The Protonic Equilibrium Accompanying the Acylation of Chymotrypsin and Subtilisin*

Joel Keizer† and Sidney A. Bernhard

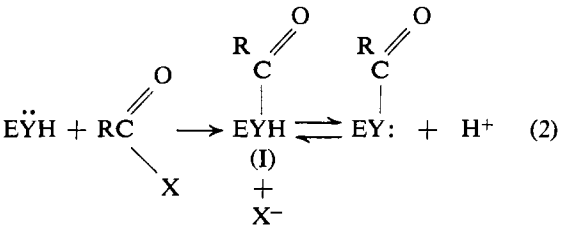
ABSTRACT: The number of protons consumed or released as a consequence of acylation of the active sites of α-chymotrypsin and the bacterial proteinase subtilisin has been examined over a wide range of pH, utilizing the acylating reagent *N*-β-(3-indole)acryloylimidazole. After correction for the protonic equilibrium associated with the imidazole product (ImH + H^+ $\rightleftharpoons$ ImH_2^+) according to the equation, AcIm + EH_m → $AcEH_{m-(n+1)}$ + ImH^0 + nH^+. The yield of protons/(equivalent of reaction), *n*, varies between 0 and −1, depending on the pH. These results can be quantitatively correlated with the disappearance of one proton-dissociable enzyme constituent residue, and the perturbation in pK_A of a second such residue as a consequence of acylation, above pH 6. At lower pH (~4 there is an apparent perturbation in still another pK_A upon acylation. The common results with two enzymes of grossly different structure suggest that these changes in protonic equilibria are related to the catalytic mechanism.

These *stoichiometric* reactions directly involve a proton-dissociable species in the acyl enzyme with pK_A identical with pK_D (the "apparent" pK_A governing catalytic hydrolysis of the acyl enzyme), a variable which is dependent on both the structure of the acyl moiety and on the particular enzyme. These results eliminate some previously postulated models of catalysis. Allowable models of catalysis are discussed.

Previously (Bender *et al.*, 1962; Caplow and Jencks, 1962) and in an accompanying communication (Bernhard *et al.*, 1966b), the influence of pH on the first-order specific rate of hydrolysis of acylchymotrypsins has been discussed. The pH dependence is described by eq 1

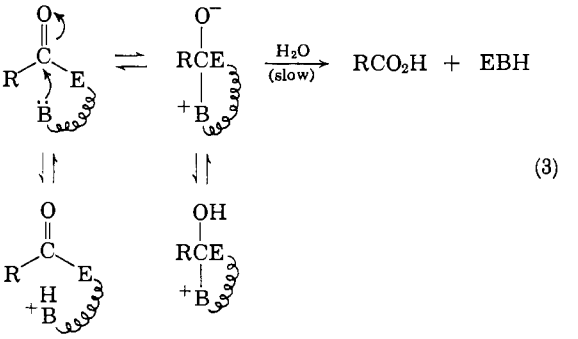
$$k_{(obsd)} = \frac{k_d K_D}{K_D + [H^+]} \qquad (1)$$

where K_D is an "apparent" protonic dissociation constant.[1] The magnitude of this dissociation constant depends on the particular electronic structure of the acyl group (Bernhard *et al.* (1966b) *vide supra*). The variability in the dissociation constant is sizable and is regularly dependent on the electronic structure of the acyl group. It is significant to inquire into the origins of this variability, since it may presumably bear on the chemical nature of the participating amino acid residues involved in the enzymic catalysis. Essentially, two types of mechanisms have been proposed for the origins of the variability of K_D with acyl structure: (a) a chemical "coupling" of the acyl group with a dissociable protonated atom in acyl enzyme I, as given in eq 2, and (b) the formation of an intermediate which alters



$$\ddot{E}YH + RC(=O)X \longrightarrow \underset{(I)}{EYH}\,X^- \rightleftharpoons EY: + H^+ \qquad (2)$$

the protonic equilibrium in the reaction pathway from acyl enzyme to products; for example, eq 3. Bruice and Schmir (1959) suggest a variety of such intermediates on the basis of precedents in the catalyzed hydrolysis of esters in homogeneous solution. Under such



$$\xrightarrow[\text{(slow)}]{H_2O} RCO_2H + EBH \qquad (3)$$

conditions, the *apparent* pK_D for enzymic activity may be markedly different from the known pK_A

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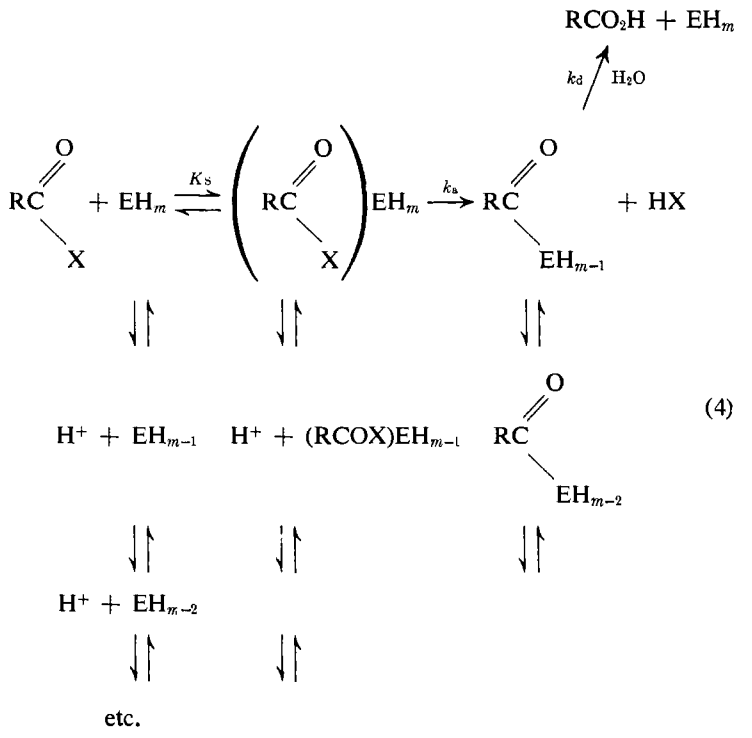
[1] Previously and in an accompanying communication, the term, "apparent" K_A or K_A(app), has been designated. Since we shall refer to a variety of titrimetrically determined K_A values we designate this apparent dissociation constant K_D, as inferred from the pH-dependent kinetic data.



