Proteolytic Enzymes: Studies of the Hydrolysis of *O*-Acetylserine Peptides as Models of the Deacylation Step

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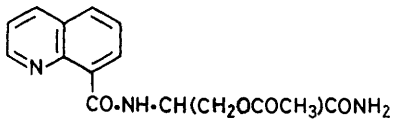
The hydrolyses of N_α-benzyloxycarbonyl-L-histidyl-(*O*-acetyl)-L-serinamide and N_α-(quinoline-8-carbonyl)-*O*-acetyl-L-serinamide have been measured over a pH range. The former compound exhibits a pH dependence with a plateau corresponding to general base catalysis by the imidazolyl function. The effective molarity of the general base catalysed reaction compared with the bimolecular analogue imidazole catalysed hydrolysis of $N_\alpha O$-diacetyl-L-serinamide is 2.3M consistent with the results of other studies with intramolecular proton transfer reactions. The effect of general base catalysis concerted with intramolecular electrophilic assistance is shown not to be a major driving force in the hydrolysis of peptide models of acetylserine proteases.

THE deacylation and acylation steps in hydrolyses catalysed by serine proteases are known to be mediated by the imidazolyl group of a histidine residue (histidine-57 in chymotrypsin);[1] it is thought that it acts as a general base to remove a proton either from the attacking serine hydroxy-group or from a nucleophile in acylation and deacylation reactions, respectively. The action of the imidazolyl function as a proton transfer agent is well documented for both inter- and intra-molecular solvolyses of carboxy-derivatives.[2] It is a reasonable assumption, which has been utilised in the past, that acylation and deacylation steps involve essentially the same mechanism so that information gained for one step is applicable to the other.[3]

Recent work has shown that the active site of serine proteases possesses an electrophilic region composed of amide NH groups which accepts the oxyanion from the formation of a tetrahedral intermediate from decomposition of the acylprotease.[4] There is little chemical

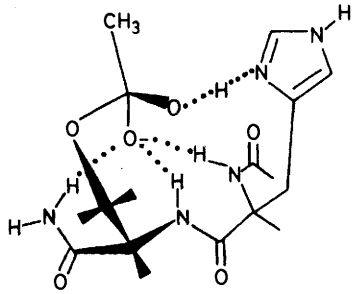
$C_6H_5CH_2O{\cdot}CO{\cdot}NHCH(CH_2C_3H_3N_2)CO{\cdot}NH{\cdot}CH(CH_2OAc)CONH_2$

(I)

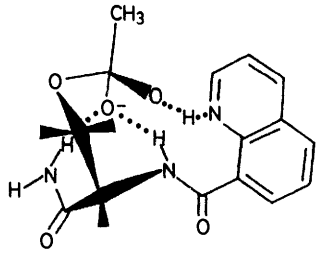


(II)

precedent for this interaction although it has been discussed recently for some phosphate and carboxylate esters.[5] The purpose of this study is to investigate the possibility of general base catalysis concerted with electrophilic assistance from amide NH groups as a major factor contributing to reactivity in acetylprotease hydrolysis. The esters we chose to study are *O*-acetyl



(III)



(IV)

Structures (III) and (IV) are tracings of photographs of models of (I) and (II) respectively to illustrate a conformation favourable for a concerted general base electrophilic process. The models were built from Kendrew atoms (Cambridge Repetition Engineers). Models built from Corey–Pauling–Koltun space-filling models (Ealing Beck Ltd.) indicate that there are no steric difficulties due to van der Waals repulsions for these conformations. The benzyloxy-group has been omitted in (III) for clarity

derivatives of serine containing peptides, N_α-benzyloxycarbonyl-L-histidyl-*O*-acetyl-L-serinamide (I) and N_α-(quinoline-8-carbonyl)-*O*-acetyl-L-serinamide (II). Both these esters are able to take up a conformation where the

[1] K. A. Walsh, Bayer Symposium No. (V), Proteinase Inhibitors, 1974, p. 1.

[2] T. C. Bruice and S. J. Benkovic, 'Bioorganic Mechanisms,' Benjamin, New York, 1966, vol. I.

[3] M. L. Bender and F. J. Kezdy, *J. Amer. Chem. Soc.*, 1964, **86**, 3704.

