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Spectrophotometric Identification of Acyl Enzyme Intermediates*

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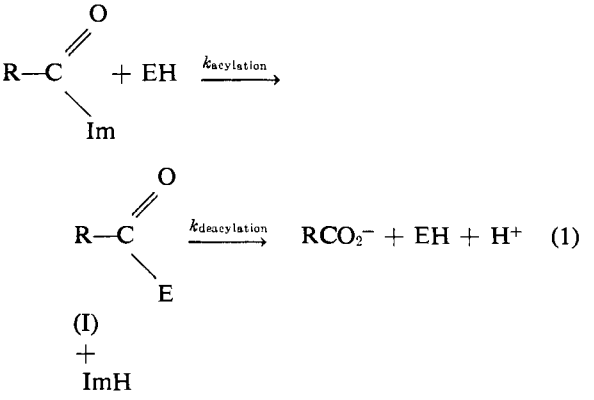
ABSTRACT: The ultraviolet spectra of a variety of β-arylacryloyl derivatives have been investigated in terms of the influence of the aryl substituent, the acyl substituent, and the solvent. The spectra of these derivatives have been compared with the corresponding spectra of the β-arylacryloyl derivatives of chymotrypsin and subtilisin, in an attempt to identify the acyl-acceptor group at the enzyme site. The "native" acyl enzyme spectrum in water differs in every case from that of a corresponding *O*-acylserine peptide in aqueous, hydrogen-bonding, or nonpolar solvents. The denatured acyl enzyme spectrum is in every case the same as that of a corresponding *O*-acylserine peptide. Of the potential amino acid acyl-acceptor side chains, only the imidazole group of histidine can form an acyl derivative which is compatible with the spectrophotometric characteristics of the corresponding native acyl enzyme. The acylimidazole spectrum in nonpolar solvents approaches that of the acyl enzyme for all β-arylacryloyl derivatives investigated. The slow rate of acyl enzyme hydrolysis at low *p*H (*p*H <4) is, however, inconsistent with the observed hydrolysis rates of acylimidazoles.

In a comprehensive set of papers, Bender and his collaborators (Bender, 1962a; Bender *et al.*, 1962a,b; Schonbaum *et al.*, 1961) demonstrated the utility of employing chromophoric acylating agents for the identification of intermediates in the chemical pathway of catalysis by proteolytic enzymes. Aside from the experimental convenience of following the rates of disappearance of substrate, appearance of enzyme-substrate intermediate, and appearance of final products individually by means of the characteristic ultraviolet absorption spectra of each of the components, the method presents a potential approach to the identification of the chemical nature of enzyme-substrate intermediates. An example reported by Bender *et al.* (1962a) serves as illustration: Table I lists the characteristic ultraviolet wavelength maxima and extinctions of a variety of derivatives of cinnamic acid. In the reaction of α-chymotrypsin with cinnamoylimidazole, an enzyme-substrate intermediate is readily detected. The kinetics of the reaction are in accord with the model of equation (1). By comparison of the data of Table I with the spectrum of the enzyme-substrate intermediate (compound I), the chemical nature of the acyl enzyme



(1)

linkage might possibly be inferred. Unfortunately, none of the model compounds of Table I corresponds very closely in its ultraviolet spectra to that of the acyl enzyme. The disparity between the spectrum of the acyl enzyme intermediate and that of the model compound, *N*-acetyl-*O*-cinnamoylserinamide (compound II), is particularly disappointing in light of the fact that an *O*-acetylserine peptide has been identified (Oosterbaan and Van Andrichem, 1958) among the proteolytic degradation products of *mono*acetyl chymotrypsin, an acyl enzyme isolated (Balls and Wood, 1956) under conditions similar to that employed in the spectral identification of cinnamoyl chymotrypsin. Wooten and Hess (1960) have found that even when non-ultraviolet absorbing acylating or phosphorylating agents are employed in the preparation of acyl or

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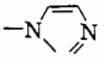
TABLE I: Spectral Characteristics of Cinnamoyl-X in H_2O.

X	λ_{max}[a] (mμ)	$\epsilon_{max} \times 10^{-4}$ (OD/M, cm)
$-OCH_3$	279	2.3
$-OCH_2CH(CONH_2)(NHCOCH_3)$	281	2.4
$-O^{(-)}$	269.5	2.0
$-OH$	279	2.2
	307	2.5
$-NH_2$	272	2.2
Chymotrypsin (*p*H 4.2)	292	1.7
Subtilisin (*p*H 4.0)	290	1.7

[a] These values are an average of the present results with those reported by Bender *et al.* (1962a).

phosphoryl enzyme, spectral changes in the region of the tryptophan and tyrosine absorption peaks of α-chymotrypsin occur. These changes, when corrected for, are such as to decrease the disparity in spectra between cinnamoyl enzyme and *O*-cinnamoylseryl peptide. Taking into account these spectral changes Bender (1962b) finds the cinnamoyl enzyme spectrum to have a maximum (cinnamoyl) absorption at 286–287 mμ. Nevertheless, a spectral disparity between enzyme and enzyme model still exists.

Bender *et al.* (1962a) cite the dependence of ultraviolet spectra on solvents, and propose that the enzyme site is a more highly polar medium than aqueous solution. Since the spectrum of the acyl enzyme exhibits a longer wavelength of maximal absorption (λ_{max}) than does the model compound (II), and since this ultraviolet absorption band has an exceedingly high oscillator strength characteristic of a strong $\pi-\pi^*$ electronic transition, it was anticipated that the environment of the acyl enzyme is one of very high polarity (> polarity of the aqueous solvent). The $\pi-\pi^*$ transitions usually occur at longer wavelengths as the dielectric constant of the medium is increased (Kasha, 1961). The spectra of cinnamic acid, cinnamide, methyl cinnamate, and cinnamoylimidazole all follow this rule in acetonitrile-water mixtures (in solutions ranging from 2 to 100% acetonitrile) and in H_2O versus isooctane solutions.[1] An environment of much higher polarity than H_2O within the enzyme site (which is demanded in order to reconcile the acyl enzyme spectrum with that of an *O*-acylserine ester) appears unlikely, but is not necessarily impossible.

[1] Unpublished results from this laboratory.

