Proteolytic Enzymes: Models for Electrophilic Assistance by NH in Acylation

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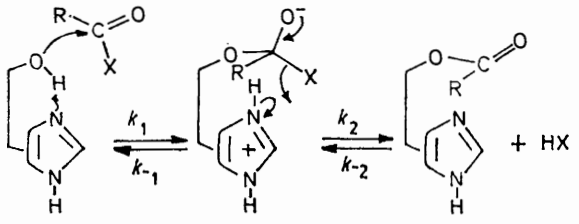
The effect of increasing dioxan content of the medium has been employed to examine the possibility of intramolecular electrophilic assistance by NH in alkaline hydrolysis of the methyl esters of 2-acetamidobenzoic (I), pyrrole-2-carboxylic (VII), and benzimidazole-4(7)-carboxylic (V) acids. Significant enhancement of rate constant is observed over isomers where no participation is possible. Enhancements increased steeply with increasing dioxan concentration in agreement with a ' built-in ' solvation by the NH of the incipient alkoxide ion (derived from the carbonyl oxygen) of the transition-state. Heterocyclic types [(VII) and (V)] exhibited maxima in their rate enhancements as the dioxan content increased. Since the NH to alkoxide bond distance in the heterocyclic types is larger than normally found for direct hydrogen-bonding the maximum is argued to arise from a water bridge acting as a relay between electrophile NH and incipient alkoxide ion of the transition-state.

GENERAL-ACID catalysis aids the departure of a strongly basic leaving group from the tetrahedral adduct during acylation of ' serine ' proteases by substrates.[1] Since this step (k_2) is by symmetry the reverse of the addition step (k_1), the acid involved is the conjugate of the base in that step [2] namely the imidazolium species. Recent



work has demonstrated the existence of electrophilic assistance at the carbonyl oxygen in the k_1 step during acylation of chymotrypsin by specific aryl ester substrates.[3a] The k_{-2} step has also been shown to involve electrophilic assistance at the carbonyl oxygen [3b] in studies on the deacylation of acylchymotrypsins. It was suggested [3a,b] that peptide NH groups of residues including and preceding serine-195 provided electrophilic assistance to stabilise the incipient oxyanion of the transition-state and to polarise the carbonyl bond of the ester in the ground-state. *X*-Ray crystallographic work of Blow [4a] and Henderson [4b] on *N*-α-formyl-L-tryptophan–chymotrypsin complex and on indolylacryloyl-chymotrypsin suggests that the carbonyl oxygen of the acid and of the ' reactive ' acyl-enzyme fit in a ' pocket ' surrounded by the peptide NH's of serine-195, glycine-193, and possibly aspartate-194. We present other arguments [3b] for the participation of these NH groups.

Possible electrophilic participation by hydroxy- and ammonium moieties in ester hydrolysis has been thoroughly investigated [5,6] but electrophilic assistance by the peptide group has not. Nucleophilic participation by amides in reactions of carboxy-derivatives is well-known [7] and except in the constrained structure of enzymes these groups prefer to act as nucleophiles utilising either the oxygen or (in preference [7b]) the nitrogen atoms. We report here a study of the following compounds as models for the electrophilic participation of NH in ester hydrolysis.

EXPERIMENTAL

Materials.—Reagent grade dioxan was purified according to the method of Vogel [8] and immediately before use was passed through an alumina column. Potassium iodide

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[2] (*a*) M. L. Bender and F. J. Kézdy, *Ann. Rev. Biochem.*, 1965, **34**, 49; (*b*) T. C. Bruice and S. J. Benkovic, ' Bio-organic Mechanisms,' vol. I, Benjamin, New York, 1966, 212ff; (*c*) W. P. Jencks, ' Catalysis in Chemistry and Enzymology,' McGraw-Hill, New York, 1969, 44ff; (*d*) S. G. Waley, *Quart. Rev.*, 1967, **21**, 379.

[3] (*a*) A. Williams, *Biochemistry*, 1970, **9**, 3383; (*b*) A. Williams and G. Salvadori, *J. Chem. Soc.* (*B*), 1971, 2401.

[4] (*a*) T. A. Steitz, R. Henderson, and D. M. Blow, *J. Mol. Biol.*, 1969, **46**, 337; (*b*) R. Henderson, *J. Mol. Biol.*, 1970, **54**, 341.

