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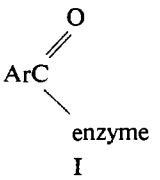
The Influence of pH on the Rate of Hydrolysis of Acylchymotrypsins*

S. A. Bernhard, E. Hershberger, and J. Keizer

ABSTRACT: The pH-dependent first-order rates of hydrolysis of a series of aroyl- and β-arylacryloylchymotrypsins have been examined. Over the range of acyl substituents examined, nonlinear structural correlations are observed in plots of log k_d *vs.* the pK_A of the corresponding carboxylic acid (where k_d is the maximal specific rate of hydrolysis at high pH). The specific rate at any pH, k_{obsd}, can be correlated (in every case examined) with k_d by the expression $k_{obsd} = k_dK_A/(K_A + [H^+])$. The magnitude of K_A, the apparent protonic dissociation constant of the acyl enzyme, is dependent upon the electronic structure of the particular acyl moiety. Plots of log K_A *vs.* the pK_A of the corresponding carboxylic acid exhibit linear structural correlations. Arguments in favor of a chemical interaction between the acyl group and an activity-linked proton dissociable residue of the enzyme are presented.

The influence of pH on the rate of hydrolysis of aroylchymotrypsins of the type I have been previously reported (Bender *et al.*, 1962; Caplow and Jencks, 1962; Bernhard *et al.*, 1965). These hydrolyses can be



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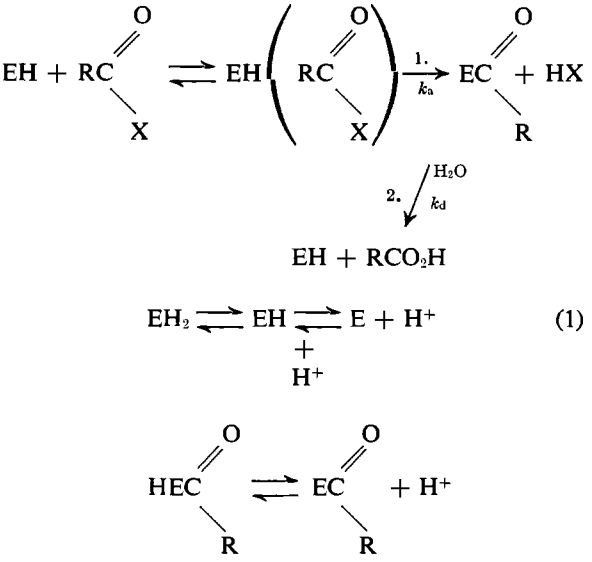
conveniently and directly followed spectrophotometrically at neutral pH, owing to the shifts in the ultraviolet spectra of the acylate anion products relative to the corresponding acyl enzymes. Previous reports concerning the influence of pH on the hydrolytic rate all agree that the hydrolytic rate–pH profile is of the pure sigmoid type illustrated in Figure 1. In an attempt to generalize the mechanistic pathway of chymotryptic hydrolysis of all substrates, the acyl enzyme model (eq 1) has been proposed (Wilson *et al.*, 1950; Hartley and Kilby, 1954; Bender, 1962), and pK_A values have been assigned to catalytic groups within the enzyme site (Bender *et al.*, 1962, 1964).



(1)

The initial *chemical* step in the catalytic sequence (step 1) has been assumed to involve two dissociating groups with pK_A values of approximately 6.7 and 8.7 (Bender *et al.*, 1962; Bender *et al.*, 1963, 1964; Bender and Kézdy, 1964). The monoprotonated species is assumed to be the catalytically active component, in correspondence with the observed "bell-shaped" pH–rate profiles in cases where there is suggestive evidence that this step is rate controlling (Bender *et al.*, 1963). This interpretation of "bell-shaped" curves as an indication of rate-controlling "acylation" has recently been

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TABLE I: Analytic Data for Acylimidazoles.