Three sets of spectrophotometric experiments for the further identification of the acyl enzyme linkage suggest themselves: (1) a spectrophotometric study of other cinnamoyl derivatives as models of cinnamoyl chymotrypsin; (2) comparison of cinnamoyl chymotrypsin with related cinnamoyl enzymes where the "solvent effect" might be anticipated to be different; (3) preparation of other chromophoric acyl groups (besides cinnamoyl derivatives) and a comparison of model compounds with acyl enzymes in these instances. Further information, vis-à-vis these three approaches, is presented in this paper.

Experimental

Preparation of Acyl Enzymes. Some variation of procedure was demanded by the relative stabilities of the various acylating agents and acyl enzymes. A more or less general procedure was as described in the following paragraphs.

A preliminary run, employing the kinetic-titrimetric method of Bender *et al.* (1962a), was made to determine the number of equivalents of active enzyme site per mg enzyme per equivalent of acylating agent. For spectral and kinetic analysis, enzyme was dissolved in acetate buffer (ionic strength 0.1 M) to about 1×10^{-3} M. The *p*H was maintained constant in the range 4.0–4.8 (depending on both the variation in the solubilities of the enzymes and the uncatalyzed rates of hydrolysis of the acylating agent). Acylating agent (0.8–0.9 equivalent), dissolved in spectrograde acetonitrile, was added to a measured volume of enzyme, and identical volumes of acetonitrile and enzyme solution were prepared as blanks. The two solutions were diluted to volume with appropriate aqueous buffer solvents for spectral measurements. When spectra in the *p*H range 4–5 were desired, more dilute solutions to enzyme in buffer were prepared, and the spectra of the acyl enzymes were determined directly by the addition of 0.8–0.9 equivalent of diluted acylating agent, following completion of the acylation reaction. Typical final concentratons of acyl enzyme were in the range of $0.2–0.5 \times 10^{-4}$ M in 0.67–1.67% aqueous acetonitrile.

Acylating Agents. Cinnamoylimidazole was prepared by the method of Schonbaum *et al.* (1961). *2-Furoylimidazole* was prepared by the method of Caplow and Jencks (1962). *3-(2-Furyl)acryloylimidazole* was prepared from the acyl chloride, following exactly the method of preparation of cinnamoylimidazole. Recrystallization of the product (cyclohexane) yielded large hexagonal crystals, mp 113–114°.

Anal. Calcd for $C_{10}H_8N_2O_2$: C, 63.9; H, 4.3; N, 14.9; O, 17.0. Found: C, 63.58; H, 4.8; N, 14.6; O, 17.1.

3-(2-Furyl)acryloyl chloride was prepared by the addition of three-times-redistilled $SOCl_2$ (1.5 equivalents) to 1.0 equivalent of dry furylacrylic acid (Aldrich Chemical Co.) which had been twice recrystallized from hot dimethylformamide-water. The mixture was stirred with a magnetic stirrer and maintained at room temperature for 3 hours. The resulting solution was evap-

orated *in vacuo*, and the solid residue was extracted with cyclohexane under reflux. The hot cyclohexane extract was decolorized with Norit and filtered and cooled to 10°. Crystals of the acyl halide separated. These were filtered and dried at 10°, and utilized without further purification in the preparation of furylacryloylimidazole (neutralization equivalent 89.0, Cl^- 22.9%).

3-(2-Furyl)acryloylimidazole was also prepared by the mixed anhydride method. To 20 mmoles of furylacrylic acid in 50 ml anhydrous dioxane at 10° were added successively 20 mmoles of isobutyl chloroformate (Eastman Red Label) and 20 mmoles of redistilled triethylamine. After 2 hours the precipitate of the amine hydrochloride was removed by filtration. To the filtrate and dioxane washings was added 20 mmoles of imidazole (Matheson, Coleman and Bell, dried *in vacuo* at 40°). The solution was allowed to stand at room temperature for 4 hours. The dioxane was removed by flash evaporation *in vacuo* and the solid mass was extracted with 100 ml anhydrous benzene at 50°. The benzene solution was then cooled to 10° and the small amount of precipitate which formed was discarded. Crystallization occurred upon addition of Skellysolve B to the benzene solution. These crystals were collected and recrystallized by the same procedure; mp 111–114°, mmp with furylacryloylimidazole prepared from the acyl halide 111–114°.

3-(3-Indole)acryloylimidazole was prepared directly from the carboxylic acid by the dicyclohexylcarbodiimide (DCC) method: 5 g of indole-3-acrylic acid (Aldrich Chemical Co.) was added to 50 ml of anhydrous ethyl acetate and vigorously stirred. The solution was filtered, the filtrate was discarded, and the neutralization equivalent of the solution was determined. To 20 mmoles of this indoleacrylic acid solution was added 20 mmoles of anhydrous imidazole and 20 mmoles of dicyclohexylcarbodiimide. After 12 hours the insoluble urea was removed by filtration and discarded. The solution was extracted exhaustively but rapidly with 0.1 M potassium phosphate buffer, *p*H 6.7 (until the aqueous phase gave a negligible ultraviolet absorbance at 315 mμ). The ethyl acetate solution was dried over anhydrous sodium sulfate, filtered, and evaporated to dryness *in vacuo* in a flash evaporator. The resulting solid was redissolved in ethyl acetate and crystallized upon the addition of Skellysolve B. Recrystallization from benzene yielded small yellow plates; mp 190°.

Anal. Calcd for $C_{14}H_{11}N_3O$: C, 70.9; H, 4.6; N, 17.7; O, 6.8. Found: C, 71.2; H, 4.2; N, 17.4; O, 7.1; mp 182° (corr).

Enzymes. α-Chymotrypsin (three-times-recrystallized) was obtained from Worthington Biochemical Corp., Freehold, N.J. Crystalline Bacterial Proteinase Novo was obtained from Novo Industri, Copenhagen, Denmark. Different batches of either enzyme gave identical spectral results.

Model Compounds

Esters. Methyl 3-(3-indole)acrylate was prepared via the mixed anhydride. 3-Indoleacrylic acid, 0.01 mole (Aldrich, recrystallized from ethyl acetate), isobutyl chloroformate, 0.01 mole (Eastman Kodak Red Label), and triethylamine (British Drug House, reagent grade) were mixed in 50 ml dioxane at 10°. After 4 hours the reaction mixture was filtered and the triethylamine salt was discarded. The filtrate was added to 50 ml of a pyridine-methanol mixture (1:1, v/v). Solvents were removed by reduced-pressure distillation and the residual filtrate was dissolved in methanol. On addition of Tris buffer (1.0 M, *p*H 8.5) with rapid stirring, an oil separated which hardened on standing. The solid was filtered and recrystallized twice from hot ethanol-water; mp 160°.