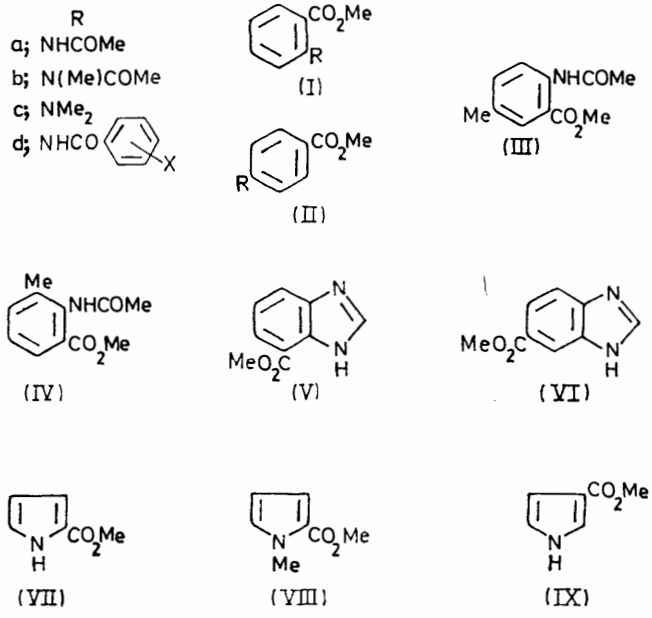
[5] (*a*) H. B. Henbest and B. J. Lovell, *J. Chem. Soc.*, 1957, 1965; (*b*) S. M. Kupchan, W. S. Johnson, and S. Rajagopalan, *Tetrahedron*, 1959, **7** 47; (*c*) S. M. Kupchan and W. S. Johnson, *J. Amer. Chem. Soc.*, 1956, **78**, 3864; (*d*) R. West, J. J. Korst, and W. S. Johnson, *J. Org. Chem.*, 1960, **25**, 1976; (*e*) T. C. Bruice and T. H. Fife, *J. Amer. Chem. Soc.*, 1962, **84**, 1973; (*f*) B. Capon and M. I. Page, *J. Chem. Soc.* (*B*), 1971, 741; (*g*) S. M. Kupchan and C. R. Narayanau, *J. Amer. Chem. Soc.*, 1959, **81**, 1913; (*h*) S. M. Kupchan, P. Slade, and R. J. Young, *Tetrahedron Letters*, 1960, **24**, 22; (*i*) H. G. Zachau and W. Karau, *Chem. Ber.*, 1960, **93**, 1830; (*j*) M. J. Allen, *J. Chem. Soc.*, 1960, 4904; 1961, 4252; (*k*) S. M. Kupchan, S. P. Eriksen, and M. Friedman, *J. Amer. Chem. Soc.*, 1962, **84**, 4159; 1966, **88**, 343; (*l*) S. M. Kupchan, S. P. Eriksen, and Y. T. Shen, *ibid.*, 1963, **85**, 350; (*m*) S. M. Kupchan, S. P. Eriksen, and Y.-T. S. Liang, *ibid.*, 1966, **88**, 347; (*n*) S. M. Kupchan, J. H. Block, and A. C. Isenberg, *ibid.*, 1967, **89**, 1189; (*o*) T. Yamika, A. Ichihara, K. Tanabe, and T. Matsumoto, *Tetrahedron*, 1965, **21**, 1031.

[6] (*a*) G. Aksnes and J. E. Prue, *J. Chem. Soc.*, 1959, 103; (*b*, B. Hansen, *Acta Chem. Scand.*, 1962, **16**, 1927; (*c*) A. Ågren) U. Hedsten, and B. Jonsson, *ibid.*, 1961, **15**, 1532; (*d*) J. A. Zaslowsky and E. Fisher, *J. Phys. Chem.*, 1963, **67**, 959; (*e*) B. Hansen and A. Flormark, *Acta Chem. Scand.*, 1963, **17**, 1481; (*f*) B. Hansen, *Svensk kem. Tidskr.*, 1963, **75**, 10; (*g*) J. A. Shafer and H. Morawetz, *J. Org. Chem.*, 1962, **27**, 2269; (*h*) L. Larsson, *Svensk kem. Tidskr.*, 1958, **70**, 405; (*i*) E. Schätzle, M. Rottenberg, and M. Thürkauf, *Helv. Chim. Acta*, 1959, **42**, 1708; (*j*) E. R. Garrett, *J. Amer. Chem. Soc.*, 1957, **79**, 5206; (*k*) G. E. Cwalina and A. Gringauz, *J. Org. Chem.*, 1961, **26**, 3344.

[7] (*a*) Ref. 2*b*, pp. 187—195; (*b*) R. M. Topping and D. E. Tutt, *Chem. Comm.*, 1966, 698; *J. Chem. Soc.* (*B*), 1967, 1346.

[8] A. I. Vogel, ' Practical Organic Chemistry,' Longmans, London, 1956, p. 177.

solution was used to test for the absence of peroxides. Column treated Analar dioxan was shown to give identical kinetic parameters. Commercial grade acetonitrile was purified according to the method of Lewis and Smyth [9] and