Compound	Calculated (%)				Observed (%)				Mp (°C)
	C	H	N	O	C	H	N	O	
p-Nitrobenzoylimidazole	55.27	3.25	19.25	22.12	54.43	3.20	18.00	24.48	120–122
p-Methoxybenzoylimidazole	65.35	4.95	13.85	15.84	64.95	5.23	13.93	16.31	182.5–184
p-Methoxycinnamoylimidazole	68.42	5.26	12.28	14.04	67.91	5.22	11.93	14.91	117–118
o-Methoxycinnamoylimidazole	68.42	5.26	12.28	14.04	67.86	5.83	11.78	12.39	140–142

questioned (Oppenheimer *et al.*, 1966). Although the available evidence indicates that for the pseudo-substrate, *p*-nitrophenyl acetate, both pK_A values are involved in the acylation reaction (Professor M. L. Bender, personal communication), the maximal velocity of hydrolysis of specific amide substrates (which are presumed to involve rate-controlling acylation) involve only the lower pK_A, and the binding of specific competitive inhibitors involve only the higher pK_A (Himoe and Hess, 1966; Bender *et al.*, 1966). Further discussion of the "acylation" step is contained in an accompanying communication (Keizer and Bernhard, 1966). The second hydrolytic (or "deacylation") step is assumed to have a "sigmoidal" pH–rate profile, as illustrated in Figure 1. (There is, at present, no controversy regarding the pH–rate dependence of this step.)

An imidazole residue of histidine has been implicated in the catalytic activity of α-chymotrypsin, both on the basis of the absolute value of the pK_A (approximately 7) derived from pH–reactivity studies of the type illustrated in Figure 1, and by a demonstration of the crucial involvement of histidine (residue 57) in the catalytic activity (Schoellmann and Shaw, 1963; Ong *et al.*, 1964). On the supposition that the pH–activity profile is a direct reflection of the state of protonation of an imidazole residue, it is surprising to note the variety of pK_A values reported in the literature (see Bender *et al.*, 1964, and references therein; Caplow and Jencks, 1962). This spread of pK_A values might be accounted for on the basis of "environmental" differences, such as differences in solvent media, and by the differences in molecular structure of the various substrates utilized in the activity measurements. Two previous communications (Caplow and Jencks, 1962; Bernhard and Tashjian, 1965) indicate that for a series of homologous acyl-α-chymotrypsins studied in identical buffer solvents, a variation in pK_A with substrate still persists. The acyl groups utilized in these latter studies are all uncharged; there is, moreover, no obvious relationship between the apparent pK_A for hydrolysis and the geometrical structure of the particular acyl group. Caplow and Jencks investigated the hydrolysis of *meta*- and *para*-substituted benzoylchymotrypsins. The primary intent of these investigators was to examine the effect of the electronic structure on maximal velocity (k_d of eq 2). In the course of such investigations, they noted a substrate-dependent variation of the "apparent" pK_A of hydrolysis.

$$k_d \text{ (obsd)} = \frac{k_d K_A}{K_A + [H^+]} \qquad (2)$$

The intent of the present investigation was to gather further information on this variation of "apparent" pK_A with the electronic and geometrical structure of particular acyl groups. Indeed, large "acyl-dependent" variations in "apparent" pK_A have been observed, and within reasonable limitations these variations can be correlated solely with the electronic nature of the acyl substituent. These results are reported and discussed below.

Experimental Section

Acylimidazoles. Preparation of acylimidazoles was carried out by one of two methods, the method chosen in each particular case being the one giving the highest yield of pure product (analyses are listed in Table I). The route requiring the least number of steps was that employing dicyclohexylcarbodiimide (DCC).[1] In this method, 0.01 mole of both acid and imidazole was dissolved in a minimum of ethyl acetate (usually 25 ml or less). A slight molar excess of DCC was added and the solution was stirred in a stoppered flask. The heavy precipitate of cyclohexylurea which formed within the first hour was filtered, and when no further precipitate formed, the reaction was considered complete. Ethyl acetate was removed under reduced pressure and the resultant solid was crystallized from cyclohexane. The second method of preparation was essentially that of Caplow and Jencks (1962). The appropriate acid chloride was prepared from the carboxylic acid and thionyl chloride. To 0.01 mole of solid acid was added a large excess of thionyl chloride and the mixture was stirred and heated to reflux for several hours. The excess thionyl chloride was removed by dis-