(4)

for any catalytically essential weak acid residue of the (acyl) enzyme protein (Bruice and Schmir, 1959). Hence, the variability of K_D may be accounted for in terms of a variability in the relative concentrations of intermediates, dependent on the particular acyl group.

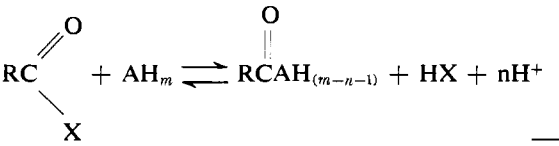
Such mechanisms should be differentiable by measurements of the stoichiometry of proton release accompanying the acylation and deacylation reactions (eq 4), where K_S is the substrate dissociation constant of the initial complex ($H_mE(RCOX)$). Mechanisms of the type illustrated by eq 2 might be expected, *a priori*, to be reflected by a protonic release upon acylation, dependent on the K_D of the particular acyl enzyme (as determined from the hydrolytic activity measurements). Moreover, the actual number of protons absorbed or liberated in the acylation reaction should indicate the type of acyl acceptor involved in the reaction; for example, among the three most plausible protein-contained acyl acceptors, the number of protons liberated, under limiting conditions of pH, is given in Table I. It should be noted that the pK_A values for particular amino acid residues in polypeptide linkage are based on directly measured or derived values from studies of model peptides. In the actual enzyme protein some of these pK values (most particularly, carboxylic acid dissociations) may be seriously perturbed by adjacent charged groups or by the polarity of the localized polypeptide environment.

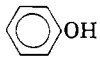
The mechanism of eq 3, on the other hand, leads to quite different expectations concerning the protonic equilibrium accompanying the acylation and deacylation reactions. Since in this mechanism the variability in apparent K_D for catalytic hydrolysis is a consequence of kinetic details occurring *after* acylation, one would not anticipate any regular correlation between the empirically determined K_D for hydrolytic activity of the acyl enzyme and the protonic equilibrium accompanying acylation. Moreover, the *number* of protons released per equivalent of reaction, either upon acylation or upon hydrolysis, should not reflect on the variability in K_D. The *rate* of hydrolysis (as determined from the proton release) should, however, be sensitive to K_D.

The results reported below deal with the protonic equilibrium accompanying the acylation reaction. These results distinguish unambiguously between the two potential mechanisms. As will be shown, the protonic equilibrium is completely in accord with the mechanism of eq 2.

For the derived results to be meaningful, certain kinetic conditions must be obtained. Specifically, (a) the two time-dependent steps in the mechanism (eq 4) must be sufficiently distinct in rate so as to yield a virtually stoichiometric quantity of the acyl enzyme intermediate (over a sufficiently long time period to permit measurement of the concomitant change in hydrogen ion concentration). The actual kinetic conditions are that $v_a \gg v_d$ ($k_aE_0 \gg\gg k_d$). (b) The pK_A values of any acid-dissociable nonenzyme products must be known. (The protonic equilibrium accompanying such acid dissociations should not overly complicate the measurement of net proton release.) On these bases, we selected the acylating agent, *N*-β-(3-indole)-acryloylimidazole, which has been discussed in the accompanying communication and elsewhere (Bernhard and Tashjian, 1965; Bernhard *et al.*, 1965).

TABLE I: Protonic Equilibrium Accompanying Acylation as a Function of Acyl Acceptor Properties.



Acyl acceptor	pK_A	n: pH << pK_A	n: pH >> pK_A
NH_3^+ (N-terminal and ϵ-amino groups)	7.5–9	1	0
CO^2H (C-terminal, β,γ-carboxylic groups)	3–6[a]	0	−1
 (histidine)	6–7	1	0
 (tyrosine)	9.5–10.5	0	−1
CH^cOH (serine hydroxyl)	13–13.6[b]	0	−1

[a] The pK_A values of carboxylic acids can be greatly perturbed by the polarity of the environment as well as by rigidly oriented proximal charges. For example, at a dielectric constant of 20 (aqueous dioxane) the dissociation constants are reduced by *ca.* three orders of magnitude (Harned and Owen, 1958). [b] Bruice *et al.* (1962) found the hydroxylic pK_A of *N*-acetylserine amide to be 13.6. Still lower pK_A values for this functional group in specific serine oligopeptides have been inferred from kinetic data (Bernhard *et al.*, 1964a).

Experimental Section

Materials. Preparation of *N-β-(3-indole)acryloylimidazole (IAI)* has previously been described (Bernhard and Tashjian, 1965). Fresh solutions of IAI (0.01 M) in spectrograde acetonitrile were prepared daily.

α-Chymotrypsin was a three-times recrystallized (Worthington Biochemical Co.) sample. It was utilized without further purification. Titration of the active sites with IAI gave a titer of 2.8 ± 0.1 × 10^4 g of enzyme/equiv of active site. Reaction solutions usually contained 3 ml of solvent (0.1 M KCl).

