Trypsin-catalyzed Hydrolysis of *N*-Benzoyl-L-arginine Ethyl Ester at Low *p*H*

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ABSTRACT: The steady-state kinetics of the trypsin-catalyzed hydrolysis of *N*-benzoyl-L-arginine ethyl ester was investigated by automatic titration in the *p*H range 3.6–4.4. The results were interpreted in terms of a three-step mechanism, which involves enzyme-substrate formation, followed by acylation and deacylation of the enzyme. The final, rate-controlling deacylation step did not appear to require any new basic group; a logarithmic plot of the apparent deacylation rate constant against *p*H was linear over the range studied. A similar plot of the apparent Michaelis constant, however, was not linear, but showed a well-defined deflection in the vicinity of *p*H 4. This was taken as an indication that a basic group in the carboxylic acid region may be necessary for enzyme-substrate complex formation or acylation. Two models, showing the possible points of enzyme-substrate interaction, are considered.

An acid-base group that functions as part of an enzymic site may be detected kinetically by investigating the *p*H dependence of the apparent rate constant and determining its intrinsic *pK* (Laidler, 1958a). From a knowledge of the *pK* values of acid-base groups in amino acids and proteins, it is possible to postulate the kind of group that is functional. Bruice and Schmir (1959) discuss some of the limitations imposed upon this method for the case of zero-order kinetics. Their work suggests that the *pK* values found may be shifted somewhat from their normal values. However, the approach has been used with some success.

A study of the tryptic hydrolysis of *N*-benzoyl-L-arginine ethyl ester (Gutfreund, 1955a) revealed a *pK* of 6.25, which is in the range of an imidazole nitrogen. A similar result, under conditions where the limitations pointed out by Bruice and Schmir (1959) are not necessarily applicable, was obtained for trypsin with *p*-nitrophenylacetate (Stewart and Ouellet, 1959). From their data, it may be said that the imidazole group is free to function in native trypsin and its acylated derivatives, but not in the Michaelis complex. Recent degradation studies (Walsh *et al.*, 1964) have disclosed that trypsinogen contains at least two histidyl residues. The authors proposed that the imidazole group of both these residues may be involved in the mechanism of catalysis.

The mechanism of tryptic catalysis has been shown (Stewart and Ouellet, 1959) to conform to the scheme proposed for chymotrypsin (Hartley and Kilby, 1954). The scheme is comprised of three consecutive elementary reactions, which can be referred to as Michaelis or enzyme-substrate complex formation, acylation, and deacylation, respectively, as shown in Figure 1.

The present work was undertaken to extend the *p*H-dependent studies of trypsin catalysis to the carboxyl region. In order to substantiate the validity of the results, the highly specific substrate *N*-benzoyl-L-arginine ethyl ester was selected over a nonspecific type. The need for this study was suggested by the fact that the amino acid sequence at the acylation site for trypsin, as well as for other hydrolytic enzymes, appears to consist of a dibasic acid residue linked to a functional seryl (Boyer, 1960). Degradation studies have shown consistently that the hydroxyl group of the seryl is active toward acylation (Cohen *et al.*, 1959). However, the carboxyl group of the dibasic acid residue, aspartyl or glutamyl, has not been identified with hydrolytic activity, although the latter possibility has been discussed (Cohen *et al.*, 1959), and the requirement of an anionic group is implicated by studies with chymotrypsin (Barnard and Laidler, 1952). Preliminary kinetic studies on chymotrypsin at low *p*H (Dickie and Stewart, 1961) added further support to this idea.

Experimental

Materials. The enzyme and substrate were purchased from Sigma Chemical Co., St. Louis, Mo. Salt-free trypsin was twice crystallized and assayed with *N*-benzoyl-L-arginine ethyl ester. The trypsin solutions, prepared by dissolving a weighed quantity in 0.001 N HCl (*p*H 3), were stored in a refrigerator at 5° and could be kept for several weeks without loss in activity. Ion-free water, used in the preparation of all solutions, was made by passing distilled water through a demineralizer unit equipped with a conductivity tester, manufactured by Ion-Exchange Products, Inc., Chicago, Ill. The water was boiled for 10 minutes to eliminate

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carbon dioxide and other dissolved gases and stored in polyethylene bottles to prevent contamination.

