Proteolytic Enzymes. Models for Hydrolyses catalysed by Papain

By **Andrew Williams,* Ernest C. Lucas,** and **Kenneth T. Douglas,** University Chemical Laboratories, University of Kent, Canterbury

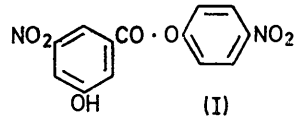
The effect of pH on the reaction of mercaptoacetic acid with 3-nitrophenyl acetate and with 4-chlorophenyl *N*-methylsulphonylglycinate gave equivocal evidence for the involvement of the unionised thiol as a nucleophile. The reaction of ethanethiol with 4-nitrophenyl 5-nitrosalicylate exhibited a sigmoid pH-dependence (pK_a = 6·00) but it was not possible to distinguish unequivocally between mechanisms involving the reaction of uncharged ester with thiolate anion or the ionised ester with thiol. The pH-dependence for the reaction of ethanethiol with 4-nitrophenyl quinoline-8-carboxylate exhibited two inflections (pK_a = 3·48 and 11·33). The alkaline limb corresponds to attack of thiolate anion on uncharged ester and the other limb involves participation of tertiary nitrogen. The sensitivity (ρ = 0·99) of the rate constant to the substituent on the phenol leaving group in the latter reaction is utilised as evidence for the reaction of thiolate anion with protonated ester. The kinetics of hydrolysis of a series of substituted phenyl formates and evidence from the literature indicates a higher selectivity to leaving group for the reaction of water as opposed to the reaction of hydroxide ion at a carboxy-centre. Arguments based on diffusion rate constants are used to eliminate the zwitterion type mechanism in papain-catalysed reactions.

ARGUMENT based on the concept of maximal rate of diffusion of species in aqueous media [1] (see also later) shows that if papain acts by virtue of an imidazolyl–thiol pair then it is the neutral form rather than the zwitterion which is responsible. Although the catalytic activity of the thiolate anion in reaction with active esters is well established [2,3] no unequivocal demonstration is available of the reactivity of a thiol group towards esters in aqueous solution. Ogilvie *et al.*[3] suggested the existence of a thiol-catalysed hydrolysis of 4-nitrophenyl acetate by 2-mercaptoethanol, glutathione, and mercaptoacetate anion but errors in the rate constants derived from small intercepts render this equivocal. Schneider's work [4] on the 4-nitrophenyl acetate reaction with 4(5)-mercaptomethylimidazole and 4(5)-(2-mercaptoethyl)imidazole provides evidence for co-operative catalysis by imidazole and thiol groups since the observed catalytic rate constants were three times the sum of the constants calculated for the individual groups. No attempt was made however to determine the proportion of reaction proceeding *via* catalysis by zwitterion or neutral species.

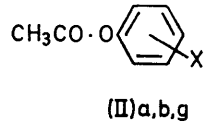
We have studied reactions of the following esters with thiols as models of the acylation reaction of papain.

EXPERIMENTAL

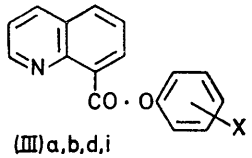
Materials.—4-Nitrophenyl 5-nitrosalicylate (I) was prepared by heating 5-nitrosalicylic acid (0·9 g) and 4-nitrophenol (0·7 g) at 120—130° for *ca.* 40 min with phosphoryl chloride (3 ml). The mixture was cooled in ice and water was added. The product was filtered, washed, dried, and recrystallised twice from AnalaR dioxan. Quinoline-8-carboxylates (III) and 4-nitrophenyl 3-nitrobenzoate (IV) were prepared by the following general method. Equimolar quantities of the acid and triethylamine (freshly distilled) were dissolved in dichloromethane and the solution cooled to −5°. Ethyl chloroformate (1 mol. equiv.) was then added and the solution stirred at −5° for 30 min. A solution or suspension of the phenol (1 mol. equiv.) in dichloromethane was then added and the solution stirred for 24 h at room temperature. The solution was washed with a little water and saturated sodium bicarbonate solution, dried ($MgSO_4$), and evaporated under reduced pressure at 50°. Aryl acetates [5] and *N*-methylsulphonylglycinates (mesylglycinates) [6] are described elsewhere. Substituted phenyl formates were prepared by adding



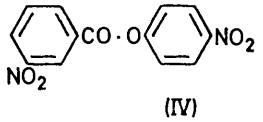
(I)



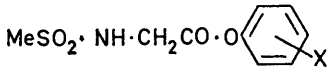
(II)a,b,g



(III)a,b,d,i



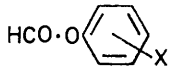
(IV)



(V)b,d,e,f,g,h

X

a, 4-Nitrophenyl
b, 3-Nitrophenyl
c, 3-Chlorophenyl
d, 4-Acetylphenyl
e, 4-Chlorophenyl
f, 4-Fluorophenyl



(VI)a,b,c,d,e,g,j,k

X

g, 4-Methoxyphenyl
h, 4-Hydroxyphenyl
i, 3,5-Dinitrophenyl
j, 4-Methylphenyl
k, H

phenol (1 mol) to a solution of formic acid (anhydrous, 1·5 mol), acetic anhydride (1·5 mol), and pyridine (0·015 mol). The solution was kept overnight at room temperature and then fractionally distilled under vacuum (water pump). A second fractionation gave pure product. The 4-nitrophenyl ester crystallised from the mixture and was recrystallised from carbon tetrachloride.

Analytical data and physical properties of the esters are recorded in Table 1; structures were confirmed by i.r. and n.m.r. spectroscopy.