Bacterial proteinase Novo (Novo Industri, Copenhagen, Denmark) was a lyophilized salt-free sample. Active site titration gave a titer of 5.6 ± 0.3 × 10^4 g/equiv of active site. Gel filtration on Sephadex G-25 resulted in a separation into active high molecular weight (>20,000) protein and a trail of peptide fragments. The active component gave a titer of 3.2 ± 0.4 × 10^4 g/active site. No differences in either kinetic or spectrophotometric (acylchromophoric) properties could be detected between purified and unpurified samples. Reaction solutions were, therefore, prepared with 5 mg of unpurified enzyme/ml of 0.1 M KCl.

Apparatus. A "Cary 31 electrometer" with input from a "Cary pH accessory switch" was connected to a Sargent recorder. Beckman glass and calomel frit-junction electrodes were attached to the pH switch for pH and millivolt readings. The pH of the reaction was determined from the difference (in millivolts) between a standard buffer and this reaction mixture to a precision of 0.02 pH. An auxiliary switch added a potentiometer to the circuit, allowing the input voltage to be reduced to an arbitrarily low level (Figure 1). The high-sensitivity scales of the Cary 31 electrometer then permitted small changes in input voltage to be measured to a precision of ±0.0002 pH (~0.01 mv).

The reaction vessel with syringe is depicted in Figure 1. Nitrogen was pumped into the vessel to maintain low millivolt drift rates at high pH. A "Faraday cage" surrounded the entire reaction and electrode assembly. This was found to be essential for the minimization of stray potentials.

Proton Release Measurements. The reaction vessel, maintained at 25.0 ± 0.1°, contained 10 ml of α-chymotrypsin or Novo in 0.1 M KCl. The pH of the resultant solution, registered in millivolts on the Cary 31 electrometer, was adjusted to a predetermined value with either 0.1 N KOH (CO_2 free) or 0.1 N HCl.

With the potentiometer switched into the circuit and the electrometer operating on a 3-mv full-scale deflection (±0.02 mv), the millivolt drift rate of the enzyme solution was recorded. A few microliters of 0.0100 N KOH (CO_2 free) were then added and the change in the millivolt reading was recorded. At all pH values, the change in millivolts was found to vary linearly with the volume of KOH added, within this 3-mv range. This reading served as a calibration, *i.e.*, Δmv/mequiv of protons.

With the same circuit arrangement of the apparatus, a few microliters of acylating agent (*ca.* 10^{-2} M) were added *via* a micrometer-operated Agla syringe. An at least twofold excess of enzyme over added acylating agent was always maintained. The change in millivolts was recorded during the first (acylation) step of the

[2] Abbreviation used: IAI, *N-β*-(3-indole)acryloylimidazole.