Equipment. The instrumental technique employed was automatic titration. The control unit was a Radiometer TTT1 equipped with a magnetic relay and an automatic temperature compensator. The titrator assembly was constructed in this laboratory. It consisted of an aluminum base, which supported a 1-ml tuberculin syringe to hold titrant, a motor-controlled feed mechanism to deliver titrant, and a four-digit counter. The reading on the counter, whose last digit represents 0.003 mm travel of the syringe plunger, was sensitive to 5×10^{-5} ml.

Procedure. The kinetic measurements were made with the apparatus described. A desired quantity of substrate was diluted with ion-free water. Sufficient sodium chloride was added to level the ionic strength effects; the final concentration was 0.1 M. The substrate solution was adjusted to the *p*H at which the reaction was to be studied, and enzyme solution at the same *p*H was added to start the reaction. The rate of addition of standard sodium hydroxide was measured by reading from a digital counter the volume added during 1-minute intervals. The final concentration of enzyme used throughout this experimental work was 0.1 g/liter, and the substrate concentration was varied from 1×10^{-4} to 2×10^{-3} M. The temperature was held at 25°.

Results

Treatment of the Rate Data. The apparent rate of hydrolysis was obtained for each run from the slope of a plot of equivalents of sodium hydroxide added versus time in seconds. The apparent rate was corrected to the true rate by the relationship:

$$\text{true rate} = \text{apparent rate}\left(1 + \frac{[H^+]}{K_a}\right) \qquad (1)$$

where $[H^+]$ is the concentration of hydrogen ion and K_a is the dissociation constant for *N*-benzoyl-L-arginine acid, which was found to be 8.1×10^{-4} in water, and is in reasonable agreement with the value in dioxane-water mixtures (Inagami and Sturtevant, 1960).

The inverse rate, under steady-state conditions for a three-step mechanism (Gutfreund, 1955b), is given by the expression:

$$\frac{1}{\text{rate}} = \frac{1}{k'_M k'_2 E_o} + \frac{K'_m}{k'_2 E_o S_o} \qquad (2)$$

where

$$k'_M = \frac{k'_3}{k'_2 + k'_3} \qquad (3)$$

$$K'_m = \frac{k'_{-1} + k'_2}{k'_1} \qquad (4)$$

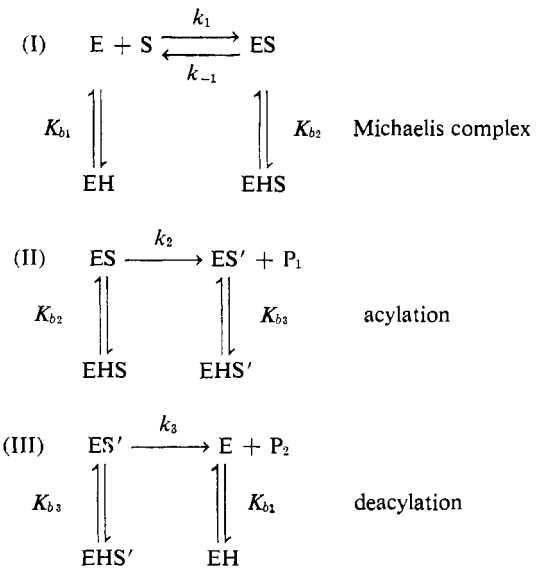

FIGURE 1: Mechanism for hydrolytic enzyme catalysis (Hartley and Kilby, 1954). E = enzyme, S = substrate, ES = enzyme-substrate complex, ES′ = acylated enzyme, P_1 = alkoxyl moeity of ester, and P_2 = acylate ion. EH, EHS, and EHS′ are the protonated forms of the enzyme, enzyme-substrate complex, and acylated enzyme, respectively, and K_{b1}, K_{b2}, and K_{b3} are the corresponding equilibrium constants of the basic groups involved.