Anal. Calcd for $C_{12}H_{10}O_2N$: C, 72.0; H, 5.0; O, 16.0; N, 7.0. Found: C, 71.7; H, 5.2; O, 16.2; N, 7.0.

Furoylcholine chloride, *furylacryloylcholine chloride*, and *cinnamoylcholine chloride* were prepared from the corresponding carboxylic acids via conversion to the mixed anhydrides by exactly the method described for the preparation of methyl indoleacrylate. The mixed anhydride filtrates (50 ml) were mixed with a suspension of 0.02 mole of dried choline chloride in 100 ml anhydrous pyridine with rapid continuous stirring. After 20 minutes the reaction mixture was warmed to reflux temperature, cooled, and filtered. Solvent was removed from the filtrate by flash evaporation and the resultant solid was redissolved in anhydrous ethanol and recrystallized twice from ethanol–ethyl acetate: furoylcholine chloride, mp 217°; furylacryloylcholine chloride, mp 215°; cinnamoylcholine chloride, mp 200°. Chloride analysis: furoylcholine chloride, calcd 15.2%; found 15.0%; saponification equivalent 236 (theor 234); furylacryloylcholine chloride, calcd 13.7%, found 13.8%; saponification equivalent 260 (theor 260); cinnamoylcholine chloride, calcd 13.2%; found 13.5%; saponification equivalent 275 (theor 270). The following were commercial samples: ethyl furoate (K & K Chemicals), ethyl furylacrylate (K & K Chemicals), methyl cinnamate (Matheson, Coleman and Bell), methyl furoate (Aldrich Chem. Co.), and *N*-acetyl-*O*-cinnamoylserinamide (Cyclo Chemical Co.).

Amides and Peptides. Amides and peptides described herein were prepared by the reaction of the corresponding acylimidazole with saturated aqueous solutions of ammonia, methylamine, glycinamide, or Tris, at a *p*H equal to the pK_A of the amine. The products were in some cases not isolated (as indicated in Table VI). At neutral and alkaline *p*H, all the acylimidazoles reported herein are stoichiometrically hydrolyzed to the corresponding acylates in the absence of other nucleophiles. It should be noted that the rates of solvolysis by these amines was $>10^2$ the hydrolysis rate at the same *p*H, in every case where the product was not isolated. The ultraviolet spectra of the acyl products were in every case identical at *p*H 2 and 9, indicative of virtually complete aminolysis (to the exclusion of hydrolysis). With furylacrylate and indoleacrylate, the extinction at 320 and 340 mμ, respectively, increased more than 4-fold upon acidification. The extinctions of the carboxylic acids are nearly maximal at these wavelengths, hence

TABLE II: Spectral Characteristics of Acyl Enzymes.[a]

Acyl Group	Chymotrypsin λ_{max} (mμ)	Chymotrypsin $\epsilon \times 10^{-4}$ (OD/M, cm)	Subtilisin λ_{max} (mμ)	Subtilisin $\epsilon \times 10^{-4}$ (OD/M, cm)
Furoyl	261	1.2	262	1.2
Indoleacryloyl	359	2.0	358	2.0
Furylacryloyl: *p*H 8.3	320	1.88		
*p*H 7.2	319	1.95		
*p*H 4.2	321	1.92	323	1.95
*p*H 3.5	320	1.80		
*p*H 8.8[b]	315	1.80		

[a] At *p*H 4.2 (0.1 M acetate) unless otherwise specified. [b] At 3°.

small amounts ($\tilde{>}2\%$) of carboxylate can be detected. The reaction with Tris proceeds via the (presumed) *O*-Tris ester (λ_{max} = 307 mμ for the furylacryloyl derivative). At 1 M Tris, *p*H 8.0, the esterification rate is >10^2-fold faster than the aminolysis although the final product appears to be virtually entirely amide. The final extinction at λ_{max} was in every case 5 ± 2% higher than the corresponding carboxylate. The amides and *N*-methylamides were isolated by extraction of the aqueous reaction mixture (neutralized to *p*H 6 with HCl) with ethyl acetate. The ethyl acetate extract was dried over Na_2SO_4, filtered, and precipitated by addition of Skellysolve B. The precipitates were recrystallized from methylene chloride–Skellysolve B mixtures.

Cinnamamide, mp 146° (lit 146°). N: calcd, 9.5%; found 9.6%.

Furylacrylamide, mp 169° (lit 169°). N: calcd, 10.2%; found, 10.0%.

2-Furamide, mp 144° (lit 143°). N: calcd, 12.5%; found, 12.5%

N-Methylcinnamamide, mp 110°. N: calcd, 8.7%; found, 9.1%.

N-Methylindoleacrylamide, mp 107°. *Amino* nitrogen: calcd, 7.0%; found, 7.0%.

Furylacryloylglycinamide, mp 180°. Acid hydrolysate: μeq/0.210 mg glycine, 1.01; NH_3, 1.05 (by Beckman-Spinco amino acid analysis).

The λ_{max} and ϵ of all of these isolated amide products were *identical* to those observed following completion of the reaction of the corresponding acylimidazoles with the amines, as described.

Anhydrides. The spectra of the mixed anhydrides were determined by reacting stoichiometric mixtures of carboxylic acid, isobutyl chloroformate, and triethylamine, 0.1 M in each component, in anhydrous prepurified dioxane at 10° for 3 hours. The reaction mixture was filtered and aliquots of the filtrate were diluted into 0.025 M phosphate buffer, *p*H 6.86, and the spectra were determined immediately. Hydrolysis was extremely slow in this solvent. Identical dilutions of the filtrate were made into 0.01 N NaOH and into 6 N NH_4OH. The concentration of total anhydride plus unreacted carboxylic acid could be determined as carboxylate from the hydroxide reaction, and the unreacted carboxylic acid could be estimated following ammonolysis by the method described. Unreacted carboxylic acid was always less than 5%.

Carboxylic Acids. These were obtained from Aldrich and recrystallized from ethanol-H_2O or from ethyl acetate (indole-3-acrylic acid). All acids gave the theoretical neutralization equivalent.