[1] Abbreviations used: DCC, dicyclohexylcarbodiimide.

tillation. The crude acid chloride (0.01 mole) was then added dropwise to a cooled mixture of 0.02 mole of imidazole in 500 ml of benzene. The mixture was stirred for at least 24 hr, filtered to remove imidazolium chloride, and the solvent was removed under reduced pressure. The resultant solid was crystallized from cyclohexane.

3,5-Dinitrobenzoylimidazole was an exceptional case in that a good preparation was not obtained by either of the above methods. This acylimidazole was prepared by mixing the acid chloride and imidazole in acetonitrile. The resultant filtered acetonitrile solution was utilized directly. All solvents were reagent grade and all carboxylic acids were purchased from Eastman Chemical Co.

Other acylimidazoles and acyl enzymes discussed herein have been previously described as follows: furoyl and benzoyl (Caplow and Jencks, 1962), cinnamoyl (Schonbaum *et al.*, 1961), furylacryloyl (Bernhard *et al.*, 1965), and indoleacryloyl (Bernhard and Tashjian, 1965).

Acyl Enzymes. The acyl enzymes were prepared from three-times recrystallized α-chymotrypsin (Worthington Biochemical Corp.). Acylations were sometimes carried out directly in 3-ml quartz ultraviolet cells. The enzyme, at a concentration of 3 mg/ml, was dissolved in the appropriate buffer solution and the acylimidazole (0.02 M in CH_3CN) was added in equimolar amount (using a molecular weight of 27,000–28,000 for the enzyme site). Often, a second method for acylation was used. The enzyme was dissolved in 0.1 M aqueous KCl (to 30 mg/ml) and a slight excess of acylimidazole was added. The enzyme, as supplied, is itself a weak buffer (pH ~4.2). Aliquots of this solution were then added to the desired buffer. This method is to be recommended at higher pH values since the rate of both deacylation and denaturation of the native enzyme are significant at elevated pH (Bender *et al.*, 1962).

Deacylation Rate Studies. With the acylating agents used, and within the concentration and pH ranges herein reported, the rate of acylation was at least two orders of magnitude faster than the deacylation rate (with the exception of furoylimidazole at pH > 8), so that the formation of the acyl enzyme intermediate was essentially complete before significant deacylation took place. This allowed analysis of the deacylation rate as a first-order process.

The rates of deacylation were followed spectrophotometrically utilizing alternatively, a Cary Model 14, a recording Beckman Model DB, or a Beckman Model DU (equipped with a Gilford optical density converter and a 10-mv recorder). "Absorbance kinetics" with these chromophoric acyl enzymes were followed, essentially as described previously (Bender *et al.*, 1962; Caplow and Jencks, 1962; Bernhard *et al.*, 1965). In several cases the spectral change on deacylation was too small to give satisfactory kinetic results. In these instances, deacylation rates were determined by utilizing the specific dye (proflavin) binding technique (Bernhard and Lee, 1964; Bernhard *et al.*, 1966). Deacylation in 2.59×10^{-4} M proflavin was followed by measuring the time-dependent binding of the dye to the emergent free enzyme at 470 mμ. The temperature of the thermostated cells was invariably 25.0 ± 0.1°. Acetate buffers (0.1 M), phosphate buffers (0.05 M), and pyrophosphate buffers (0.1 M) were used over the pH ranges, 3.8–5.7, 6.2–7.8, and 7.8–9.5, respectively. Previous studies have demonstrated that over this range of salt concentration and pH, the deacylation rate is independent of ionic strength. The pH was checked often on a Radiometer Model 4 pH meter.

