Cooperative Effects of Functional Groups in Peptides. II. Elimination Reactions in Aspartyl-(O-acyl)-serine Derivatives[1]

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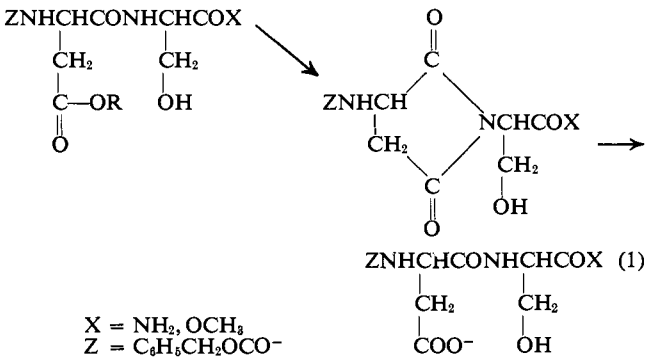
Abstract: In a previous communication the unusual hydrolytic lability of β-alkyl esters of aspartyl peptides was noted. This lability was most pronounced with blocked β-alkylaspartylserine derivatives. We have now examined the properties of related O-acylserine peptides. Such O-acyl ester derivatives are cleaved to yield acylate anion in moderately or weakly basic (aqueous) solution, the reaction rate being dependent on the nature of the substituents (R_1 and X) in compounds of the type, RCO(N–)CH(CH_2O-acyl)COX. When R = N-carbobenzyloxy-L-β-benzylaspartyl and X = OCH_3, the reaction rate is rapid (for the formation of acetate from the acetyl ester, k = 1.0 min^{-1} at pH 10.3 and 25°). The yield of acetate, although high (*ca.* 70%), is not stoichiometric. This particular reaction is shown to involve (sequentially) (a) the formation of an α,β aspartoimide (initiation), (b) the *elimination* of acetate (formation of an α,β double bond), and (c) the hydrolysis of the aspartoimide (termination). The elimination of acylate$^-$ from β-acylserine derivatives is dependent on the electron-withdrawing ability of the two substituents (R_1 and X) as evidenced by model studies of a series of substituted O-acylserine peptides. The β-elimination rate is remarkably more rapid than the hydrolysis rate when RCO(N–) is an α,β-aspartoimide and COX is an alkyl ester. A corresponding amide (X = NH_2) yields little acylate under mildly alkaline conditions. When X is an oligopeptide, the elimination rate (and yield) is notably increased over that obtained from the amide. The over-all reaction is "general base" catalyzed. Under specified conditions of base and solvent, the reaction appears to involve acylate elimination from an intermediate carbanion rather than a concerted base-catalyzed elimination (E2) mechanism (the reaction is inhibited in rate by the conjugate "general acid"). An investigation of imidazole catalysis revealed (a) that both β-elimination and hydrolysis of the β-O-acylserine ester are catalyzed by imidazole; (b) that the aspartoimide promotes both of these reactions; and (c) that the hydrolytic reaction, unlike the elimination, is relatively insensitive to the substituent X. The properties of substituted aspartylserine peptides in aqueous solution are compared with the structurally simpler N-acylserinamides. The relevance of this comparison to the mechanism of proteolytic "active serine" enzyme catalysis is discussed.

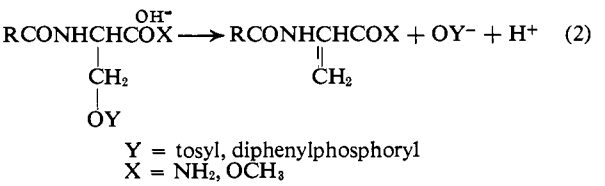
A group of proteolytic, esterolytic, and transfer enzymes which undergo specific monoacylation or monophosphorylation upon treatment with acylating or phosphorylating agents[3] has been classified as "serine enzymes" since a specific serine hydroxyl has been established as the acyl or phosphoryl acceptor. The course of catalytic solvolysis of acyl derivatives by these enzymes apparently involves acylation of this active serine followed by solvolytic deacylation—processes which are presumably mediated *via* "general base" and/or "general acid" catalysis by an imidazole or conjugate imidazolium of histidine.[4]

A number of these "serine enzymes" contain an adjacent aspartyl residue in an aspartyl–seryl sequence.[3] Motivated by this finding, a previous communication described the properties of β-esters of aspartylserine peptides. The β-carbonyl position of the aspartyl residue is exceedingly susceptible to hydrolysis and its enhanced reactivity is due to cooperative intramolecular catalysis involving the peptide bond and serine hydroxyl. The β-ester hydrolysis involves a two-step reaction (eq 1), cyclization to the imide followed by imide hydrolysis.

Since the above-mentioned enzyme reactions are presumed to involve O-acylserine in the catalytic pathway,



it would be of interest to study the properties of aspartyl-(O-acyl)-serine derivatives. "Active" esters of serine peptides have been reported previously. These are esters of strong acids and serine. O-Diphenylphosphorylserine derivatives[5] and O-*p*-toluenesulfonylserine derivatives[6-8] undergo rapid β-elimination to the corresponding acid and dehydroalanine derivative in the presence of a strong base (eq 2).



(1) Paper I in this series: S. A. Bernhard, A. Berger, J. H. Carter, E. Katchalski, M. Sela, and Y. Shalitin, *J. Am. Chem. Soc.*, **84**, 2421 (1962).

(2) On leave of absence from the Department of Biophysics, The Weizmann Institute of Science, Rehovoth, Israel.

(3) See *e.g.*, (a) P. E. Boyer, *Ann. Rev. Biochem.*, **29**, 15 (1960); (b) C. Milstein and F. Sanger, *Biochem. J.*, **79**, 456 (1961).

(4) (a) G. Schoellmann and E. Shaw, *Biochemistry*, 2, 252 (1963); (b) E. B. Ong, E. Shaw, and G. Schoellmann, *J. Am. Chem. Soc.*, **86**, 1271 (1964).

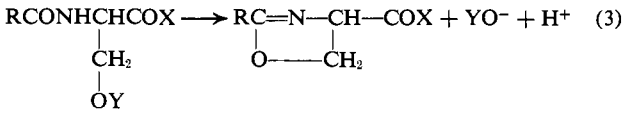
(5) G. Riley, J. H. Turnbull, and W. Wilson, *J. Chem. Soc.*, 1373 (1957).

(6) I. Photaki, *J. Am. Chem. Soc.*, **85**, 1123 (1963).

(7) L. Benoiton, R. W. Hanson, and H. N. Rydon, *J. Chem. Soc.*, 824 (1964).

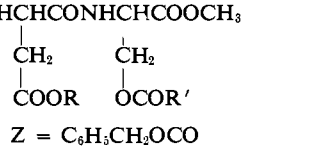
(8) D. Strumeyer, W. N. White, and D. E. Koshland, *Proc. Natl. Acad. Sci. U. S.*, **50**, 931 (1963).

With a weaker base (potassium acetate), formation of oxazoline takes place[7,9] in ethanol.



Reactions involving poorer leaving groups (Y), such as acyl or aminoacyl which are typical of hydrolytic enzyme substrates, have not been explored except for N,O-diacetylserinamide, for which Anderson,[10] *et al.*, reported an enhanced saponification rate (about eightfold) of this acetyl ester over that for ethyl acetate. This has been reported to be in accord with the more acidic character of the serine hydroxyl,[11,12] although quantitative interpretations of the acceleration are subject to a variety of mechanistic considerations.[13]

We have now studied the reactivity of other O-acylserine derivatives. Peptides of type I served as models; other related peptides were studied for comparison.



$Z = C_6H_5CH_2OCO$

I, R = $C_6H_5CH_2$; R′ = CH_3 (DL-serine)
II, R = $C_6H_5CH_2$; R′ = CH_3 (L-serine)
IV, R = CH_3; R′ = $C_5H_5CH{=}CH$ (DL-serine)

Experimental Section

β-Benzyl-N-carbobenzyloxy-L-aspartyl-L-serine methyl ester (bencasme) was prepared according to Bernhard, *et al.*[1]

O-Acetyl-DL-serine Methyl Ester Hydrochloride. DL-Serine methyl ester hydrochloride[14] (3.1 g, 0.02 mole) was dissolved in a minimal volume of concentrated hydrochloric acid to which 3 ml of glacial acetic acid was added. To the chilled solution, 5 ml of acetyl chloride was added dropwise. After standing at room temperature for 1 hr, an excess of ether was added. The precipitate which formed was thoroughly washed several times with absolute ether and then dried, yield 9%. *Anal.* Calcd for $C_6H_{12}NO_4Cl$: N, 7.1; acetyl, 21.7; neut equiv, 197.5. Found: N, 7.0; acetyl, 21.3; neut equiv, 197, as determined by anhydrous titration in ethanol with sodium methoxide solution using thymol blue as indicator.[15]

β-Benzyl-N-carbobenzyloxy-L-aspartyl-(O-acetyl)-DL-serine Methyl Ester (I). To a solution of 3.57 g (0.01 mole) of β-benzyl-N-carbobenzyloxy-L-aspartate[16] in 20 ml of ethyl acetate, a solution of 0.01 mole of O-acetyl-DL-serine methyl ester hydrochloride and 0.01 mole of triethylamine in 20 ml of chloroform was added, followed by 0.01 mole of N,N′-dicyclohexylcarbodiimide (DCC) (Aldrich Chemical Co.).

After standing overnight at room temperature, a few drops of acetic acid were added to the reaction mixture. The insoluble urea derivative was filtered off, and the filtrate was washed successively with dilute HCl solution, phosphate buffer (pH 6), and distilled water. The organic layer was dried over anhydrous sodium sulfate, and then evaporated *in vacuo*, yielding white crystals. The material was dissolved in ethyl acetate and recrystallized by addition of petroleum ether, yield 75%, mp 88°. *Anal.* Calcd for $C_{25}H_{28}N_2O_9$: C, 60.0; H, 5.6; N, 5.6; acetyl, 8.6. Found: C, 60.5; H, 5.6; N, 5.7; acetyl, 8.7.