only those fractions showing minimal aromatic u.v. absorption were employed. The following substances were prepared by standard methods: 4-acetamidobenzoic acid (X), methyl 4-acetamidobenzoate (IIa), methyl 2-acetamidobenzoate (Ia), methyl 4-(*N*-methylacetamido)benzoate (IIb), methyl 2-(*N*-methylacetamido)benzoate (Ib), methyl 4-(*NN*-dimethylamino)benzoate (IIc), and methyl 2-(*NN*-dimethylamino)benzoate (Ic). 2-Benzamidobenzoates (Id) were prepared using the Schotten–Baumann reaction: methyl 2-aminobenzoate (1·51 g), prepared by the action of diazomethane on anthranilic acid, was dissolved in dry pyridine (15 ml) and to this solution was added the substituted benzoyl chloride (10 mmol) in small portions during 0·5 h with vigorous stirring. The mixture was stored overnight at room temperature, evaporated to dryness *in vacuo*, and the solid partially dissolved in ice–water. The products were filtered, dried, and recrystallised from methanol. Methyl 2-acetamido-5-methylbenzoate (III) was prepared from 2-amino-5-methylbenzoic acid *via* the 3,1-benzoxazine which was hydrolysed and methylated with diazomethane. Methyl 2-acetamido-3-methylbenzoate (IV) was prepared in a similar manner to the 5-methyl isomer (III). Methyl pyrrole-2-carboxylate (VII) was a gift from Dr. M. V. Sargent and it had m.p. 74—75° (lit.,[10] m.p. 70—72°). Methyl 1-methylpyrrole-2-carboxylate (VIII) was prepared by adding a solution of methyl pyrrole-2-carboxylate (1·05 g) in sodium-dry toluene (5 ml) to a suspension of molecular potassium in refluxing dry toluene (0·35 g in 25 ml). After allowing the mixture to reflux for 24 h (with vigorous stirring) the solvent was distilled and the residue treated with methyl iodide (10 ml); the mixture was refluxed for a further two days. The solution was cooled, filtered, and, after evaporation, distilled *in vacuo*. Methyl pyrrole-3-carboxylate (IX) was prepared according to the method of Rapoport. Methyl benzimidazole-4(7)-carboxylate (V) was synthesised from 3-nitro-2-aminotoluene (from Aldrich) *via* the following series of reactions. 3-Nitro-2-aminotoluene (7·6 g) was mixed with granulated tin (20 g) and concentrated hydrochloric acid (50 ml) was added slowly during 0·5 h with swirling and cooling. The mixture was then warmed on a hot-plate until the yellow colour had disappeared (*ca.* 1 h). Sodium hydroxide solution (20%) was then added until the mixture was alkaline to litmus and then the solution was boiled until most of the white precipitate had dissolved. The solution was extracted with chloroform (3 × 50 ml) and the extract was dried (Na_2SO_4), and evaporated; the residue of 2,3-diaminotoluene (XI) was allowed to crystallise. 2,3-Diaminotoluene (1·5 g) was warmed at 100° for 1 h with formic acid (1·6 g). The product was cooled and diluted with a little water; potassium hydroxide (1N) was then added until the solution was alkaline to litmus. A crystalline white precipitate of 4(7)-methylbenzimidazole (XII) was filtered off and crystallised from water after clarification with active charcoal. 4(7)-Methylbenzimidazole (1·3 g) was mixed with water (20 ml) and potassium permanganate (3·6 g) was added slowly to the mixture whilst it was warmed gently on a water-bath. After 1 h the addition was complete; care is necessary with this reaction as it becomes violent if the permanganate is added too quickly. The suspension of manganese dioxide was filtered, and the residue was washed with hot water; the filtrate was acidified with 2N-sulphuric acid. Crystalline benzimidazole-4(7)-carboxylic acid sulphate salt (XIII) was precipitated from solution. Benzimidazole-4(7)-carboxylic acid sulphate (4 g) was finely powdered with phosphorus pentachloride (1 g) in a boiling tube. Chloroform (2 ml) was added and the mixture was warmed on a steam-bath. After 0·5 h of warming (replenishing the chloroform occasionally) the mixture was cooled and rubbed with a glass rod. The mixture solidified and was mixed with n-heptane; it was then filtered off and washed with n-heptane. The solid acid chloride was added to methanol (5 ml) and the methyl ester hydrochloride (V) was precipitated with acetone; it was filtered off and dried. Methyl benzimidazole-5(6)-carboxylate was prepared from 3,4-diaminobenzoic acid. 3,4-Diaminobenzoic acid (7·5 g from Aldrich) was warmed on a hot-plate at 100° with formic acid (15 ml). After 1 h the solution was diluted by half with water and neutralised with sodium hydroxide (1N). The sulphate salt of the acid (XIV) was precipitated by addition of 1N-sulphuric acid until the solution was acid to litmus. Methyl benzimidazole-5(6)-carboxylate (VI) was prepared from the acid *via* the acid chloride as for the 4(7)-isomer.

The structures of all the compounds used here were confirmed using i.r. (Unicam SP 200) and n.m.r. (Perkin-Elmer 60 MHz R.10 machine) spectroscopy.

Kinetics.—Hydrolyses were followed by observing the change in u.v. absorption (Tables 2 to 5) of the species using a Unicam SP 800 spectrophotometer fitted with a Smith's Servoscribe recorder. Reactions of methyl benzimidazole-4(7)-carboxylate and the 5(6)-isomer over a pH-range were followed also by the following method. Aliquot portions (1 ml) of the hydrolysing mixture were withdrawn at intervals and incubated at 100° with freshly prepared hydroxylamine buffer [1 ml, prepared from 4M-hydroxyl-

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

[10] F. P. Doyle, M. D. Mehta, G. S. Sach, and J. L. Pearson, *J. Chem. Soc.*, 1958, 4458.

amine hydrochloride, 3·5M-sodium hydroxide, 2M-tris-(hydroxymethyl)aminomethane, 10 : 11 : 4 v/v, and ethylenediaminetetra-acetic acid to 10^{-4}M] for 10 min. The intensity of the colour at 540 nm produced by the addition of 4 ml ferric chloride solution (20% in 0·3M-hydrochloric acid) was determined in a Unicam SP 600UV spectrophotometer using 1 cm cells. The solution was topped up to 6 ml with distilled water to combat evaporation. It was found that a suitably calibrated E.E.L. spectrometer gave identical kinetic results.

Hydroxide ion was kept in large excess over substrate in order to obtain pseudo-first-order kinetics. The data were analysed *via* the Guggenheim [11] or infinity methods. Stock solutions of substrates were in acetonitrile whose final concentration was 0·4% (v/v). pK_a values were measured using a Radiometer Titratigraph, type SBR2c, with Titrator 11 attachment and pH-meter-25.

