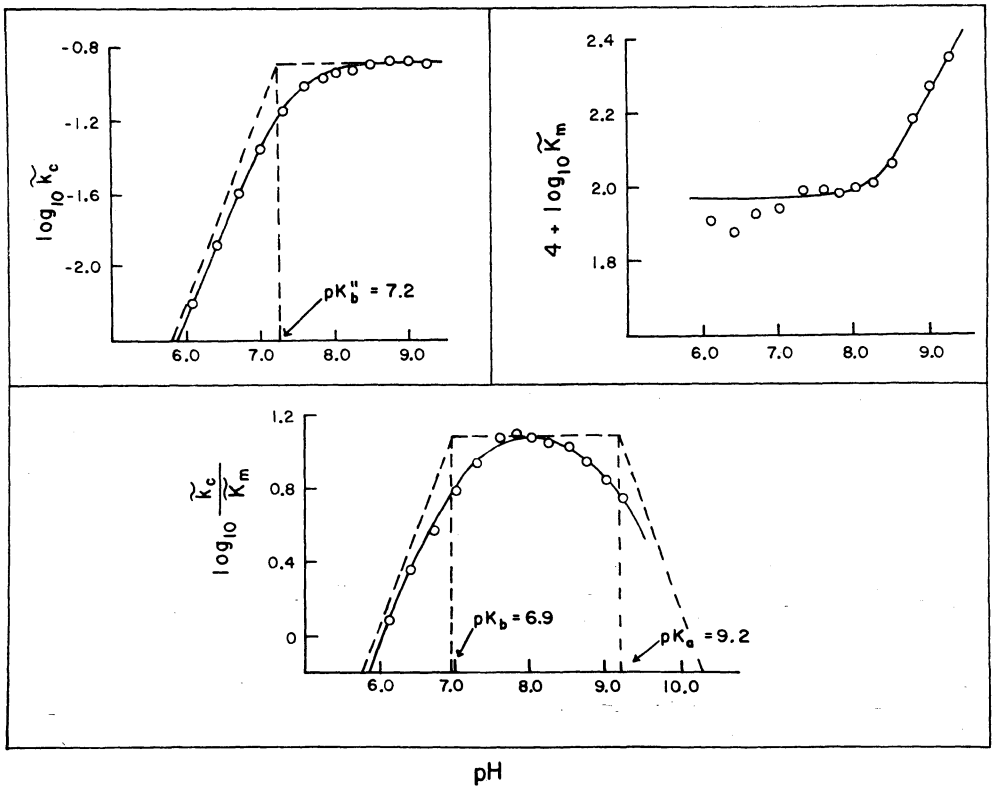


FIG. 4. Plot of $\log_{10} \tilde{k}_c$, $\log_{10} \tilde{K}_m$, and $\log_{10} \tilde{k}_c/\tilde{K}_m$ for the α-chymotrypsin-catalyzed hydrolysis of *N*-benzoyl-L-alanine methyl ester in water. $T = 20.0$ °C; 0.10 *N* NaCl.

In the present work, done with a pH-stat, no buffer was employed.

The data represented in Fig. 7 clearly correspond to the case in which the ionizing groups are cationic–neutral. The lower pK remains constant at about 6.9, but the upper one is increased markedly as dioxane is added. The pK values in the various mixtures are summarized in Table I.

TABLE I

pK values in the various solvent mixtures of dioxane and water

% dioxane (by volume)	Dielectric constant	pK_b	pK_a
0	80.38	6.93	9.2
6.67	74.5	6.90	10.0
13.3	69.0	6.95	10.4
26.7	57.0	6.95	>11.0

The group of lower pK has previously been postulated to be an imidazole group, on the basis of studies in which specific imidazole inhibitors were used (14), and more recently from a comparison of the amino acid sequences in chymotrypsinogen and trypsinogen (15–20). The present results are in agreement with this conclusion, the ionization in question being of the type

$$\text{N}^+\text{—H} \rightarrow \text{N} + \text{H}^+,$$

Neutral group

the active species being the neutral group.