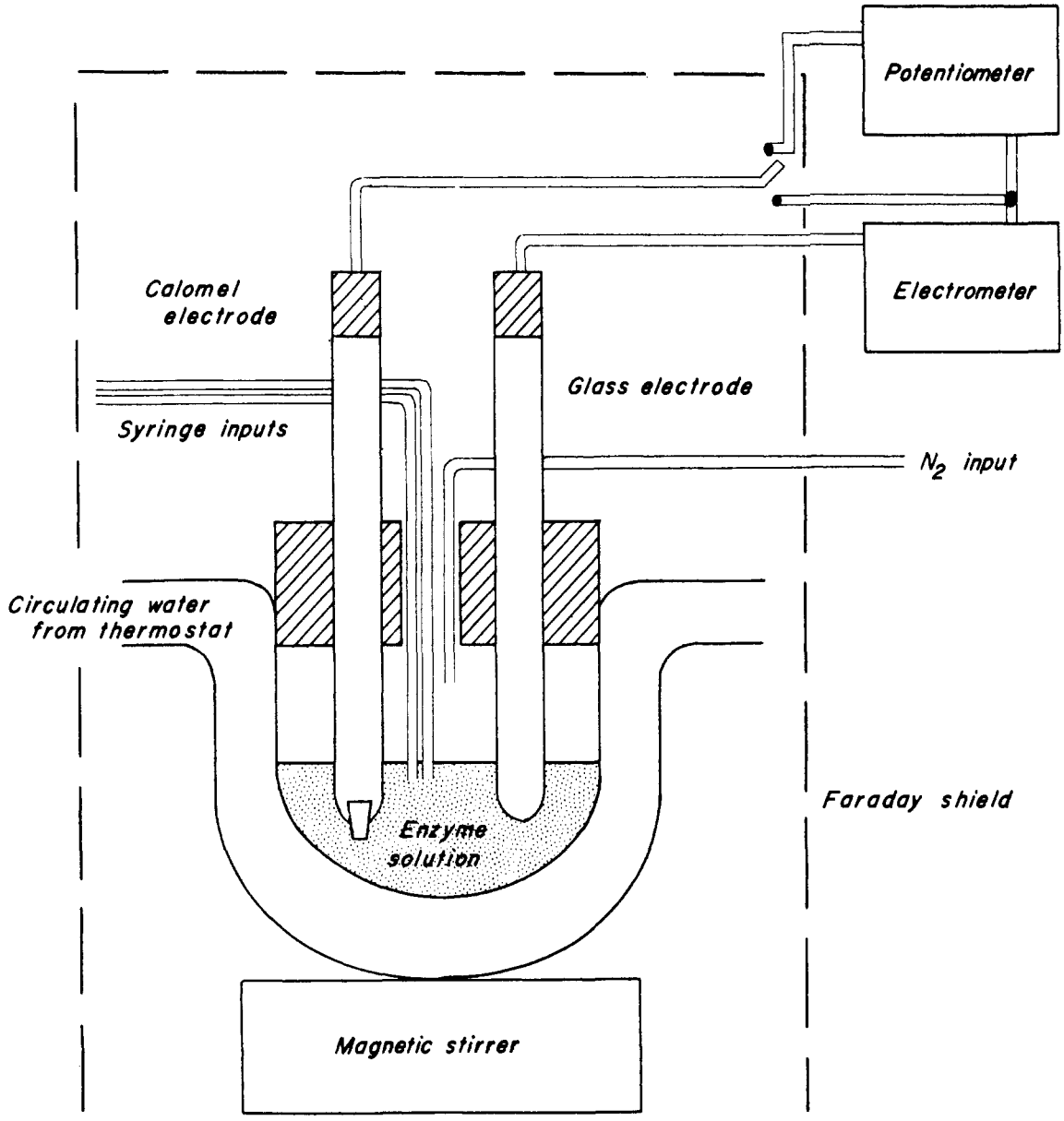


FIGURE 1: The reaction vessel and electronic equipment for measuring the release of protons in the acylation reactions described in the text are shown schematically. Polyethylene tubes conduct NaOH, HCl, substrate solution, and nitrogen into the vessel. With the potentiometer switched out of the circuit, direct measurements of pH can be made; with the potentiometer switched into the circuit, small changes in electrode voltage can be measured very accurately.

reaction. (At all pH values, the first step was at least 100 times faster than the second with α-chymotrypsin, and at least 20 times faster with Novo subtilisin.)

The ratio of milliequivalents of protons released/milliequivalent of acylating agent was obtained after correction for the pH-dependent association of protons with imidazole. A pK_A of 7.05 was taken for imidazolium$^+$ based on potentiometric titrations of low concentrations of imidazole in the same solvent and at the same temperature.

Results and Discussion

The kinetics of the reaction of α-chymotrypsin with IAI is accurately described by eq 4. With this acylating reagent, the rate of acylation is exceedingly faster than the subsequent rate of hydrolysis (or deacylation) of the acyl enzyme intermediate (Bernhard and Tashjian, 1965). Moreover, at any pH, the rate of deacylation is sufficiently slow so that the protonic equilibrium accompanying the acylation reaction can be studied in

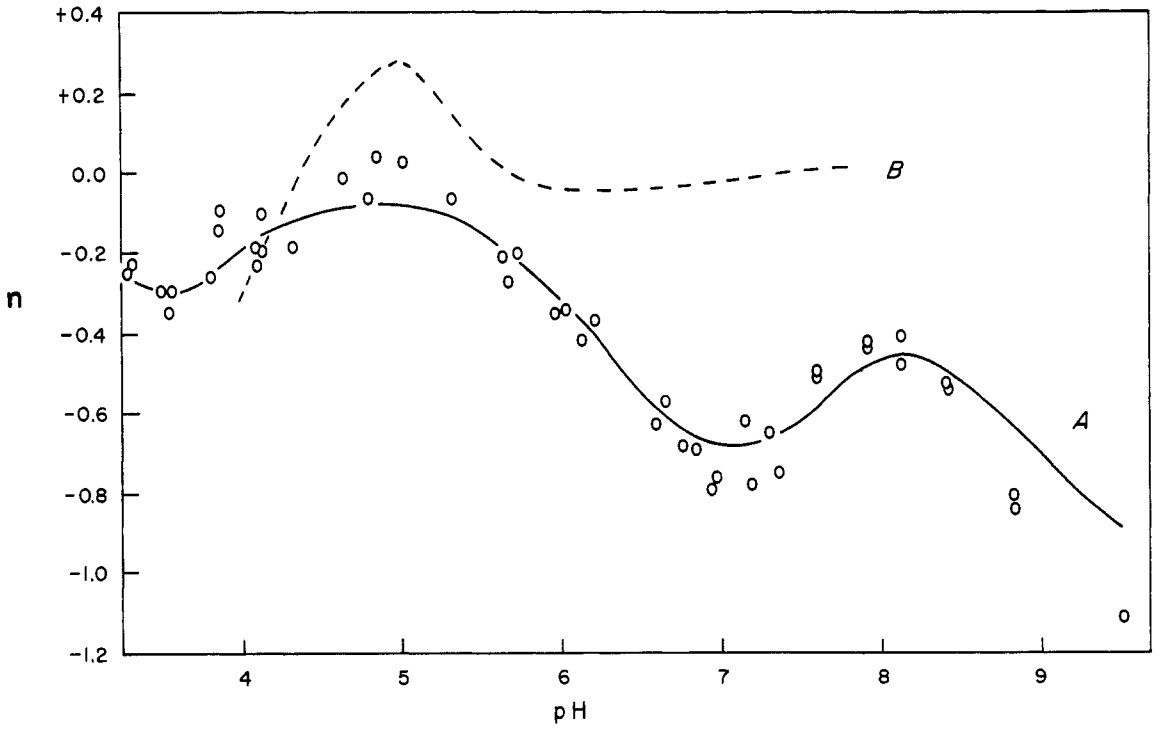
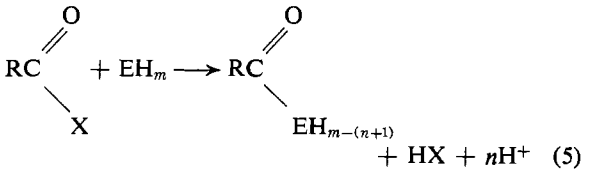


FIGURE 2: The protonic equilibrium accompanying indoleacryloylation of α-chymotrypsin. The circles are values of the coefficient "*n*" (see text for definition). Measurements were made in 0.1 M KCl at 25° using the electrometer technique described in the text. The solid line, A, is a theoretical line for $n = -\alpha_{8.7} + \alpha_{3.8} - \alpha_{3.3} + \alpha_{7.7} - \alpha_{6.3}$. ($\alpha = K/(K + [H^+])$; the subscript on α is the requisite pK_A.) The broken line, B (data of Fahrney and Gold, 1963), for phenylmethane–sulfonyl-α-chymotrypsin is included for comparison.

isolation over the requisite time range (dictated by the response time of the experimental measuring system) (Figure 1). These facts allow us to determine unambiguously the number of protons released concomitant with the acylation reaction. In all experiments reported herein, the enzyme concentration is in excess of the substrate. The buffering capacity of the enzyme is not, however, excessive. Hence, the number of protons released per mole of substrate can be accurately determined.