The first-order deacylation rate constants, k_{obsd}, were usually obtained from plots of log $(A_t - A_\infty)$ *vs.* t (where A_t is the absorbence at time t). With very slow reactions, first-order "Guggenheim plots" were used (Guggenheim, 1926). In all cases, the reaction was first order over at least 4 half-lives.

pK_A *Determinations.* The pK_A values for the chromophoric carboxylic acids were determined spectrophotometrically with a Cary Model 14 spectrophotometer. The spectra of carboxylic acid and carboxylate anion were measured at the corresponding extremes of pH (at concentrations of 10^{-4}–10^{-5} M). Solutions of intermediate pH (0.1 M acetate buffers) were scanned. The degree of dissociation (α) at the intermediate pH values could then be calculated, utilizing two extremes of wavelength and the isosbestic point (Flexser *et al.*, 1935). The experimental pK_A values thus derived, rather than literature values (determined in a variety of solvents), were used throughout.

Results and Discussion

In correspondence with all previous reports, the rate of hydrolysis of each acyl enzyme reported herein exhibits a sigmoidal pH dependence, as illustrated in Figure 1. From such data, two parameters can be

TABLE II: Influence of Acyl Structure on Solution Equilibrium and Enzymic Rate Parameters.

Acyl Group	pK_A of Corresponding Carboxylic Acid	pK_A (app) for Deacylation	k_d (min^{-1})
3,5-Dinitrobenzoyl	2.80	6.70	4.56
p-Nitrobenzoyl	3.2	6.86	0.051
Benzoyl	4.20	7.49	0.039
p-Methoxybenzoyl	4.40	7.34	0.0072
2-Furoyl	3.16	6.90	0.094
Cinnamoyl	4.39	7.32	0.793
p-Methoxycinnamoyl	4.34	7.26	0.195
β-(2-Furyl)acryloyl	4.45	7.26	0.143
β-(3-Indole)acryloyl	4.95	7.68	0.113
o-Methoxycinnamoyl	4.46	6.95	0.013

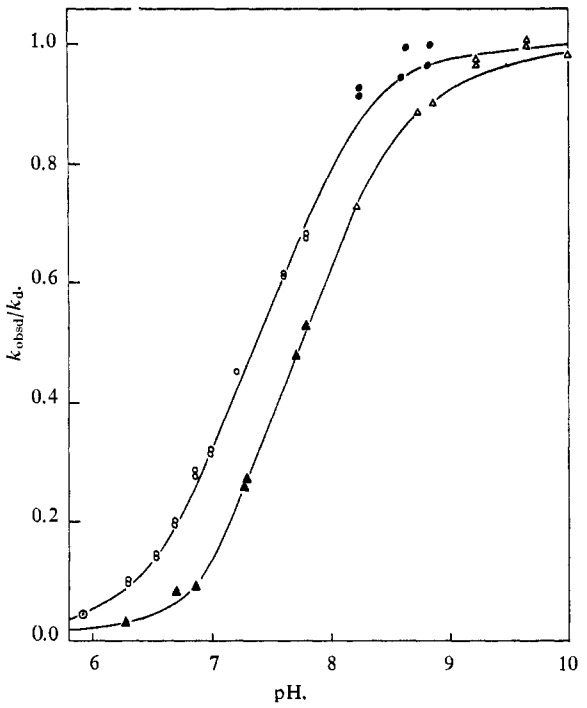
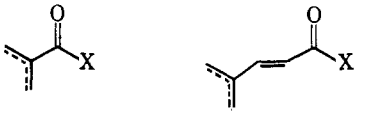


FIGURE 1: Rates of deacylation of indole acryloyl-α-chymotrypsin at 25° in phosphate buffer, 0.05 M (▲) and pyrophosphate buffer, 0.10 M (Δ); rates of cinnamoyl-α-chymotrypsin in acetate buffer, 0.1 M (⊙); phosphate buffer, 0.05 M (O); and in pyrophosphate buffer, 0.1 M (●).

derived (eq 2), namely, the apparent pK_A and the maximal first-order rate of hydrolysis (k_d). These parameters are tabulated in Table II. A few representative plots of the derived data, illustrative of our methods, are shown in Figures 2 and 3. Note that our particular choice of acyl derivatives was made primarily on the criterion of rigid structure. In every case, the π-resonance system extends over the entire acyl portion of the molecule, and is directly chemically coupled to the acyl–enzyme linkage. The planar geometries "α" to the carbonyl–enzyme linkage are of two distinct types, namely an aroyl type (II) and a β-arylacryloyl type (III).