[1] E. C. Lucas and A. Williams, *Biochemistry*, 1969, **8**, 5125.
[2] J. R. Whitaker, *J. Amer. Chem. Soc.*, 1962, **84**, 1900.
[3] J. W. Ogilvie, J. T. Tildon, and B. S. Strauch, *Biochemistry*, 1964, **3**, 754.
[4] (*a*) F. Schneider, *Z. Physiol. Chem.*, 1967, **348**, 1034; (*b*) F. Schneider, E. Schaich, and H. Wenck, *ibid.*, 1968, **349**, 1525; (*c*) F. Schneider and H. Wenck, *ibid.*, 1969, **350**, 1653.
[5] A. Williams and R. A. Naylor, *J. Chem. Soc.* (*B*), 1971, 1967.
[6] A. Williams, E. C. Lucas, and A. R. Rimmer, *J.C.S. Perkin II*, 1972, 621.

Cysteine (AnalaR grade), ethanethiol, and mercaptoacetic acid were from B.D.H. Mercaptoacetic acid was distilled before use. Acetonitrile was purified by the method of Lewis and Smyth [7] and only those fractions with minimal aromatic u.v. absorption were used. Dioxan (AnalaR grade) was passed through an alumina column immediately before use and stored in bottles covered with aluminium foil. Potassium iodide was employed to demonstrate the absence of peroxides. All other materials and buffers were of analytical reagent grade.

Methods.—Ester hydrolyses were followed spectroscopically at the appropriate wavelengths using a Unicam SP 800 instrument coupled with a Smith's Industries Servoscribe recorder. Conditions were arranged so that first-order kinetics were obtained in ester and pseudo-first-order rate constants were derived using the Guggenheim method.[8] Hydrolyses of aryl formates were followed at 0·09M ionic strength (10% CH_3CN) and 25° using a pH-stat (Radiometer SBR2c coupled with a titrator II and a pH meter 25). A solution of the aryl formate in acetonitrile (*ca.* 0·1M, 1 ml) was diluted to 10 ml with 0·1M-sodium chloride (9 ml) in the thermostatted reaction cell of the pH-stat. Nitrogen (passed through water at 25°) was passed slowly over the surface of the stirred mixture and the pH was held constant by the addition of sodium hydroxide (0·02M). The burette stroke was equivalent to 0·5 ml. For pH values below 4 (for 4-nitrophenyl ester) the reaction was followed at 350 nm using an SP 800 instrument.

Linear plots of the experimental data were subjected to least-squares analysis to obtain parameters of the best fit line. The Kent 'On-line' system was employed in these calculations using Dartmouth Basic Language (the assistance of Dr N. J. Bridge and Mr K. T. Taylor is gratefully acknowledged) and a central Elliott 4130 computer.

TABLE 1

Analytical and physical properties of materials [a]

Substrate	M.p. or *b.p.*[k] (*T*/°C)	Lit. m.p. or *b.p.*[k] (*T*/°C)	Found (%) C	H	N	Formula	Calc. (%) C	H	N
(IV)	143·5—145	145—145·5 [b]							
(I)	203—205	204—205 [c]							
(IIIa)	127—128		65·4	3·4	9·7	$C_{16}H_{10}N_2O_4$	65·3	3·4	9·5
(IIIb)	134—135		65·2	3·4	9·7	$C_{16}H_{10}N_2O_4$	65·3	3·4	9·5
(IIId)	128—130		74·0	4·7	5·0	$C_{18}H_{13}NO_3$	74·3	4·5	4·8
(IIIi)	188—189		56·6	2·8	12·4	$C_{16}H_9N_3O_6$	56·6	2·7	12·4
(VIa)	71—73	72—73 [d,e]							
(VIb)	38—40	37·5—38·5 [d]							
(VIc)	*108*(30) [l]		54·1	3·1		$C_7H_5ClO_2$	53·9	3·2	
(VId)	*101*(0·015)	*120*(2) [e,f]							
(VIk)	*76*(30) [g]	*68*(13) [d]							
(VIj)	*101*(30) [h]	*55*—57(1) [d]							
(VIg)	*131*(30) [j]	*126*(16) [e]							
(VIe)	*110*(30) [i]	*75*(4) [e]							

[a] Analyses were carried out on a Hewlett–Packard 185 analyser by Mrs. M. J. Clark and Mr. G. Powell. M.p.s determined with a Kofler Thermospan instrument. [b] Ref. 9. [c] G. V. Jadhar and R. M. Thakker, *Chem. Abs.*, 1950, **44**, 584*a*. [d] I. Maramatsu, S. Sofuku, M. Tsuji, and K. Hagitani, *Nippon Kagaku*, 1965, **86**, 113. [e] A. Van Es and W. Stevens, *Rec. Trav. chim.*, 1965, **84**, 1247. [f] A. Van Es and W. Stevens, *ibid.*, 1965, **84**, 1294. [g] n_D^{25} 1·54094 (lit.,[d] 1·509). [h] n_D^{25} 1·5062 (lit.,[d] 1·509). [i] n_D^{25} 1·5299 (lit.,[e] 1·5286). [j] n_D^{25} 1·5195. [k] Boiling points have pressure (Torr) in parentheses. [l] n_D^{25} 1·5295.

TABLE 2

Determination of pK_a values

Ethaneth iol (pK_a = 11·33 ± 0·04) [c,d]

pH	6·8	6·8	11·72	11·72	11·15	11·15	13·3
$OD_{250\ nm}$	0	0	0·82	0·78	0·48	0·50	1·18
pH	13·3	13·3					
$OD_{250\ nm}$	1·15	1·16					

(IIIa) (pK_a = 3·48 ± 0·01) [a,d]

pH	1·4	1·4	1·4	3·25	3·25	3·25	3·62
$OD_{320\ nm}$	1·21	1·22	1·20	0·96	1·00	0·96	0·84
pH	3·62	3·62	5·4	5·4	5·4		
$OD_{320\ nm}$	0·82	0·83	0·56	0·56	0·56		

Mercaptoacetic acid (pK_a = 10·35 ± 0·01) [a,b]

pH	9·9	10·07	12·24	10·48	10·74	11·5	12·5
$OD_{240\ nm}$	0·16	0·20	0·26	0·36	0·43	0·56	0·6

[a] 25°, 0·1M-Ionic strength. [b] 10% Acetonitrile. [c] 25°, 0·133M-Ionic strength. [d] 34% Dioxan (v/v).