Since the actual number of equivalents of protons released is dependent in this instance on the protonic dissociation of product (a pH variable), we shall define the coefficient, *n*, as "the number of equivalents of protons released *solely* as a consequence of the transformation of enzyme to acyl enzyme," according to eq 5. This coefficient, *n*, is plotted as a function of pH in Figure 2A.



Each of the "indoleacryloylation" points in Figure 2A represents an average of both repeated additions of substrate to the same enzyme solution and also reruns of the experiment with new solutions of substrate and enzyme. Note the odd contour of the pH–proton release profile. A variety of other, seemingly anomalous, profiles for other acylations and analogous reactions have been previously reported (Fahrney and Gold, 1963; Erlanger *et al.*, 1963; Bernhard, 1963; Gutfreund and Sturtevant, 1956). For comparison, we have included the data of Fahrney and Gold in Figure 2B. Differences in the pH *vs.* "*n*" profile reflect differences in the equilibrium protonic dissociations of the various enzyme–substrate products (*i.e.*, acyl enzymes), as will be discussed below. These results are not unexpected in view of the variability in the apparent pK_A (pK_D) for catalytic hydrolysis, reported in an accompanying communication (Bernhard *et al.*, 1966b, *vide infra*).

That the reaction measured is indeed acylation of the enzyme can be substantiated on the basis of a large variety of precedents arising from studies of reactions of acylating agents with α-chymotrypsin (Bender *et al.*, 1962; Balls and Wood, 1956; Oosterbaan and van Adrichem, 1958; Caplow and Jencks, 1962).

The stoichiometric formation of an acyl enzyme in the reaction of IAI with α-chymotrypsin has previously been demonstrated (Bernhard and Tashjian,

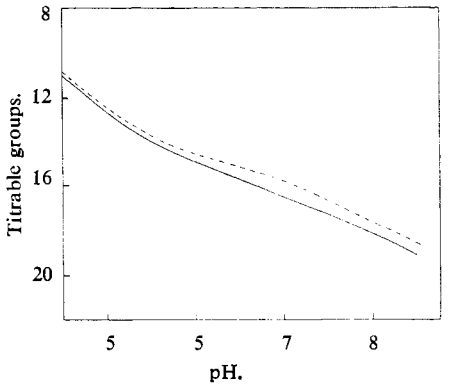


FIGURE 3: Schematic interpretation of Figure 2 in terms of titration curves. The solid line (data of Marini and Wunsch, 1963) is an actual titration curve for α-chymotrypsin in 0.15 M KCl at 20°. The broken line is obtained by adding the coefficient "*n*" for indoleacryloyl-α-chymotrypsin (see Figure 2A) at each pH to the solid line, and represents, schematically, the titration curve of indoleacryloyl α-chymotrypsin.

1965). This acylation reaction gives rise to a chromophoric acyl enzyme with readily discernible visible, near-ultraviolet spectral chracteristics. That no carboxylic acid product (eq 4) is formed over the time course of the presumed acylation reaction is readily ascertained by spectrophotometric examination of the reaction products. The acyl enzyme intermediate and the carboxylate product are sufficiently different in spectra so that small amounts (*ca.* 2–3%) of carboxylate can be measured in the presence of the acyl enzyme. No carboxylate product could thus be observed over the time period required for measurement of the initial step of proton release *via* the potentiometric experiments.

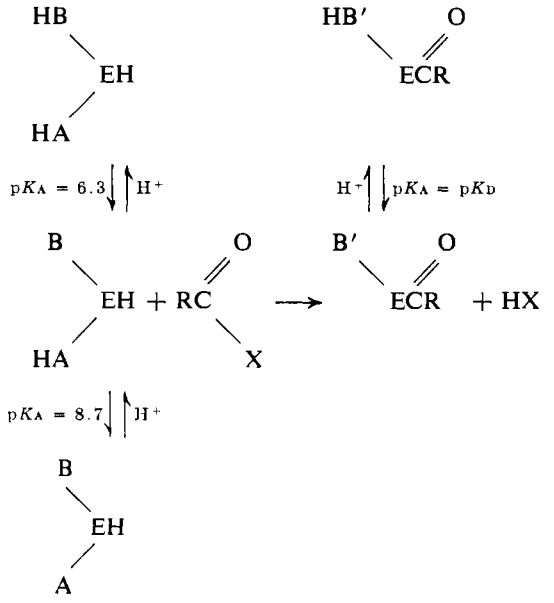
With IAI and α-chymotrypsin, the reaction system we are observing is clearly that described by eq 4. Since the starting enzyme is the same in both the reactions illustrated in Figure 2, and since any protonic equilibria associated with free (nonenzyme) products have been substracted prior to construction of Figure 2, the differences between the two profiles illustrated must arise from differences in the two acyl enzymes. The protonic equilibria described in Figure 2 can be alternatively visualized in the schematic representation of Figure 3, in which two titration curves, one for enzyme and the other for acyl enzyme, are shown. Figure 2 must of necessity represent the difference between these two titration curves. The titration curve for the free enzyme is taken from an actual experiment previously reported (Marini and Wunsch, 1963). The data of Figure 2A demand the requisite acyl enzyme titration curve. Such acyl enzyme titration experiments would be exceedingly difficult to carry out, owing to the hydrolytic lability of the native acyl enzyme. Moreover, the technique we have employed permits a great magnification of the *difference* between the titration curves of enzyme and acyl enzyme at any particular pH. Titration curves of the type schematized in Figure 3 have been reported for *stable* diisopropylphosphoryl enzymes *vs.* native enzymes (Moon *et al.*, 1965). Nevertheless, the quantitative distinctions between the titration curves of enzyme and diisopropylphosphoryl enzyme would be much more precisely measured by our present (difference) technique. The acylating reagent we utilized (IAI), moreover, follows the pathway of eq 4, a pathway presumed to be relevant to the catalytic hydrolysis of true substrates.