and in general,

$$k'_i = k_i/(1 + [H^+]/K_{b_i}) \qquad (5)$$

except for k'_{-1}. In the case of k'_{-1},

$$k'_{-1} = k_{-1}/(1 + [H^+]/K_{b_2}) \qquad (6)$$

The equations (5) and (6) give the relationship between the true rate constant k_i and the apparent rate constant k'_i. The manner in which the k_i and K_{b_i} constants are assigned to the three-step mechanism is shown in Figure 1. Since the results for a series of *N*-benzoyl-L-arginine esters indicate that deacylation is rate controlling (Neurath and Schwert, 1950), $k'_2 > k'_3$, and

$$\frac{1}{\text{rate}} = \frac{1}{k'_3 E_o} + \frac{K'_m}{k'_2 E_o S_o} \qquad (7)$$

This means that a plot of 1/rate versus $1/S_o$ should give a straight line whose intercept is $1/k'_3 E_o$ and slope is $K'_m/k'_2 E_o$. This equation was used to evaluate the data shown in Table I.

As a matter of convenience, the data are plotted in Figures 2, 3, and 4, according to well-established methods (Laidler, 1958a). Figure 2 is a plot of the deacylation rate constant k'_3 on logarithmic scale versus *p*H. Since the plot is linear in this *p*H range no

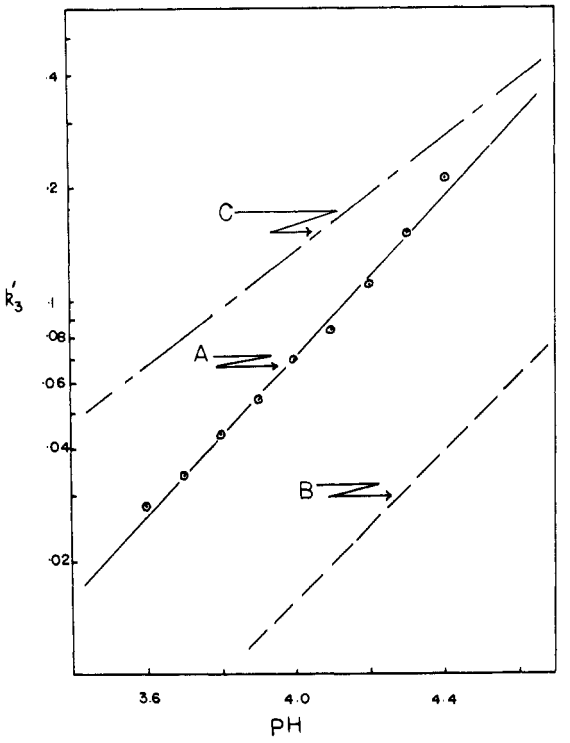

FIGURE 2: Plot of the apparent deacylation rate constant, k'_3, versus pH, log scale. (A) Trypsin-*N*-benzoyl-L-arginine ethyl ester system; (B) theoretical slope of unity; (C) chymotrypsin-*N*-acetyl-L-tryptophan ester systems (Kézdy and Bender, 1964a,b).