The pK_a values of mercaptoacetic acid, ethanethiol, and 4-nitrophenyl quinoline-8-carboxylate were determined spectroscopically (Table 2) under the conditions of the kinetic experiments. Measurements of pH were carried out using a Radiometer-25 pH-meter calibrated using 'Soloid' standard buffers. Concentrations of thiolate anions were calculated at the pH, ionic strength, and temperature of the medium using the respective ionisation constants (Table 2) and equation (1). Concentrations of

$$[R{\cdot}S^-] = [R{\cdot}SH_{total}]/(1 + [H^+]/K_a) \qquad (1)$$

the ionic species present in buffered cysteine solutions were calculated from equations and data given in the Appendix.

RESULTS

Reaction of ethanethiol with substituted phenyl quinoline-8-carboxylates (III) was pseudo-first order in ester owing to the large excess over ester of thiol concentration. Nucleophilic attack of thiol on the 4-nitrophenyl nucleus (IIIa) was eliminated as a mechanism by the spectroscopic observation that the theoretical quantity of 4-nitrophenol was liberated during reaction. There is little doubt that the reaction being measured is the production of the thiol-ester because although thiolester was not demonstrated in this particular study its formation can be inferred by analogy with other work.[2] The pH-profile of the pseudo-first-order rate constants (Table 3) for reaction of ethanethiol with (IIIa) when corrected for a small background rate

[7] G. L. Lewis and C. P. Smyth, *J. Chem. Phys.*, 1939, **7**, 1085.
[8] E. A. Guggenheim, *Phil. Mag.*, 1926, **2**, 538.

constant exhibits two plateaux (Figure 1) and the results fit the theoretical equation (2) where k_1 and k_2 are rate

TABLE 3

Reaction of ethanethiol with substituted phenyl quinoline-8-carboxylates [a] [8]

pH	Buffer composition	$10^3 k_{EtSH}$/l mol^{-1} s^{-1}
	(IIIa) [d]	
1·20	Chloride [b]	0·021 ± 0·001
1·75	Chloride [c]	0·022 ± 0·002
2·65	Chloride [c]	0·258 ± 0·002
3·32	Chloride [b]	0·719 ± 0·010
3·64	Formate [c]	1·32 ± 0·02
4·65	Formate [b]	2·04 ± 0·02
5·40	Formate [b]	2·00 ± 0·04
6·82	Phosphate [b]	2·30 ± 0·01
7·60	Phosphate [b]	2·27 ± 0·01
8·45	Phosphate [b]	3·57 ± 0·03
8·86	Phosphate [b]	8·94 ± 0·06
9·30	Borate [b]	18·2 ± 0·3
9·85	Borate [b]	51·3 ± 0·3
10·88	Carbonate [b]	551 ± 3
11·20	Carbonate [b]	866 ± 6
11·30	Carbonate [b]	1060 ± 10
12·65	Hydroxide [b]	2050 ± 15
	Uncatalysed rate constant	$10^4 k$/s^{-1}
1·62	Chloride [c]	0·123 ± 0·001
3·25	Formate [b]	0·121 ± 0·001
3·64	Formate [c]	0·234 ± 0·005
8·86	Phosphate [b]	0·159 ± 0·002
11·1	Carbonate [b]	2·92 ± 0·03
12·1	Carbonate [b]	22·2 ± 0·2
12·65	Hydroxide [b]	107 ± 1
	(IIIb) [e]	
6·85	Phosphate [b]	1·15 ± 0·01
6·85	Phosphate [b]	1·18 ± 0·01
5·50	Formate [b]	1·21 ± 0·01
	(IIId) [f]	
6·85	Phosphate [b]	0·95 ± 0·01
	(IIIi) [g]	
5·4	Formate [b]	7·71 ± 0·01
6·85	Phosphate [b]	12·7 ± 0·1
10·43	Borate [b]	2800 ± 10

[a] 25°, Rate constants corrected for uncatalysed rate constant. [b] Ionic strength 0·133M. [c] Ionic strength 0·067M. [d] Parameters derived from these data [equation (2)]: k_1, 2 × 10^{-3} l mol^{-1} s^{-1}; k_2, 2 l mol^{-1} s^{-1}; K_1, $10^{-3\cdot48}$; K_2, $10^{-11\cdot33}$. [e] k_1 = 1·17 ± 0·01 × 10^{-3} l mol^{-1} s^{-1}. [f] k_1 = 0·95 ± 0·01 × 10^{-3} l mol^{-1} s^{-1}. [g] k_1 = 7·71 ± 0·1 × 10^{-3} l mol^{-1} s^{-1}. [8] Log^{10} k_1 = 0·99σ − 3·57 (r = 0·987).

constants calculated assuming reaction of thiol and thiolate anion with neutral ester; K_1 and K_2 refer to the ionisation

$$k_{obs}/[EtSH] = \frac{k_1}{(1 + [H^+]/K_1)} + \frac{k_2}{(1 + [H^+]/K_2)} \quad (2)$$

of quinolinium ion and thiol respectively. Values of k_1 for a number of phenol leaving groups obeyed a good Hammett-σ relationship (which is illustrated later in Figure 5c); measurements on 4-acetylphenyl ester were made at only one pH value (6·85) and it is reasonably assumed that this is on the plateau pH region for each ester.

The spectrum of acetophenone in buffers used in these experiments was identical in the presence and absence of ethanethiol. Thus thioacetal and hemithioacetal formation does not render the kinetic results for the 4-acetylphenyl ester meaningless.