Protonic Equilibria above pH 6. The "roller-coaster" contour of Figure 2A, and its striking dissimilarities with Figure 2B, clearly exclude the possibility that the difference in titration curve (as schematized in Figure 3) can arise from chemical perturbation of a single dissociable species on the enzyme protein. Within the pH range 6.0–9.5, two points of inflection are notable in Figure 2A. Consequently, the perturbation (in pK_A) of a minimum of two dissociable species upon acylation is required for the over-all reaction of eq 4. It is entirely possible that many dissociable groups are perturbed as a consequence of this acylation reaction; particularly since it has been reported that acylation of the enzyme site results in, or is a consequence of, a conformational change of the protein (Oppenheimer *et al.*, 1966; Moon *et al.*, 1965; Bernhard *et al.*, 1966a; Bernhard and Lee, 1964; Havsteen and Hess, 1963; Wooten and Hess, 1960; Bender *et al.*, 1962). Nevertheless, the rather sharp maxima and minima observed, as noted both in Figure 2 and elsewhere (Erlanger *et al.*, 1963), are indicative of a discrete number, rather than a continuum of perturbable species. Therefore, and for simplicity, we have attempted to fit the experimental data to a model of an enzyme catalyst containing two pK_A-perturbable acidic species which participate in the acylation reaction.

Two facts were of primary importance to our analysis. (1) The equivalents of protons liberated (*n*), varies between −1 (at high pH) and a maximum value of 0 (at lower pH values). (2) The high pH "tail" of the profile in Figure 2A exhibits the behavior to be expected if, upon acylation, there is a loss of a dissociable acid with $pK_A \sim 8.7$. A pK_A of about this magnitude has been implicated in the catalytic configuration (Bender and Kèzdy, 1964; Bender *et al.*, 1963, 1964) and/or the conformational stability of the binding site (Himoe *et al.*, 1965; Himoe and Hess, 1966; Bender *et al.*, 1966). We note (Figure 2) that at moderately low pH (pH ~6) virtually no protons are liberated in either of the acylation reactions. In the intervening pH range (pH 6.5–8.0), the yield of protons changes in a fashion dependent on the particular acyl moiety. Since this yield is invariably negative at more elevated pH, any model involving the perturbation of a *single* dissociable species in this pH range must be in the direction $pK_A^{acyl\text{-}E} > pK_A^{E}$. Since there is no *acyl-dependent* distinction in the pK_Avalues of the reactants, it follows that $pK_A^{acyl\text{-}E}$ must be differently perturbed in the two acyl enzymes illustrated in Figure 2. We first attempted to fit the data of Figure 2A to a perturbation

in the pK_A of a single dissociable species with $\Delta pK_A = pK_A^{acyl\text{-}E} - pK_A^{E}$, by assuming the disappearance of a second dissociable acid with $pK_A \sim 8.7$ upon acylation. The rather sharp contours in Figure 2A present a good case for extracting the requisite ΔpK_A from the available data.

Our best fit of equation to experimental data (solid line, Figure 2A) was obtained for a pK_A^{E} of 6.3 and a ΔpK_A of +1.4 pH units. It should be noted that pK_A^{E} and ΔpK_A are independent variables in fitting the experimental data. The $pK_A^{acyl\text{-}E}$ of 7.7 thus required is not without significance. It is precisely the apparent pK_D governing the hydrolytic reactivity of this acyl enzyme (*vide infra*). If only one enzyme proton is involved in the actual acylation reaction, the yield of protons released (n) is calculable from eq 6 on the basis of a loss of an acid (HA) with pK_A 8.7 and a perturbation in the pK_A of a second acid (BH) with $pK_A^{E} = 6.3$, concomitant with acylation.



(6)

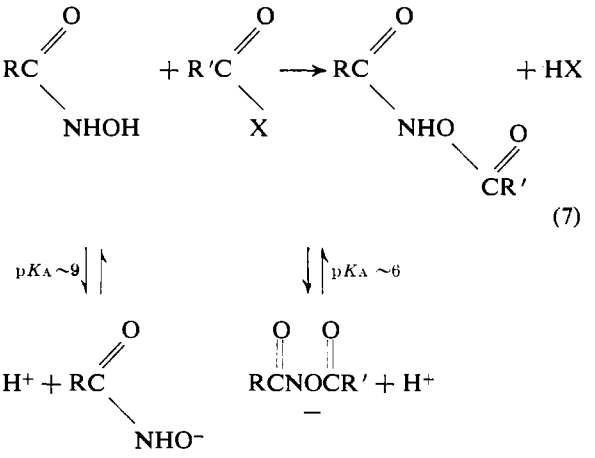
$$n = \alpha_D - \alpha_{6.3} - \alpha_{8.7} = \frac{K_D}{K_D + [H^+]} - \frac{K_{BH}}{K_{BH} + [H^+]} - \frac{K_{HA}}{K_{HA} + [H^+]}$$

From the formal symmetry of equation 6, a *perturbation* of a dissociable group with $pK_A = 8.7$ (BH) to a $pK_A^{acyl\text{-}E} = 7.7$, and the *loss* of a second dissociable species with $pK_A = 6.3$ (AH), concomitant with acylation, would lead to identical behavior in the variability of n with pH. Hence, these two symmetrical alternatives are indistinguishable.