O-Acetyl-L-serine methyl ester hydrochloride was prepared by the method employed for the DL isomer. *Anal.* Calcd for $C_6H_{12}NO_4Cl$: acetyl, 21.7; neut equiv, 197.5. Found: acetyl, 22.3; neut equiv, 200.

β-Benzyl-N-carbobenzyloxy-L-aspartyl-(O-acetyl)-L-serine methyl ester (II) was prepared from β-benzyl-N-carbobenzyloxy-L-aspartate and O-acetyl-L-serine methyl ester hydrochloride by the method employed for the DL serine isomer, yield 65%, mp 89–90°. *Anal.* Calcd for $C_{25}H_{28}N_2O_9$: C, 60.0; H, 5.6; N, 5.6; acetyl, 8.6. Found: C, 60.3; H, 5.9; N, 5.8; acetyl, 8.2.

β-Benzyl-N-acetyl-L-aspartate. β-Benzyl aspartate (10 g) was suspended in 50 ml of glacial acetic acid containing 7 ml of acetic anhydride. On heating, the suspension dissolved. The resultant solution was refluxed for 5 min and then cooled. Upon addition of petroleum ether, an oil formed, which solidified on triturating with ether. The solid was washed with petroleum ether and dried, yield 90%, mp 158–159°. *Anal.* Calcd for $C_{13}H_{15}O_5N$: N, 5.28; acetyl, 16.2; neut equiv, 265. Found: N, 5.18; acetyl, 16.4; neut equiv, 268, as determined by anhydrous titration with sodium methoxide.

β-Benzyl-N-acetyl-L-aspartyl-(O-acetyl)-L-serine Methyl Ester. β-Benzyl-N-acetylaspartate (2.65 g, 0.01 mole) dissolved in dioxane was coupled with O-acetyl-L-serine methyl ester by the DCC method, as in the preparation of O-acetyl-bencasme, yield 55%, mp 89°. *Anal.* Calcd for $C_{19}H_{24}N_2O_8$: C, 56.0; N, 6.85; H, 5.64; acetyl, 21.1. Found: C, 56.6; N, 7.0; H, 6.0; acetyl, 19.8.

N-Acetyl-L-α-aspartyl-(O-acetyl)-L-serine Methyl Ester. β-Benzyl-N-acetyl-L-aspartyl-(O-acetyl)-L-serine methyl ester (1 g) was dissolved in methanol containing a few drops of acetic acid. Debenzylation was effected by catalytic hydrogenolysis using Pd-C as a catalyst. The catalyst was removed by filtration and the solvent by flash evaporation. The resulting oil solidified on trituration with petroleum ether and ether, yield 83%, mp 114°. *Anal.* Calcd for $C_{12}H_{18}N_2O_8$: N, 8.8; acetyl, 27. Found: N, 8.6; acetyl, 25.7.

O-Acetyl-DL-serinamide hydrochloride was prepared from serinamide·HCl (Cyclo Chemical Corp.) by the method employed in preparing O-acetylserine methyl ester. *Anal.* Calcd: neut equiv, 182.5. Found: neut equiv, 180.

β-Methyl-N-carbobenzyloxy-L-aspartyl-(O-acetyl)-DL-serinamide (III) was prepared from β-methyl-N-carbobenzyloxy-L-aspartate (Cyclo Chemical Corp.) and O-acetylserinamide hydrochloride using DCC as a coupling agent as in the preparation of O-acetyl-bencasme, yield 40%, mp 137°. *Anal.* Calcd for $C_{18}H_{23}N_3O_8$: C, 52.9; H, 5.6; N, 10.5. Found: C, 53.7; H, 5.8; N, 10.2.

N-Carbobenzyloxy-O-cinnamoyl-DL-serine Methyl Ester. N-Carbobenzyloxy-DL-serine methyl ester (0.01 mole, prepared by the general method of Wolman, *et al.*[17]) was dissolved in tetrahydrofuran and refluxed for 0.5 hr with 1.3 equiv of cinnamoyl chloride (Matheson Coleman and Bell). The solvent was removed, and the resulting material was dissolved in ethyl acetate and washed with sodium bicarbonate and water. The organic layer was dried over anhydrous sodium sulfate. Upon addition of petroleum ether, N-carbobenzyloxy-O-cinnamoyl-DL-serine methyl ester separated as an oil, which was solidified upon triturating with ether and petroleum ether, yield 85%, mp 81°. *Anal.* Calcd for $C_{21}H_{21}NO_6$: C, 65.8; H, 5.5; N, 3.66. Found: C, 65.4; H, 5.8; N, 3.8.

O-Cinnamoyl-DL-serine methyl ester hydrobromide was prepared by dissolving N-carbobenzyloxy-O-cinnamoyl-DL-serine methyl ester in dioxane and saturating it with gaseous HBr.[18] The solute was concentrated by flash evaporation. On addition of absolute ether it precipitated. It was exhaustively washed with ether and dried, yield 90%, mp 154° (dec pt), ϵ_{282} 20,500 M^{-1} cm^{-1}. *Anal.* Calcd for $C_{13}H_{16}NO_4Br$: C, 47.3; H, 4.85; N, 4.24; neut equiv, 330. Found: C, 47.9; H, 5.0; N, 4.5; neut equiv, 335.

β-Methyl-N-carbobenzyloxy-L-aspartyl-(O-cinnamoyl)-DL-serine methyl ester (IV) was prepared from β-methyl-N-carbobenzyloxy-

(9) G. L. Schmir and C. Zioudrou, *Biochemistry*, 2, 1305 (1963).
(10) B. M. Anderson, E. H. Cordes, and W. P. Jencks, *J. Biol. Chem.* 236, 455 (1961).
(11) T. C. Bruice, T. H. Fift, J. J. Bruno, and N. E. Brandon, *Biochemistry*, 1, 7 (1962).
(12) W. P. Jencks and M. Gilchrist, *J. Am. Chem. Soc.*, 84, 2910 (1962).
(13) Although the base-catalyzed transacylation of active esters (*p*-nitrophenylacetate) with other alcohols and phenols is straightforwardly interpretable in terms of the pK_a of the acceptor alcohol (see, for example, T. C. Bruice and S. Benkovic, "Bioorganic Mechanisms," W. A. Benjamin and Co., New York, N. Y., 1966, pp 99–102), nonlinear structure–reactivity correlations are observed in both the OH^- and imidazole (free base) catalyzed hydrolysis of acetate esters of alcohols and phenols of diverse acidities (J. F. Kirsch and W. P. Jencks, *J. Am. Chem. Soc.*, 86, 837 (1964)).
(14) R. A. Boissonnas and S. Guttmann, *Helv. Chim. Acta*, 41, 1852 (1958).
(15) J. S. Fritz and N. M. Lisicki, *Anal. Chem.*, 23, 589 (1951).
(16) A. Berger and E. Katchalski, *J. Am. Chem. Soc.*, 73, 4084 (1957).
(17) Y. Wolman, P. M. Gallop, A. Patchornik, and A. Berger, *ibid.*, 84, 1889 (1962).
(18) D. Ben Ishai and A. Berger, *J. Org. Chem.*, 17, 1569 (1952).

L-aspartate (Cyclo Chemical Corp.) and O-cinnamoyl-DL-serine ester hydrobromide similarly to O-acetyl-bencasme. It was isolated as a viscous oil, yield 32%, ϵ_{282} 20,000 M^{-1} cm^{-1}. *Anal.* Calcd for $C_{26}H_{28}N_2O_4$: C, 60.9; N, 5.46. Found: C, 60.2; N, 5.8.

O-Cinnamoyl-L-serinamide Hydrobromide. N-Carbobenzyloxy-O-cinnamoyl-L-serinamide (2 g) (Cyclo Chemical Corp.) was dissolved in tetrahydrofuran, and the solution was saturated with gaseous hydrogen bromide. After several hours, the resulting viscous mixture was dissolved in ethanol. A white precipitate formed upon addition of ether. The precipitate was washed exhaustively with absolute ether and dried, yield 70%, mp 220° (dec pt), ϵ_{282} 20,500 M^{-1} cm^{-1}. *Anal.* Calcd for $C_{12}H_{15}N_2O_3Br$: neut equiv, 322. Found: neut equiv, 317.

β-Methyl-N-carbobenzyloxy-L-aspartyl-(O-cinnamoyl)-L-serinamide (V) was prepared by the procedure used for the corresponding methyl ester derivative, yield 50%, mp 163–164°, ϵ_{282} 20,000 M^{-1} cm^{-1}. *Anal.* Calcd for $C_{25}H_{27}N_3O_8$: C, 60.4; H, 5.43; N, 8.45. Found: C, 59.8; H, 5.9; N, 8.42.

β-Benzyl-N-carbobenzyloxy-L-aspartyl-(O-carbobenzyloxyglycyl)-L-serine Methyl Ester (VI). Bencasme (0.92 g, 0.002 mole) and 0.002 mole of carbobenzyloxyglycine were dissolved in 10 ml of dioxane, and 1 equiv of DCC was added. After standing overnight, the solution was acidified with acetic acid, and the precipitated urea compound was filtered off. The filtrate was poured onto a chilled 0.1 *M* aqueous HCl solution. The resultant precipitate was extracted into ethyl acetate and washed with phosphate buffer (pH 7) and with water. The organic solution was dried over anhydrous sodium sulfate, evaporated to a small volume at 40°, and precipitated with petroleum ether, yield 70%, mp 108–110°. *Anal.* Calcd for $C_{33}H_{34}N_3O_{11}$: N, 6.5. Found: N, 6.7.

Copoly-L-aspartyl-(O-acetyl)-L-serine was prepared by Mr. I. Jacobson of the Department of Biophysics, The Weizmann Institute of Science, Rehovoth, Israel. It was prepared by removal of the benzyl groups of β-phenylaspartyl-(O-acetyl)-serine copolymer in HBr–glacial acetic acid. *Anal.* Calcd for 1:1 copolymer: acetyl, 18.4. Found: acetyl, 15.9.