Reaction of ethanethiol with 4-nitrophenyl 5-nitrosalicylate (I) was not carried out below pH 5 because the rate of intermediate thiolester formation became comparable with its rate of decomposition. As a result the optical density at 325 nm rose to a maximum and then

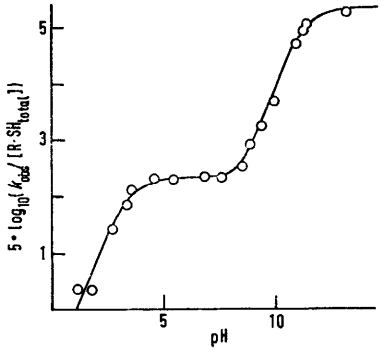


FIGURE 1 pH Profile of $k_{obs}/[EtSH_{total}]$ for reaction of ethanethiol with 4-nitrophenyl quinoline-8-carboxylate (IIIa). Line calculated from equation (2) using data from Table 3

decreased making the accurate measurement of a final reading difficult. The reaction above pH 5 was pseudo-first-order in ester and varied with pH (Table 4 and Figure 2) according to equation (3) where K_1 is the dissociation

$$k_{obs}/[EtSH] = k_{EtSH}/(1 + [H^+]/K_1) \quad (3)$$

constant of the phenol group and k_{EtSH} is the rate constant for reaction of thiol with phenolate anion.

TABLE 4

Reaction of ethanethiol with 4-nitrophenyl 5-nitrosalicylate (I) [a,b]

pH	Buffer	$10^2 k_{EtSH}$/l mol^{-1} s^{-1}
11·25	Carbonate	4·63 ± 0·03
10·40	Carbonate	4·44 ± 0·04
8·90	Phosphate	4·28 ± 0·03
7·52	Phosphate	3·59 ± 0·03
6·83	Phosphate	3·58 ± 0·03
5·90	Acetate	1·82 ± 0·03
5·55	Acetate	1·19 ± 0·01
5·07	Acetate	0·484 ± 0·002

[a] 25°, Ionic strength 0·13M with 34·4% dioxan (v/v); initial ester concentration 9·6 × 10^{-5}M, thiol concentration, 0·103M. [b] Parameters derived from these data fitted equation (3) where K_a = 6·00 and k_{EtSH} = 4·40 × 10^{-2}M l mol^{-1} s^{-1}.

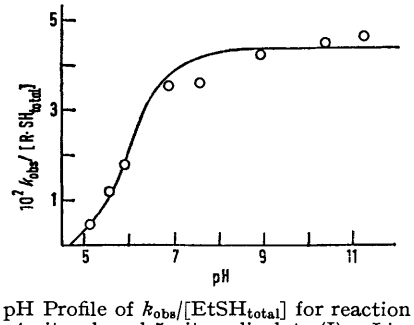


FIGURE 2 pH Profile of $k_{obs}/[EtSH_{total}]$ for reaction of ethanethiol with 4-nitrophenyl 5-nitrosalicylate (I). Line calculated from equation (3) and data from Table 4

Assuming thiol and thiolate react intermolecularly with active esters the theoretical kinetic equation is (4) where K_1 is the dissociation constant of the thiol and k_1 and k_2

respectively the parameters for reaction with thiol and thiolate. A plot of the left hand side of equation (4) *versus*

$$\frac{k_{obs}/[R{\cdot}SH_{total}]}{(1 + K_1/[H^+])} = k_1 + k_2K_1/[H^+] \quad (4)$$

$1/[H^+]$ has slope k_2K_1 and intercept k_1 on the vertical axis. Figures 3 and 4 illustrate the plots for reaction of mercaptoacetic acid with 3-nitrophenyl acetate and 4-chlorophenyl mesylglycinate. Experimental data for these hydrolyses and values of k_1 and k_2 are given in Tables 5 and 6. Table 5 includes values of k_2 for reaction of mercaptoacetic acid

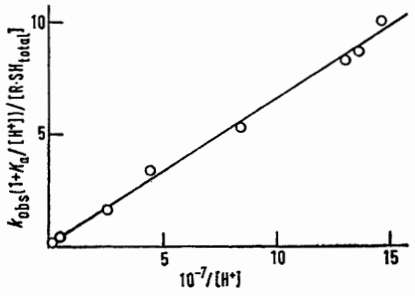


FIGURE 3 Plot of $k_{obs}/[R{\cdot}SH_{total}]/(1 + K_a/[H^+])$ *versus* $1/[H^+]$ for reaction of mercaptoacetic acid with 3-nitrophenyl acetate (IIb). Line is theoretical [equation (4)] and data from Table 5

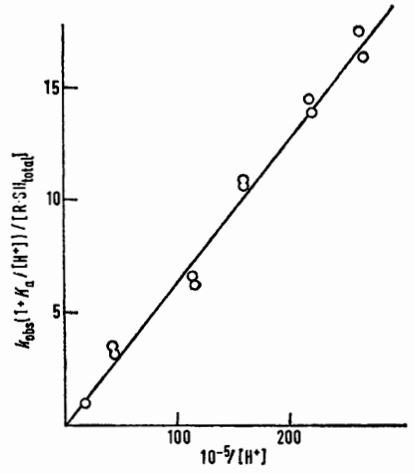


FIGURE 4 Plot of $k_{obs}/[R{\cdot}SH_{total}]/(1 + K_a/[H^+])$ *versus* $1/[H^+]$ for reaction of mercaptoacetic acid with 4-chlorophenyl mesylglycinate (Va). Line is theoretical [equation (4)] and data from Table 6

with 4-methoxy and 4-nitrophenyl acetates and the variation with σ gives a Hammett ρ of 2·3. Table 6 also indicates the effect of buffer on the reaction between mercaptoacetic acid and 4-chlorophenyl mesylglycinate.