The group of pK ≈ 9 (in water) has previously been postulated to be the α-ammonium group of the N-terminal isoleucine residue, the evidence being that, when this group is acetylated, α-chymotrypsin is inactivated (21–24). From this study, the group appears to be neutral because of the very considerable effect of dielectric constant on the ease of its ionization. It is possible, however, in view of the proposal that this group plays a

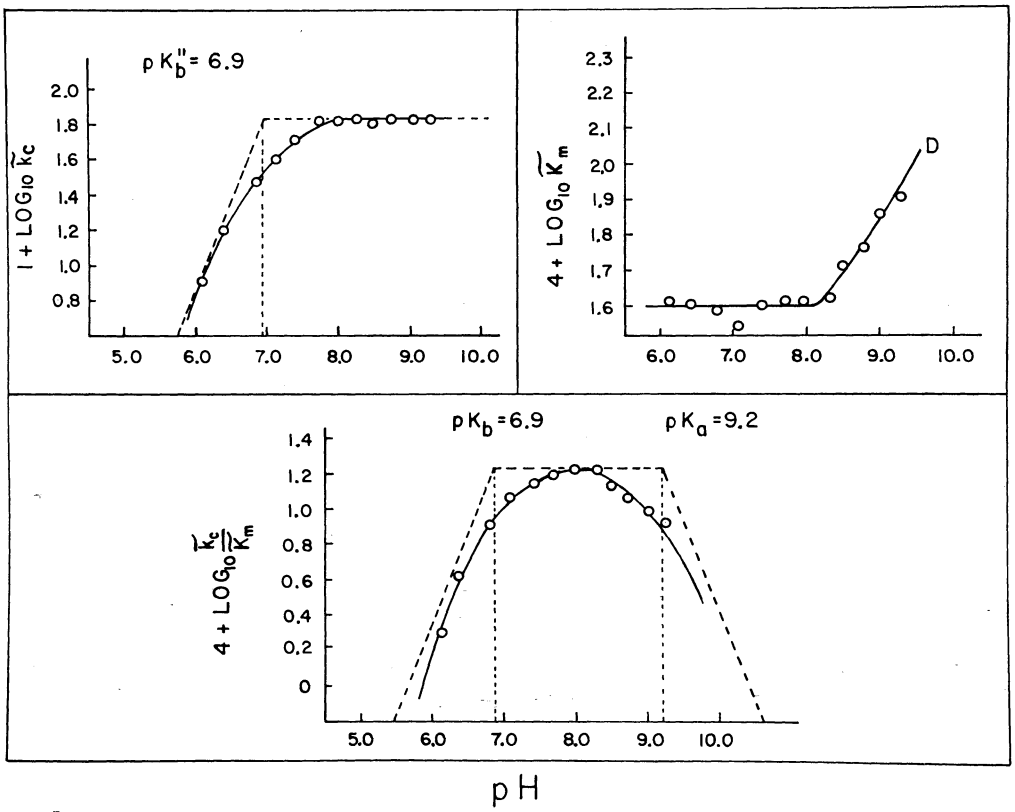


FIG. 5. Plot of $\log_{10} \tilde{k}_c$, $\log_{10} \tilde{K}_m$, and $\log_{10} \tilde{k}_c/\tilde{K}_m$ for the α-chymotrypsin-catalyzed hydrolysis of *N*-benzoyl-D-alanine methyl ester in water. $T = 20.0$ °C; 0.10 *N* NaCl.

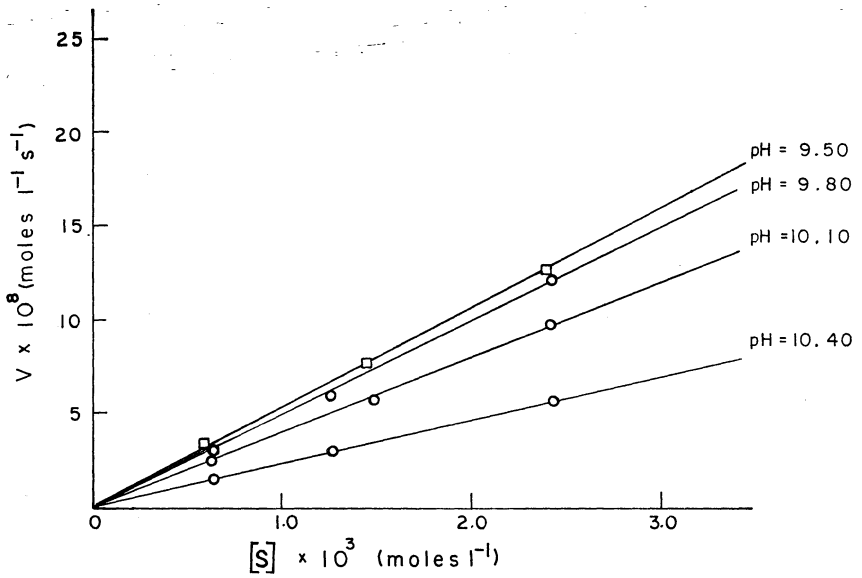


FIG. 6. Plots of rates against [S] for the hydrolysis of *N*-benzoyl-L-alanine methyl ester in dioxane (13.3%) – water (v/v).