γ-Benzyl-N-acetyl-L-glutamate was prepared from γ-benzyl-L-glutamate similarly to the preparation of the analogous aspartate derivative, yield 80%, mp 105–107°. *Anal.* Calcd for $C_{14}H_7NO_5$: N, 5.0; acetyl, 15.3; neut equiv, 279. Found: N, 4.8; acetyl, 15.0; neut equiv, 280.

γ-Benzyl-N-acetyl-L-glutamyl-(O-acetyl)-L-serine methyl ester was prepared from N-acetyl-γ-benzyl-L-glutamate and O-acetyl-L-serine methyl ester hydrochloride similarly to the β-benzyl-N-acetylaspartyl-(O-acetyl)-serine methyl ester, yield 50%, mp 99–100°. *Anal.* Calcd for $C_{20}H_{26}N_2O_8$: C, 56.9; H, 6.9; N, 6.6; acetyl, 20.4. Found: C, 57.6; H, 6.5; N, 6.7; acetyl, 19.5.

N-Carbobenzyloxyglycyl-(O-acetyl)-DL-serine methyl ester was prepared from carbobenzyloxyglycine and O-acetyl-DL-serine methyl ester hydrochloride by a procedure similar to that for O-acetyl-bencasme, yield 40%, mp 63°. *Anal.* Calcd for $C_{16}H_{20}N_2O_7$: N, 8.0; acetyl, 12.8. Found: N, 8.3; acetyl, 12.5.

N-Benzoyl-DL-serine methyl ester was prepared by dissolving 5 g of N-benzoyl-DL-serine[19] in 20 ml of methanol and saturating it with HCl for several minutes. The solution was poured into a large excess of water and the precipitate was extracted into ethyl acetate. The extract was washed with bicarbonate solution and water, dried over anhydrous sodium sulfate, filtered, and evaporated. An oily material resulted which solidified upon standing at room temperature several days, yield 80%, mp 60°. *Anal.* Calcd for $C_{11}H_{13}NO_4$: C, 59.1; H, 5.8, N, 6.3. Found: C, 59.2; H, 5.7; N, 6.3.

***p*-Nitrophenyl-Δ-oxazoline** was prepared according to Zioudrou and Schmir.[20]

N-Carbobenzyloxy-(O-acetyl)-DL-serylglycine Ethyl Ester. N-Carbobenzyloxy-(O-acetyl)-DL-serine[21] (0.01 mole) in ethyl acetate was added to a solution of 0.01 mole of glycine ethyl ester hydrochloride (Aldrich Chemical Co.) in chloroform containing an equivalent of triethylamine. An equivalent of DCC was then added. The isolation of the resulting dipeptide was similar to the isolation of the above-mentioned dipeptides, yield 70%, mp 71°. *Anal.* Calcd for $C_{17}H_{22}N_2O_7$: C, 55.8; H, 6.01; N, 7.65. Found: C, 56.1; H, 6.1; N, 7.9.

N-Carbobenzyloxy-(O-acetyl)-DL-serydiglycyl ethyl ester was prepared by coupling N-carbobenzyloxy-(O-acetyl)-serine with diglycine ethyl ester hydrochloride using DCC as the condensing reagent, similarly to the preparation of N-Z-(O-acetyl)-serylglycine ethyl ester, yield 70%, mp 79–80°. *Anal.* Calcd for $C_{19}H_{25}N_3O_8$: N, 9.9. Found: N, 9.7.

N-Carbobenzyloxy-(O-diphenylphosphoryl)-DL-serine ethyl ester and N-carbobenzyloxy-(O-diphenylphosphoryl)-DL-serylglycine ethyl ester were prepared by the method of Riley, *et al.*[5]

Solvents and Reagents. The organic solvents employed in kinetic experiments were all "spectro grade" (Matheson Coleman and Bell). At higher concentration, commercial imidazole (Matheson Coleman and Bell) had a significant absorbancy at 300 mμ ($\gtrsim$ 1 *M*). It was suspended in benzene and refluxed in the presence of active charcoal. The mixture was filtered hot, and on cooling, imidazole crystallized; the crystals were filtered and dried. Transparent solutions (at 300 mμ) could thus be prepared.

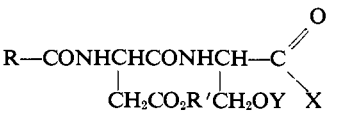
"pH-Stat" Kinetics. The following is a typical kinetic hydrolysis experiment. The reactant (0.01 mole) was dissolved in 1.0–5.0 ml of organic solvent (dioxane or acetonitrile) in a thermostated reaction vessel. Aqueous KCl solution was added to a final volume of 10.0 ml, and to an ionic strength of 0.2. Electrodes were immersed into the solution, and the desired pH was maintained by a pH-stat (radiometer-type TTT1a autotitrator) coupled to a recorder (Ole Dich, Copenhagen). Blank rates (which were usually negligible) were subtracted from the total measured rate.

Spectrophotometric Kinetics. A Cary Model 14 recording spectrophotometer was used in all measurements reported here. Sample and reference cell compartments were thermostated at 25.0°. A small volume (0.02–0.2 ml) of substrate in dioxane or acetonitrile was added to the solvent medium in the sample cell at zero time. The deacylation of cinnamoyl derivatives was followed by the disappearance of cinnamate ester at 300 mμ. Dehydroalanine formation was followed at 240 mμ.

When the solvent itself had an appreciable absorbance (*e.g.*, imidazole solutions), the reference cell contained an identical solution but for the exclusion of substrate solute.

Results

Hereafter we shall utilize the following abbreviated terminology for aspartylseryl peptides.



Order of symbols: (O–Y) (β-aspartyl ester) (aspartylseryl) (X)
(as)

Y = H, no symbol; Y = acyl, O-acyl; R′ = benzyl, ben; R′ = methyl, met; R = $C_5H_5CH_2O$, c; X = NH_2, a; X = OCH_3, me

For example, $C_6H_5CH_2OCONHCH(CH_2CO_2CH_3)CONHCH(CH_2OCOCH_3)CONH_2$ = O-acetyl-metcasa

Reactions of Aspartyl-(O-acyl)-serine Peptides. The time course of reaction of O-acetyl-bencasme in 1:2 dioxane–water (v/v) at pH 10.5 was followed in a pH-Stat. Results are shown in Figure 1A. Following an initial lag phase, a rapid, virtually first-order release of protons was observed. For comparison, the previously described reaction of bencasme under identical conditions is given (Figure 1B). The rate of proton release is slower with the O-acetyl derivative than with the free hydroxyl derivative. Moreover, the O-acetyl derivative liberates 1.7 equiv of protons/mole of peptide, while only 1 equiv of protons is released in the reaction of bencasme. The terminal first-order specific rates of reaction of O-acetyl-bencasme, under various conditions of pH, are listed in Table I. The reaction rate is proportional to OH^- concentration. The DL compound undergoes reaction at the same rate as the L compound.

(19) S. P. L. Sorensen and A. C. Andersen, *Z. Physiol. Chem.*, **56**, 250 (1908).
(20) C. Zioudrou and G. L. Schmir, *J. Am. Chem. Soc.*, **85**, 1123 (1963).
(21) M. Frankel and M. Halman, *J. Chem. Soc.*, 2735 (1952).

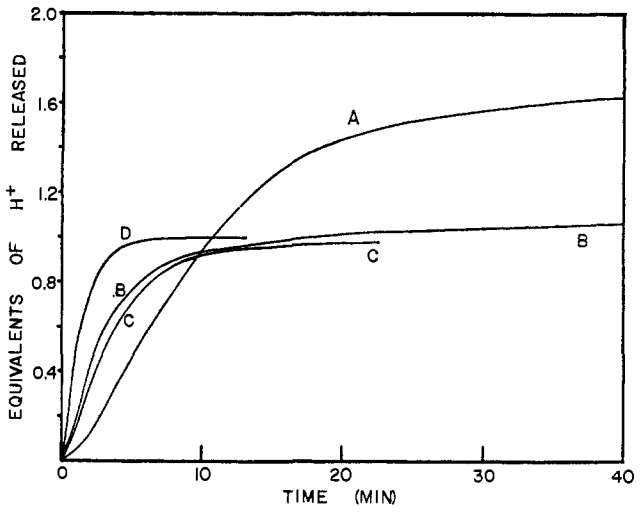


Figure 1. Proton release in the reaction of 1.5×10^{-3} M substrate in dioxane–water (1:2 v/v) at pH 10.5, $\mu = 0.2$, 25°, measured in the pH-stat. A, β-benzyl-N-carbobenzyloxy-L-aspartyl-(O-acetyl)-DL-serine methyl ester (I); B, β-benzyl-N-carbobenzyloxy-L-aspartyl-DL-serine methyl ester; C, β-methyl-N-carbobenzyloxy-L-aspartyl-(O-acetyl)-DL-serinamide (III); D, β-methyl-N-carbobenzyloxy-L-aspartyl-L-serinamide.

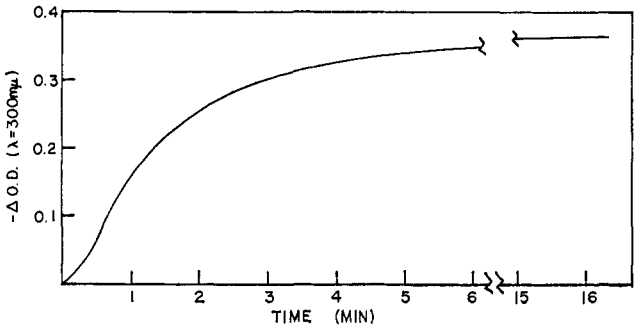


Figure 2. Decinnamoylation of β-methyl-N-carbobenzyloxy-L-aspartyl-(O-cinnamoyl)-DL-serine methyl ester (IV), 0.5×10^{-4} M in phosphate buffer, pH 10.27, $\mu = 0.2$ at 25°, 1-cm path.