TABLE 1

Analytical and physical properties of substrates [a]

Compound	M.p. (lit. m.p.) (*t*/°C, ‡ b.p.)	Found (%) C	H	N	Formula	Calc (%) C	H	N
(Ia)	100—101 (98—99) [b]							
(IIa)	126—128 (128) [c]							
(Ib)	193—195 (193) [d]							
(IIb)	39—40	64·1	6·0	6·6	$C_{11}H_{13}NO_3$	63·8	6·3	6·8
(Ic)	141—142/15 mm (130—131/11·5 mm) ‡, [e]							
(IIc)	101—103 (102) [f]							
(Id) (4-H)	93—95 (99—100) [g]							
(Id) (4-Me)	110—110·5 (100) [h]							
(Id) (4-MeO)	102—104 (113) [h]	67·1	5·0	4·9	$C_{16}H_{15}NO_4$	67·4	5·3	4·9
(Id) (4-Cl)	133—134 (138) [i]	62·0	4·4	4·7	$C_{15}H_{12}ClNO_3$	62·2	4·2	4·8
(Id) (4-Br)	149—150	53·8	3·5	4·0	$C_{15}H_{12}BrNO_3$	53·9	3·6	4·2
(Id) (3,4-di-NO_2)	144—146	52·4	3·2	12·0	$C_{15}H_{11}N_3O_7$	52·2	3·2	12·2
(Id) (3,5-di-NO_2)	157—157·5	52·0	3·3	12·1	$C_{15}H_{11}N_3O_7$	52·2	3·2	12·2
(III)	87·5—88·5	64·0	6·5	7·0	$C_{11}H_{13}NO_3$	63·8	6·3	6·8
(IV)	109—112	63·6	6·1	6·6	$C_{11}H_{13}NO_3$	63·8	6·3	6·8
(VIII)	46—47/0·6 mm (62/1 mm) ‡, [j]							
(IX)	86—87 (86—87) [k]							
(V) [q]	250 (dec)	49·4	4·1	13·6	$C_9H_9N_2O_2Cl$ [l]	48·0	4·5	14·0
(XI)	60—60·5 (63—64) [m]							
(XII)	143—145 (145) [n]							
(XIII) [q]	250 (dec)	44·4	3·9	12·9	$C_{16}H_{16}N_4O_4S$ [o]	43·7	3·6	12·7
(XIV) [q]	250 (dec)	44·3	3·6	12·7	$C_{16}H_{16}N_4O_4S$ [p]	43·7	3·6	12·7
(VI) [q]	250 (dec)	48·3	4·3	12·5	$C_9H_9N_2O_2Cl$	48·0	4·5	14·0

[a] Analyses were by Mrs. M. J. Clark and Miss F. Duckworth of this laboratory using a Hewlett–Packard 185 analyser. M.p.s were determined using a Kofler Thermospan instrument. [b] D. T. Zentmyer and E. C. Wagner, *J. Org. Chem.*, 1949, **14**, 967. [c] G. W. K. Cavill and J. M. Vincent, *Chem. and Ind.*, 1948, **67**, 25. [d] P. Grammaticakis, *Bull. Soc. chim. France*, 1950, 158. [e] R. Willstätter and R. Kahn, *Chem. Ber.*, 1904, **37**, 408. [f] E. Bischoff, *Chem. Ber.*, 1889, **22**, 343. [g] E. Erdmann and H. Erdmann, *J. prakt. Chem.*, 1901, **63**, 261. [h] H. Stephen and G. Wadger, *J. Chem. Soc.*, 1956, 4420. [i] L. Legrand, *Bull. Soc. chim. France*, 1960, 337. [j] F. P. Doyle, M. D. Mehta, G. S. Sach, and J. L. Pearson, *J. Chem. Soc.*, 1958, 4458. [k] H. Rapoport and C. D. Willson, *J. Org. Chem.*, 1961, **26**, 1102. [l] Chlorine analysis: found 16·7, calc. 17·8%. [m] S. Gabriel and A. Thieme, *Chem. Ber.*, 1919, **52**, 1081. [n] H. Hübbner and R. Schüpphaus, *Chem. Ber.*, 1884, **17**, 777. [o] Sulphur analysis: found 7·4, calc. 7·3%. [p] Sulphur analysis: found 7·2, calc. 7·3%. [q] Thin layer chromatography and n.m.r. spectroscopic criteria showed these substances to be pure despite the poor analyses.

TABLE 2

Alkaline hydrolysis of methyl 2-acetamidobenzoate (Ia) and the *para*-isomer (IIa) in dioxan–water mixtures [a]

Dioxan (v %) [d]	$k_{obs} \times 10^3$/s^{-1} [b,c] *ortho*	*para*	*ortho*/*para* Ratio
0	36·4	10·6	3·45
10	35·7	7·63	4·68
20	33·6	5·18	6·49
30	31·5	3·96	7·95
40	27·6	2·53	10·91
50	25·7	1·61	15·96
60	23·0	1·15	20·0
65	18·9	0·73	24·9
45	k^H/k^D 0·799	$k/{}^Hk^D$ 0·899	

[a] 25°, 0·2M-Ionic strength. [b] Values are average of duplicate measurements in 0·2M-NaOH. [c] Reaction followed at 280 nm for (Ia), 320 nm for (IIa). [d] % Dioxan in water by volume.

TABLE 3

Alkaline hydrolysis of benzoate esters [a]

Ester	λ/nm [c]	Dioxan (v %)	$k_{obs} \times 10^3$/s^{-1} [b]	*o*/*p* Ratio
(Ib)	225	0	14·4	
		50	13·8	
(IIb)	235	0	29·1	0·49
		50	16·5	0·83
(Ic)	350	0	0·0777	
		50	0·0265	
(IIc)	325	0	0·433	0·18
		50	0·0432	0·61
(IV)	290	0	5·75	
		50	3·68	
(III)	325	0	21·5	
		50	16·4	

[a] 25°, 0·2M-Ionic strength. [b] Average of duplicate measurements in 0·2M-NaOH. [c] Wavelength used in kinetic study.