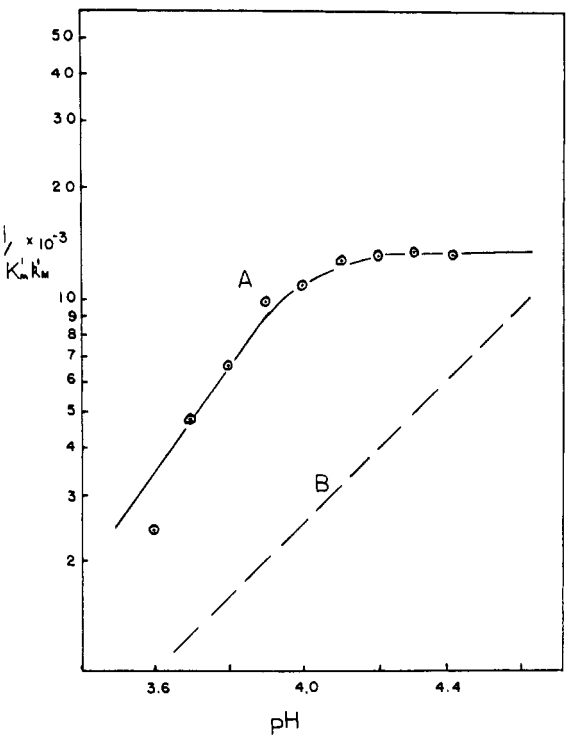

FIGURE 3: Plot of the apparent Michaelis constant, $1/K'_mk'_M$ versus pH, log scale. (A) Trypsin-*N*-benzoyl-L-arginine ethyl ester system; (B) theoretical slope of unity.

TABLE 1: Influence of pH on the Steady-state Kinetic Constants for the Trypsin-catalyzed Hydrolysis of *N*-Benzoyl-L-arginine Ethyl Ester.

pH	k'_3 ($\times 10^2$)	K'_m/k'_2 ($\times 10^4$)	$K'_mk'_M$ ($\times 10^5$)
4.4	21.5	3.58	7.70
4.3	15.2	5.02	7.64
4.2	10.9	7.10	7.73
4.1	8.3	9.68	8.03
4.0	6.9	13.5	9.32
3.9	5.4	20.7	11.1
3.8	4.4	34.1	15.0
3.7	3.4	62.4	21.1
3.6	2.8	150.	41.5

new ionizable group is involved in deacylation. However, when log $1/K'_mk'_M$ is plotted against pH as illustrated in Figure 3, a functional group does appear with a pK 4, as exemplified by a change of slope from zero to approximately unity. It should be noted that the plot in Figure 4 is independent of the corrections made according to equation (1).

Discussion

The influence of the hydrogen-ion concentration on the tryptic hydrolysis of *N*-benzoyl-L-arginine ethyl ester at moderate pH values was investigated previously under steady-state conditions (Gutfreund, 1955a). Since the initial substrate concentration was 3×10^{-3} M, $S_o > K'_mk'_M$ (see Table I), equation (2) under this condition reduces to:

$$\text{rate} = k'_Mk'_2E_o \qquad (8)$$

But $k'_2 > k'_3$ for esters, so that equation (8) simplifies further:

$$\text{rate} = k'_3E_o \qquad (9)$$

It is seen from Figure 1 that k'_3 is the rate constant for deacylation, and its pH dependence can be used to determine K_{b_3}. This ionization constant, K_{b_3}, therefore, will be related to a "free" functional basic group in acyl-trypsin, which aids in the removal of the acyl group (*N*-benzoyl-L-arginyl) from the enzyme surface. The reported pK of 6.25 was assigned to an imidazole group (Gutfreund, 1955a). The existence of two imidazole groups in trypsinogen is indicated by degradation studies (Walsh *et al.*, 1964). Since at low pH the present results in Figure 2 for deacylation are linear

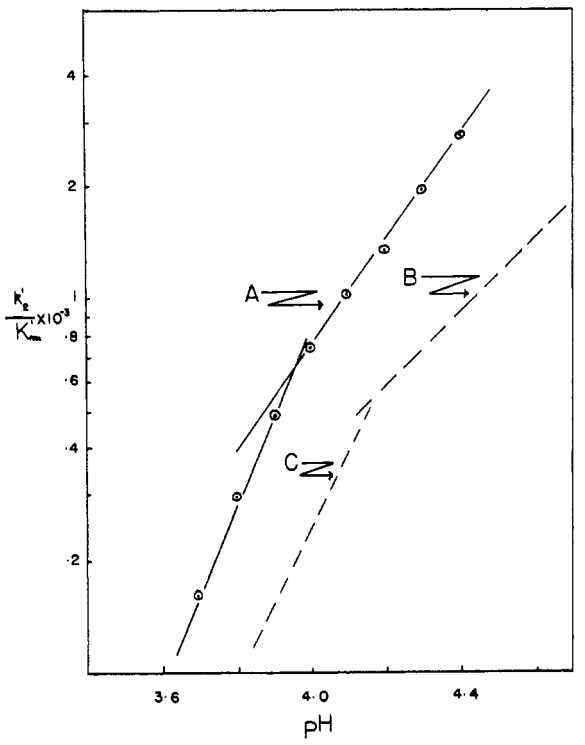

FIGURE 4: Plot of k'_2/K'_m versus pH on logarithmic scale. (A) Trypsin-*N*-benzoyl-L-arginine ethyl ester; (B) theoretical slope of unity; (C) theoretical slope of two.