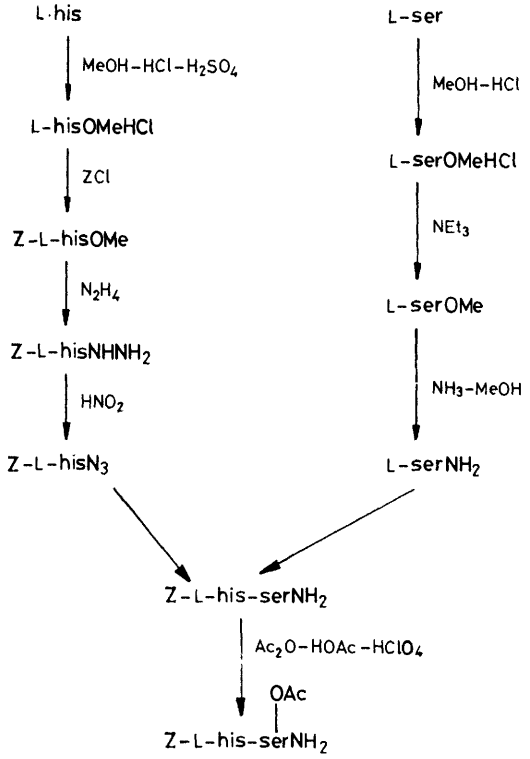
[4] (*a*) T. A. Steitz, R. Henderson, and D. M. Blow, *J. Mol. Biol.*, 1969, **46**, 337; (*b*) R. Henderson, C. S. Wright, G. P. Hess, and D. M. Blow, Cold Spring Harbor Symposium on Quantitative Biology, 1971, No. 36, p. 63; (*c*) J. D. Robertus, J. Kraut, R. A. Alden, and J. J. Birktoft, *Biochemistry*, 1972, **11**, 4293; (*d*) D. A. Matthews, R. A. Alden, J. J. Birktoft, S. T. Freer, and J. Kraut, *J. Biol. Chem.*, 1975, **250**, 7120; (*e*) R. Huber, D. Kukla, W. Steigemann, J. Deisenhofer, and A. Jones, ref. 1, p. 497; (*f*) D. M. Blow, *ibid.*, p. 473; (*g*) R. Huber, 6th Harden Conference, Wye, 1974; (*h*) D. M. Blow, J. Janin, and R. M. Sweet, *Nature*, 1974, **249**, 54; (*i*) R. M. Sweet, H. T. Wright, J. Janin, C. H. Chothia, and D. M. Blow, *Biochemistry*, 1974, **13**, 4212.

[5] (*a*) A. Williams and R. A. Naylor, *J. Chem. Soc.* (*B*), 1971, 1973; (*b*) A. Williams and G. Salvadori, *J.C.S. Perkin II*, 1972, 883.

general base may remove a proton concerted with electrophilic action by the amide NH protons.

EXPERIMENTAL

Materials.— N_α-Benzyloxycarbonyl-L-histidyl-(*O*-acetyl)-L-serinamide was prepared *via* the Scheme. Originally it had been anticipated that there would be difficulty in acetylating the serine hydroxy-group in the presence of a histidyl residue and it was planned to acetylate N_α-benzyloxycarbonyl-L-serinamide, deprotect, and couple with N_α-benzyloxycarbonyl-L-histidine. However, in our hands, the amide was obtained in poor yield and quality. Since a method exists for acetylating hydroxy-groups in the presence of amino-functions [6] the former route was not utilised and acetylation of the dipeptide was the preferred route. Extreme difficulty was encountered in the coupling step between azide and serine methyl ester and synthesis was finally achieved by a modification of a literature route.[7,8]



SCHEME Z = Benzyloxycarbonyl

The preparation of N_α-benzyloxycarbonyl-L-histidyl-L-serine methyl ester was attempted using the method of Holley and Sondheimer.[7] L-Serine methyl ester hydrochloride (5.44 g, 38 mmol) in ether (120 ml) was cooled in ice and cold K_2CO_3 solution (45 ml; 50% aqueous solution) added; the mixture was equilibrated and the ethereal solution separated and stored in the cold over anhydrous $MgSO_4$ while the azide was prepared. A solution of N_α-benzyloxycarbonyl-L-histidine hydrazide (9.6 g, 30 mmol) in HCl (90 ml, 1N) was mixed with ethyl acetate (120 ml) and cooled in an ice-bath. A cold solution of sodium nitrite (2.11 g) in water (7.5 ml) was added and after 2 min cold potassium carbonate (36 ml; 50% aqueous solution) and the mixture equilibrated. The ethyl acetate solution was separated and the aqueous layer extracted with ethyl acetate (15 mol). Combined extracts were dried over $MgSO_4$ at 0°. The solution of the L-serine methyl ester was filtered through a sintered glass funnel and the azide passed through the same filter into the filtrate. The mixture was kept for 2 days at 0° and then at room temperature for a further 2 days. The solvent was removed *in vacuo* and the oil lyophilised from dioxan–water. The small amount of yellow solid product was recrystallised from ethanol–water and had m.p. *ca.* 80° (lit.,[7] 140—142°).