The cysteine-catalysed hydrolysis of substituted phenyl mesylglycinates (Table 7) can arise from catalysis by four separate species (see Appendix). The species depicted as $NH_3^+{\cdot}SH$ is assumed to be inactive and the data can be analysed by equation (5). In these experiments the term for $NH_2{\cdot}S^-$ has too much error to be reliable and the only accurate term could arise from catalysis by either $NH_3^+{\cdot}S^-$ or $NH_2{\cdot}SH$. The term $k_{NH_3^+{\cdot}S^-}$ has a Hammett-σ ρ value of 1·65 at 25°.

$$k_{obs} = [NH_3^+{\cdot}S^-]k_{NH_3^+{\cdot}S^-} + [NH_2{\cdot}S^-]k_{NH_3{\cdot}S^-} \quad (5)$$

Ethanethiol reacted with 4-nitrophenyl acetate with a rate constant (derived from measurements at pH 8·6 and 8·8

TABLE 5

Reaction of mercaptoacetic acid with substituted phenyl acetates [a]

pH	$10k_{obs}/[R{\cdot}SH_{total}]$/l mol^{-1} s^{-1}
	(IIb) [b]
5·95	0·0903 ± 0·007
6·42	0·243 ± 0·007
7·40	1·68 ± 0·03
7·66	3·29 ± 0·07
7·92	5·36 ± 0·07
8·12	8·40 ± 0·05
8·13	8·68 ± 0·11
8·17	9·88 ± 0·007
	(IIa) [c]
7·9	1·0 ± 0·03
7·6	0·524 ± 0·003
7·43	0·35 ± 0·04
	(IIg) [d]
7·7	0·0199 ± 0·0004

[a] 0·1M-Ionic strength, 25°. Phosphate buffer. [b] Parameters from equation (4) are $k_1 = 8·85 \pm 9·5 \times 10^{-4}$ l mol^{-1} s^{-1} and $k_2 = 14·5 \pm 0·2$ l mol^{-1} s^{-1}. Concentration of thiol, 0·104M. [c] k_2 [equation (4)] = 25·8 ± 0·5 l mol^{-1} s^{-1}. Concentration of thiol, 0·109M. [d] k_2 [equation (4)] = 0·085 ± 0·005 l mol^{-1} s^{-1}. Concentration of thiol, 0·20M.

TABLE 6

Reaction of mercaptoacetic acid with 4-chlorophenyl mesylglycinate (Ve) [a]

pH	$10^2k_{obs}/[R{\cdot}SH_{total}]$/l mol^{-1} s^{-1}
6·23	1·64
6·66	5·25
6·66	5·15
7·05	11·4
7·05	10·8
7·21	17·7
7·21	17·8
7·34	24·0
7·34	23·2
7·42	27·3
7·42	29·0
7·00 [b,c]	3·83
7·00 [b,d]	3·85
7·00 [b,e]	3·92

[a] 25°, 0·1M-Ionic strength. Parameters fitting equation (4): $k_1 = (-13·2 \pm 40·8) \times 10^{-2}$ l mol^{-1} s^{-1}, $k_2 = (2·42 \pm 0·05) \times 10^{-2}$ l mol^{-1} s^{-1}. Phosphate buffer. [b] Ionic strength 2·86M. [c] 1M-Buffer. [d] 0·5M-Buffer. [e] 0·05M-Buffer.

TABLE 7

Reaction of cysteine with substituted phenyl mesylglycinates [a]

Ester	λ/nm	pH	10^2k_{obs}/s^{-1}	$k_{NH_3^+{\cdot}S^-}$/l mol^{-1} s^{-1}
(Ve)	281	7·45	1·57	
		7·23	1·04	
		6·71	0·308	23 ± 0·4
(Vb)	355	6·68	2·15	
		6·91	3·00	
		7·08	5·43	179 ± 17
(Vd)	300	7·07	2·98	
		6·71	1·25	
		6·91	1·85	103 ± 11
(Vh)	290	7·37	0·177	
		7·08	0·0958	
		7·23	0·132	2·98 ± 0·008
(Vg)	295	7·39	0·268	
		7·07	0·134	
		7·23	0·195	4·42 ± 0·13
(Vf)	290	7·07	0·380	
		6·75	0·192	
		7·23	0·541	12·3 ± 0·07

[a] 25°, Ionic strength 0·2M, phosphate buffers.

at 25°) of 25·9 l mol^{-1} s^{-1}. This value is close to the one predicted from the work of Ogilvie *et al.* [equation (8), ref. 3].

$$k_{obs} = k_{H_2O} + k_{OH}[OH^-] \quad (6)$$

Hydrolysis of the aryl formates obeyed the rate law (6) and an example of the fit of data (Table 8) to a theoretical line is given (Figure 6). The individual rate constants (k_{H_2O} and k_{OH}) fitted good Hammett-σ relationships (ρ 1·58, r 0·99; ρ 1·09, r 0·99, respectively) and these are illustrated in Figure 5.

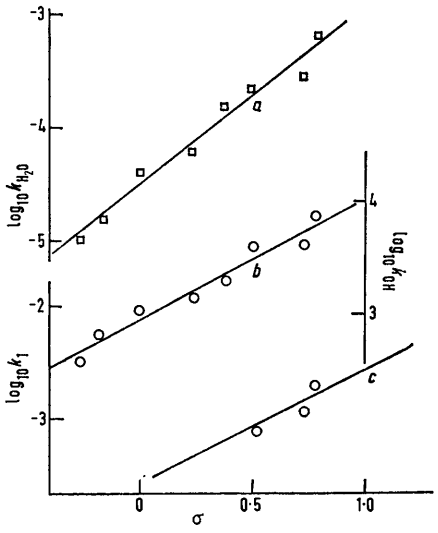


FIGURE 5 Dependence of rate constants on Hammett-σ. *a*, Reaction of water with substituted phenyl formates (Table 8); *b*, reaction of hydroxide ion with substituted phenyl formates (Table 8); and *c*, reaction of ethanethiol with substituted phenyl quinoline-8-carboxylates (k_1, Table 3). Hammett-σ values from G. B. Barlin and D. D. Perrin, *Quart. Rev.*, 1966, **20**, 75; lines are theoretical