Spectra. All spectra were recorded in a Cary Model 14 ultraviolet spectrophotometer. Optical density maxima were in the range 0.5–1.5 and slit widths were in the range 0.08–0.30 mm depending on the reference solutions. The noise level never exceeded 1% of the maximal OD.

Results and Discussion

Comparison of the Ultraviolet Spectra of Chromophoric Acyl Enzymes with the Presumed O-Acylserine Analogs. The spectral characteristics of various acyl enzymes are listed in Table II. All of the acyl enzymes, with the exception of furoyl enzymes, were sufficiently stable in the *p*H 4 range to determine precisely the optical density maxima. In principle, the spectra might have been complicated by the consecutive acylation-deacylation reaction scheme (equation 1). However, at these lower *p*H values, the two reactions are so widely separated in rate that measurements of the acyl enzyme intermediate spectra posed no problems. The furoyl case is exceptional in two ways, namely, the relative rates of acylation and deacylation with α-chymotrypsin are more nearly equal, and the uncatalyzed hydrolysis of furoylimidazole is significant in this *p*H range. Two expedients were taken to determine the spectrum of furoyl chymotrypsin: (1) the *p*H was raised to 5.74, and (2) the spectra of the reaction mixture (furoylimidazole plus α-chymotrypsin) was followed as a function of time. In this way, a clear extrapolation to the acyl enzyme intermediate spectrum was possible. In the reaction of subtilisin with furoylimidazole this extrapolation was less difficult to achieve.

Since chymotrypsin and subtilisin are optimally

active at *p*H 8 and the activity of the enzymes is apparently correlated with the basic component of an acid with pK_A near 7, it was of interest to attempt to determine the ultraviolet spectrum of the acyl enzyme intermediate in this *p*H range. This was achieved in the case of furylacrylyl chymotrypsin by interpolation of the time-dependent optical density at one fixed wavelength, following the mixing of furylacrylylimidazole with chymotrypsin, to the time of maximal acyl enzyme formation. Independent kinetic experiments on the rates of acylation and deacylation indicate that at the time of maximum acyl enzyme formation, 98% of the acyl group is chemically bound to the enzyme. The spectrum of furylacryloyl chymotrypsin at *p*H 8 was reconstructed from such optical density–time studies by a series of kinetic experiments at different wavelengths. In each experiment, the reaction was allowed to proceed to completion of deacylation and the spectrum of furylacrylate$^{\ominus}$ ion was measured. Any slight differences in the concentration of acyl group from experiment to experiment could then be corrected for by normalizing the optical density of the acyl enzyme to the optical density maximum of furylacrylate$^{\ominus}$. A similar experiment with indoleacryloyl chymotrypsin has recently been reported (Bernhard and Tashjian, 1965).

It has now been well established by chemical analysis that the ultimate site of specific acylation in α-chymotrypsin (Oosterbaan and Van Andrichem, 1958; Naughton *et al.*, 1960), and a host of related enzymes, is the hydroxyl oxygen of a unique serine residue located within a specific amino acid sequence, namely, glycyl-aspartyl-seryl-glycine. This result, however, has been established only by chemical analysis of the denatured and degraded constituent peptides. In the intact native enzyme there exists the possibility that the acyl group is attached to another more reactive functional group. It has been demonstrated that following specific phosphorylation of the active site of subtilisin by diisopropylfluorophosphate (Sanger and Shaw, 1960) a single serine hydroxyl group has been phosphorylated. Once again, this result was obtained following denaturation and degradation of the enzyme to constituent peptides. In the case of subtilisin, the specific serine residue is found in the peptide sequence threonyl-seryl-methionyl-alanine, a considerably different primary amino acid sequence. Recently it has been demonstrated in this laboratory (Noller and Bernhard, 1965) that specific acylation of Novo subtilisin yields an *O*-acylated serine on denaturation and degradation of the enzyme. The spectra of the native acyl enzymes, both with subtilisin and with chymotrypsin (Table II), are in *no* case correlated with the corresponding spectra of *O*-acylserine esters in H_2O (Table III). It is noteworthy that the two rather different enzymes exhibit nearly identical acyl enzyme spectra. Although the two enzymes perform the same chemical function (the hydrolysis of acyl derivatives), they differ widely in regard to substrate specificity, peptide sequence about the active site, and gross amino acid composition. In addition they differ considerably in tertiary structure (conformation) in that chymotrypsin has only a small amount of helix content, whereas subtilisin is considerably helical (approximately 40% based on optical rotatory dispersion studies).[2] Subtilisin is considerably more soluble than chymotrypsin at neutral *p*H, and contains no disulfide bridges. Although these structural considerations do not necessarily exclude the possibility that the two enzymes share a very similar spatial array of functional groups in the vicinity of the specific serine hydroxyl, and that the peculiar spectral characteristics of the acyl enzyme are, in both cases, a consequence of this similar configuration, the possibility that the acyl group in this active native acyl enzyme resides elsewhere than at the serine oxygen should not be excluded.

TABLE III: λ_{max} (mμ) of Chromophoric Acyl Esters in Water.

	Alcohol		
Acyl Group	*N*-Acetyl-serin-amide	*N*-Carbobenzyloxy-Thr-Ser-Met-Ala Methyl Ester[a]	Methanol
Cinnamoyl	281		278
Furylacryloyl	309	308	306
Indoleacryloyl	335	334	329
Furoyl			254

[a] This analog of the "active serine" sequence in subtilisin is described elsewhere. These results are for the *mono*furylacrylated and *mono*indoleacrylated *serine* esters (Bernhard *et al.*, 1964).

Correlation of the Ultraviolet Spectra of Denatured Acyl Enzymes with O-Acylserine Analogs. In an attempt to distinguish between a "perturbed serine ester" and an active acyl group otherwise chemically bound, the native acyl enzymes were denatured but not degraded, and the spectra of the denatured acyl enzymes were determined, as is summarized in Table IV. In every case

TABLE IV: λ_{max} of Acyl Enzymes in 0.01 M Sodium Dodecyl Sulfate.[a]

Acyl Group	Chymotrypsin (mμ)	Subtilisin (mμ)
Cinnamoyl	280	280
Furylacryloyl	309	309
Indoleacryloyl	334	334

[a] *p*H 4.2 (0.1 M acetate–acetic acid).