The preparation was repeated paying special attention to cooling; all apparatus was precooled before mixing reagents. A third preparation involved limiting the final coupling solution volume to 100 ml. Poor products were obtained in these modifications. The cause of the poor yield was discovered to be the absence of partitioning of the L-serine methyl ester into the ether layer in the initial part of the synthesis and we point out this error in Holley and Sondheimer's report.[7] Probably these workers used an organic phase different from the one quoted (ether). The following modification gave excellent results: L-serine methyl ester hydrochloride (3.76 g, 25 mmol) was suspended in dichloromethane (100 ml) and triethylamine (2.5 ml, 25 mmol) added. The mixture was stirred for 0.5 h and then ether added (300 ml). The precipitate was filtered and the filtrate evaporated to give an oil which had an n.m.r. spectrum corresponding to the L-serine methyl ester free base. The ester was taken up in dichloromethane (50 ml) and stored at 0°. *N*-Benzyloxycarbonyl-L-histidyl azide was prepared on a 25 mmol scale as described above and reacted with the amino-ester. The solid yellow product was filtered and recrystallised twice from ethanol. The crystals had m.p. 133—136° (lit.,[7] 140—142°) (Found: C, 54.5; H, 5.5; N, 14.3. Calc. for $C_{18}H_{22}N_4O_6$: C, 55.4; H, 5.7; N, 14.4%). Unfortunately aminolysis of the protected dipeptide with aqueous ammonia gave material 15% low in carbon and 1% high in nitrogen for the amide. The product had m.p. 125—128° and gave two spots on t.l.c. (conditions as in Table 1) with R_F 0.07 and 0.35.

TABLE 1

T.l.c. data [a]

Compound	R_F	Ninhydrin	Pauly	Serine
Z-L-his-L-serOCH$_3$	0.4	—	+	+
Z-L-hisNHNH$_2$	0.27	—	+	—
L-serOCH$_3$HCl	0.25	+	—	+
Z-L-his-L-serNH$_2$	0.33	—	+	+
Z-L-his-L-(*O*-acetyl)serNH$_2$	0.37	—	+	+
Quinoline-8-carbonyl-L-serOCH$_3$	0.62 [b]	—	—	+
Quinoline-8-carbonyl-L-serNH$_2$	0.53 [b]	—	—	+

[a] Eluant n-butanol–acetic acid–water (4 : 1 : 1). [b] Detected by u.v. light.

N_α-Benzyloxycarbonyl-L-histidyl-L-serinamide was prepared by the following alternative technique. An ethyl acetate solution of N_α-benzyloxycarbonyl-L-histidyl azide was prepared as above on a 30 mmol scale, cooled to 0°, and mixed after filtering through a sintered filter with

[6] W. Sakami and G. Toennies, *J. Biol. Chem.*, 1942, **144**, 203.
[7] R. W. Holley and E. Sondheimer, *J. Amer. Chem. Soc.*, 1954, **76**, 1326.
[8] R. F. Fischer and R. R. Whetstone, *J. Amer. Chem. Soc.*, 1954, **76**, 5076.

L-serinamide (3.12 g, 30 mmol) dissolved in dimethylformamide (50 ml) cooled to 0°. The mixture was allowed to stand at room temperature for two days, then filtered from a small amount of insoluble material, and evaporated *in vacuo*. The residue was dissolved in hot methanol and on partial evaporation the solution commenced to deposit crystals. The solution was kept at 0° to complete the crystallisation and the product recrystallised from methanol. The crystals had m.p. 126—130° (Found: C, 54.0; H, 5.6; N, 18.5. Calc. for $C_{17}H_{21}N_5O_5$: C, 54.4; H, 5.6; N, 18.7%). The equivalent weight was determined by titration with 10 mM-HCl and Methyl Orange indicator to be 360 (calc. 375). T.l.c. (see Table 1) exhibited one spot negative to ninhydrin and positive to Pauly's reagent.