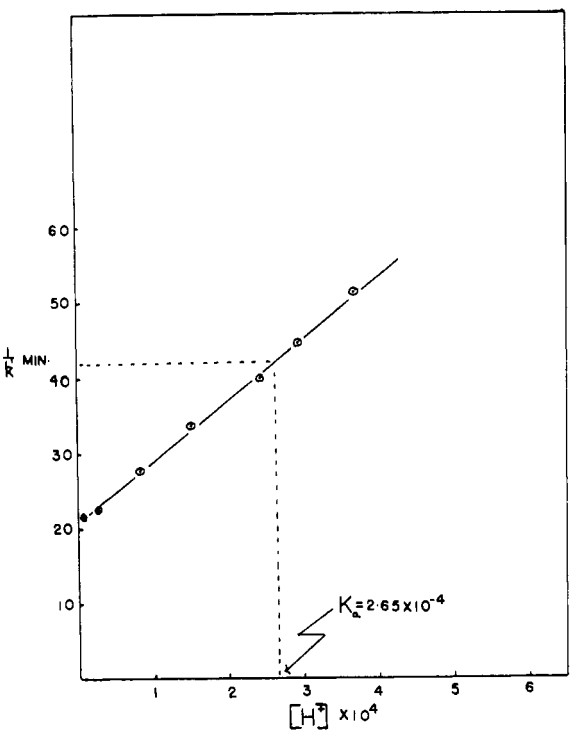

FIGURE 5: Kinetic determination of the K_a of the intramolecular, catalytic, carboxyl group in chloroacetylsalicylic acid (J. Knoblich, unpublished results, Jamestown College, Jamestown, N.D., 1963). The apparent rate constant, k', is for spontaneous hydrolysis in 10% dioxane-water solution.

and conform to a slope near unity as predicted by theory (Laidler, 1958a), no basic group, other than the imidazole suggested by Gutfreund, is involved in the final stage of tryptic catalysis. This finding is supported by similar deacylation studies at low pH for chymotrypsin (Kézdy and Bender, 1964a,b; see Figure 2).

The foregoing result should be contrasted with the plot in Figure 3 of log $1/K'_m k'_M$ versus pH. This plot shows a deflection in the vicinity of pH 4, which suggests an additional basic group, besides imidazole, is functional in either the enzyme or the enzyme-substrate complex.

The values of K'_m/k'_2 given in Table I are plotted as the log k'_2/K'_m versus pH in Figure 4. This plot at pH 4 shows a change of slope from approximately one to two, which conforms to theory (Laidler, 1958a). It has been suggested (Gutfreund, 1955a; Laidler, 1958b) that k'_2/K'_m plot may deflect at pH 6–7, because of the functional imidazole group. This belief is supported by the present study. Above pH 4 the value of $K'_m k'_M$ becomes constant. The limiting value in Table I is 7.7×10^{-5} M, which is in good agreement with K_m (classical) 8×10^{-5} M determined at pH 8 (Neurath and Schwert, 1950). Since $K'_m k'_M = K'_m k'_3/k'_2$ = constant, and k'_3 deflects at pH 6.25, it follows that a plot of log k'_2/K'_m versus pH will deflect at 6.25. Thus besides a basic group at pK 4, a second ionizable group with a pK of 6.25 is inherent in the constants k'_2/K'_m. Since k'_2/K'_m is a composite of rate constants, the assignment of these pK values to the scheme in Figure 1 is not a straightforward matter. However, without further consideration, since k'_3 is not involved, the assignment can be narrowed down to the native enzyme and the enzyme-substrate complex.