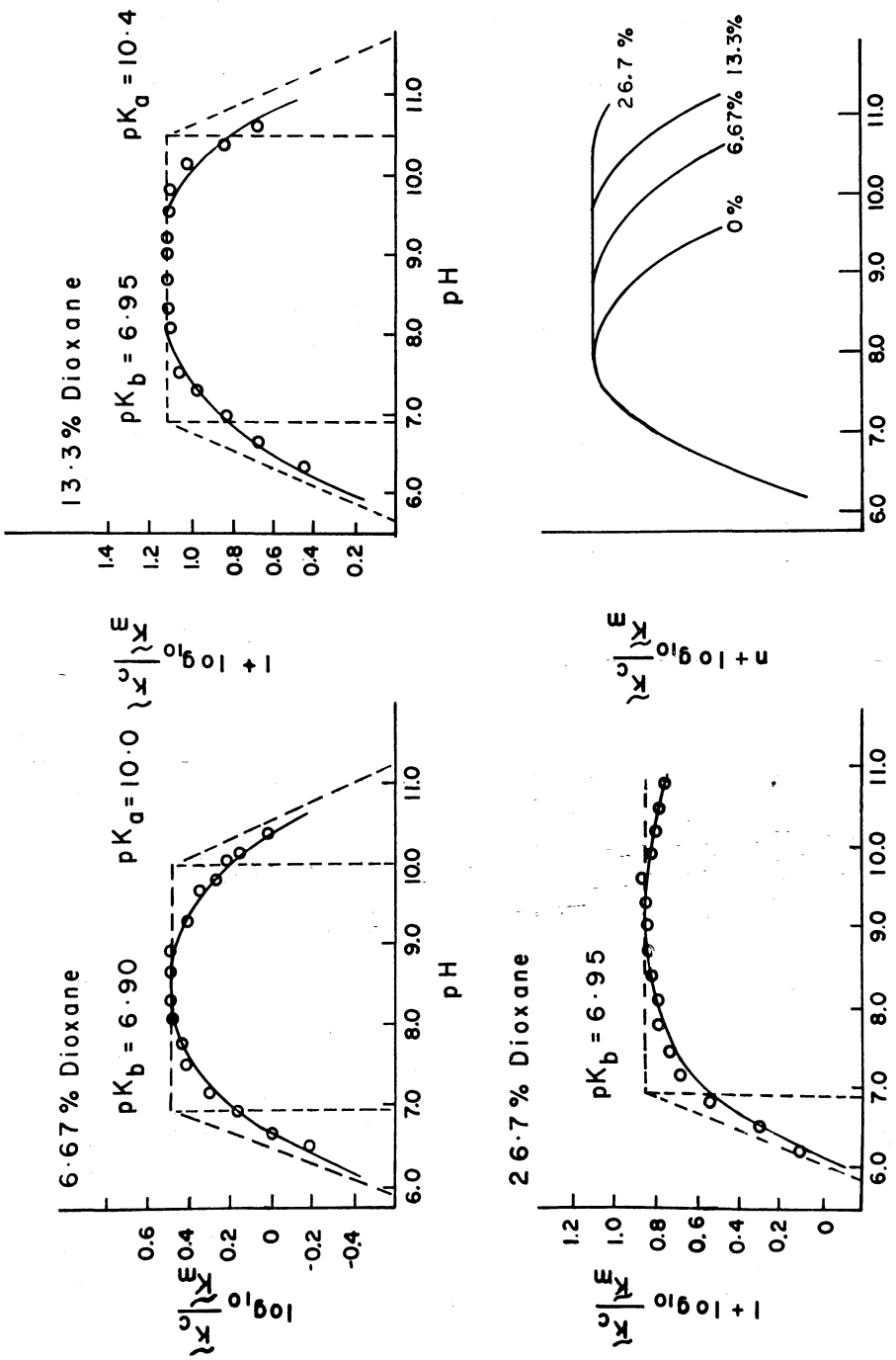


FIG. 7. Logarithm of $k_c/\bar{K}_m$ plotted against pH for *N*-benzoyl-L-alanine methyl ester in dioxane (6.67, 13.3, and 26.7%) – water (v/v), and the superposition of these curves. T = 20.0 °C; 0.10 *N* NaCl.