The results presented provide strong evidence for the formation of a weak acid with pK_A equal to pK_D (Bernhard *et al.*, 1966b) upon acylation. The good quantitative fit of model (eq 6) to experimental data (Figure 2A) demonstrates that the formation of this weak acid is *stoichiometric* with acyl enzyme formation. Hence, the variability of pK_D with acyl structure (previously noted) does not arise from the formation of variable extents of pH-dependent intermediates in steps *following* acylation of the enzyme. The protonic equilibria are consistent with any of the models shown in Figure 4.

Schemes I and II (Figure 4) differ in that the acyl acceptor is involved in the pH-dependent protonic equilibrium in Scheme I, but not in Scheme II. Scheme II is preferred for the following reasons. (1) The site of acylation is almost certainly the serine (residue 197) hydroxyl group. Although the acidity of such hydroxyl groups is strikingly greater than usual aliphatic hydroxyls, there is no evidence for the extreme perturbation of pK_A values to the requisite high acidity ($pK_A = 8.7$) demanded by the experimental data. (2) The NH_3^+-terminal group (of isoleucine) in all active chymotrypsins, formed by specific proteolysis of the zymogen, has been shown to be an essential group in defining the active site conformation (Oppenheimer *et al.*, 1966; Himoe *et al.*, 1965; Himoe and Hess, 1966). An apparent pK_A of the requisite magnitude is associated with this group in otherwise fully ϵ-*N*-acetylated active δ-chymotrypsin. This acidic group becomes nondissociable upon phosphorylation of the native enzyme (over the measurable range of pH < 10).

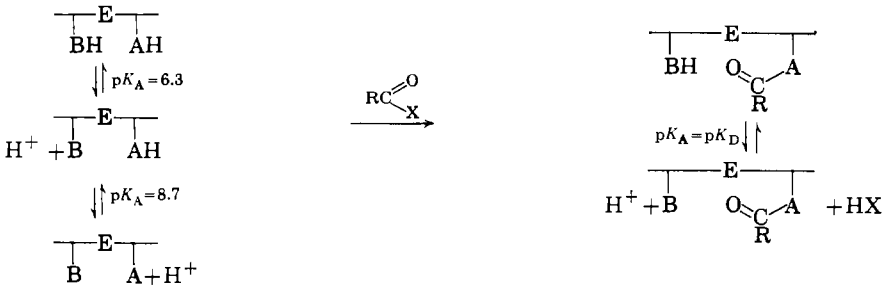
Scheme II is also to be preferred over Scheme III, in which both the acceptor and the acylated product are dissociable acids with differing pK_A values. Of the known protein-contained acceptors, none would lead to the requisite ΔpK_A in the pH range 6–9. Moreover, the predicted value of n would be inconsistent with our present observations. A perturbation of pK_A in the observed direction *via* Scheme III is conceivable, in principle (without reference to the known protein acceptors), on the basis of reaction precedents; for example, in the acylation of *N*-hydroxamic acids (eq 7) (Bernhard *et al.*, 1964b).



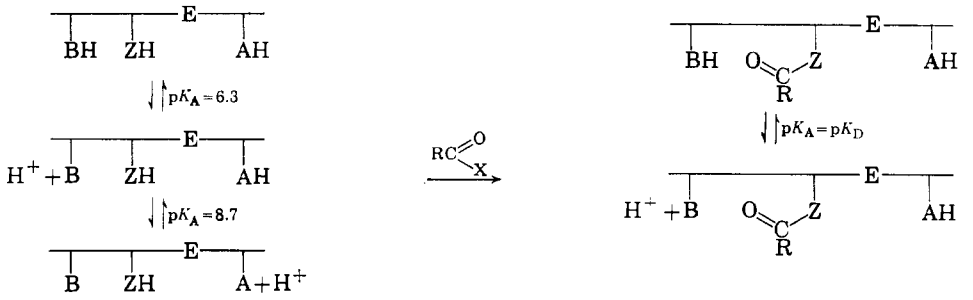
(7)

For the above-mentioned reasons, we prefer the model of Figure 5 to explain the protonic equilibrium accompanying acylation. The pK_A of the terminal NH_3^+

SCHEME I



SCHEME II



SCHEME III

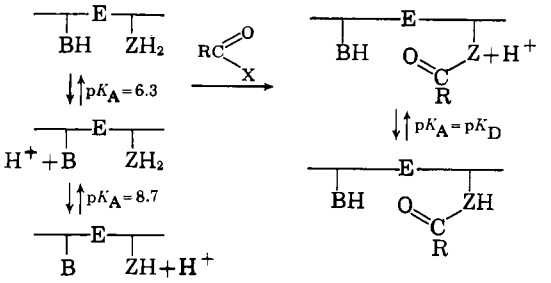


FIGURE 4.

is higher, by approximately one pH unit, than that usually associated with an α-NH_3^+. A plausible explanation is that an interaction of this cation with carboxylate anion is weak in the native enzyme and the interaction becomes stronger upon acylation. Such an interaction would depend on the polarity of the environment, a factor which might vary if the extent of hydration of the site were to change upon acylation.

Protonic Equilibria at Low pH. Small changes in n, concomitant with acylation, are notable in the low pH range (Figure 2A). These changes are readily explainable on the basis of a shift in pK_A of still another acidic group, from 3.3 in the native enzyme to 3.8 in the acyl enzyme. It would be difficult to distinguish the uniqueness of a single dissociable species from these data. The results are, however, suggestive of a change in the environment (and presumably in the polypeptide conformation) of dissociable carboxylic acid residues.