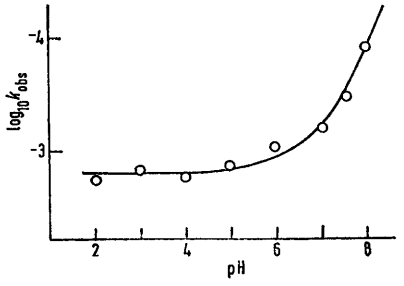


FIGURE 6 pH Dependence of the hydrolysis rate constant for 4-nitrophenyl formate. Line is theoretical from data of Table 8

DISCUSSION

Ogilvie and Schneider [3,4] found equivocal evidence for reaction of 4-nitrophenyl acetate with thiol and Whitaker's systematic study [2] does not report the possibility of this reaction. We considered that this reaction should be studied with a thiol of high pK_a at low pH since the main difficulty in measuring reactions with thiols and active esters is the incursion of the thiolate component. Despite this approach with mercaptoacetic acid and 3-nitrophenyl acetate and 4-chlorophenyl mesylglycinate the intercepts due to thiol catalysis carried errors as large as the intercepts themselves (Tables 5 and 6 and Figures 3 and 4). The possibility of utilising general base catalysis to enhance the thiol component was investigated but phosphate buffer did not noticeably affect the rate of reaction of mercaptoacetic acid with 4-chlorophenyl mesylglycinate (Table 6). Thus, although thiols probably react with activated esters the reaction is not detectable in useful magnitude for the esters studied here.

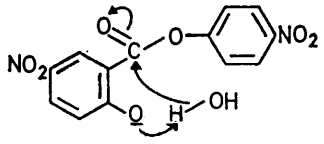
TABLE 8

Hydrolysis of substituted phenyl formates over a pH range [a]

Ester	σ [d]	$10^5k_{H_2O}$/s^{-1} [b]	10^3k_{HO}/ l mol^{-1} s^{-1} [c]
(VIa)	0·78	64 ± 3·5	7·24 ± 0·1
(VIb)	0·72	28 ± 0·1	4·21 ± 0·6
(VIc)	0·37	14·7 ± 0·1	2·0 ± 0·1
(VId)	0·5	21·5 ± 1	3·9 ± 0·06
(VIk)	0	3·86 ± 0·11	1·07 ± 0·1
(VIe)	0·23	6·05 ± 0·3	1·38 ± 0·1
(VIg)	−0·27	1·03 ± 0·01	0·0377 ± 0·03
(VIj)	−0·17	1·55 ± 0·03	0·063 ± 0·01

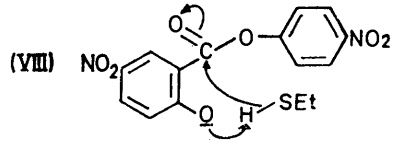
[a] 0·09M-Ionic strength, 25°. [b] $\log_{10}k_{H_2O} = 1·58\sigma - 4·51$ (r = 0·987). [c] $\log_{10}k_{HO} = 1·09\sigma + 2·95$ (r = 0·990). [d] σ Values from ref. in Figure 5.

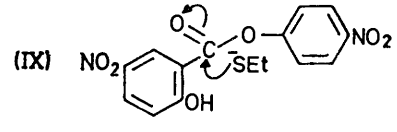
Bender *et al.*[9] have shown that in the pH-range 6—10 4-nitrophenyl 5-nitrosalicylate (I) probably hydrolyses *via* intramolecular general base-catalysed attack of phenoxide ion on the attacking water (VII). Thiolysis of this ester by ethanethiol has a pH-dependence (Figure 2) which indicates involvement of the phenol.



(VII)

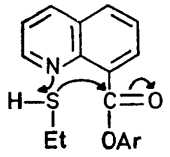
It can be calculated that the rate constant for attack of thiolate anion on uncharged ester is 9·19 × 10^3 l mol^{-1} s^{-1} and it is known that anions attack the unionised ester some 2—6 times faster than 4-nitrophenyl 3-nitrobenzoate;[9] thus we can estimate a rate constant for reaction with uncharged ester as 2—6 × 778 = 1·6—4·7 × 10^3 l mol^{-1} s^{-1} which although lower than that calculated from the observed rate constant does not enable a clear cut decision to be made between mechanisms (VIII) and (IX).

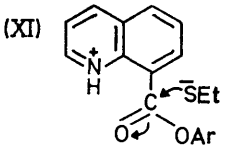




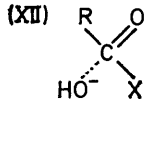
[9] M. L. Bender, F. J. Kézdy, and B. Zerner, *J. Amer. Chem. Soc.*, 1963, **85**, 3017.

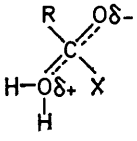
Reaction of ethanethiol with activated esters of quinoline-8-carboxylic acid is a good potential model for the reaction of papain with activated esters since the potential general base is a tertiary nitrogen with pK_a close to that for papain (4). The higher pK_a derived from the pH-profile corresponds to that for ethanethiol and the mechanism in the alkaline region of pH (Figure 1) involves attack of thiolate anion on uncharged ester. The mechanism in the low pH-region is ambiguous [(X) and (XI)] but consideration of the Hammett selectivity

(X) 

(XI) 

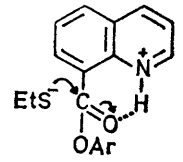
to substituent on the phenyl leaving group points to involvement of thiolate anion (XI). Attack of an anion at an aryl ester (XI) should have a lower sensitivity to change in substituents on the phenol leaving group than has attack of unionised nucleophile (X); the anion, a stronger base, will be more reactive and hence bond formation to carbonyl carbon will not be so advanced (XII) in the transition state as with the weaker nucleophile (XIII).