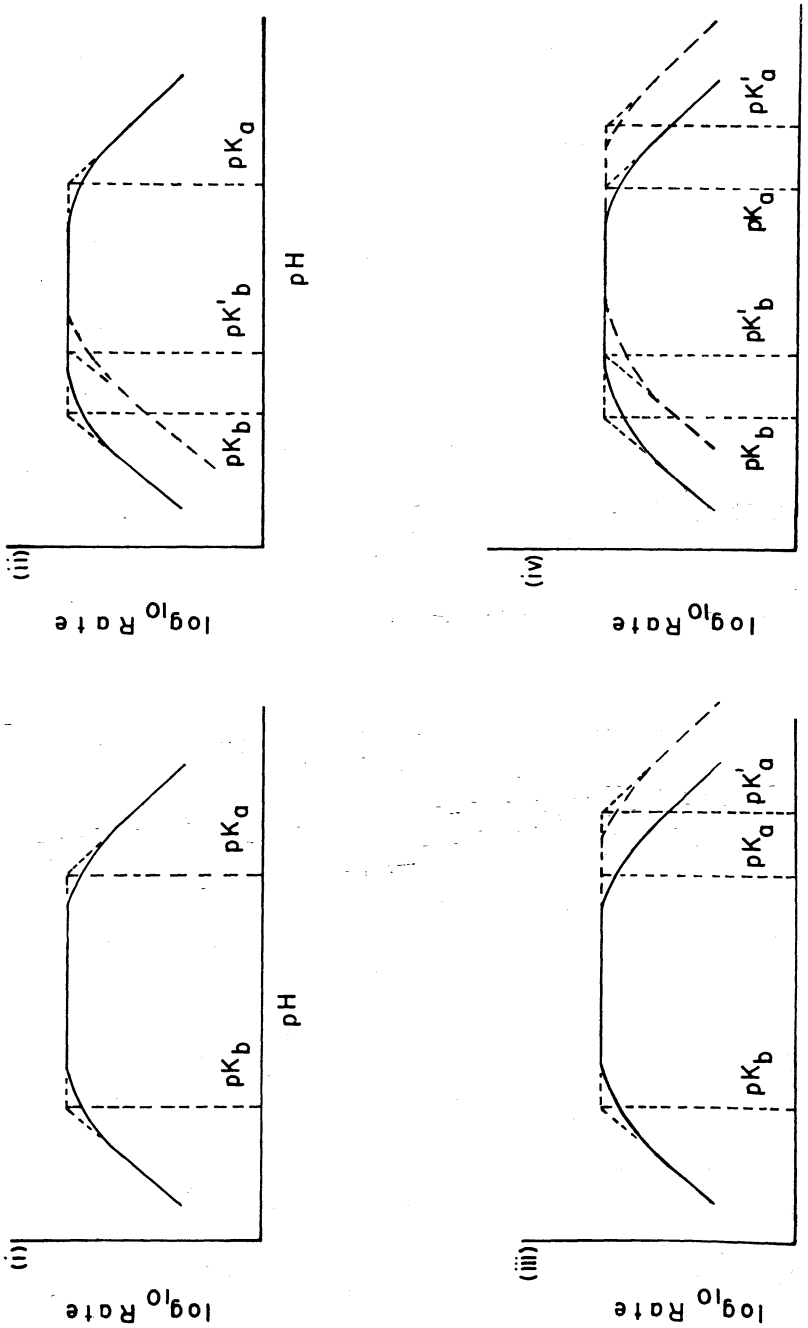


FIG. 8. Theoretical pH (water) – activity curves for the various possible ionizing pairs in water (solid lines) and in a dioxane–water mixture (broken lines).

conformational role (25), that a mixed solvent could perturb the pK_a of a cationic group if the group were only partially accessible to the solvent.