[2] Unpublished results from this laboratory, and in conjunction with Dr. Elkan Blout.

the spectrum of the denatured acyl enzyme is virtually identical to the spectrum of the corresponding model acylserine ester. Thus denaturation alone suffices to convert the spectrum of a native acyl enzyme to that corresponding to an *O*-acylserine ester. Although this result does not preclude the possibility of acyl transfer during the course of denaturation, it makes unlikely the possibility that acyl transfer occurs as a consequence of peptide bond rupture. The denatured enzymes, listed in Table IV, all contained the acyl group chemically bonded to the protein, as was evident in all cases by the inability to resolve the two species on repeated passages through Sephadex G-25 or G-50, and in the case of the subtilisin, by the isolation of *O*-acylserine peptides (Noller and Bernhard, 1965). Some attempts were made to investigate the question of whether the change in the spectrum of the acyl enzyme upon denaturation is linked to a change in enzyme configuration on denaturation and/or to the activity of the enzyme, i.e., to the ability of the acyl enzyme to undergo deacylation. The denaturation of α-chymotrypsin, subtilisin, and their monocinnamoyl and monofurylacryloyl derivatives by urea and by sodium dodecyl sulfate was studied. Under the conditions of denaturation employed, the kinetics of denaturation of the native enzyme could be conveniently followed. In correspondence with a previous report by Bender *et al.* (1962b), we found the acyl enzyme to be far less susceptible to denaturation than the nonacylated enzyme. Thus, for example, at *p*H 5 the kinetics of urea denaturation are rapid with both of the nonacylated enzymes, whereas the rates of denaturation of the acyl enzymes are slow and equal to the rates of deacylation (i.e., denaturation of the acyl enzyme does not occur appreciably in this solvent; denaturation of the enzyme occurs rapidly subsequent to deacylation). Since the term "denaturation" is subject to a variety of interpretations, we adopted different criteria of "denaturation" and followed the course of "denaturation" according to each criterion separately. The criteria were (1) the OD at 235 mμ, which in the case of the two free enzymes and each of the two cinnamoyl and furylacryloyl enzymes is to a large extent due to the conformation of the polypeptides; (2) the OD at 287 mμ, which in the case of two nonacylated enzymes and the furylacryloyl enzymes is due primarily to the change in spectral properties of the tryptophan residues in the enzyme preparation; (3) the specific activities of the enzymes; and (4) the change in the spectrum of the chromophoric acyl enzymes. This final criterion has been established as a consequence of the studies reported herein. It can only be applied in situations where "denaturation" occurs at a much more rapid rate than deacylation.

For these two enzymes and their various acyl derivatives, no distinctions have been found in the rates of "denaturation," which are dependent on the criterion of "denaturation" selected; i.e., the diverse processes accompanying denaturation of the enzyme are all kinetically coupled to the conversion of an active acyl enzyme spectrum to an inactive acyl enzyme spectrum. The alkaline hydrolysis of the denatured acyl enzyme is similar in rate and *p*H dependence to that of model *O*-acylserine peptides (Bender *et al.*, 1962b; Noller and Bernhard, 1965). We can now define more rigorously alternatives for the spatially proximal environment surrounding the active acyl group: Either a very specific set of perturbing forces (arising as a consequence of a specific geometry of functional groups within the polypeptide chain) gives rise to an unusual spectrum for an "active" serine ester, or the active acyl group is chemically bonded to some functional group in the enzyme *other* than the serine hydroxyl but is *necessarily* transferred to the serine hydroxyl when the specific native polypeptide conformation is destroyed. Intuitively, the first of these possibilities seemed somewhat more likely, and for this reason we first investigated the effects of solvents on chromophoric acyl ester spectra. Solvents which might reflect the presumed environment of the active acyl group were selected.

Effect of Solvent Environment on the Ultraviolet Spectra of Chromophoric Acyl Derivatives. The electronic transitions responsible for the strong near-ultraviolet absorptions in the cinnamoyl and furylacryloyl derivatives are π-π^* transitions. Such electronic transitions usually (but not always) are of higher energy (shorter wavelength) in solvents of lower polarity (Kasha, 1961; Kosower, 1958; McConnell, 1952). This shift to lower wavelength is observed with both the cinnamoyl and the furylacryloyl derivatives. Hence, no arguments which are based on the "hydrocarbonlike" nature of the environment of the active site will offer a valid explanation for the peculiar spectra observed with active chromophoric acyl enzymes, assuming that the acyl linkage is to a serine hydroxyl oxygen. Bender *et al.* (1962a) have examined the ultraviolet spectrum of a cinnamoyl ester in 10 M lithium chloride. This is a solvent of presumed greater polarity than water; it should therefore result in an increase in the wavelength of maximal optical density. Indeed, this is the observed result with methyl cinnamate. However, the spectrum of the ester, even in 10 M lithium chloride, is not nearly so far displaced to higher wavelengths, relative to the pure aqueous spectrum, as is the spectrum of the active acyl enzyme relative to the aqueous spectrum of the ester. With the furylacryloyl and indoleacryloyl enzymes, the spectra exhibit much greater shifts to higher wavelengths compared to the corresponding *O*-acylserine esters in water. Another possible perturbation of the ester spectrum might arise from intramolecular hydrogen bonding. We therefore measured the spectra of some model furylacryloyl and cinnamoyl esters in 9 M urea and 4 M imidazole-imidazolium mixtures at various *p*H values. These results are listed in Table V. Although there is a shift in wavelength concomitant with the transfer of the chromophores from water to the above-mentioned solvents, the wavelength shifts are not of sufficient magnitude to reconcile the active acyl enzyme spectrum with that of a "hydrogen bond–solvated" ester. Moreover, both with subtilisin and with chymotrypsin, the maximal extinction of the acyl enzyme is

TABLE V: Spectra of Chromophoric Esters in Hydrogen Bonding Solvents.

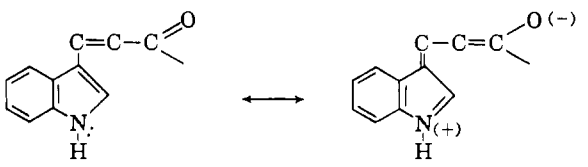
Solvent	Ester		
	Methyl Cinnamate	*N*-Acetyl-*O*-cinnamoyl Serinamide	Ethyl Furylacrylate
	λ_{max} and $\epsilon \times 10^4$ (in parentheses)[a]		
Urea, 8.7 M	281 (2.33)	282.5 (2.31)	308 (2.34)
Guanidine hydrochloride, 5 M			309 (2.33)
Imidazole, 4 M (free base)	281.5 (2.06)	283 (2.18)	308 (2.27)
2 M Imidazole + 2 M imidazolium chloride	282 (2.03)	283 (2.20)	309 (2.24)
4 M Imidazolium chloride	282 (2.13)	282 (2.25)	308 (2.26)

[a] In units of mμ and OD/M, cm, respectively.

considerably lower than that of the corresponding acyl ester, whereas the above-mentioned solvents lead to either small increases or no changes in the extinctions of the model compounds relative to their extinctions in water.