In light of the differences in the reactions of the two above-mentioned peptides it seemed desirable to prepare an O-acyl derivative of bencasme in which the kinetic fate of the O-acyl group could be followed more conveniently. Toward this end we prepared β-methyl-N-carbobenzyloxyaspartyl-(O-cinnamoyl)-serine methyl ester (IV). Any deacylation of the cinnamoyl ester to yield cinnamate ion can readily be detected by the difference in ultraviolet absorption spectra between the ester and the carboxylate anion. That rapid deacylation of IV does occur at moderate pH is illustrated in Figure 2. The shape of this curve is similar to that of Figure 1A. After an induction period, a first-order reaction (in release of cinnamate ion) takes place. The spectrum of the final product (Figure 3) establishes that most of the cinnamate ester has been converted to cinnamate ion. On the other hand, when β-benzyl-N-carbobenzyloxy-L-aspartyl-(O-cinnamoyl)-L-serinamide (V) was added to a buffered solution at pH 11, the release of cinnamate ion, as judged by the decrease of the 3000-A absorbancy, was found to be first order in the starting peptide, with no induction period. The OH^- catalyzed specific rate is $k = 20$ M^{-1} min^{-1}, which is very much slower than that of the serine methyl ester analogs, but comparable to that reported for the hydrolysis of N-acetyl-O-cinnamoylserinamide.[22] An O-acetyl analog, β-methyl-N-Z-Asp-(O-acetyl)-Ser-NH_2,

Table I. Rate of Proton Release from O-Acetylserine Derivatives as Measured in the pH-Stat, $\mu = 0.2$, 25°

Compound	Solvent composition dioxane–water (v/v)	pH	$100k$, min^{-1}
β-Benzyl-N-Z-L-Asp-(O-acetyl)-DL-Ser-OCH_3 (I)	1:2	10.0	4.7
	1:1	10.5	4.7
	1:2	10.5	13.5
	1:4	10.5	39
	1:2	11.0	58
	1:1	11.5	28
β-Benzyl-N-Z-L-Asp-(O-acetyl)-L-Ser-OCH_3 (II)	1:9	9.5	17.5
	1:2	10.0	4.7
β-Methyl-N-Z-L-Asp-(O-acetyl)-DL-Ser-NH_2 (III)	1:2	10.5	23

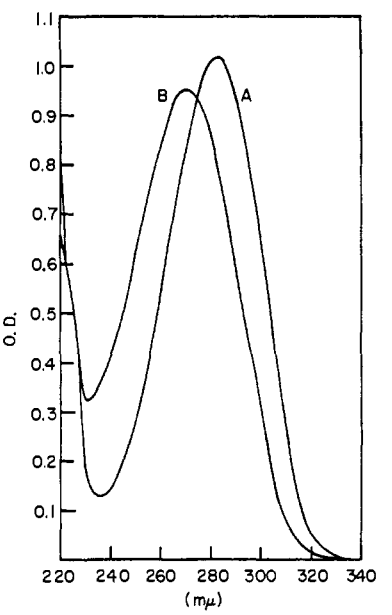


Figure 3. Ultraviolet spectra of β-methyl-N-carbobenzyloxy-L-aspartyl-(O-cinnamoyl)-DL-serine methyl ester (IV). A, initial spectrum, pH 6.8; B, after decinnamoylation.

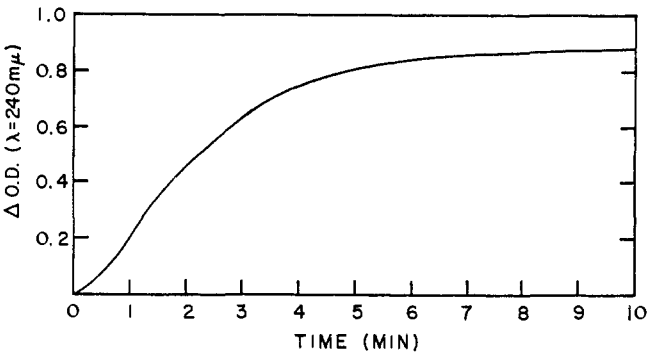


Figure 4. Change of optical density at 240 mμ during the reaction of β-benzyl-N-carbobenzyloxy-L-aspartyl-(O-acetyl)-DL-serine methyl ester (I) (3.2×10^{-4} M, 1-cm path) in dioxane–water (1:2 v/v), phosphate buffer, pH 10.9, $\mu = 0.2$ at 25°.

(22) M. L. Bender, G. R. Schonbaum, and B. Zerner, *J. Am. Chem. Soc.*, 84, 2540 (1962).

releases *1* equiv of H^+ rapidly, although at a slower rate than metcasa (Figure 1).

It has previously been shown that imide reaction intermediates (eq 1) exhibit weak, albeit measurable, absorption bands in the 240-mμ wavelength region.[1] The change in absorbancy of O-acetyl-bencasme in 1:2 (v/v) dioxane–water was studied in this wavelength region in anticipation of once again identifying such an imide intermediate. Contrary to expectations, much greater changes in optical density, and an initial lag phase, were observed (Figure 4). The shape of the kinetic curve is essentially the same as that observed for the release of cinnamate from the O-cinnamoyl analog (Figure 2), and unlike the transient OD–time curve associated with the time course of imide formation.[1] The initial reaction mixture does not absorb at 240 mμ (Figure 5). On incubation in mildly alkaline solution the absorption increases after an induction period (Figure 4), indicating that some chromophoric group is produced during the reaction (presumably the β-olefin *via* dehydration of the serine hydroxyl). The molar extinction (ϵ_{240}) of the final product is 3300–4000 M^{-1} cm^{-1}. The terminal first-order rate of formation of this product in various solvents is given in Table II. When an excess of mercaptoethanol solution is added to the reaction product, the absorbancy at 240 mμ decreases rapidly, the rate being first order in $\Delta\epsilon_{240}$, and first order in mercaptoethanol. The second-order specific rate constant is 8.4 M^{-1} min^{-1} at pH 7.2. Addition of excess aqueous dimethylamine solution (pH 10.7) also caused a decrease in the absorbancy at 240 mμ, with a second-order specific rate constant of 7 M^{-1} min^{-1}. Similar treatment of a chromophoric oxazoline, *p*-nitrobenzoyloxazoline, with mercaptoethanol or dimethylamine gave no change in the ultraviolet spectrum.

Table II. Rate of Reaction of O-Acyl-bencasme as Measured by Change of Absorbancy in Phosphate and Pyrophosphate Buffers, μ = 0.2, 25°

Compound	% dioxane in water	pH	100*k*, min^{-1}
β-Benzyl-N-Z-L-Asp-(O-acetyl)-DL-Ser-OCH_3[a] (I) (O-acetyl-bencasme)	3.2	9.0	3.8
	3.2	10.3	100
	3.2	10.92	350
	6.4	10.78	280
	10.0	9.6	15
	10.0	10.86	200
	20.0	10.53	64
	33.0	10.8	35
	50.0	10.8	14
β-Benzyl-N-Z-L-Asp-(O-acetyl)-L-Ser-OCH_3[a] (II)	10.0	10.86	185
β-Methyl-N-Z-L-Asp-(O-cinnamoyl)-DL-Ser-OCH_3[b] (IV)	3.2	9.8	19
	3.2	10.27	58
	3.2	10.8	175

[a] Measured by change of absorbancy at 240 mμ. [b] Measured by change of absorbancy at 300 mμ.

The aqueous, alkaline (pH 10.5) reaction product derived from O-acetyl-bencasme was subjected to total hydrolysis by refluxing with 6 *N* HCl for 24 hr. Aspartic acid was recovered quantitatively, whereas only 25% of the initial serine, and in addition 0.75 equiv of NH_3, were found. No other amino acid could be identified.

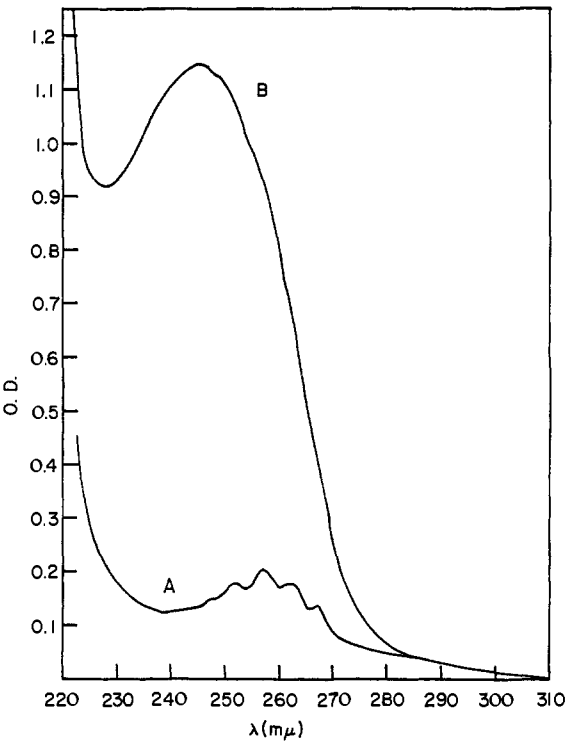


Figure 5. Ultraviolet spectra of β-benzyl-N-carbobenzyloxy-L-aspartyl-(O-acetyl)-DL-serine methyl ester (I) (3.2×10^{-4} *M*, 1-cm path) in dioxane–water (1:2 v/v), μ = 0.2 at 25°. A, at pH 6.8; B, after reaction at pH 10.9.

Similar acid hydrolysis of the original reactant (O-acetyl-bencasme) resulted in a recovery of over 95% of the constituent amino acid components.

Hydrogenolysis of the alkaline reaction products of O-acetyl-bencasme (see Experimental Section) followed by acid hydrolysis, as above, yielded aspartic acid quantitatively, serine in 30% yield, and alanine in 70% yield.

When O-acyl-bencasme was dissolved in a 1 *M* potassium acetate solution in anhydrous ethanol, an increase of the absorbancy at 240 mμ took place. The spectrum of the resulting product was similar to that shown in Figure 5. On addition of small amounts of acetic acid to the reacting mixture, the reaction rate dropped considerably.