N_α-Benzyloxycarbonyl-L-histidyl-L-(*O*-acetyl)serinamide was prepared by a modification of the method of Sakami and Toennies [6] for the preparation of *O*-acyl compounds in the presence of free amino-groups. A solution of N_α-benzyloxycarbonyl-L-histidyl-L-serinamide (1 g) was prepared in a perchloric acid–acetic acid mixture [5 ml of a solution of 60% perchloric acid (10 ml) and acetic acid (153 ml)]. Acetic anhydride (1.6 g) was then added dropwise with stirring and cooling and the mixture allowed to stand for 1 h. Water (0.1 ml) was then added to destroy excess of anhydride, the mixture kept for a further 1 h and then diethylamine (0.45 ml) added to basify the mixture. The product was precipitated as an oil with an excess of dry ether and the oil partially solidified on cooling. The ether was decanted and the oil dissolved in methanol (2 ml), cooled, and ether slowly added while the oil was rubbed. The oil solidified in *ca.* 0.5 h and the solid was filtered off, washed with ether, and dried. Recrystallisation from methanol–ether gave *crystals*, m.p. 161—165° (Found: C, 51.5; H, 5.6; N, 15.6. $C_{19}H_{25}N_5O_6,1.5H_2O$ requires C, 51.3; H, 5.9; N, 15.8%); peptides containing bound water are well known.[9] T.l.c. results are given in Table 1; an i.r. spectrum (ν 1 730 cm^{-1}) and a positive hydroxamic acid test confirm the presence of an ester linkage.

N_α-(Quinoline-8-carbonyl)-L-serine methyl ester was prepared by dissolving quinoline-8-carboxylic acid (18.7 g, 100 mmol) in dichloromethane (100 ml) with triethylamine (14 ml, 100 mmol) cooling to −5° with an ice–salt bath and adding, with stirring, ethyl chloroformate (9.6 ml, 100 mmol). A solution of L-serine methyl ester hydrochloride (15.6 g, 100 mmol) in dichloromethane (50 ml) was treated with triethylamine (14 ml, 100 mmol) and added slowly to the stirred solution of acid which was then kept (with stirring) overnight at room temperature. The material was extracted with dilute HCl, dried with Na_2SO_4, evaporated *in vacuo* to yield a residue which was recrystallised from methanol–water. The *product* had m.p. 97—100° (Found: C, 61.8; H, 5.1; N, 10.2. $C_{14}H_{14}N_2O_4$ requires C, 61.5; H, 4.8; N, 10.3%). Table 1 records the t.l.c. data.

N_α-(Quinoline-8-carbonyl)-L-serinamide was prepared by stirring the methyl ester (1.4 g) with methanol saturated with ammonia gas (300 ml) in a sealed flask at room temperature for two days. The excess methanol and ammonia were removed *in vacuo* until 30 ml solution were left. The solution was then kept at 0° for a day and the resultant precipitate filtered off, washed with methanol, and then ether. Recrystallisation from methanol–ether gave material, m.p. 234—236° (Found: C, 60.1; H, 5.2; N, 16.3. $C_{13}H_{13}N_3O_3$ requires C, 60.2; H, 5.0; N, 16.2%). T.l.c. data are given in Table 1.

N_α-(Quinoline-8-carbonyl)-L-(*O*-acetyl)serinamide was prepared by warming N_α-(quinoline-8-carbonyl)-L-serinamide (0.6 g) with pyridine (30 ml) using a water-bath until dissolution was complete. Acetic anhydride (2 ml) was then added and the mixture stirred at room temperature for 4 h; the excess of pyridine and acetic anhydride was removed *in vacuo* and the resulting oil solidified by rubbing with ether in the cold. The suspension was filtered, the residue washed with ether, and the product recrystallised from methanol. The *material* had m.p. 180—182° (Found: C, 59.4; H, 5.1; N, 14.3. $C_{15}H_{15}N_3O_4$ requires C, 59.8; H, 5.0; N, 14.0%). The i.r. spectrum (ν 1 725 cm^{-1}) and a positive hydroxamic acid test confirm the presence of the ester link.

Other materials were of analytical reagent grade from B.D.H. or Fisons Ltd. and where these were not available bench grade material was redistilled or recrystallised before use. Acetonitrile was purified by the method of Lewis and Smyth [10] and dioxan (analytical reagent grade) was purged of peroxides by passage through an activated alumina column; [11] potassium iodide solution was used to test for the absence of peroxides. Water used throughout the investigation was doubly distilled from glass.

Methods.—Structures of the compounds synthesised above were confirmed by n.m.r. spectroscopy using a Perkin-Elmer R10 60 MHz instrument or by i.r. spectroscopy (Perkin-Elmer 237 or 257 grating machines) using Nujol mulls.