RESULTS

All the esters hydrolysed in alkali to yield a stoicheiometric amount of the acid as determined by the observation

[11] E. A. Guggenheim, *Phil. Mag.*, 1926, **2**, 538.

of well-defined isosbestic wavelengths * by repetitive scanning of the u.v. spectrum during hydrolysis; the product spectra were identical with those of equivalent concentrations of the respective acids under the same conditions. Rate constants were pseudo-first-order up to 80%

TABLE 4

Alkaline hydrolysis of heterocyclic methyl esters [a]

Dioxan (v %)	$k_{obs} \times 10^3/s^{-1}$ (V) [b]	(VI) [c]	Ratio (V)/(VI)
0	2·25	0·392	5·7
10	1·41	0·192	7·3
30	0·57	0·061	8·3
40	0·38	0·030	12·7
50	0·26	0·021	12·4
60	0·16	0·019	8·4

	(VII) [d]	(IX) [e]	(VIII) [f]	VII/IX
0	5·05	1·13	4·58	4·48
10	3·60	0·72	3·30	5·0
20	2·86	0·53	2·38	5·4
30	2·30	0·35	1·63	6·57
40	1·54	0·24	1·16	6·42
50	1·15	0·18	0·9	6·39
60	0·92	0·15	0·64	6·13

[a] Rate constants from duplicate runs, 0·2M-NaOH, 25°. [b] 315 nm. [c] 310 nm. [d] 295 nm. [e] 265 nm. [f] 275 nm.

TABLE 5

Alkaline hydrolysis of methyl esters of substituted 2-arylamidobenzoic acids (Id) [a]

Substituent	λ/nm [c]	$k_{obs} \times 10^3/s^{-1}$ [b]
4-MeO	330	0·770
4-Me	300	0·841
Unsubstd.	300	0·830
4-Cl	300	1·045
4-Br	300	1·024
3,4-$(NO_2)_2$	325	1·46
3,5-$(NO_2)_2$	310	1·29

[a] 25°, 0·01M-Ionic concentration, 20% (v/v) in dioxan–water. [b] Duplicate measurements in 0·01M-NaOH. [c] Wavelength employed to measure kinetics.

of the hydrolysis and were determined under a variety of conditions of solvent (Tables 2—5 and Figure 1), pH (Table 6 and Figure 2) and temperature (Table 7). Second-order rate constants were obtained by division by the alkali concentration.

The alkaline hydrolysis of methyl 2-arylamidobenzoates (Id) was only slightly sensitive to Hammett σ ($\rho = 0·132$, $r = 0·953$). At 100° the pH-dependence of the hydrolysis of methyl benzimidazole-4(7)-carboxylate (V) exhibits acid and alkaline limbs and a plateau region from pH 2·5 to 5·5 while at 25° a sigmoid dependence is observed in the pH range 11—14 corresponding to the ionisation of the imidazole secondary nitrogen (Figure 2). The ionisation constant obtained was not of the highest accuracy since it was impossible in our system to study the reaction at pH values higher than 13·7.

* This method is not absolutely certain (see Ch. Chylewski, *Angew. Chem. Internat. Edn.*, 1971, **10**, 195; H. L. Schäfer and O. Kling, *Angew. Chem.*, 1956, **68**, 667; G. Körtum, 'Kolorimetrie, Photometrie, und Spectrometrie,' Springer, Berlin, 1962, p. 32).

At 25° 2- and 4-acetamidobenzoic acids in 20% dioxan–water (v/v) had pK_a values 4·55 ± 0·01 and 5·18 ± 0·01 respectively.

Table 8 includes n.m.r. data for $CDCl_3$ solutions of methyl 2(and 4)-acylamidobenzoates; the absorption frequency for (Ia) is independent of concentration indicating existence of intramolecular hydrogen-bonding. Table 9 includes i.r. data for different media aimed at demonstrating the existence of intramolecular hydrogen-bonding in (Ia). Table 10 collects the deuterium oxide solvent isotope effects on the alkaline hydrolysis of (Ia) and (IIa).

TABLE 6

Hydrolysis of methyl benzimidazole-4(7)-carboxylate over a pH-range [a]

Buffer (concn./M)	pH	$k_{obs} \times 10^6/s^{-1}$ [b]
	(100°)	
HCl (0·12)	0·95	8·2
Glycine (0·06)	1·85	1·4
Glycine (0·096)	2·89	0·48
Glycine (0·14)	3·67	0·609
Acetate (0·12)	4·00	0·703
Phosphate (0·15)	5·67	0·703
Phosphate (0·15)	6·76	16·7
	(70°)	
Borate (0·15)	8·6	34·0
Borate (0·15)	8·6	0 [5(6)-ester]
Borate (0·15)	9·22	123
Borate (0·15)	9·22	115
Borate (0·15)	9·22	43·5 [5(6)-ester]
Borate (0·15)	9·60	98·0 [5(6)-ester]
Borate (0·15)	10·00	224 [5(6)-ester]
	(25°)	
Hydroxide	11·29	135
Hydroxide	11·99	500
Hydroxide	12·29	1020
Hydroxide	13·3	2250
Hydroxide	13·7	2870

[a] 0·2M-Ionic strength; kinetics except where stated as in Table 4. [b] Kinetics except in hydroxide buffers were followed using the hydroxamic assay method.