The alkaline reaction of β-methyl-N-carbobenzyloxy-aspartyl-(O-acetyl)-serinamide at 240 mμ showed a slow increase in absorbancy. The second-order (OH^- catalyzed) specific rate is 50 M^{-1} min^{-1}, which is essentially the specific rate found for N,O-diacetyl-serinamide.[10] Similar slow rates were found for other O-acetylserinamide and peptide derivatives (see Table III). These rates are about 100-fold slower than the rate of imide formation in β-alkylaspartylserine derivatives.[1]

A transient increase in absorbancy at 240 mμ during the reaction of bencasa is observed, whereas the O-acyl derivatives show a stable increase in absorbancy which can be decreased by the addition of amines. These facts indicate that O-acylserinamide derivatives undergo elimination to the corresponding dehydroalanine derivatives.

Reaction of Other Derivatives of O-Acetylserine Methyl Ester. The reactions of various peptides

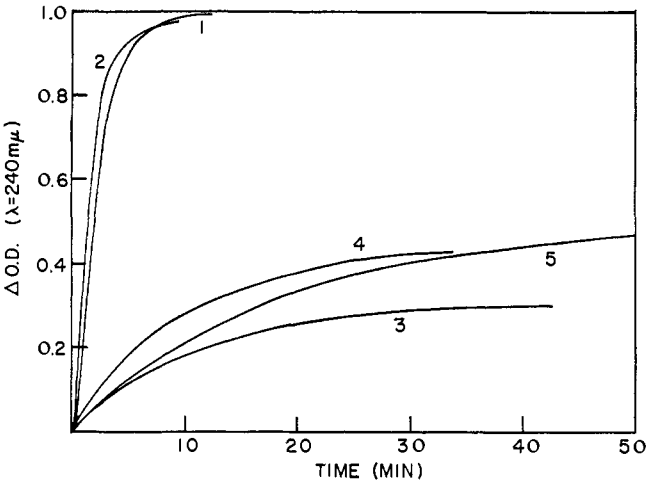


Figure 6. Change of optical density during the reaction of 3.2×10^{-4} M substrate solution in dioxane–water (1:2 v/v), phosphate buffer, pH 11.2, μ = 0.2 at 25°. (1) β-Benzyl-N-carbobenzyloxy-L-aspartyl-(O-acetyl)-DL-serine methyl ester (I). (2) β-Benzyl-N-acetyl-aspartyl-(O-acetyl)-L-serine methyl ester. (3) N-Acetyl-α-aspartyl-(O-acetyl)-L-serine methyl ester. (4) N-Carbobenzyloxy-glycyl-(O-acetyl)-DL-serine methyl ester. (5) N-Carbobenzyloxy-O-acetyl-DL-serine methyl ester.

containing the O-acetylserine methyl ester residue but lacking a β-aspartyl *ester* component were investigated spectrophotometrically at 240 mμ. N-Acetyl-α-aspartyl-(O-acetyl)-serine methyl ester undergoes a much slower first-order reaction uncomplicated by an initial lag period (Figure 6). Other peptides lacking the β-aspartyl ester group behave similarly. The ratio of elimination products to hydrolysis product can be estimated from the final optical density at 240 mμ. Results are listed in Table IV.

When the O-acylserine group is replaced by a strongly electron-withdrawing group(diphenylphosphoryl)(Table V), the elimination reaction is similar in rate to that of O-acyl-bencasme. However, N-acyl-O-diphenylphosphorylserinamide derivatives undergo elimination at nearly the same rate as the analogous O-acylserine esters. Aminoacyl groups at the O-serine facilitate elimination to a small extent relative to the O-acylserine derivatives. Rates of elimination as a function of the leaving groups are illustrated in Table V.

Table III. Elimination Rates[a] of O-Acetylserinamide and Peptide Derivatives

Compound	$100k$, min^{-1}	ϵ_{240}	% elimination[b]
β-Methyl-N-Z-L-Asp-(O-acetyl)-DL-Ser-NH_2	4.7	250	5
N-Z-(O-acetyl)-DL-Ser-Gly-OC_2H_5	8.7	750	15
N-Z-(O-acetyl)-DL-Ser-Gly_2-OC_2H_5	7	1800	36
Copoly[-L-Asp-(O-acetyl)-L-Ser]	7	200	4

[a] In dioxane–water 1:9 (v/v), pH 11.0 in 0.02 M carbonate buffer, μ = 0.2, 25°, as measured by rate of change of OD at 240 mμ. [b] Assuming ϵ_{240} for dehydroalanine derivatives is 5000.

Table IV. Rate of Elimination of Derivatives of O-Acetylserine Methyl Esters[a]

Compound	$100k$, min^{-1}	ϵ_{240}	% elimination[b]
N-Z-O-acetyl-DL-Ser-OMe	5	1800	36
N-Z-Gly-(O-acetyl)-L-Ser-OMe	10	1700	34
β-Benzyl-N-Z-Asp-(O-acetyl)-L-Ser-OMe	64	3300	66
β-Benzyl-N-acetyl-L-Asp-(O-acetyl)-L-Ser-OMe	84	3400	68
N-Acetyl-L-Asp-(O-acetyl)-L-Ser-OMe	7	1200	25
γ-N-Acetyl-L-Glu-(O-acetyl)-L-Ser-OMe	10	1600	32
N-Z-O-acetyl-DL-Ser-OH	Negligible		

[a] In dioxane–water 1:2 (v/v), at pH 11.2 in 0.02 M phosphate buffer, μ = 0.2, 25°, determined by change of absorbancy at 240 mμ. [b] Assuming molar extinction of 5000 for dehydroalanine derivatives.

Table V. Elimination Rates of Various O-Acyl and Phosphorylserine Derivatives[a]

Compound	$100k$, min^{-1}
β-Benzyl-N-Z-L-Asp-(O-acetyl)-L-Ser-OMe (II)	62.6
β-Benzyl-N-Z-L-Asp-O-(N-Z-gly)-L-Ser-OMe (IV)	84.3
N-Z-(O-diphenylphosphoryl)-DL-Ser-OC_2H_5	10
N-Z-(O-diphenylphosphoryl)-DL-Ser-Gly-OC_2H_5	3.3

[a] In dioxane–water 1:2 (v/v) at pH 11.0 in 0.002 M phosphate buffer, μ = 0.2, 25°, as measured by the change of OD at 240 mμ.

Imidazole Catalysis. Cinnamate ion is released from O-cinnamoyl-bencasme in 2 M imidazole (pH 7.3) at a rate 6.7-fold faster than that which occurs in the absence of imidazole at the same pH. O-Cinnamoyl-bencasa, which releases cinnamate very slowly at this pH in the absence of imidazole, undergoes decinnamoylation at a rate comparable to that for O-cinnamoyl-bencasme in 2 M imidazole (~500 times faster than in the absence of imidazole). When the reaction of O-acetyl-bencasme in 2 M imidazole at pH 7.3 was followed spectrophotometrically at 240 mμ, an initial increase in absorption followed by a decrease occurred. The maximum change in absorbancy was about 15% of that found for total OH^- catalyzed reaction. This suggested the formation and disappearance of dehydroalanine during the course of reaction.

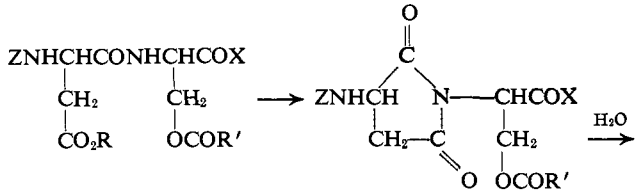
Indeed when the eliminated product of the OH^- catalyzed reaction of O-acetyl-bencasme was made 2 M in imidazole (pH ~7.3), a drop in the absorbancy at 240 mμ with a first-order specific rate of 6×10^{-3} min^{-1} occurred.

O-Cinnamoylserinamide derivatives lacking the β-aspartyl ester group have a much slower decinnamoylation rate in imidazole buffers. Thus N-carbobenzyloxyaspartyl-(O-cinnamoyl)-serinamide, obtained by the selective hydrolysis of the benzyl ester linkage in O-cinnamoyl-bencasa, and N-carbobenzyloxyglycyl-(O-cinnamoyl)-serinamide undergo decinnamoylation at a rate corresponding to about 0.1 of that for the β-aspartyl ester (O-cinnamoyl)-serine derivative.

Discussion

Hydrolysis of O-Acetyl-bencasme. With the carbobenzyloxy-β-esters of aspartyl peptides, an intermediate in the hydrolysis of the β-ester was identified as the aspartoimide.[1] With the O-acetyl esters of aspartyl-serine peptides (Figure 1), a lag in the production of

protons is again presumably due to the formation of an imide intermediate, the sequence of reactions being given by eq 4.

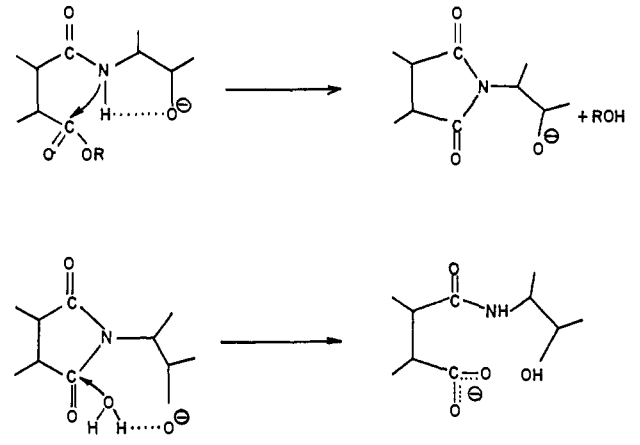


hydrolysis products + $H^{\oplus}$ (4)

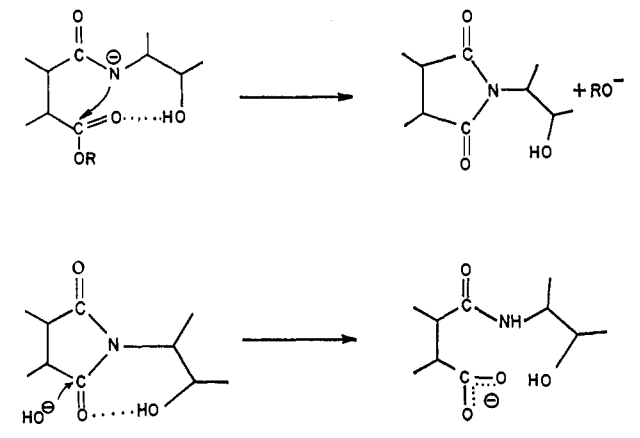
The rate of proton release from O-acetyl-bencasme is about threefold slower than from bencasme, and similar to the rate from O-acetyl-bencasa. Kienhuis[23] obtained similar results with Z-Gly(β-benzyl)aspartyl-(O-acetyl)-Ser-Gly-NH_2 and the corresponding unsubstituted serine peptide.