It might be anticipated that the presumed involvement of an imidazole residue (histidine) in catalysis reflects the spatial proximity of this residue to the serine hydroxyl. At 4 M concentrations, however, neither imidazole nor imidazolium[⊕] perturbs the spectrum of a serine ester derivative significantly. We can only conclude from these solvent-spectra experiments that the unusual acyl enzyme spectrum, if it is a perturbed serine ester spectrum, arises from an as yet undefined type of perturbation reflecting *neither* the polarity nor the hydrogen-bonding properties of the microscopic environment of the bound acyl group.

It is not necessarily surprising that the λ_{max} of these β-arylacryloyl derivatives can shift to substantially shorter wavelengths in nonpolar solvents (relative to water), but may not shift to proportionally higher wavelengths in solvents of very high polarity. The extent of the wavelength shift of a chromophore in a particular solvent is dependent on at least two factors: (1) a "chemical effect" dependent on the extent to which the solvent stabilizes particular ground-state resonance forms, e.g.,

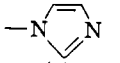
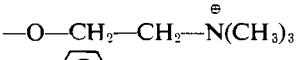
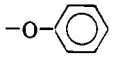


(2) a "polarizability effect" dependent on the resultant of the interaction of the polar solvent with the electronic transition moment. The "chemical effect" may be nearly completely saturated in aqueous solution; i.e., the chromophore may be as "red-shifted" as is possible. Indeed, this is essentially the situation with the related chromophores in conjugated unsymmetrical polymethine dyes (e.g., $R_2N{-}CH{=}CH{-}CH{=}O$) where large blue shifts (in solvents more polar than H_2O) are observed (see for example Brooker *et al.*, 1951).

To a first-order approximation, the perturbation of λ_{max} due to the "polarizability effect" should be small and nearly independent of the nature of the β-arylacryloyl derivative in the series of compounds considered herein. The present results with aqueous denaturants and salts are consistent with an assignment of the small changes in λ_{max} to this effect, but are inconsistent with the assignment of λ_{max} of the native acyl enzyme to an "environmentally perturbed" serine ester.

Studies of Other Acyl Derivatives as Models of the (Native) Acyl Enzyme Linkage. Consider the alternative hypothesis—that the active acyl residue is not chemically bonded to the oxygen of serine. We may then inquire as to whether other chromophoric acyl derivatives have spectral properties more closely correlated with those observed for the acyl enzymes. On the assumption that in chymotrypsin and in subtilisin there are only twenty types of amino acid side chains, among these twenty only a limited number might be expected to be acceptors of an acyl group. These potential acyl acceptors are as follows: (1) the alcoholic hydroxyl groups of serine and threonine; (2) the carboxylic groups from aspartic and glutamic acids and the C-terminal end of the polypeptide chain; (3) the phenolic hydroxyl of tyrosine; (4) the imidazole group of histidine; (5) the amino groups of lysine and the N-terminal end of the chain; and (6) the pyrrole nitrogen of tryptophan. The last of these would appear to be extremely unlikely owing to its very low basicity and its expected very low nucleophilicity. The first possibility has already been discussed. As to the other four possibilities, a study of model compounds should be enlightening. We have therefore prepared some model acyl derivatives of these four types of functional groups.

TABLE VI: λ_{max} (mμ) of Various R—C(=O)—X Derivatives in Water.[a]

—X	R—C(=O)— Cinnamoyl	Furyl-acryloyl	Indole-acryloyl	Furoyl
	307 (2.5)	340 (2.7)	378 (3.0)	280 (1.7)
—O$^{(-)}$	269.5 (2.0)	292 (2.4)	313 (2.5)	245 (1.2)
—OH	279 (2.2)	308 (2.5)	330 (2.7)	255 (1.2)
—NH_2	272 (2.2)	299 (2.5)		250 (1.4)
—$NHCH_3$	272 (2.2)	300[b]	320[c] (2.6)	
	273[b] (2.2)	302 (2.5)	322[b] (2.6)	
	281 (2.3)	308 (2.5)		256 (1.3)
	285[c]	315 (2.6)		
	290[d]			
	289[b] (2.3)	316[b] (2.5)		265[b]

[a] Molar extinction $\times 10^{-4}$ in parentheses. [b] Compound was not isolated. For purity analysis see Experimental. [c] For *N*-acetyl-*O*-cinnamoyl tyrosinamide. Results of Bender *et al.* (1962a). [d] Results of Bender *et al.* (1962a).

Although the formation of an amide via an ϵ-amino group of lysine or the N-terminal amino acid in the polypeptide chain seems an unlikely possibility on the basis of the known low reactivity of amides, model amides were studied in light of the apparent lack of correlation between reactivity and thermodynamic stability of acyl enzyme intermediates. Acyl enzymes are formed to an appreciable extent from esters of carboxylic acids plus enzyme (Bender and Zerner, 1962). The spectral data, however, clearly eliminate amides as possible acyl enzyme intermediates. The spectra of amides and peptides are farther removed from that of the acyl enzyme than are the spectra of esters. Anhydrides, phenyl esters, and imidazoles all absorb at wavelengths significantly greater than those of the aliphatic esters. Among this group of three derivatives (Table VI), the anhydrides and phenyl esters both absorb maximally at wavelengths which are sometimes considerably lower than those of the corresponding acyl enzyme, whereas the acylimidazoles absorb at wavelengths significantly higher. Since solvent studies indicate that both hydrogen-bonding solvents and solvents of high polarity result in only minor changes in the ultraviolet spectrum from that observed for the same chromophore in water, it appears likely that large solvent-dependent spectral shifts can be achieved only in the direction of *lowering* the wavelength of maximal absorption from that observed in water. The λ_{max} is lowered in solvents of low polarity (relative to H_2O). The absorption spectra of *no* model acyl derivative listed in Table VI correlates with that of the corresponding acyl enzyme. It follows from the as yet unproven assumption that *the acyl linkage to the enzyme is via a conventional amino acid side chain* that the enzyme acyl group must be linked to a substituent (X) such that the λ_{max} of acyl-X in water would occur at higher wavelengths than that observed in the polypeptide environment of the active site. This restricts the possible conventional chemical linkages to only one type, namely, acylimidazoles.