II III

Aside from facilitating experimental observation, this particular selection of acyl derivatives affords a fairly wide range of variation in electronic substituent effects. These substituent effects are reflected in the pK_A values of the corresponding carboxylic acids (X = OH) listed in Table II. It should be noted that these carboxylic acid pK_A values were determined in solvents approximating those used in the hydrolytic activity experiments, and therefore are not true pK_A values (extrapolated to infinite dilution and zero ionic strength).

A casual perusal of Table II indicates that the maximal rates of the hydrolytic reactions falls into at least two classes, depending on whether the geometry is as in II, or as in III, if electronic contributions are used as a criterion of hydrolytic reaction velocity. Note that the aroyl derivatives of type II are very considerably slower in hydrolytic rate than the β-arylacryloyl derivatives. Attempts to correlate the electronic effects of the substituents (as reflected in the pK_A values of the carboxylic acids) with the maximal rates of hydrolytic reaction (Hammett, 1940) are illustrated in Figure 4. A good deal of scatter is observed, even when the two classes of derivatives (II and III) are treated separately. Nevertheless, the slope for type II derivatives is in qualitative agreement with that obtained by Caplow and Jencks (1962) for substituted benzoylchymotrypsins. Surprisingly, when a similar Hammett plot (Hammett, 1940) is derived for the correlation of electronic substituent effects (as reflected in the pK_A values of the carboxylic acids) with the apparent pK_A values for the hydrolytic reactions, a remarkably good linear plot is obtained (see Figure 5). A "ρ" value of +0.4 is obtained from the slope of Figure 5. Note that a variation of approximately 1 pH unit has been observed in the "apparent" pK_A for hydrolysis. Some significant modification of the supposition that "a simple imidazolium$^+$ protonic dissociation governs the hydrolytic rate" is demanded by these findings; the results of Figure 5 are in no way explainable on the basis of the size, polarity, or Van der Waal's contributions of the particular R group (other than by the way such factors perturb the corresponding pK_A values of the carboxylic acids). The pK_A values of nitrogen bases of the type

$$BH^+ \rightleftharpoons B + H^+$$

are notably insensitive to the solvent environment. The pK_A of imidazolium cation is virtually insensitive (±0.25 pH) to solvents ranging in dielectric constant from 18 to 80 (J. H. Carter and S. A. Bernhard, unpublished results). The two acyl groups leading to the largest predictable Van der Waal's interaction with the protein environment (indoleacryloyl and 3,5-dinitrobenzoyl) are at the opposite extremes with regard to pK_A values for hydrolysis.

Four alternative potential explanations for the above-noted, large and regular effect of electronic structure of the acyl group on the apparent pK_A for hydrolysis have occurred to us. (1) "The imidazolium$^+$ residue at the active site (presumably the histidine residue no. 57) is chemically perturbed by the acyl group esterified to a serine (residue no. 197) hydroxyl oxygen." (2) "Upon acylation of the serine hydroxyl, a protein conformational change occurs, perturbing (presumably *via* changes in the detailed proximity of fixed charges) the pK_A of the imidazolium$^+$ residue." The observed variation in this pK_A would presumably be a reflection of the variable degree of conformational change induced by the specific acylation. (3) "The '*apparent*' pK_A