Blocking of the serine hydroxyl (by acylation) decreases the rates of imide formation and hydrolysis. The serine hydroxyl may function as a general base (Scheme I) or a general acid catalysis (Scheme II) by a

Scheme I



Scheme II



mechanism suggested previously.[24-26]

Although the reaction rates of the O-acetyl derivatives are slower than the free hydroxyl compounds, the hydroxide-catalyzed rate of reaction is much faster than that found in the hydrolysis of monofunctional esters. The rate of reaction of O-acetyl-bencasme is about 600 times faster than the rate of hydrolysis of ethyl acetate and about 80-fold faster than the hydrolysis of N,O-diacetylserinamide under similar conditions.

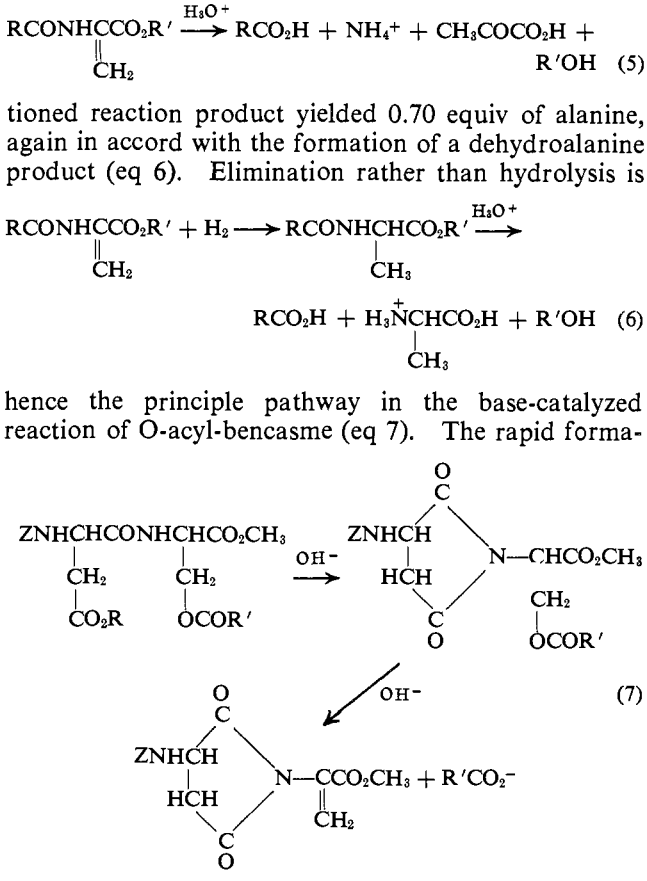
Bencasme consumes 1 equiv of alkali in the rapid hydrolytic reaction whereas the O-acetyl derivative consumes approximately 1.7 equiv, indicating a more complex reaction pathway in the latter. In the case of O-acetyl-metcasa only 1 equiv of alkali is rapidly consumed (Figure 1). The methyl esters of serine derivatives do not hydrolyze at comparable rates (bencasme, for example, undergoes rapid hydrolysis at the β-aspartyl position under conditions where the serine methyl ester is only slightly hydrolyzed). It is unlikely that the presence of the O-acetyl group would considerably promote the hydrolysis of the serine methyl ester. On the other hand, the saponification of N,O-diacetylserinamide is only eight times faster than ethyl acetate,[10] which is about 80-fold slower than O-acetyl-bencasme. In order to distinguish which of the two (serine) esters was involved in the above-mentioned reaction of O-acetyl-bencasme, we prepared the corresponding O-cinnamoyl-bencasme. The difference between a cinnamoyl ester and cinnamate anion is readily detected by the difference in ultraviolet spectra of the two compounds.[22] N-Acetyl-O-cinnamoylserinamide has λ_{max} 282 mμ and ϵ_M 22,000 (M^{-1} cm^{-1}), while cinnamate ion absorbs maximally at 269 mμ with ε 20,000. Figures 2 and 3 demonstrate that O-cinnamoyl-bencasme undergoes decinnamoylation. The spectrum shifts from cinnamate ester to cinnamate anion. Thus the second ester group which is deacylated in O-acyl-bencasme is at the acylserine bond rather than the serine alkyl ester bond. The hydroxyl ion catalyzed specific rate of decinnamoylation of cinnamoyl-bencasme is ~2700 M^{-1} min^{-1} (in 3.2% dioxane in water) which is 130-fold faster than the hydrolysis rate of N-acetyl-O-cinnamoylserinamide. Again this ultimately pseudo-first-order decinnamoylation (Figure 2) takes place after an induction period, presumably due to the prior formation of an aspartoimide intermediate.

β-Elimination in O-Acetyl-bencasme. On the basis of the decinnamoylation reaction with the chromophoric reactant, O-cinnamoyl-bencasme, it is reasonable to assume that deacetylation of O-acetyl-bencasme occurs in mildly alkaline solution, and that unanticipated spectral changes in the 240 mμ range (Figure 4) are concomitant with this reaction. The difference in molar extinction between reactant and final products is $\Delta\epsilon_{max} \simeq 3300$ (at 240 mμ). The hydrolysis of either O-acetylserine esters or β-aspartyl aliphatic esters does not involve such an absorbancy change. The resultant difference spectrum (Figure 5), following deacetylation of O-acetyl-bencasme, is characterisitic of α-amino acrylate derivatives, which typically have ϵ_{240} ~5000.[5] The final product undergoes chemical reactions characteristic of α-amino acrylate derivatives. These compounds add thiols or amines to saturate the double bond, thereby losing the strong absorption at 240 mμ.[27]

The amino acid composition of the pH 10 reaction product of O-acetyl-bencasme is in accord with the known instability of dehydroalanine derivatives toward

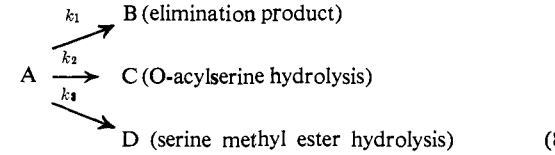
(23) H. Kienhuis, Ph.D. Dissertation, Leiden University, 1962.
(24) Y. Shalitin and S. A. Bernhard, *J. Am. Chem. Soc.*, **86**, 2291 (1964).
(25) M. L. Bender, F. J. Kézdy, and B. Zerner, *ibid.*, **85**, 3017 (1963).
(26) T. C. Bruice and T. H. Fife, *ibid.*, **84**, 1973 (1962).
(27) J. P. Greenstein and M. Winitz, "Chemistry of the Amino Acids," John Wiley and Sons, Inc., New York, N. Y., 1961, p 856.

hydrolysis (eq 5). Hydrogenation of the above-mentioned reaction product yielded 0.70 equiv of alanine, again in accord with the formation of a dehydroalanine product (eq 6). Elimination rather than hydrolysis is hence the principle pathway in the base-catalyzed reaction of O-acyl-bencasme (eq 7). The rapid formation of an imide intermediate enhances the elimination of the O-acyl group and is responsible for the initial lag.



The importance of the imide in enhancing the rate of elimination is illustrated by the relative rates of reaction of Z-glycyl-(O-acetyl)-serine methyl ester and N-acetyl-α-aspartyl-(O-acetyl)-serine methyl ester. Both these compounds undergo β elimination at about 10% of the rate found for O-acetyl-bencasme but without an initial lag period (see Figure 6 and Table IV).

The fact that other O-acetylserine esters undergo elimination to a lesser extent (25–35%) is due to the rates of competitive reactions, *viz.*, normal hydrolysis of the acetylserine ester, and the hydrolysis of the serine methyl ester group (yielding serine carboxylate which does not undergo elimination). The specific rate of hydrolysis of N,O-diacetylserinamide is 48 M^{-1} min^{-1}, while that of the N-acylamino acid esters, methyl hippurate, and N-benzoylserine methyl ester are 180 M^{-1} min^{-1} (in H_2O)[28] and 120 M^{-1} min^{-1} (in 1:2 v/v dioxane–water), respectively. The kinetic course of these reactions, assuming that all three potential pathways are very much more rapid once cyclization to the imide has occurred, is given by eq 8.



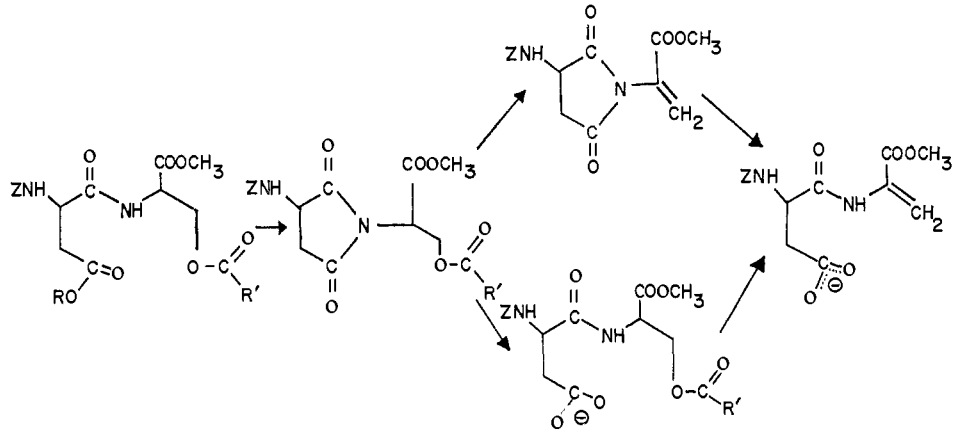
$$[A]_t = A_0 e^{-(k_1 + k_2 + k_3)t} = A_0 e^{-kt}$$

$$[B]_t = \frac{k_1}{k} A_0 (1 - e^{-kt}) = B_\infty (1 - e^{-kt})$$

where A_0 = initial reactant concentration and $B_\infty = [B]_{t=\infty}$. The true first-order constants (k_1) are obtained by multiplying the apparent rate constants, k, by the fractional yield of the particular product (*e.g.*, B_∞/A_0).