T.l.c. was carried out using either pre-made plates from Merck Ltd. or plates prepared by the technical services department of this laboratory. Only silica gel supports were used and compounds were 'spotted' with 5 λ pipettes and separation achieved by the ascending solvent technique.[12a] Spots were developed by u.v. light or, for the peptides, by spraying the dried plates with a reagent and drying in a hot air stream. Ninhydrin spray (B.D.H.) was used to identify the free amino-groups in unprotected peptides and amino-acids. The imidazolyl side chain was detected with the Pauly reagent; [12b] equal volumes of a 10% solution of sodium carbonate and a fresh solution of (A) were mixed carefully. (A) was prepared by adding 1 part of a 1% solution of 4-sulphanilic acid in 10% concentrated HCl to 1 part of a cooled 5% solution of sodium nitrite. The reagent gives a deep orange to brown colour for imidazolyl containing spots. The test for serine involved the four reagents: (a) periodic acid (H_5IO_6), 40% in water (2 ml); (b) pyridine (2 ml); (c) acetone (100 ml); (d) ammonium acetate (15 g)–acetic acid (0.3 ml)–acetylacetone (1 ml) in water (100 ml). Dry chromatograms were sprayed with a mixture of (a)—(c) and then dried. A yellow colour develops when the dried plates are sprayed with (d) if the spots contain a serine residue.

M.p.s were determined using a Kofler hot stage Thermospan apparatus. Some compounds were determined in capillary tubes in a Gallenkamp MF-370 instrument.

Microanalyses were carried out by Mr. G. M. Powell of this laboratory using a Hewlett–Packard model 185 CHN

[9] J. P. Greenstein and M. Winitz, 'Chemistry of the Amino-acids,' Wiley, New York, vol. 2.

[10] G. L. Lewis and C. P. Smyth, *J. Phys. Chem.*, 1939, **7**, 1085.

[11] A. I. Vogel, 'Practical Organic Chemistry,' Longman, London, 1956, p. 177.

[12] (*a*) I. Smith, 'Chromatographic and Electrophoretic Techniques,' Heinemann, London, 3rd edn., 1969; (*b*) R. M. C. Dawson, D. C. Elliott, W. H. Elliott, and K. M. Jones, 'Data for Biochemical Research,' Oxford University Press, Oxford, 1969.

analyser. Under ideal conditions the standard error is ±0.3% absolute.

Determinations of pK_a were carried out using the following combination of equipment from Radiometer of Copenhagen: Autoburette ABU11, Titrator TTT60, Titratigraph REA 160, Digital pH-meter PHM 62, Servograph pen drive REA 310, and glass electrode GK 2321c together with a stirred, thermostatted cell. The pH-meter was standardised at the appropriate temperature prior to use with standard buffers (accurate to ±0.01 units) prepared from powders supplied by E. I. L. Titrations were performed in dioxan–water or aqueous solutions.

Kinetics.—Rates of hydrolysis were measured spectrophotometrically with a Unicam SP 800 machine fitted with a repetitive scanning attachment (SP 825) or a Beckman DB-G instrument equipped with a linear-logarithmic converter. Data were recorded automatically with a Servoscribe recording potentiometer. Repeat scanning between pre-set wavelengths with the Unicam machine was used to determine the stoicheiometry of the reaction, the most suitable wavelength for rate measurements, and the nature of the products. Conditions were adjusted so that pseudo first-order rate constants were observed: buffer (2.5 ml) in a silica cell was equilibrated for *ca.* 15 min in the thermostatted cell compartment of the instrument. The temperature was maintained within ±0.1° of the stated temperature with a Gallenkamp Thermostirrer. A portion of substrate (20—200 λ of a stock solution in acetonitrile, dioxan, methanol, or *NN*-dimethylformamide) was added either direct or on the flattened tip of a glass ' plumper '. The solution was mixed, the cell sealed with a Teflon stopper, and the recorder activated. The disappearance of the ester was also measured by the formation of the hydroxamic acid followed by colour development with iron(III) chloride.[13] A portion of the solution under observation (0.3 ml) was mixed with hydroxylamine solution [0.3 ml of a mixture of 3.5M-NaOH and 4M-NH_3OHCl (5 : 4 v/v)] diluted with water (1 ml) and heated at 100° for 10 min. The mixture was cooled, acidified with 3N-HCl (0.2 ml) and treated with iron(III) chloride (1 ml) in 0.1N-HCl, kept for 10 min, and the optical density read at 540 nm. First-order rate constants were estimated from plots of $A_\infty - A_t$ *versus* time on two-cycle semilogarithmic graph paper.

A Radiometer pH-meter 25b (Copenhagen) or a Pye Dynacap instrument was used to determine pH. A sodium ion correction was employed for the alkaline pH region and a B class electrode was also used. The pH of the buffer solutions was measured after reaction directly in the silica cell. Buffers used in this work were: carbonate (pH 9—11), borate (pH 8—10), phosphate (pH 6—8), and acetate (pH 4—6).