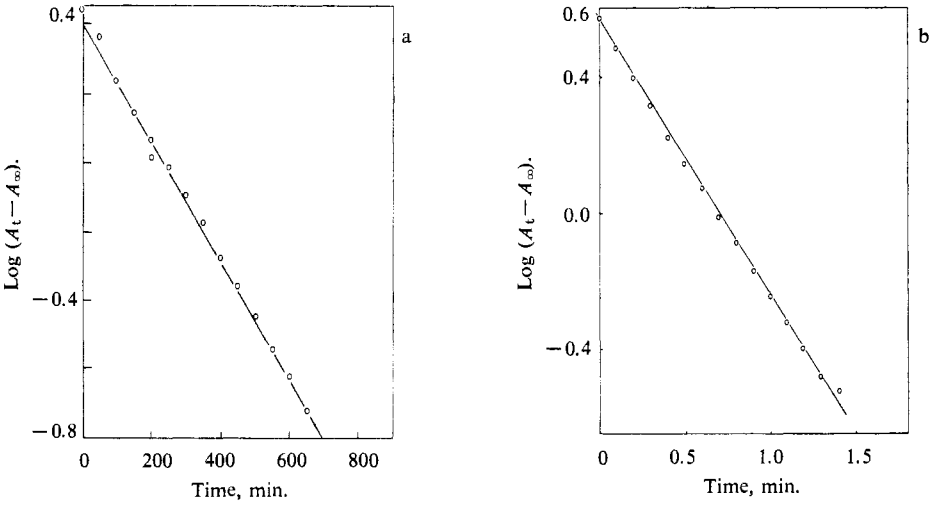


FIGURE 2: (a) Deacylation of *p*-methoxybenzoyl-α-chymotrypsin (a) and 3,5-dinitrobenzylchymotrypsin (b). (a) At 25° in pH 8.07 phosphate buffer, 0.05 M. The reaction rate was followed by the spectral change accompanying hydrolysis of the acyl enzyme. (b) At 25° in pH 6.54 phosphate buffer, 0.05 M. The reaction rate was followed spectrophotometrically by the appearance of proflavin–enzyme complex (see Experimental Section).

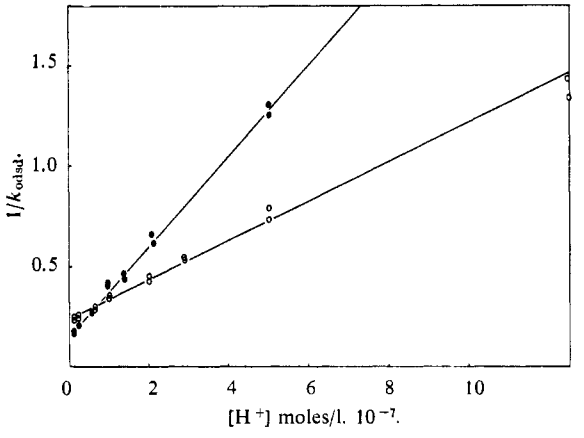


FIGURE 3: Determinations of $pK_{deacylation}$ and k_d for cinnamoyl-α-chymotrypsin (●) and 3,5-dinitrobenzoyl-α-chymotrypsin (○).

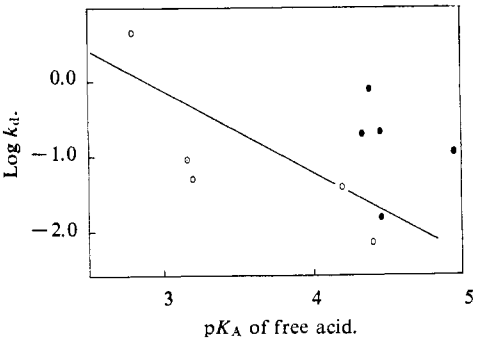
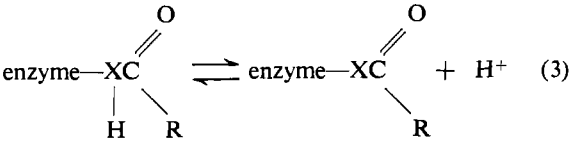