TABLE 7

Temperature dependence of alkaline hydrolysis of methyl esters [a]

Ester	$\Delta H^{\ddagger}$ [c] (kcal mol^{-1})	$\Delta S^{\ddagger}$ [c] (e.u.)	T/K	$k_{obs} \times 10^2/s^{-1}$ [b]
(IIa)	18·8 ± 1·0	−2·0 ± 3·4	298	0·60
			303	1·29
			308	1·86
			313	2·56
(Ia)	11·7 ± 0·2	−22·8 ± 0·7	298	3·15
			303	4·46
			308	5·81
			313	7·4
(V)	12·4 ± 0·4	−25·2 ± 1·3	298	0·263
			303	0·380
			308	0·564
			313	0·785
(VI)	14·9 ± 0·2	−19·8 ± 0·7	298	0·0667
			303	0·101
			308	0·147
			313	0·202

[a] 0·2M-Ionic strength, 100% water. [b] Duplicate measurements in 0·2M-NaOH. [c] Values for 30° for bimolecular rate constant.

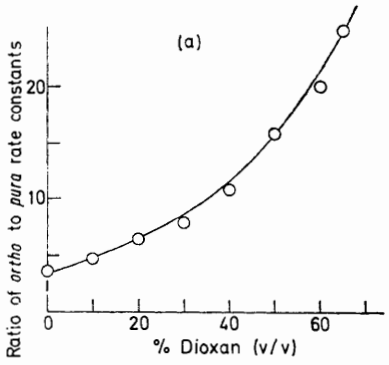


FIGURE 1 (a) Variation with dioxan concentration of the *ortho*/*para* ratio for alkaline hydrolysis of methyl 2(and 4)-acetamidobenzoates; data from Table 2

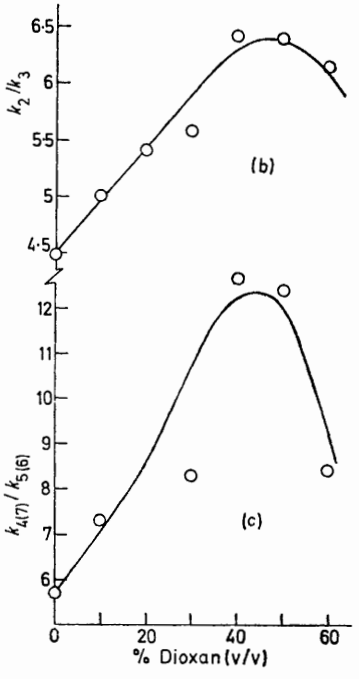


FIGURE 1 (*b*) Variation with dioxan concentration of the ratio k_{VII}/k_{IX} for alkaline hydrolysis; data from Table 4; (*c*) variation with dioxan concentration of the ratio k_V/k_{VI} for alkaline hydrolysis; data from Table 4

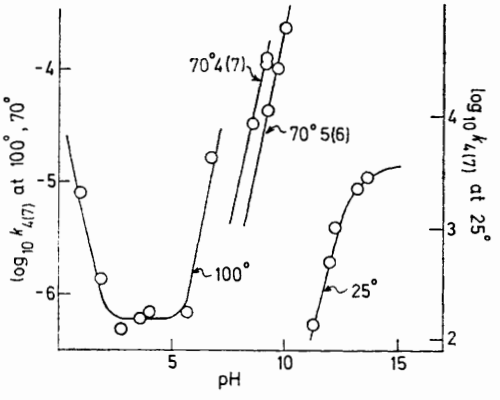


FIGURE 2 pH-Dependence of hydrolysis of methyl benzimidazole-4(7)-carboxylate (V); data from Table 6

TABLE 8

Nuclear magnetic resonance data for solutions of methyl 2(and 4)-acylamidobenzoates

Compd.	Conc. (M) (in $CDCl_3$)	τ [a]
(Ia)	50% (w/v)	−1·05
	0·948	−1·02
	0·0948	−1·02
(IIa)	50% (w/v)	+1·16
	5% (w/v)	+1·66
(Id) (H)	0·24	−2·83
(Id) (4-Me)	0·24	−2·28
(Id) (4-MeO)	0·24	−2·0

[a] Relative to the tetramethylsilane absorption at τ = 10

TABLE 9

I.r. absorption frequencies of 2(and 4)-acylaminobenzoates under a variety of conditions

Compd.	Solvent	Conc. (M) $\times 10^2$	λ/cm^{-1} [a]	λ/cm^{-1} [b]	*para*−*ortho* (cm^{-1}) (NH)	(CO)
(Ia) (H–N)	CCl_4	3·02	3320	1695		
		0·48	3320	1695		
		0·024	3320	1695		
	$CHCl_3$	0·024	3320	1695		
	Nujol		3320	1690		
(IIa) (H–N)	CCl_4	0·025	3418	1727	98	32
	$CHCl_3$	0·025	3418			
	Nujol		3418	1690		
(Ia) (D–N)	$CDCl_3$	0·025	2470			
	Nujol		2410			
(IIa) (D–N)	$CDCl_3$	0·025	2548		78	
	Nujol		2482		72	

[a] NH (or ND) frequency. [b] CO (ester) frequency.

TABLE 10

Effect of deuterium oxide solvent on alkaline hydrolysis of methyl 2(and 4)-acetamidobenzoates [a]

Compd.	(Ia) (H)	(IIa) (H)	(Ia) (D)	(IIa) (D)
$k_{obs} \times 10^3$/s^{-1} [b]	28·4	2·71	35·6	3·03

[a] 25°, 0·2M-NaOH (or NaOD), 45% (v/v) dioxan–water (or D_2O). [b] Average of duplicate measurements.