RESULTS AND DISCUSSION

The hydrolyses of esters (I) and (II) as followed spectrophotometrically exhibited excellent pseudo-first-order kinetics up to *ca.* 80% of the total reaction. Good isosbestic wavelengths were observed in the hydrolysis of the quinoline derivative but no crossings were seen in the imidazolyl case over the wavelength range used. In both cases the first-order kinetics were observed over the whole time scale confirming either simple 1 : 1 stoicheiometry or an intermediate present in a concentration below the analytical limits. The rate constants were also measured by observing the ester decay using the hydroxamic acid assay and good agreement obtained with the spectroscopic method (Tables 2 and 3 and Figure). A complication in the kinetics occurred with the benzyloxycarbonylhistidine peptide at high pH

TABLE 2

Hydrolysis of N_α-benzyloxycarbonyl-L-histidyl-L-(*O*-acetyl)serinamide [f,h,i]

pH	$10^6 k_{obs}/s^{-1}$ [a]	$\lambda_{kinetic}$/nm
10.77	7 100	245
10.24	2 400	245
10.00	1 500 [g]	
9.64	460	245
8.55	61	245
7.98	30	245
7.52	10	250
7.10	8.7	250
6.51	8.1	255
5.40	2.0	255
5.72	3.1	255
10.71	1 400 [b]	254
10.58	1 100 [b]	254
10.30	600 [b]	254
6.50	250 [c]	260
6.50	210 [d]	260
6.50	180 [e]	260
4.80	≤1.0	256
5.92	5.4	255
6.01	7.8	255
6.30	6.6	253
6.80	11	250
7.03	6.8 [g]	
9.09	160	245
9.41	363	245
9.70	830	245
10.51	4 900	245

[a] 49.5°; 20% dioxan–water (v/v); ionic strength made up to 0.1M with NaCl. [b] 25°. [c] 75.5°. [d] 66.6°. [e] 58.8°. [f] pK_a in 20% dioxan–water 5.7 (50°); 6.25 (25°); pK_a of the product dipeptide at 25° in the same solvent is 6.8. [g] Measured using the hydroxamic acid assay. [h] Arrhenius parameters for the plateau rate constant: $\Delta H^\ddagger$ −9 kcal mol^{-1}, $\Delta S^\ddagger$ −54 cal mol^{-1} K^{-1} at 50°. [i] k_1 (49.5°) 7.9 × 10^{-6} s^{-1}.

TABLE 3

Hydrolysis of N_α-(quinoline-8-carbonyl)-L-(*O*-acetyl)-serinamide [a,c]

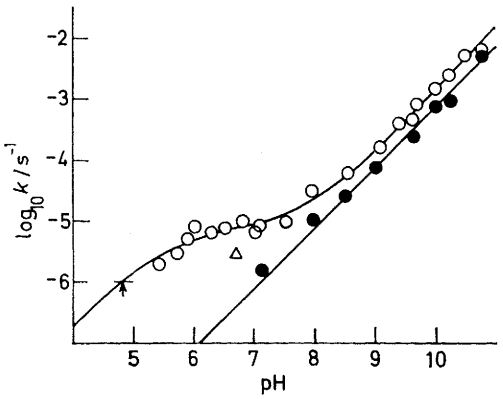
pH	$10^6 k_{obs}/s^{-1}$	$\lambda_{kinetic}$/nm
10.77	5 300	329
10.24	890	329
10.00	750 [b]	
9.64	240	329
8.5	25	330
9.0	79	329
7.98	10	331
7.10	1.5	333
6.50	Too slow to measure satisfactorily	

[a] 49.5°; 20% dioxan–water (v/v); ionic strength made up to 0.1M with NaCl. [b] Measured using the hydroxamic acid assay. [c] pK_a in 20% dioxan–water was too small to measure. The titration curve (addition of HCl) followed that for the titration of the solvent under the conditions of the kinetic experiments.

[13] (*a*) S. Hestrin, *J. Biol. Chem.*, 1949, **180**, 249; (*b*) F. Lipman and L. C. Tuttle, *ibid.*, 1946, **159**, 21; (*c*) W. P. Jencks, *J. Amer. Chem. Soc.*, 1958, **80**, 4581; (*d*) W. P. Jencks and J. M. Regenstein, ' Handbook of Biochemistry,' ed. H. A. Sober, Chemical Rubber Co., Cleveland, 2nd edn., 1970; (*e*) M. L. Bender, E. J. Pollack, and M. C. Neveu, *J. Amer. Chem. Soc.*, 1962, **84**, 595; (*f*) S. L. Johnson, *Adv. Phys. Org. Chem.*, 1967, **5**, 237; (*g*) M. Komiyama, J. R. Roesel, and M. L. Bender, *Proc. Nat. Acad. Sci. U.S.A.*, 1977, **74**, 23; (*h*) W. P. Jencks, ' Catalysis in Chemistry and Enzymology,' McGraw-Hill, New York, 1969.