It has been found, both in this laboratory (Bernhard and Lee, 1964) and elsewhere (Foster and Cochran, 1963), that when a chromophore is bound to the active site of the enzyme without forming covalent bonds, the effect of the enzyme-site environment on the spectrum of the chromophore is that anticipated from the transfer of the chromophore from water to a "hydrocarbon-like" solvent. We have therefore examined the spectra of four chromophoric acylimidazoles in cyclohexane and in acetonitrile. The change in λ_{max} with solvent is

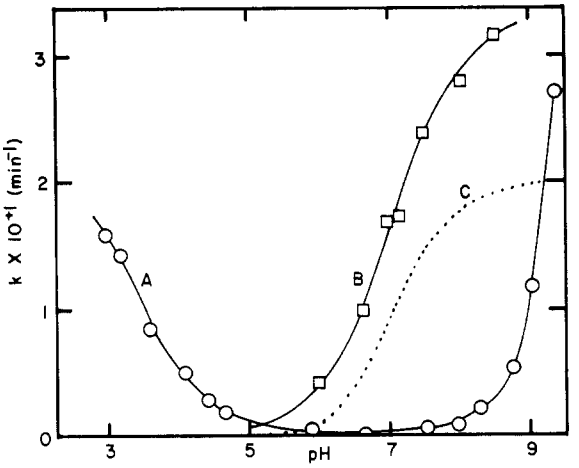


FIGURE 1: Hydrolysis of "active" cinnamoyl derivatives. (A) Cinnamoylimidazole, acid, neutral, and basic hydrolysis. (B) Cinnamoylimidazole in 1 M imidazole at an ionic strength of 1.0 M. $k = k_{obs} - k_{H_2O}$. (C) Cinnamoyl chymotrypsin. Data of Bender *et al.* (1962b); k is in arbitrary units, k_{max} (high *p*H) = 0.75 min^{-1}.

striking in each case. The spectra of all four chromophoric acylimidazoles in cyclohexane (Table VII) very closely approximate the corresponding spectra of native acyl enzymes in water. It should be noted that these large solvent-dependent spectral shifts observed with the acylimidazoles are in no way peculiar to imidazole derivatives. The same magnitude of displacement of λ_{max} to lower wavelengths is observed, in the transfer from water to cyclohexane, with the other chromophoric acyl derivatives herein described. The spectra of the acylimidazoles are not very different in anhydrous acetonitrile from those observed in cyclohexane. Although there is a small shift to higher wavelengths in acetonitrile, the spectra are very much more like those in cyclohexane than like those in water. Hence, if the enzyme site can be compared to a "hydrocarbonlike" environment (for which there is some presumptive evidence), the spectrum of an acyl-*N*-(imidazolyl)histidine at the active site should resemble the spectrum of an acylimidazole in a solvent of low dielectric constant.

TABLE VII: Spectra of Acylimidazoles in Organic Solvents.

Acyl Group	λ_{max} (mμ)	
	Cyclohexane	Acetonitrile
Furoyl	263	
Cinnamoyl	294, 295[a]	294
Furylacryloyl	322	326
Indoleacryloyl	358	364

[a] In isooctane; data of Bender *et al.* (1962a).

A Comparison of the Chemical Properties of Acylimidazoles with Acyl Enzymes. The hydrolysis of cinnamoyl chymotrypsin and cinnamoylimidazole is illustrated in Figure 1. At acid and alkaline *p*H ranges, cinnamoylimidazole is far more reactive than the cinnamoyl enzymes, whereas at *p*H values near neutrality the acyl enzymes are far more reactive. The nonenzymic *p*H-rate profile is the composite of the three reactions

$$RCOImH^{\oplus} + OH^{\ominus} \rightarrow \text{products} \quad (2)$$

$$RCOImH^{\oplus} + H_2O \rightarrow \text{products} \quad (3)$$

$$RCOIm + OH^{\ominus} \rightarrow \text{products} \quad (4)$$

whereas the enzymic processes are entirely in accord with the model

$$RCOE + H_2O \rightarrow \text{products}$$
$$RCOE + H^{\oplus} \rightleftharpoons RCOEH^{\oplus},\ K_A = 10^{-7}\ M \quad (5)$$

or the kinetically equivalent expression (6).

$$RCOEH^{\oplus} + OH^{\ominus} \rightarrow \text{products}$$
$$RCOEH^{\oplus} \rightleftharpoons RCOE + H^{\oplus},\ K_A = 10^{-7}\ M \quad (6)$$

At or near neutrality, reaction (2) is the predominant pathway in the hydrolysis of acylimidazoles.

The *p*H dependence of the hydrolysis of acyl enzyme, at neutral and at high *p*H, is consistent with the *p*H dependence of the hydrolysis of an acylimidazole, on the assumption that the rate-controlling step involves general-base catalysis by imidazole (histidine). The hydrolysis of acylimidazoles is general-base catalyzed (Jencks and Carriuolo, 1958). Imidazole is an effective base catalyst for the reaction, and only the uncharged base is catalytic (see Figure 1). Near neutrality, in 2 M imidazole solutions, the imidazole-catalyzed pathway predominates.

The surprising selectivity of alcohol over H_2O in the solvolysis of acylimidazoles is noteworthy (Jencks and Carriuolo, 1958; Bernhard *et al.*, 1964). This selectivity is exhibited to a similar extent with acyl enzymes (Bender, *et al.*, 1964). In 2 M ammonia at *p*H 9.5, furylacrylate anion is the principal product of reaction with furylacrylolyl chymotrypsin, whereas in 2 M methanol at the same *p*H, the methyl ester is the principal initial product (Table VIII). In the reaction of acylimidazoles with alcohols, the chemical nature of the alcohol is of prime importance in determining the reaction rate. At *p*H 9.5, equal total concentrations of methanol and phenol react at nearly the same rate (Bernhard *et al.*, 1964). This contrasts with the reactions of alcohols with active esters, in which the rate of reaction is more closely (but not necessarily linearly) related to the base

TABLE VIII: Reactions of Furylacryloyl Chymotrypsin with Nucleophiles at *p*H 9.5.