For systems of the trypsin or chymotrypsin–amino acid ester type, there is reason to believe that K'_m is at times the equilibrium constant for the enzyme-substrate complex (Neurath and Hartley, 1959). This means that $k'_{-1} > k'_2$ and

$$\frac{K'_m}{k'_2} = \frac{k'_{-1}}{k'_1} \cdot \frac{1}{k'_2} \qquad (10)$$

If the pK vaues of the functional groups occur in k'_{-1}, then according to equation (5) a plot of log K'_m/k'_2 versus pH would be in order rather than k'_2/K'_m as in Figure 4. This infers that either k'_1 or k'_2, or both, must be pH dependent. In order to discuss this situation further, it is necessary to draw on information concerning K'_m and k'_2 obtained from transient-phase studies with substrates other than *N*-benzoyl-L-arginine ethyl ester, and to examine systems involving chymotrypsin catalysis, which parallel trypsin.

The ratio K'_m/k'_2 may be resolved by transient-phase

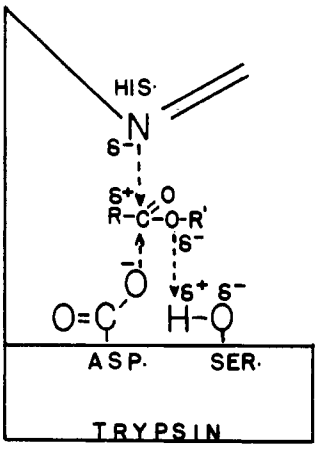

FIGURE 6: Enzyme-substrate complex model I. Large alphabetical letters represent the enzyme, small letters the substrate, and dashed arrows the direction of nucleophilic attack.

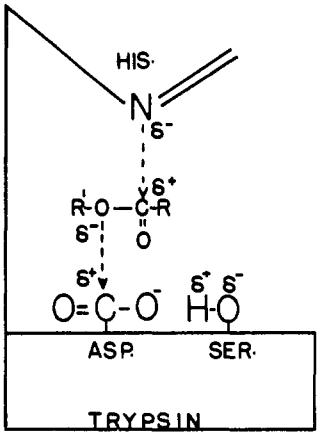

FIGURE 7: Enzyme-substrate complex model II. Large alphabetical letters represent the enzyme, small letters the substrate, and dashed arrows the direction of nucleophilic attack.

kinetics (Ouellet and Stewart, 1959). This has been done for trypsin using the substrate *p*-nitrophenylacetate (Stewart and Ouellet, 1959). It was found that k'_2 was *p*H independent within experimental error, but K'_m was dependent upon a basic group (*pK* 6.9) such as imidazole. In this instance, a plot of K'_m versus $[H^+]$ was linear, so that the ionizable group must be free to function in the native enzyme, i.e., k'_1 is *p*H dependent. There is no reason to believe, however, that this work with *p*-nitrophenylacetate should validate work with *N*-benzoyl-L-arginine ethyl ester. Similar work in the literature on *p*-nitrophenylacetate with chymotrypsin cannot be used in support as the evidence is conflicting. The initial study of chymotrypsin with *p*-nitrophenylacetate indicated that the acetylation rate constant k_2 was *p*H independent (Gutfreund and Sturtevant, 1956a), whereas similar work by the same authors (Gutfreund and Sturtevant, 1956b) with a different substrate, 2,4-dinitrophenylacetate, indicated that k'_2 was *p*H dependent. Recently, the *p*-nitrophenylacetate system was reinvestigated (Kézdy and Bender, 1962), and both k'_2 and K'_m were reported to be *p*H dependent. Therefore the imidazole group may be functional not only in the native enzyme and acyl-enzyme, but also in the enzyme-substrate complex. This latter behavior brings ester studies into line with amide studies (Gutfreund and Sturtevant, 1956a).