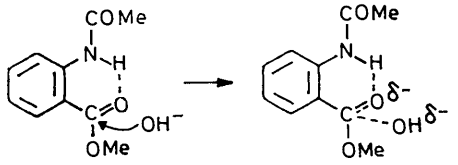
DISCUSSION

Amide Neighbouring Group.—The rate constants for alkaline hydrolysis of (Ia) and (IIa) decrease with decreasing water content of the medium (Table 2) in accord with a polar transition-state.[12] Quantitative evaluation of the variation of the rate constant with, for example, dielectric constant is not meaningful. Although the *ortho*/*para* ratio is only 3·45 in water it rises to about 25 in 65% dioxan and thus satisfies the criterion of Capon [13] for the existence of intramolecular catalysis in the case of (Ia).

Enhancement is probably not due to steric release from a crowded ground-state because alkaline hydrolysis of (Ib) and (Ic) is slower than (IIb) and (IIc) respectively; these results indicate that the enhancements for (Ia) and (IIa) are *theoretically* larger than observed. We propose that enhancement is caused by stabilisation of the

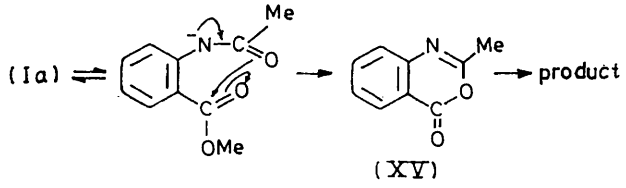
[12] M. L. Bender and W. A. Glasson, *J. Amer. Chem. Soc.*, 1959, **81**, 1590.
[13] B. Capon, *Quart. Rev.*, 1964, **18**, 45.

transition-state by hydrogen-bonding with the *ortho*-amide and by polarisation of the ground-state by the amide interacting with the ester carbonyl oxygen. This mechanism is possible since a ' Dreiding ' model of the tetrahedral *intermediate* indicates a distance of *ca.* 0·12 nm between the oxyanion and the amido-proton



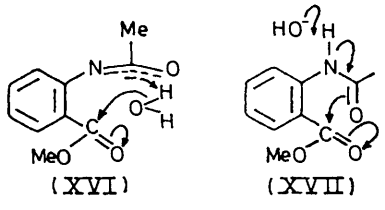
well within the expected range for a hydrogen-bond.[14] Hydrogen-bonding is probably the explanation of the greater acidity of the *ortho*-acetamidobenzoic acid as opposed to the *para*-isomer. Peltier [15a] suggested the existence of intramolecular hydrogen-bonding between amide NH and carbonyl oxygen of the ester in (Ia) for non-aqueous solutions and this is confirmed by n.m.r. evidence (ref. 15*b* and Table 8). I.r. absorption data (Table 9) for (Ia) and (IIa) and their *N*-deuterio-forms point to intramolecular hydrogen-bonding in (Ia) between ester carbonyl oxygen and amide NH. Work on hydrogen-bonding in different solvents is not however very relevant to the ground-state in water [5d,13] possibly owing to the greater solvating power of the latter solvent.

A mechanism involving a 3,1-benzoxazin-4-one (XV)

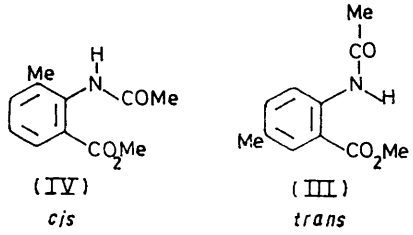


(XV)

is possible and the alkaline hydrolysis of the intermediate proceeds at a rate [16a] fast enough to accommodate the hydrolysis of (Ia). However the prior ionisation of (Ia) would indicate a greater effect of solvent than for the *para*-isomer. General-base catalysis as in (XVI) is also excluded by the greater solvent effect expected and also on account of the *inverse* solvent deuterium isotope



effect on alkaline hydrolysis. The greater reactivity of the deuteroxide ion in deuterium oxide is probably due to its greater basicity and hence nucleophilicity than hydroxide ion.[16b] Attack of hydroxide ion concerted with formation of the benzoxazine (XVII) does not predict an inverse deuterium isotope effect. The benzoxazin-4-one mechanisms would also predict a faster rate constant for the alkaline hydrolysis of methyl 2-acetamido-3-methylbenzoate (IV) than the 5-methyl

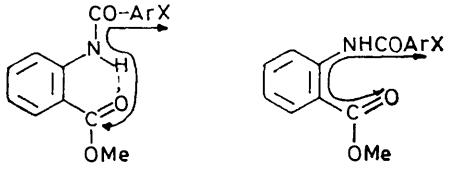


(IV) *cis* (III) *trans*

isomer (III) because the amido carbonyl would spend less time *trans* to the ester owing to steric hindrance of the 3-methyl group.

It is probable that for better leaving groups than methanol the 3,1-benzoxazin-4-one type mechanism operates.[17]

The low positive Hammett sensitivity for the alkaline hydrolysis of (Id) (Table 5) is not easily interpreted as it would be predicted by the postulated interaction or by transmission of polar effects *via* the anthranilic nucleus. Alkaline hydrolysis of the corresponding *para*-esters



(IId) exhibits a negative Hammett selectivity ($\rho = -0{\cdot}28$, unpublished work of A. Williams). The explanation of this selectivity is not clear and is being investigated but the difference between *ortho*- and *para*-selectivities (0·41) is in good agreement with the postulate of electrophilic assistance in the *ortho*-case (Id) not possible in the *para* (IId).

Arrhenius parameters (Table 7) indicate that enhancement of the *ortho*-isomer results from a favourable enthalpy of activation but the entropy of activation favours the *para*-form. Whilst Arrhenius parameters can be equivocal mechanistic tools for solution reactions the lower enthalpy of activation for the *ortho*-form possibly reflects polarisation of the ester carbonyl bond by the electrophilic amide.