values: a strong peak appeared in the u.v. spectrum at $\lambda_{max.}$ 308 nm with a pseudo-first-order rate constant 6.4×10^{-5} s^{-1} (at 0.1M-NaOH; 50°). The observed rate constant is far below that for the ester hydrolysis (see Table 2) and is probably due to cleavage of the benzyloxycarbonyl–amido link since the same spectral change occurred with the dipeptide N_α-benzyloxycarbonylhistidylserinamide.

The pH dependences of the ester hydrolyses are illustrated in the Figure; the histidyl derivative possesses a plateau region up to pH 7.5 and at low pH the rate falls off as the imidazolyl group is protonated. The quinolinoyl ester shows no plateau region in the pH-profile for hydrolysis and this is consistent with a low rate constant predicted from the pK_a of the quinolinyl group being lower than that of imidazolyl. Measurements of rate constants below *ca.* 10^{-6} s^{-1} are somewhat uncertain in this system and the value at pH 4.8 for the



The pH dependence (49.5°; 0.1M ionic strength) for the hydrolysis of N_α-benzyloxycarbonyl-L-histidyl-(*O*-acetyl)-L-serinamide (I) (○) and N_α-(quinoline-8-carbonyl)-(*O*-acetyl)-L-serinamide (II) (●). Lines are theoretical from data in Tables 2, 4, and 5. Hydrolysis of (I) in D_2O(△)

imidazolyl compound (see Figure) is an upper limit. The general rate law followed by the hydrolysis is (1) where

$$k = k_1/(1 + a/_{H}K_a) + k_{OH}[OH] \qquad (1)$$

the plateau parameter is k_1 [absent in ester (II)] and k_{OH} is the hydroxide term.

The pK_a of the imidazolyl compound (5.7 at 50 and 6.25 at 25°) is in the region expected for histidine derivatives.[13d]

The action of the imidazolyl group in (II) is probably as a general base catalyst involving a rate-limiting proton transfer; the deuterium oxide solvent isotope effect ($k_1^{H_2O}/k^{D_2O} = 2.5$) confirms this.[13e,f] Komiyama *et al.*[13g] find a value for the isotope effect of 3.0 for a similar reaction and we would not expect an ester with a poor leaving group (an alcohol) to be susceptible to nucleophilic attack by the imidazolyl function.[13h]

The effective molarity of the intramolecular general base catalysed hydrolysis (2.3M) compared with the intermolecular reaction between imidazole and *NO*-diacetylserinamide is of the order to be expected for an intramolecular proton transfer.[14a]

Fox and Jencks [14b] argued that concerted push–pull catalysis is not an easily observable process because assistance at the nucleophilic extremity of the substrate reduces selectivity at the electrophilic end; thus the termolecular reaction will not be any more efficient than the uncatalysed bimolecular one (after due allowance has been made for entropy differences). If it is proposed that the concerted action of a general base and an electrophile provides significant driving force for the deacylation of an acyl serine protease then a model system combining these components should exhibit an enhancement over systems where no such assistance may occur. Such an enhancement would be a lower limit due to entropy considerations which in an intermolecular process would be insurmountable. Provided the entropy barrier is decreased as in an intramolecular reaction a major enhancement due to concerted catalysis ought to show up. Perusal of Table 4 indicates that the present

TABLE 4

Neutral hydrolysis of some acetate esters

Compound	k/s^{-1}
N_α-Benzyloxycarbonyl-L-histidyl-L-(*O*-acetyl)serinamide	7.9×10^{-5} (100°) [a]
	2.3×10^{-6} (25°) [a]
N_α-*O*-Diacetyl-L-serinamide–imidazole	1.83×10^{-4} l mol^{-1} (100°) [b]
Acetylchymotrypsin	6.93×10^{-3} (25°) [c]
Acetyltrypsin	9.9×10^{-3} (25°) [c]
4-(2-Acetoxyethyl)imidazole	3.3×10^{-6} (78°) [d]
Acetylsubtilisin	0.31 [f]
2-Cinnamoyloxy-5-imidazolylbicyclo-[2.2.1]heptane	6.4×10^{-7} (60°) [13g]
2-Cinnamoyloxy-5-imidazolylbicyclo-[2.2.2]octane	1.8×10^{-7} (60°) [13g]