The prime concern of this paper is to characterize, not the imidazole, but rather another basic group whose *pK* of 4 is in the carboxylic acid region. Since for the present trypsin system this basic group appears in the ratio K'_m/k'_2, which increases with decrease in *p*H according to Table I, it follows that the *pK* of this group must be in k'_1 or k'_2, or both. Work on the transient-phase kinetics of 2,4-dinitrophenylacetate with chymotrypsin at low *p*H has revealed a *pK* of 4 in K'_m, but not in k'_2 (Stewart *et al.*, 1963). Since in this case a plot of K'_m versus $[H^+]$ was linear, then according to the inverse of equation (5), this functional group must be free in the native chymotrypsin.

An examination of the deflection in Figure 4, and equation (10) show that there is a *pK* of 4 intrinsic in k'_1 or k'_2. Since work with chymotrypsin did not result in a *p*H-dependent k_2 at low *p*H (Stewart *et al.*, 1963), a tentative assignment can be made to k'_1. This would mean that a basic group with a *pK* of 4 is free to function in the native enzyme.

For two reasons, this basic group may be assigned to a carboxyl: (a) carboxyl groups in proteins display *pK* values in the range 3.0–4.7 (Cohn and Edsall, 1943), and (b) several hydrolytic enzymes have a dibasic acid condensed in sequence with the functional seryl residue (Cohen *et al.*, 1959).

Model systems involving intramolecular carboxyl catalysis seem to lend some support to the above low-*p*H studies. For example, the compound *o*-chloroacetylsalicylic acid undergoes hydrolysis to yield chloroacetic acid and salicylic acid. If the *o*-carboxyl group of salicylic acid enhances hydrolysis, then a plot of $1/k'$ versus $[H^+]$ according to the inverse of equation (5) should produce a straight line with a slope of $1/kK_a$ and an intercept of $1/k$, where k' is the apparent rate constant and k the true rate constant for the rate-controlled step, and K_a is the ionization constant for the *o*-carboxylic acid group in *o*-chloroacetylsalicylic acid. The plot in Figure 5 gives the kinetic results for chloroacetylsalicylic acid hydrolysis in 10% dioxane-water mixtures at several hydrogen ion concentrations (J. Knoblich, unpublished results, Jamestown College, Jamestown, N.D., 1963). These results, obtained by automatic titration methods, give a pK_a of 3.58. This value is in good agreement with the actual pK_a of chloroacetylsalicylic acid, which has been reported as 3.62 in 10% dioxane-water mixtures (Marko, 1964). Similar agreement for an intramolecular functional carboxyl group has been reported for the hydrolysis of phthalamic acid (Bender *et al.*, 1958).

If enzymic *p*H studies are as valid as those for model systems involving an intramolecular catalytic carboxyl group, then it is possible to write some plausible complexes to account for the involvement of an ionizable carboxyl group. It should be realized, however, that *p*H studies on enzymes may be related to changes in structural conformation, and, alone, they should not be taken as positive evidence. Outside support is obtained for the present finding from several sources. (a) The deacylation kinetics as shown in Figure 2 is perfectly normal. (b) An aspartyl residue is known to exist at what is believed to be part of the active site of trypsin. (c) Trypsin is not denatured at low *p*H and can be stored at *p*H 3 for long periods without loss in activity. (d) Chymotrypsin, which behaves similarly to trypsin, exhibits the same phenomenon at low *p*H.

In summary, it appears that both an imidazole group and a carboxyl group are required for enzyme-substrate formation. Therefore the initial reaction in Figure 1 may be written in a more detailed manner. As shown in Figures 6 and 7, the substrate could react with the enzymic site in at least two ways. In the first model, illustrated in Figure 6, the carbonyl carbon of the substrate interacts with the imidazole nitrogen and the carboxylate anion oxygen; this could lead to an anhydride intermediate. The second model, in Figure 7, is an interchange mechanism that involves the concerted interaction of the carboxyl carbon and imidazole nitrogen of the enzyme on the substrate ester bond. Although the first model is the more conventional one, both models should be considered until further experimental evidence is available. In any event, the serine oxygen of either model can act as a nucleophilic center and become acylated during the next stage of catalysis.

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