*Temperature Dependence of the Hydrolysis of N-Benzoyl-*D*- and -*L*-Alanine Methyl Esters*

Hein and Niemann (26) were the first to demonstrate the stereospecificity of α-chymotrypsin towards *N*-benzoyl-D- and -L-alanine methyl esters. From the pH dependence exhibited by these substrates, it appears that the rate-limiting step is deacylation, with the L-antipode being hydrolyzed approximately 17 times faster than the D-antipode, in spite of the fact that there is no chemical difference between the two forms of acyl enzyme. It was therefore considered to be of interest to determine whether this difference is associated with different energies or entropies of activation.

Figure 9 shows a plot of $\log_{10} k_3$ against $1/T$, and there is seen to be a break at 25 °C, with an apparent lowering of the activation energy at higher temperatures. This type of behavior has often been observed in enzyme reactions, and may be attributed to several factors (27). The possibilities relevant to the present case are: (*i*) the reaction might involve two intermediates, with each reaction step showing a different temperature coefficient; (*ii*) the reaction might be accompanied by the rapid and reversible denaturation of the enzyme.

Two intermediates are known to be involved, the reaction scheme being

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} ES \xrightarrow{k_3} E + P_2.$$
$$\searrow P_1$$

The kinetic parameters are given by

$$[1] \qquad k_c = \frac{k_2 k_3}{k_2 + k_3}$$

and

$$[2] \qquad K_m = \frac{k_3}{k_2 + k_3} \times \frac{k_{-1} + k_2}{k_1}.$$

The rate constants k_2 and k_3 may have different activation energies, so that one reaction becomes rate limiting in the low-temperature region and the other in the high-temperature region. If such were the case, the experimental curve at the inflection point is expected to lie 0.3 log unit below the intersection point of the individual Arrhenius lines. Figure 9 shows that the change in slope at high temperature is inconsistent with this explanation, the change of slope being much too sharp. Furthermore, if this explanation were applied, a similar inflection would be found in K_m. Figure 10 shows that such is not the case; K_m is independent of temperature over the entire temperature range studied for both the D- and L-compounds.

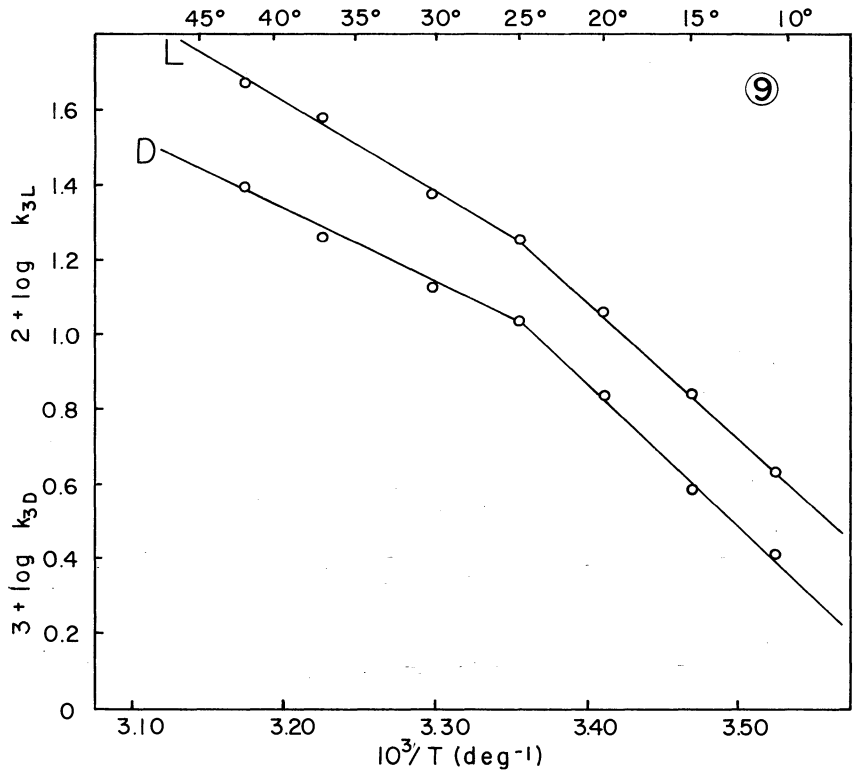
The second possibility is that the reaction is accompanied by a rapid reversible denaturation of the enzyme molecule. Bender and co-workers (1) have studied the irreversible denaturation of α-chymotrypsin and found it to be first order in enzyme concentration.