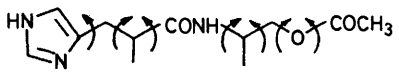
[a] Estimated from the temperature dependence of the pH-independent rate constant (see Table 2). [b] See footnote *d* of Table 5. [c] A. Williams and G. Salvadori, *J. Chem. Soc.* (*B*), 1971, 2401. [d] T. C. Bruice and J. M. Sturtevant, *J. Amer. Chem. Soc.*, 1959, **81**, 2860. [e] M. L. Bender, J. V. Killheffer, and F. J. Kezdy, *J. Amer. Chem. Soc.*, 1964, **86**, 5331. [f] Value is for k_{cat} for 4-nitrophenyl acetate (L. Polgar and M. L. Bender, *Biochemistry*, 1967, **6**, 610) and is assumed to be equal to the deacylation rate constant.

imidazolyl derivative is not much more reactive than is 4(5)-(2-acetoxyethyl)imidazole to hydrolysis so far as the water reaction is concerned. The two esters are not strictly comparable on account of differences in basicity of the imidazolyl function and leaving group ability but these factors should not necessitate correction in excess of say 10-fold.

The hydrolysis of acetyl serine proteases is over 3 000-fold larger than that of the present model (Table 4); we must seek therefore an explanation for the reactivity other than the concerted postulate. If the contribution to the entropy of activation of a free rotation is taken to be *ca.* 4 cal mol^{-1} K^{-1} then our model could, in principle, be accelerated *ca.* 10^6-fold merely by destroying the seven degrees of rotational freedom available [see (V)].

[14] (*a*) M. I. Page, *Chem. Soc. Rev.*, 1973, **2**, 295; (*b*) J. P. Fox and W. P. Jencks, *J. Amer. Chem. Soc.*, 1974, **96**, 1436.

This is an upper limit as it is unlikely that the bonds specified have complete rotational freedom. Moreover, the loss of entropy upon freezing an internal rotation is partially compensated by a favourable enthalpy change



(V)

of *ca.* 0.5 kcal mol^{-1}.[15] Complete freezing of internal rotations could occur for the substrate group in acetyl serine proteases by virtue of close packing of the acetyl group in the active site. Probably the $COCH_3$ bond is somewhat free to rotate since the phenylpropionyl group confers higher reactivity without a concomitant electronic effect.[16] Presumably the binding of the phenyl group in the tosyl pocket[17] tends to reduce rotation. The hydrolysis of acetylsubtilisin (Table 4) is some 10^5-fold faster than the present model: the 10^6-fold acceleration that might be expected by 'freezing' the model ester implies that the deacylation of this acetyl enzyme is close to its maximum theoretical limit for the acetyl group.

Maugh and Bruice[18] concluded that there was no evidence for intramolecular general base–general acid or nucleophilic–general acid catalysis for the hydrolysis of esters in water. The models so far studied do not possess the supposed active site constituents of the serine proteases which the present ones do.

There is no evidence (see Table 5) that the alkaline hydrolysis of the two peptide esters [(I) and (II)] exhibit an enhanced rate over hydrolyses where no interaction by amido NH is possible. This is in concert with the results of the alkaline hydrolysis of a series of *O*-acylglycolamides previously reported.[19]

TABLE 5

Alkaline hydrolysis rate constants for some acetate esters

Ester	k_{OH}/l mol^{-1} s^{-1} [a]
N_α-*O*-Diacetyl-L-serinamide	0.815 [d]
Ethyl acetate	0.11 [d]
Acetylcholine	0.95 [d]
N_α-Benzyloxycarbonyl-L-histidyl-L-(*O*-acetyl)serinamide	11,[b,c,f] 3.0 [c,e,g]
N_α-(Quinoline-8-carbonyl)-L-(*O*-acetyl)-serinamide	5.8 [b,c,f]

[a] 25° except where stated. [b] 49.5°. [c] This work. [d] B. M. Anderson, E. H. Cordes, and W. P. Jencks, *J. Biol. Chem.*, 1961, **236**, 455. [e] Zero dioxan concentration. [f] pK_w = 13.89 (from H. S. Harned and L. D. Fallon, *J. Amer. Chem. Soc.*, 1939, **61**, 2374). [g] pK_w = 14.00 (from H. S. Harned and R. A. Robinson, *Trans. Faraday Soc.*, 1940, **36**, 973).

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[18] T. Maugh and T. C. Bruice, *J. Amer. Chem. Soc.*, 1971, **93**, 3237.
[19] J. A. Boudreau and A. Williams, *J.C.S. Perkin II*, 1977, 1221.