Solvent (H_2O-X)[a] X =	λ_{max} of Product[b]	λ_{max} of Furyl-acryloyl-X
	292	292
NH_3 (2 M)[c]	292	299
CH_3OH (2 M)	305	306

[a] 0.1 M pyrophosphate buffer. [b] Furylacryloyl chymotrypsin concentration was 0.67×10^{-4} M in all experiments. [c] Adjusted to *p*H 9.5 with HCl.

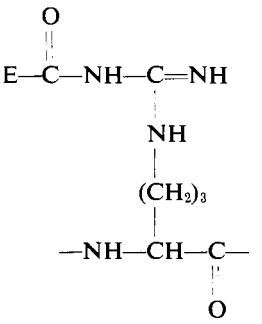
strength of the conjugate alkoxide ion (Bruice *et al.*, 1962; Jencks and Gilchrist, 1962; Kirsch and Jencks, 1964). A tetrapeptide analog of the (partial) active site sequence of subtilisin, *N*-carbobenzyloxy-L-threonyl-L-seryl-L-methionyl-L-alanine methyl ester, at concentrations of 1×10^{-3} M, competes effectively with water for reaction with furylacrylolylimidazole at *p*H 8.5, the product of the competitive reaction being the corresponding *O*-furylacryloylserine ester (Bernhard *et al.*, 1964). It is not implausible to assume that within the enzyme site, an *intra*molecular reaction of an acylimidazolium$^{\oplus}$ (or a chemically related group) with a hydroxyl group of a serine peptide might occur in preference to an *inter*molecular reaction with the aqueous solvent. The *O*-acylseryl peptide, invariably found following denaturation and degradation of the acyl enzyme, might be the result of a transfer of the acyl group from a reactive linkage to a nearby serine, rather than the result of a stabilization, owing to destruction of the catalytic deacylation apparatus, of a preexistent active serine ester. The role of the serine hydroxyl in the "serine proteases" may hence be quite different from that previously envisaged.

The behavior of acyl enzymes at low *p*H argues against the identification of the native acyl enzyme linkage as an acylimidazole; there is only a small solvent effect on the hydrolysis rate of an acylimidazole at low *p*H, where reaction (3) predominates. Thus it would be anticipated that at low *p*H an acylhistidyl enzyme would hydrolyze at a rate at least comparable to that of an acylimidazole. The specific rate of hydrolysis of furylacryloyl chymotrypsin at *p*H 3.5 is 1.7×10^{-4} min^{-1}, whereas the specific rates of hydrolysis of furylacryloylimidazole, in water and in 50% dioxane (at the same measured *p*H of 3.5) are 5.5×10^{-2} and 3.8×10^{-2} min^{-1}, respectively.

Conclusions

Attempts to correlate the spectral and chemical properties of acyl enzymes with acyl derivatives, in which the acyl acceptor site is an analog of a side chain of a constitutive amino acid of the enzyme, have thus far led to ambiguous conclusions; arguments have been presented for the exclusion of all such acyl derivatives as potential models of the native acyl enzyme. In order to maintain the hypothesis that the acyl-acceptor linkage is via a conventional side chain, we are left with two seemingly unattractive rationalizations, namely, (1) perturbations of the *O*-acylserine spectrum occur in the native acyl enzyme due to peculiar *chemical* environmental effects of a type not as yet observed with model compounds in homogeneous solution; (2) the native acyl enzyme linkage is via an acylimidazole, but the dimensions of the enzyme site at low *p*H are such as to exclude the approach of a water molecule, and hence to exclude the possibility of reaction of an acylimidazolium$^{\oplus}$ with H_2O.

Alternatively, the acyl enzyme derivative which is spectrophotometrically observable when the enzyme is in its native configuration may result from an acyl linkage to an *unusual* amino acid side chain. No new suggestions as to the nature of such unusual acyl acceptors are offered herein. Erlanger (1960) has suggested that a monoacylated guanidine (compound III), derived from arginine and glutamate or aspartate, may be a constituent of the active site.



(III)

Such a derivative (compound III) might have chemical properties similar to an acylimidazole. The pK_A of dicarbobenzyloxyarginine is near neutrality. The α,-δ,ω-triacyl derivative of arginine undergoes facile hydrolysis to the diacyl derivative in mildly alkaline solution. Recently Viswanatha (1964) has postulated an *N*-alkoxyarginine (formed via serine and arginine) as a constituent of the active site. The present results suggest that alternatives to an *O*-acylserine ester linkage in the *active* acyl enzyme should not be summarily rejected.

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Isolation and Structural Determination of Chromophoric Acyl Peptides from Subtilisin Enzymes*

Harry F. Noller and Sidney A. Bernhard

ABSTRACT: Stable monofurylacryloyl derivatives of two subtilisin enzymes were prepared. Following proteolytic digestion, isolation and structural determination of furylacrylyl peptides showed the acyl group to be present as *O*-furylacryloylserine in the sequence Asp-(NH_2)·Gly·Thr·Ser·Met. The ultraviolet spectra of the denatured furylacrylyl enzymes and furylacryloyl peptides were similar to each other but different from the spectra of the undenatured acyl enzymes. The rates of base-catalyzed deacylation of denatured furylacryloyl enzymes and furylacryloyl peptides were those expected for furylacryloyl esters. Owing to its distinct absorption spectrum, the furylacrylyl group was of great utility both as a label to facilitate isolation of acyl derivatives and as a spectral probe of the chemical nature of acyl linkages.

The enzymes collectively known as "subtilisins" constitute a group of alkaline proteases which can be isolated in crystalline form from various strains of *Bacillus subtilis*. Much of the information which has been obtained regarding their isolation, purification, chemical and physical characterization, and enzymic specificity has been reviewed by Hagihara (1960). Mechanistically, these enzymes have become of interest since the observation that they are inactivated by reaction with 1 mole of DFP per mole of enzyme (Güntelberg and Ottesen, 1954; Ottesen and Schellman, 1957; Matsubara and Nishimura, 1958). More recently, the observation of acyl intermediates during the catalysis of various hydrolytic reactions (Bernhard *et al.*, 1965) similar to those which have been observed for chymotrypsin-catalyzed reactions has served to emphasize the similarities between these and other "serine pro-

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