It is unlikely that enhancement in the alkaline hydrolysis of methyl 2-acetamidobenzoate (Ia) com-

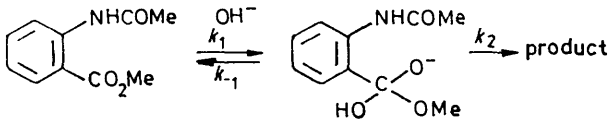
[14] *Cf.* G. C. Pimentel and A. L. McClellan, ' The Hydrogen-bond,' W. H. Freeman and Co., San Francisco, 1960, p. 260.

[15] (*a*) D. Peltier and A. Pichevin, *Bull. Soc. chim. France*, 1960, 1141; D. Peltier, A. Pichevin, and A. Bonnin, *ibid.*, 1961, 1619; (*b*) I. D. Rae, *Canad. J. Chem.*, 1968, **46**, 2589.

[16] (*a*) A. Williams and G. Salvadori, *J. Chem. Soc.* (*B*), 1971, 1105; (*b*) F. A. Long, *Proc. Nat. Acad. Sci. U.S.A.*, 1960, **84**, 596.

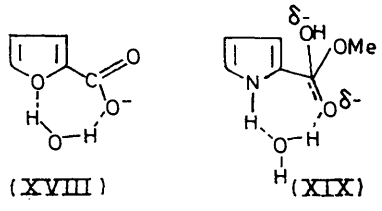
[17] (*a*) M. E. Aberlin and C. A. Bunton, *J. Org. Chem.*, 1970, **35**, 1825; (*b*) J. de Jersey, P. Willadsen, and B. Zerner, *Biochemistry*, 1969, **8**, 1959; J. de Jersey, A. A. Kortt, and B. Zerner, *Biochem. Biophys. Res. Comm.*, 1966, **25**, 383.

pared with the *para*-isomer is due to electrophilic assistance in the breakdown of the tetrahedral intermediate



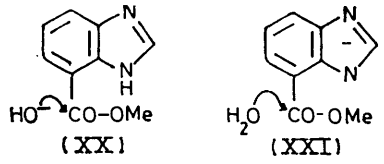
(k_2 step) because the analogous step (k_{-1}) should also be assisted by electrophiles. Bender [18] has shown that the tetrahedral intermediate in alkaline hydrolysis of methyl benzoate partitions to products and reactants in the ratio 2·6 : 1. Thus the overall rate constant [$k_{obs} = k_1 \cdot k_2/(k_{-1} + k_2)$] is essentially that for the addition step.

Assistance by Heterocyclic NH.—It has been suggested that the well known lability of methyl pyrrole-2-carboxylate to alkali compared with the 3-isomer is due to hydrogen-bonding from the pyrrole NH to the incipient alkoxide ion of the transition-state.[19] A Dreiding model of the tetrahedral intermediate shows the N–$O^{\delta-}$ distance to be *ca.* 0·32 nm in excess of that expected for a stable hydrogen-bond.[14] Price[20] suggested the acid strengthening effect in 2-carboxylic acid derivatives of furan and thiophen to be due to participation of a water molecule



in the structure of the conjugate base (XVIII). By analogy it is possible that the near tetrahedral transition-state is stabilised by a bridging water molecule (XIX). Dreiding models of the tetrahedral intermediate confirm this possibility. The greater sensitivity to solvent composition of the alkaline hydrolysis of methyl benzimidazole-4(7)-carboxylate and methyl pyrrole-2-carboxylate compared with their 5(6)- and 3-isomers is in accord with the ' bridging ' theory as is the observation of a maximum in the enhancement ratio as the dioxan concentration is increased (Figures 1b and c). The dissociation constant for formation of the complex must be relatively high since 50% dioxan, the composition of maximal effect, corresponds to a water concentration much larger than the ester. Similar cyclic transition-states involving water bridges have been suggested for the acid hydrolysis of esters, amides, and anilides [21] and represent one interpretation of the phenomenon of solvent sorting.[5f]

The alkaline region for the hydrolysis of methyl benzimidazole-4(7)-carboxylate [(V) Figure 2] is due to either attack of hydroxide ion on neutral species (which ionises) or of water on the ionised species and the former mechanism is preferred. Ambiguity of mechanism also



holds for the ' neutral ' plateau region which could be due to attack of water on neutral ester or hydroxide ion on the protonated ester. Acid-catalysed hydrolysis of (V) is less efficient than that of uncharged methyl benzoate because of the positive charge held at acid pH.

[1/1915 *Received*, 19*th October*, 1971]

[18] M. L. Bender, H. Matsui, R. J. Thomas, and S. W. Tobey, *J. Amer. Chem. Soc.*, 1961, **83**, 4193.
[19] M. K. A. Khan and K. J. Morgan, *Tetrahedron*, 1965, **21**, 2197.
[20] C. C. Price and E. A. Dudley, *J. Amer. Chem. Soc.*, 1956, **78**, 2197.
[21] (*a*) K. J. Laidler and P. A. Landskroener, *Trans. Faraday Soc.*, 1956, **52**, 200; (*b*) E. Tommila, A. Koivisto, J. P. Lyyra, K. Antell, and S. Heino, *Ann. Acad. Sci. Fennicae, Ser. All.*, 1952, **47**, 3; (*c*) Ya. K. Syrkin and I. I. Moiseev, *Uspekhi Khim.*, 1958, **77**, 717; (*d*) C. A. Lane, *J. Amer. Chem. Soc.*, 1964, **86**, 2521; C. A. Lane, M. F. Chenng, and G. F. Dorsey, *ibid.*, 1968, **90**, 6492.