For the case in which $k_2 \gg k_3$,

$$[3] \qquad k_c = \frac{k_3}{1 + K},$$

where K, equal to $[E_{inactive}]/[E_{active}]$, is the constant for the equilibrium between the active and inactive enzyme. It is possible to calculate K from the amount the observed value lies below the expected value. A plot of log K against $1/T$ (Fig. 11) gives $\Delta H = 25$ kcal/mole and $\Delta S = 80$ e.u., which are reasonable values for denaturation phenomena. It is therefore reasonable to conclude in this case that the break in the plot of log k against $1/T$ (see Fig. 9) is due to a rapid reversible denaturation.

Of greater interest is the fact that in the low-temperature region the plots are linear and give activation energies of 16.2 ± 0.3 and 16.5 ± 0.6 kcal/mole for the D- and L-antipodes, respectively. This is a rather surprising result, in that it might have been expected that groups on the D-antipode may cause steric interference; there would then be a more highly strained activated complex and a higher activation energy. The difference in the rates of deacylation must therefore be due to a less favorable entropy of activation for the D-antipode.



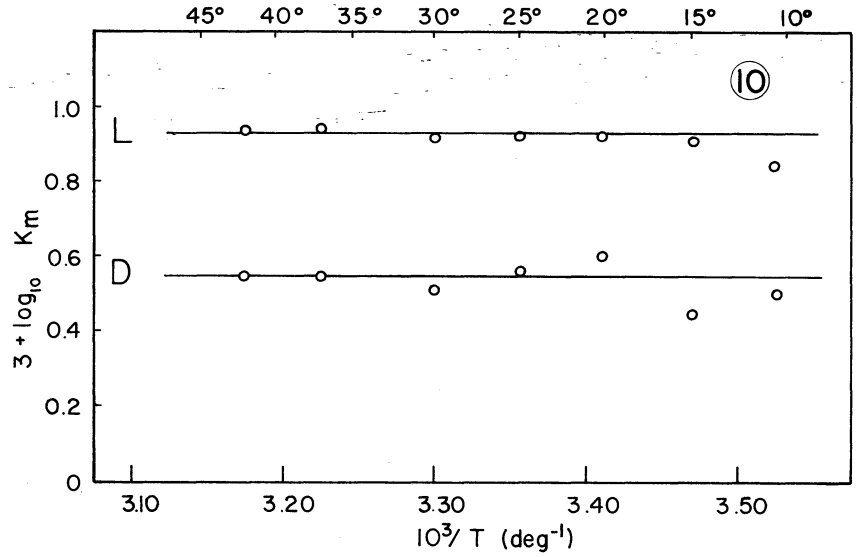


FIG. 9. Plot of $\log_{10} k_3$ against $1/T$ for *N*-benzoyl-D- and -L-alanine methyl esters.
FIG. 10. Plot of $\log_{10} K_m$ against $1/T$ for *N*-benzoyl-D- and -L-alanine methyl esters.

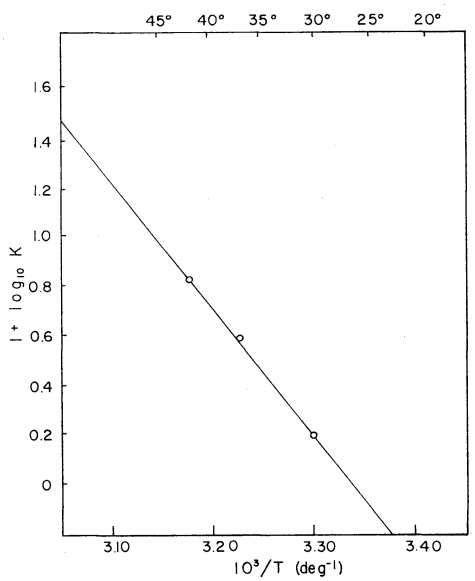


FIG. 11. Plot of $\log_{10} K$ against $1/T$.

The calculated entropies of activation are −9.6 e.u. and −14.1 e.u. for the L- and D-antipodes, respectively.

Since there is no chemical difference between the two substrates, the entropies of the ground states are the same in both reactions. The difference in entropy of activation must therefore arise from the different conformations of the enzyme when it is interacting with the D- and L-substrates in the activated complexes. In the activated complex of the D-antipode the enzyme is probably in a more extended form.

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