The role of the two imide carbonyl groups is presumably to enhance formation of the α-(seryl) carbanion by electron-withdrawal. The carboxyl-terminal methyl ester group further facilitates formation of such a carbanion, as is emphasized by the far greater extent of elimination (relative to hydrolysis) in esters as compared to amides (Tables III and IV). Imide hydrolysis has been shown to be a rapid process in all such compounds.[2] If the imide is hydrolyzed before elimination takes place the resultant aspartyl-(O-acyl)-serine peptide is relatively stable. These reaction pathways are summarized in Scheme III.

Scheme III



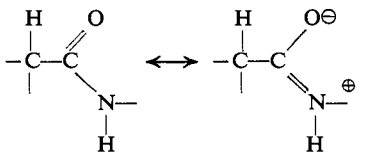
The Effect of the Leaving Group on the Rate and Extent of Elimination. Serine derivatives with strongly acidic groups attached to the hydroxyl undergo facile elimination. Riley, *et al.*,[5] found that N-Z-(O-diphenylphosphoryl)-serine ethyl ester and N-Z-(O-diphenylphosphoryl)-serylglycine ethyl ester undergo rapid elimination in strongly basic aqueous–alcoholic solution. Similar results have been reported with N-Z-(O-tosyl)-serylglycine ethyl ester.[6] Samuel and Silver[29] elegantly demonstrated that even the negatively charged

(28) S. A. Bernhard, W. C. Coles, and J. F. Nowell, *J. Am. Chem. Soc.*, 82, 3043 (1960).
(29) D. Samuel and B. L. Silver, *J. Chem. Soc.*, 289 (1963).

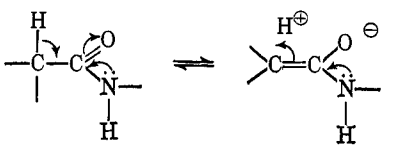
serine phosphate undergoes elimination in concentrated alkali although the reaction rate is low.

The specific rate of β elimination from N-Z-(O-diphenylphosphoryl)-serine methyl ester is one-sixth the rate of O-acetyl elimination from O-acetyl-bencasme. The effect of the proximal imide residue in the latter compound apparently compensates for the very much poorer (acetyl) leaving group; N-Z-(O-diphenylphosphoryl)-serylglycine ethyl ester undergoes elimination at a much greater rate than the O-acetyl analog, the strongly electron-withdrawing diphenylphosphoryl group presumably stabilizing the carbanion. The elimination of an O-aminoacyl group is somewhat faster than the elimination of an acetyl group (Table V).

The Influence of Serine Carboxyl Substituents on the Pathway of Reaction. The rate of "deacylation" of acylserine derivatives is 100-fold slower for amides than for esters. The slow deacylation rate for a series of O-acylserinamides is comparable to the (ester) hydrolysis rate reported for N,O-diacetylserinamide. However, the extent of elimination varies from 4 to 40% (as estimated from the ϵ_{240} value). Electron-withdrawal by amides is much poorer than esters, presumably due to resonance of the form,



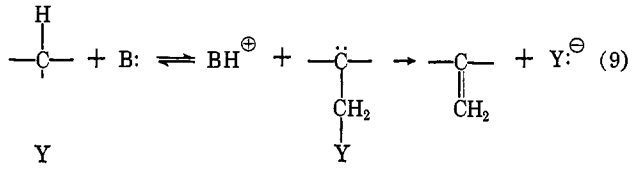
hence opposing the elimination of an α-proton (carbanion formation).



Elimination occurs in serinamides and serine peptides only with "better" leaving groups such as diphenylphosphoryl and tosyl groups. In O-acylserinamide derivatives, two parallel reactions occur: hydrolysis and elimination (as in eq 8). If the rate of elimination (k_1) is of the order of, or less than, the rate of hydrolysis (k_2), the observed over-all specific rate (k) will not differ considerably from the specific rate of hydrolysis, whereas a large variation in the extent of elimination can occur depending on the value of k_1. With O-acetyl-metcasa, the extent of elimination is only 5%. Hence the over-all apparent specific rate of reaction is not notably different from the specific rate of hydrolysis. With N-Z-(O-acetyl)-serylglycine ethyl ester, the extent of elimination increases to 36%. The over-all specific rate is only twice that found for O-acetyl-bencasa. The fact that aspartyl-(O-acetyl)-serine copolymers undergo elimination to some extent suggests that the base-catalyzed deacylation of poly(β-benzyl)-aspartyl-(O-acetyl)-serine reported by Adler, *et al.*,[30] is an elimination of acetate rather than a hydrolysis of the acetyl ester.

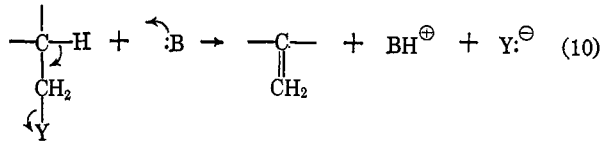
It has been reported that when N-hippuryl-(O-tosyl)-serinamide is refluxed in a methanolic solution of potassium acetate, 2-benzamidomethyl-4-carbamoyl-Δ^2-oxazoline is formed, whereas starting with the corresponding serine methyl ester, similar treatment results in the formation of the corresponding dehydroalanine derivative.[7] N-Acetyl-O-diphenylphosphorylserinamide under similar conditions yields N-acetylserinamide.[9] Schmir and Zioudrou interpret this latter reaction as a two-step process involving the intermediate formation of the oxazoline followed by rapid hydrolysis. Dehydroalanine derivatives could not be detected in this reaction; the assumption of an oxazoline intermediate which also could not be detected is therefore indirect.

The Mechanism of β Elimination. β Elimination may occur *via* two pathways, involving an Ecb mechanism or an E2 mechanism (eq 9 and 10). The Ecb mechanism involves the intermediate formation of a carbanion followed by departure of the group Y, whereas in the E2 mechanism, the processes are concerted. These mechanisms can be distinguished by the dependence of the reaction rate on the conjugate acid, BH^+.



(9)

E_{CB} mechanism



(10)

E_2 mechanism

The presence of this acid would lower the reaction rate (by lowering the concentration of the carbanion) according to the Ecb mechanism, but have no effect on the reaction rate according to the E2 mechanism.[31] The addition of small amounts of acetic acid to an ethanolic solution of potassium acetate results in a decreased rate of elimination from O-acetyl-bencasme. This is in accord with the Ecb mechanism, the much rarer type of elimination mechanism.[32]

Imidazole Catalysis. Imidazole catalyzes the decinnamoylation of both O-cinnamoyl-metcasme (IV) and O-cinnamoyl-metcasa (V) (as observed by the absorbancy change at 300 mμ). The reaction rates for the two compounds are not very different. This is in contrast to the hydroxyl ion catalyzed reaction in which the former undergoes a much faster β elimination, suggesting either alternative pathways (E2 and Ecb), or variability in relative rates within the Ecb mechanism, dependent on catalyst and substrate. The imidazole-catalyzed deacylation (in 2 *M* imidazole, pH 7.3) of the β esters IV and V are eightfold and 500-fold more rapid than the reactions in the absence of imidazole.

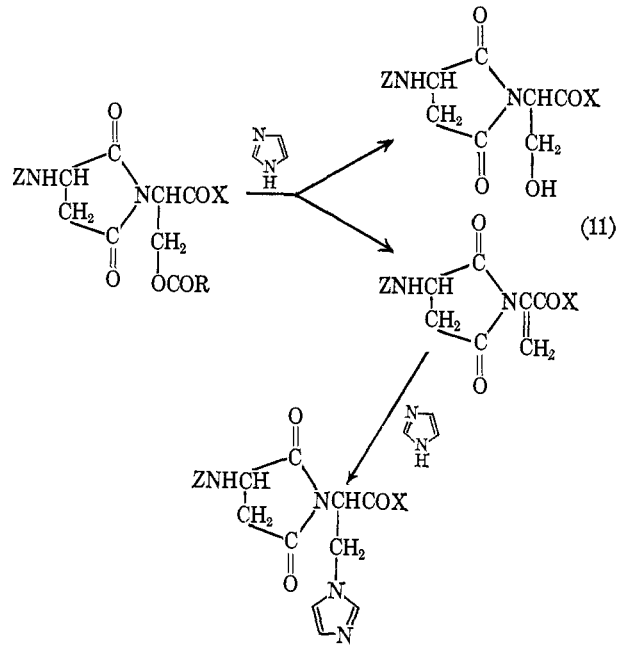
(30) A. J. Adler, G. D. Fasman and E. R. Blout, *J. Am. Chem. Soc.*, **85**, 90 (1963).

(31) (a) J. Hine, "Physical Organic Chemistry," McGraw-Hill Book Co., Inc., New York, N. Y., 1962, p 186; (b) E. S. Gould, "Mechanism and Structure in Organic Chemistry," Holt, Rinehart and Winston, Inc., New York, N. Y., 1959, p 478.