(XII) 

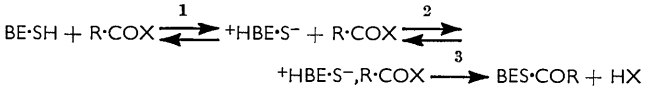
(XIII) 

This effect is supported by the lower Hammett ρ for hydroxide (1·09) as opposed to water (1·58) attack at substituted phenyl formates. The Taft selectivity to reaction of hydroxide with ethyl esters (2·45) [10] is also lower than that for general base-catalysed attack of water on these esters (3·5).[11] Reaction of hydroxide with substituted phenyl diphenylphosphinates has a lower ρ value (1·55) than the corresponding imidazole-catalysed reaction with water (2·88, this reaction is general-base catalysed).[5] The selectivity for reaction of ethanethiol with substituted phenyl quinoline-8-carboxylates (0·99) is lower than that observed for attack of thiolate anions on aryl esters (1·65 for $NH_3^+ \cdot S^-$ on aryl mesylglycinates; 2·3 for $^-S \cdot CH_2CO_2R$ on aryl acetates; 1·7 for $NH_3^+ \cdot S^-$ on aryl acetates [12]).

The rate constant for reaction between ethanethiolate and quinolinium ester can be calculated to be $1{\cdot}42 \times 10^5$ l mol^{-1} s^{-1}. This value is some $5{\cdot}5 \times 10^3$-fold higher than the rate constant for reaction of ethanethiolate anion with 4-nitrophenyl acetate. This enhancement is undoubtedly to a large extent due to the high reactivity of a positively charged system. Some of the reactivity could be due to intramolecular electrophilic participation by the NH^+ on the carbonyl oxygen atom (XIV) and the low Hammett selectivity is in agreement with this hypothesis.

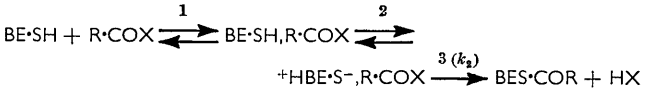
(XIV) 

The only convincing piece of evidence that papain acts as an imidazolyl–thiol pair rather than as an imidazolium–thiolate pair has been recently criticised.[13] We use here the concept of a maximal rate constant (caused by rate-determining diffusion of reactants and products to and from a reaction centre) [14] to eliminate a number of postulated mechanisms. From kinetic pK_a values it can be calculated that less than 10^{-4} of the



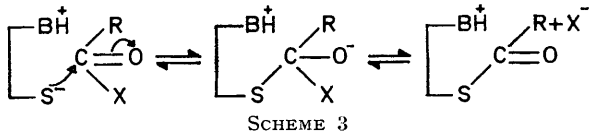
Scheme 1

total enzyme is present as free zwitterion thus the overall rate constant for steps 2 and 3 in Scheme 1 must exceed 10^{11} l mol^{-1} s^{-1} to accommodate the observed overall rate constant of $2{\cdot}6 \times 10^7$ l mol^{-1} s^{-1} for the combination of 4-nitrophenyl N_α-benzyloxycarbonyl-L-lysinate and free BE·SH to give acyl enzyme.[15] This value is in excess of the maximal rate constant attainable for enzyme systems *ca.* 10^9 l mol^{-1} s^{-1} owing to diffusion becoming rate-limiting.[14] It could be argued that the zwitterion is formed *after* formation of the enzyme–substrate complex (Scheme 2).



Scheme 2

The proportion of ' bound ' zwitterion is less than 10^{-4} of the total enzyme thus the rate of formation of product acyl enzyme is $(10^4\ k_2) \times$ [BE·SH] × [R·COX]. Since the rate constant has the value $2{\cdot}6 \times 10^7$ l mol^{-1} s^{-1} for 4-nitrophenyl N_α-benzyloxycarbonyl-L-lysinate [15] k_2 must approximate to 10^{11} s^{-1}; the latter is in excess of the maximal for fragmentation unimolecular reactions (10^9 s^{-1}).[14] Scheme 3 is thus not operative for this ester.

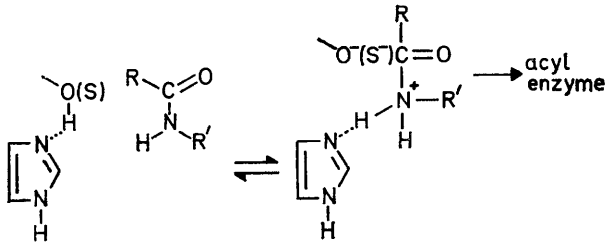


Scheme 3

The microscopic reverse of Scheme 3 for acylation by acids (deacylation) is also eliminated by a separate diffusion argument (X = OH^-). Rate constant for

[10] R. W. Taft, ' Steric Effects in Organic Chemistry,' ed. M. S. Newman, Wiley, New York, 1956, 556.
[11] W. P. Jencks and J. Carriuolo, *J. Amer. Chem. Soc.*, 1961, **83**, 1743.
[12] G. Lowe and A. Williams, *Biochem. J.*, 1965, **96**, 202.
[13] A. R. Fersht, *J. Amer. Chem. Soc.*, 1971, **93**, 3504.
[14] M. Eigen and G. G. Hammes, *Adv. Enzymol.*, 1963, **21**, 1.
[15] M. L. Bender and L. J. Brubacher, *J. Amer. Chem. Soc.*, 1966, **88**, 5880.

hydrolysis of N_α-benzyloxycarbonyl-L-lysylpapain is 38·3 s^{-1} at 25°.[16] If the mechanism involved reaction of ions OH^- and $^+$HBE·SCOR the calculated rate constant is 3·83 × 10^{11} l mol^{-1} s^{-1} ($K_a \times 38{\cdot}3/K_w$) which is too



SCHEME 4

fast to be accommodated by diffusion of reactants to the reaction centre.[14] Similar evidence has been cited to eliminate mechanisms involving attack of serine-195 alkoxide in α-chymotrypsin-catalysed reactions.[17]

Pre-equilibrium protonation of amide or ester by papain as in the Wang mechanism [18] for α-chymotrypsin (essentially Scheme 4) can be postulated. This mechanism was eliminated by us [19] using a diffusion argument; this was recently confirmed by Fersht who was able to estimate pK_a values for *N*-protonation of amides (*ca.* 17) [13] and hence obtain more accurate estimated rates.[20] The mechanism of Scheme 4 for papain would give impossibly high rate constants.