FIGURE 4: "Bronsted plot" of the log of the optimal specific rate of deacylation of acyl enzyme (k_d) *vs.* the pK_A of the corresponding carboxylic acid. (○) substituted benzoylchymotrypsins (data of Caplow and Jencks, 1962). (●) substituted acryloylchymotrypsins. Caplow and Jencks demonstrate a linear relationship between log k_{obsd} at pH 7.07 and the pK_A of the corresponding carboxylic acid. As is evident from the present figure, the previously observed linearity is fortuitous.

observed in the kinetic experiments is a reflection of a reaction mechanism involving at least one other intermediate (beyond that shown in eq 1); the transformation of the acyl enzyme to this intermediate, and/or the rate of reaction of this intermediate, being pH dependent in a manner different from that of the presumably unperturbed imidazolium$^+$ residue." This explanation is a specific example of the arguments of Bruice and Schmir (1959) concerning the influence of pH-dependent preequilibrium intermediates on apparent pK_A values for activity. Such arguments allow for apparent pK_A values considerably different from that of any discernible catalytically active species, by the formation of stoichiometrically minute quantities of essential pH-sensitive intermediates in the catalytic reaction pathway. (4) "The acyl enzyme, presumed to be an acylserine ester, is in actuality an acyl-XH derivative with a finite dissociation constant" (eq 3).



(3)

Arguments 2 and 3 can be disposed of relatively easily for the following reasons. A substrate-induced conformational change of variable extent would be anticipated to be a function of the *geometrical structure*

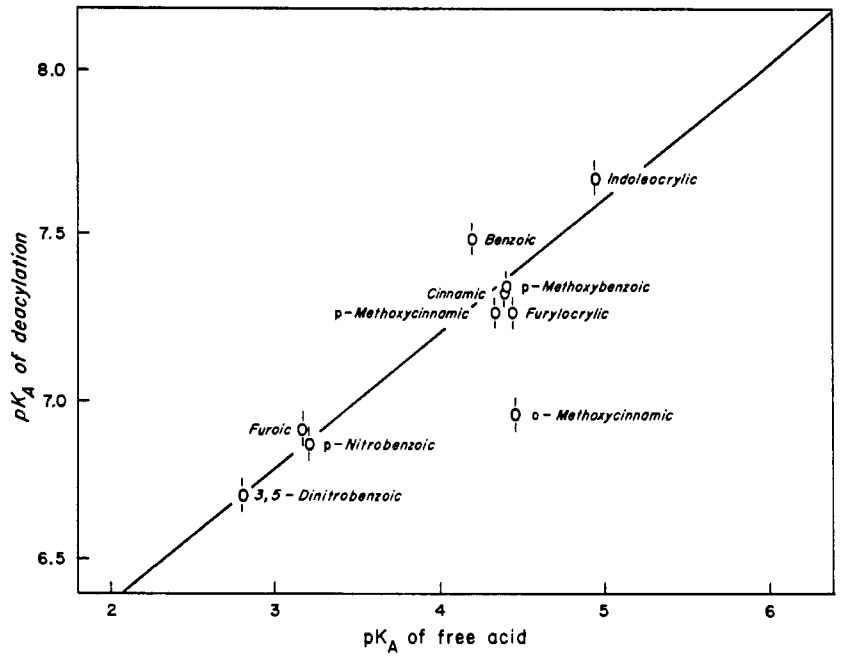
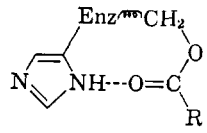


FIGURE 5: Variation of the observed pK_A of deacylation of acylchymotrypsins with the pK_A of the corresponding carboxylic acids. A nonlinear structural correlation might have been anticipated for *o*-methoxycinnamoylchymotrypsin due to the large steric factor introduced by the *o*-methoxy substituent. Spectrophotometric comparisons of the *o*-methoxy-, *p*-methoxy-, and unsubstituted cinnamic acids indicate that the phenyl and acryloyl systems are not coplanar in the *o*-methoxy derivative. k_d for the *o*-methoxycinnamoyl enzyme is also much smaller than for the para derivative, suggesting that the noncoplanar phenyl–acryloyl system interferes sterically with the catalytically optimal configuration of the active site of the acyl enzyme.