(32) A referee has suggested that the observed catalysis may be primarily due to base catalysis by the lyate (ethoxide) ion, and hence that the decreased catalysis observed upon the addition of acetic acid is the result of the reduced lyate concentration. We believe this explanation to be unlikely in view of the very much slower rate of elimination observed in aqueous solutions at comparable or higher concentrations of hydroxide ion.

This is presumably owing to the formation of an aspartoimide intermediate, which is imidazole catalyzed as well. Anderson, *et al.*,[10] have reported an imidazole-catalyzed reaction of N,O-diacetylserinamide at pH 7.1 and 100°. At the same pH, at room temperature, no measurable reaction was observed. Kirsch and Jencks[13] report the value of 7.5×10^{-6} M^{-1} min^{-1} for the imidazole-(free base) catalyzed hydrolysis of ethyl acetate, about 300-fold slower than that found for O-cinnamoyl-metcasa. The rate of imidazole-catalyzed ester hydrolysis is dependent on the nature of the alcohol moiety; for instance the specific rates for phenyl and *p*-nitrophenyl acetate are 0.52 and 30 M^{-1} min^{-1}, respectively. Thus O-cinnamoyl-bencasa is much less reactive than "active acetates," but much more reactive than ethyl acetate.

The straightforward hydrolysis of O-acetylserine esters involves no spectral change in the 240-mμ region. The initial increase in absorbancy at 240 mμ during the imidazole-catalyzed reaction of O-acetyl-bencasme might be interpreted as the formation of a dehydroalanine derivative. In the hydroxyl ion catalyzed reaction, the dehydroalanine spectrum is stable. Since addition to the double bond occurs with aliphatic amines, the decline in absorbancy suggests the possibility of imidazole addition to the double bond. Indeed, when the eliminated product from the hydroxyl ion catalyzed reaction of O-acetyl-bencasme is made 2 *M* in imidazole (pH 7.3), the 240 mμ absorption is quenched with a pseudo-first-order specific rate of 6×10^{-3} min^{-1}. Imidazole appears to act both as a catalyst and as a reactant in the reaction of these O-acylserine derivatives (see eq 11). Making use of the extinction of the dehydroalanine derivative (at 240 mμ) and the specific rates of decinnamoylation of O-cinnamoyl-bencasme and of the presumed addition of imidazole to the above-mentioned dehydroalanine product, a much higher extinction than that observed at 240 mμ would be anticipated. An explanation for this discrepancy may involve the validity of the assumption that the addition of imidazole to the β-dehydroalanyl carbon occurs at the same rate in aspartylserine as in aspartoimidylserine peptides. The enhanced electron-withdrawing power of the aspartoimidyl group should increase the electrophilicity on the β-(dehydroalanyl) carbon. If this effect is large, the specific rate of disappearance of absorbancy at 240 mμ, due to imidazole addition to the double bond, could be much faster than the rate of formation of the dehydroalanyl intermediate, and could result in a much lower apparent extinction owing to the small concentration of unsaturated intermediate that would obtain. When the final products in the imidazole-catalyzed hydrolysis of O-acetyl-bencasme were degraded (by total acid hydrolysis) and chromatographed on a 15-cm column at pH 5.28 in a Beckman-Spinco amino acid analyzer, a ninhydrin-positive component was detected on the chromatogram with a mobility virtually identical with that obtained for histidine.[33] Addition of imidazole to the carbon of the dehydroalanyl derivative should yield 3-(1-imidazolyl)alanine (eq 11), an isomer of histidine in which the β carbon is attached to nitrogen rather than to carbon of the imidazole ring. Since such a derivative would have both the same structure, and essentially the same pK_a as histidine, it should appear at about the same position on the chromatogram.



By using a 150-cm column, the "histidine-like" compound was separated from a histidine marker. From the ninhydrin color value of this component, it can be estimated that 75% of the original compound reacts *via* a pathway involving elimination followed by addition of imidazole. The fact that, even after a prolonged incubation of O-acyl-bencasme with imidazole at pH 7.3, the yield of the "histidine-like" compound remains 75% and the yield of serine is 25%, indicates an imidazole-catalyzed *hydrolysis* of O-acetyl-bencasme which is much more marked than that found in simple acetate esters.

Speculation on the Chemistry of the Active Site of Serine Proteases. The unanticipated facile β elimination of an aspartyl-O-acylserine ester derivative is suggestive of the possibility that the "deacylation" of proteolytic O-acylserine enzymes is in actuality a β-acylate elimination rather than a straightforward hydrolysis. This possibility is negated, however, by the following experimental facts. (1) In the presence of competitive nucleophilic reagents (in addition to water) such as methanol,[34] hydroxylamine,[28,35] and amines,[36] the competitive acyl products (ester, hydroxamate, and peptide) can be formed in substantial yield by a transfer reaction. (2) The catalytic hydrolysis of a large excess of acetyl-L-tyrosine ethyl ester (over the equivalence of chymotrypsin) in H_2O^{18} does not result in the incorporation of O^{18} into the catalytically specific serine hydroxyl.[37] Such incorporation would be demanded by an elimination–rehydration mecha-

(33) Y. Shalitin, S. A. Bernhard, and N. Shaltiel, to be published.

(34) (a) A. K. Balls and H. N. Wood, *J. Biol. Chem.*, **219**, 245 (1956); (b) M. L. Bender, G. E. Clement, C. R. Gunter, and F. J. Kédzy, *ibid.*, **238**, PC3143 (1963).

(35) (a) M. Coplow and W. P. Jencks, *ibid.*, **238**, PC1907 (1963); (b) R. M. Epand and I. B. Wilson, *ibid.*, **238**, PC3138 (1963); (c) F. J. Kédzy, G. E. Clement, and M. L. Bender, *ibid.*, **238**, PC3141 (1963).

(36) (a) R. B. Johnston, M. J. Mycek, and J. S. Fruton, *ibid.*, **187**, 205 (1950); (b) M. Brenner, H. R. Müller, and R. W. Pfister, *Helv. Chim. Acta*, **33**, 568 (1950); (c) Y. Levin, A. Berger, and E. Katchalski, *Biochem. J.*, **63**, 308 (1956).

(37) J. A. Cohen, R. A. Oosterbaan, and F. Berends, personal communication.

nism. (3) Elimination of tosylate from monotosyl-chymotrypsin yields an inactive "dehydro enzyme" in which the specific serine residue is converted to the corresponding dehydroalanine.[8] However, it could be argued that under these unusual conditions the enzyme is inactivated.

The imidazole-catalyzed deacylation of O-acylserine esters and serine peptides is perhaps of greater pertinence to the mechanism of enzyme action. Aspartoimidyl-O-acylserine peptides are more susceptible to imidazole catalysis than are other O-acylserine derivatives. Many serine proteases contain the active site sequence, aspartylserine. At neutrality it might be anticipated that the free energy of ionization of the β-carboxylic acid would provide sufficient "driving force" to prohibit formation of consequential fractions of aspartoimide. In a medium of high negative charge, or in a medium of low polarity, the ionization of the β-carboxylic acid might be greatly repressed. It is known that negatively charged molecules are poorly bound to the active site of α-chymotrypsin, and evidence has been presented that the "environment" of the active site is one of low polarity.[38,39] Under such conditions a neutral β-carboxylic acid would be anticipated to cyclize to the corresponding imide to an appreciable extent. In absolute methanol, comparable fractions of aspartoimide and β-methylaspartyl ester are formed from aspartylserine peptides at equilibrium.[40] The equilibrium constant, k = [methyl ester]·[H_2O]/([carboxylic acid][methanol]) is near unity.

The new results reported herein demonstrate the strong influence of an aspartoimidyl residue on an O-acylserine ester. This arises from the strong electron-withdrawing properties of the imide group. Although the model compounds studied react *primarily* by an elimination pathway, imidazole-catalyzed deacylation occurs to a significant extent *via* direct hydrolysis. The effectiveness of imidazole-catalyzed hydrolysis of esters is dependent on the acid strength of the corresponding alcohol. Electron withdrawal by the β-imide tends to increase the acid strength of the serine hydroxyl and thus make such serine esters more susceptible to catalyzed hydrolysis by imidazole. In addition, this enhanced acidity of the serine hydroxyl would result in a greater fraction of seroxide anion in such peptides. The nucleophilicity of seroxide anion has been frequently invoked in the mechanism of proteolytic enzyme action. The deacylation of O-cinnamoyl-bencasa in 2 *M* imidazole at pH 7.3 is about 300-fold slower than the deacylation rate of cinnamoyl-chymotrypsin.[39] It has been postulated that a bimolecular reaction involving substrate and catalyst in the concentration range of 10–1000 *M* would occur at a pseudo-first-order rate equal to an analogous intramolecular reaction. For example, the acetate-catalyzed hydrolysis of phenyl-acetate in 8 *M* acetate has the same pseudo-first-order rate as that for the intramolecularly catalyzed hydrolysis of aspirin anion;[41] *p*-nitrophenol acetate undergoes hydrolysis in "350 *M*" acetate at the same rate as a copolymer of acrylate anion and *p*-nitrophenyl methacrylate.[42] The first-order intramolecular rate of hydrolysis of propyl-γ-4-imidazolyl thiobutyrate is the same as the pseudo-first-order rate of hydrolysis of ethyl thioacetate in "500 *M*" imidazole.[43] Many similar results have been obtained in comparisons of inter- and intramolecular catalysis. Thus the intermolecular rate of imidazole catalysis of O-acetyl-bencasa may well be a sufficiently plausible model of the enzyme-catalyzed intramolecular reaction of O-acetyl- or O-cinnamoyl-chymotrypsin. The constants for deacylation of specific substrates of α-chymotrypsin are as much as 2×10^4-fold faster than the rate of deacylation of cinnamoyl-chymotrypsin. Obviously, a more sophisticated approach is called for in order to explain the mechanism of these very much faster reactions.

Acknowledgment. This work was supported by grants from the National Science Foundation and the U. S. Public Health Service.

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(42) From M. L. Bender, *Chem. Rev.*, 60, 89 (1960).
(43) T. C. Bruice and S. J. Benkovic, *J. Am. Chem. Soc.*, 81, 5444 (1959).