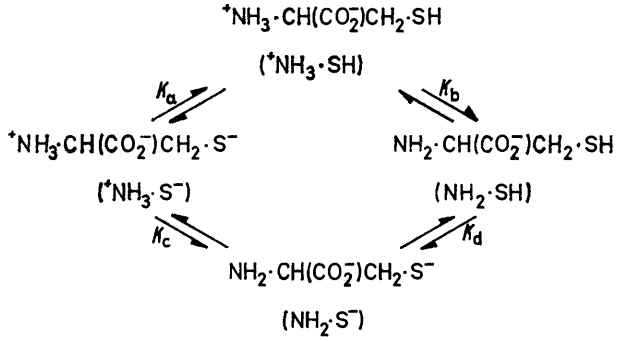
The diffusion argument is only valid for those substrates which are highly reactive; less reactive substrates could give rates less than diffusion rates and the argument against Schemes 1—3 is then by analogy assuming a constant mechanism throughout.

Attack of thiol on esters and amides in papain-catalysed reactions continues to defy chemical simulation. Base- and acid-catalysed attack of thiol at other unsaturated systems is however known in water and in non-aqueous systems.[21] It is considered likely that proximity effects coupled with electrophilic participation at the carbonyl oxygen atom [6] of the substrate favour general base-catalysed attack of the thiol in the enzyme reaction. A further problem with papain is the lack of reactivity with specific substrates and with 4-nitrophenyl acetate [6] as the pH rises above the pK_a of the thiol. This lack of thiolate reactivity could be caused by a conformational change of the enzyme at this pH but the pH invariant inhibition and binding constants for inhibitors and substrates [1] argue against this. Solvation effects (either intramolecularly with donors on the enzyme or with solvent water) could depress reactivity relative to the thiol which is only solvated by possible hydrogen bonding with the imidazolyl nitrogen atoms of histidine-159.

[16] A. Williams, E. C. Lucas, A. R. Rimmer, and H. C. Hawkins, *J.C.S. Perkin II*, 1972, 627.
[17] M. L. Bender and F. J. Kézdy, *J. Amer. Chem. Soc.*, 1964, **86**, 3704.
[18] (*a*) J. H. Wang, *Science*, 1968, **161**, 328; (*b*) J. H. Wang and L. Parker, *Proc. Nat. Acad. Sci. U.S.A.*, 1967, **58**, 2451; (*c*) L. Parker and J. H. Wang, *J. Biol. Chem.*, 1968, **243**, 3729.
[19] A. Williams, *Biochemistry*, 1970, **9**, 3383.
[20] A. R. Fersht and Y. Requena, *J. Amer. Chem. Soc.*, 1971, **93**, 7079.

APPENDIX

In the neighbourhood of pH 6—10 cysteine is an equilibrium mixture of four ions (Scheme 5).[22] Equations (7)—



SCHEME 5

(10) connect fractions of total cysteine, present in the form of different ions, with hydrogen ion concentration.

$$[NH_2{\cdot}SH](1 + [H^+]/K_b + K_d/[H^+] + K_a/K_b) = [\text{total cyst}] \quad (7)$$

$$[^+NH_3{\cdot}S^-](1 + [H^+]/K_a + K_c/[H^+] + K_b/K_a) = [\text{total cyst}] \quad (8)$$

$$[NH_2{\cdot}S^-](1 + [H^+]/K_d + [H^+]/K_c + [H^+]^2/K_bK_d = [\text{total cyst}] \quad (9)$$

$$[^+NH_3{\cdot}SH](1 + K_a/[H^+] + K_b/[H^+] + K_aK_c/[H^+]^2 = [\text{total cyst}] \quad (10)$$

Graphius and Neilands [22a] give values of K_a, K_b, K_c, and K_d at a defined ionic strength and these are used in this study.

The University of Kent (E. C. L.) and the Government of Northern Ireland (K. T. D.) are thanked for studentships.

[1/1411 *Received*, 10*th August*, 1971]

[21] (*a*) R. E. Barnett and W. P. Jencks, *J. Amer. Chem. Soc.*, 1969, **91**, 2358; (*b*) E. Campaigne, 'Organic Sulphur Compounds,' ed. N. Kharasch, Pergamon, Oxford, 1961, **1**, 134; (*c*) B. Dumchovsky, F. B. Zienty, and W. A. Vredenburgh, *J. Org. Chem.*, 1966, **31**, 865; (*d*) F. B. Zienty, B. D. Vineyard, and A. A. Schleppnik, *J. Org. Chem.*, 1962, **27**, 3140; (*e*) B. Dumchovsky, B. D. Vineyard, and F. B. Zienty, *J. Amer. Chem. Soc.*, 1964, **86**, 2874.
[22] (*a*) M. A. Graphius and J. B. Neilands, *J. Amer. Chem. Soc.*, 1955, **77**, 3389; (*b*) L. R. Ryklan and L. L. A. Schmidt, *Arch. Biochem.*, 1944, **5**, 89; (*c*) R. E. Benesch and R. Benesch, *J. Amer. Chem. Soc.*, 1955, **77**, 5877.