Comparison with Subtilisin. The same acylation and deacylation reactions occur readily with the bacterial proteinase, subtilisin (Bernhard and Tashjian, 1965) It is of interest to compare the protonic equilibrium associated with the acylation of subtilisin with the results obtained for the chymotrypsin reaction. Although acylation occurs at a uniquely reactive serine residue in both enzymes (Noller and Bernhard, 1965), both the sequence of amino acid residues about the reactive serine and the primary and secondary structures of the two enzymes differ widely. The dependence of n on pH in the subtilisin reaction is illustrated in Figure 6. The same type of "roller-coaster" profile as was obtained with chymotrypsin is to be noted. The principal difference between the two enzymes is a shift in the pH scale. Once again, the higher pH data (pH > 6) can be fitted quite well to the model of eq 6, with $pK_1^E = 6.7$, $pK_2^E = 8.7$, and $pK_D = 8.2$. Since pK_D was undetermined previously, we have carried out the pH-dependent *deacylation* velocity studies as described in the accompanying communication. Results are illustrated in Figure 7. Within the limits of

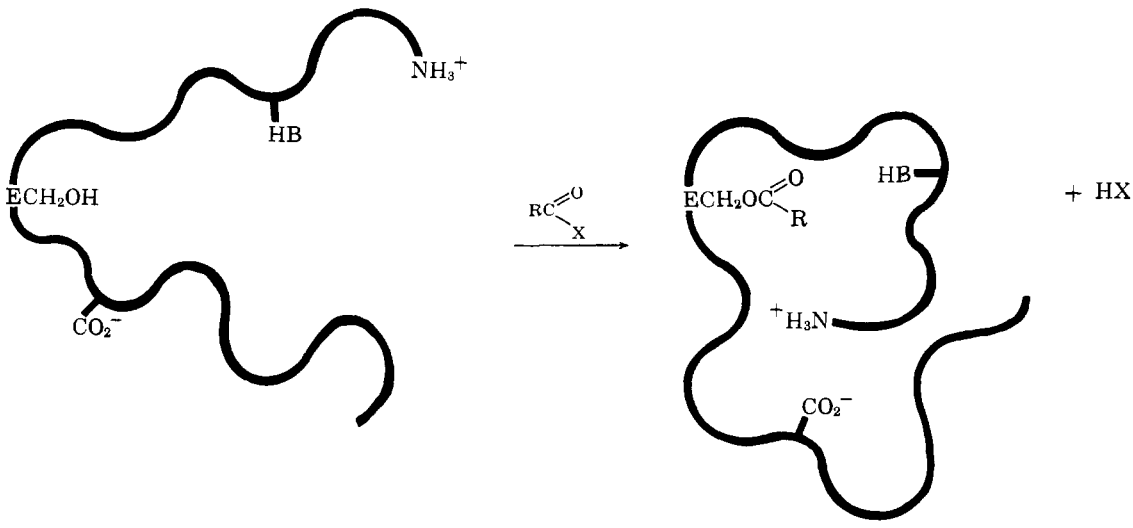


FIGURE 5.

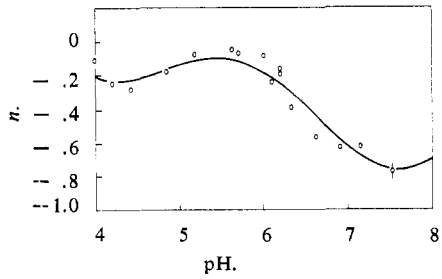


FIGURE 6: The protonic equilibrium accompanying indoleacryloylation of subtilisin. The circles are values of the coefficient "*n*." The solid line is a theoretical line for $n = -\alpha_{8,7} + \alpha_{4,5} - \alpha_{4,1} + \alpha_{8,2} - \alpha_{6,7}$. See legend of Figure 2 for details.

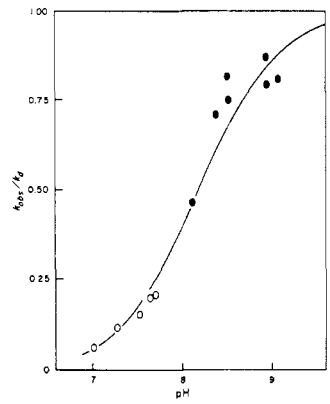


FIGURE 7: The dependence on pH of the deacylation of indoleacryloyl Novo, at 25°, as measured spectrophotometrically. The solid line is a theoretical line for $pK_d = 8.2$, and $k_d = 0.40$ min^{-1}. Phosphate buffer, 0.1 M (○) and pyrophosphate buffer, 0.1 M (●).

precision (±0.1 pH), pK_D is identical in the acylation and deacylation experiments.

At lower pH values, smaller but significant changes in *n*, entirely analogous to the chymotrypsin results, are again observed. Although we are hesitant to suggest specific mechanisms for the origins of these low pH variations in *n*, the similarity of results with two structurally quite different enzymes suggests that these observations are in some way relevant to the mechanism of catalysis.

We are less hesitant to comment on the very similar behavior of acylchymotrypsins and subtilisins at higher pH. The magnitude and location of the common perturbation of pK^E to $pK^{acyl\text{-}E}$, the common disappearance of a second pK^E, the common change in stability of the protein upon acylation (Wooten and Hess, 1960; Bender *et al.*, 1962; Bernhard *et al.*, 1965) all suggest a specific, stoichiometric, and *dynamic* involvement of proton-dissociable groups in the mechanism of catalysis. Our present results, as well as the results of Oppenheimer *et al.* (1966) and Bernhard *et al.* (1966a), suggest that structural changes in the enzyme protein concomitant with enzyme–substrate compound formation are essential to the catalytic mechanism, and are reflected in the protonic equilibria which we have described.

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