(Koshland, 1958) as well as of the electronic structure. There appears to be no systematic dependence of the pK_A of hydrolysis on the geometrical shape or size of the acyl group. Therefore, it seems unlikely that conformational changes can account for the variation of pK_A, although there is now considerable suggestive evidence that such conformational changes of the enzyme protein do occur during the course of catalytic reaction (Oppenheimer *et al.*, 1966; Moon *et al.*, 1965; Bernhard *et al.*, 1966; Bernhard and Lee, 1964; Havsteen and Hess, 1963; Wooten and Hess, 1960; Bender *et al.*, 1962). The third argument would imply that there is some significant distinction between the stoichiometry of the protonic equilibrium, and the kinetically derived pK_A; *i.e.*, the protonic equilibrium between free enzyme and acylated enzyme would be that predicted from the stoichiometric reaction sequence, whereas the kinetically derived "apparent" pK_A for hydrolysis would be anticipated to be considerably different. Relevant experiments have actually been carried out, and are described in an accompanying communication (Keizer and Bernhard, 1966). The results reported therein clearly demonstrate that the stoichiometry of the protonic equilibrium involved in the acylation reaction is dependent on the formation of an acyl enzyme intermediate with pK_A identical with the "apparent" pK_A derived from the *kinetics* of the subsequent hydrolysis.

We are thus left with the choice between arguments 1 and 4. Some weakly suggestive arguments in favor of the latter have been previously proposed (Bernhard *et al.*, 1965); namely, the spectra of the β-arylacryloyl enzymes are all markedly different from that of model acylated serine peptide esters containing the same acyl group. The acyl-transfer rate from reactive acyl derivatives (such as acylimidazoles) to a serine hydroxyl has been demonstrated to be remarkably rapid, in particular serine-containing peptide sequences (Bernhard *et al.*, 1964) (hence, arguing for the plausibility of rapid acyl transfer accompanying the denaturation and degradation of acylchymotrypsin to the chemically identified acylserine ester). Nevertheless, the invariant chemical and spectral identifications of denatured chymotrypsins as acylserine esters, independent of the denaturation procedures (Balls and Wood, 1956; Oosterbaan and van Andirchem, 1958; Naughton *et al.*, 1960; Noller and Bernhard, 1965), places the weight of evidence in favor of a native acylserine ester.

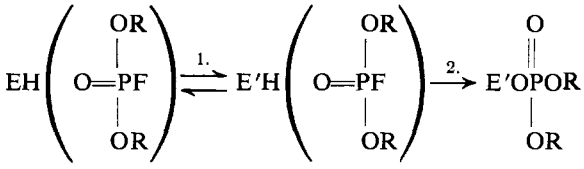
The only mechanistic precedents favoring argument 1 involve hydrogen bonding or proton transfer between the acylcarbonyl oxygen and the proton of an imidazolium residue (IV). To the extent that hydrogen bonding is a covalent phenomenon, there should be a correlation between the electronegativity of the carbonyl oxygen (and hence the pK_A of the carboxylic acid) and the pK_A of the imidazolium$^+$ group. This correla-



IV

tion is qualitatively in agreement with experimental observations. We cannot estimate the magnitude of such a perturbation on the pK_A of imidazolium$^+$ (*i.e.*, we cannot predict the "ρ" value for this dissociation). Complete proton transfer (the formation of the protonated ester) seems intuitively unreasonable at neutral pH, even in the unknown environment of the enzyme site.

Whatever the detailed mechanism is, by which the pK_A of a weak acid at the catalytic site is altered upon acylation, it is clear that this perturbation arises *via* covalent forces between the acyl group and the proton donor. It is of interest to note that in the reaction of diisopropylfluorophosphate with chymotrypsin, two reaction steps have been detected in the presumed reaction (eq 4)



(4)

(Moon *et al.*, 1965), and that analogous changes in pK_A appear to be correlated with the first rather than the second step in the reaction sequence.

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