Hydrolyses of *O*-Acylglycolamides as Models of the Deacylation Step in the Mechanism of Action of Serine Proteases: Function of the Oxyanion Pocket

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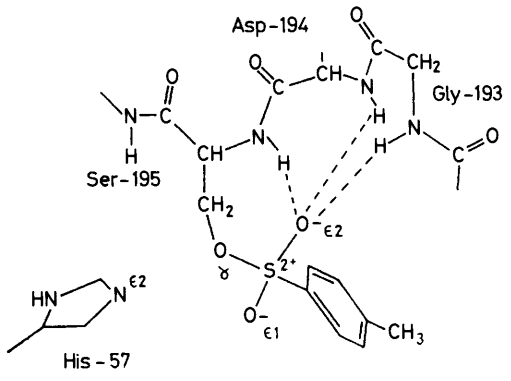
Hydrolyses of 54 *O*-acylglycolamides in aqueous base have been studied as models for the hydrolysis of acylated serine proteases. The essential feature of these models is the presence of amide residues capable of intramolecular hydrogen bonding with the carbonyl oxygen of the ester in a way similar to that proposed for the mechanism of action of serine proteases. No significant enhancement due to electrophilic participation is observed for the reactivity of the models as compared with that expected for normal substituent effects; this is explained by the strained nature of the interacting conformer as compared with that where amide and ester moieties are remote from each other. The action of the oxyanion pocket in the active site of proteases does not povide a major contribution to enzymic activity apart from an entropic factor in solvation; the electrophilic interaction is necessary to allow the formation of the oxyanion and hence the tetrahedral intermediate in the close-packed environment of the active site which would otherwise be non-polar.

THE mechanism of hydrolyses catalysed by serine proteases involves formation of an acyl–enzyme intermediate where the serine hydroxy-group is acylated, followed by decomposition of this ester. Both steps are assisted by the imidazolyl side chain of a histidine residue, probably

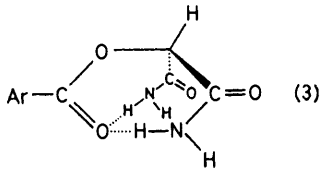


(1)

Tracing of a photograph of a model of the active site region of tosylchymotrypsin built by using Kendrew models (Cambridge Repetition Engineers) and co-ordinates from J. J. Birktoft, B. W. Matthews, and D. M. Blow, *Biochem. Biophys. Res. Comm.*, 1969, **36**, 131; nomenclature as in J. T. Edsall, P. J. Flory, J. C. Kendrew, A. M. Liquori, G. Nemethy, G. N. Ramachandran, and H. A. Scheraga, *J. Mol. Biol.*, 1966, **15**, 399; a similar diagram is illustrated in P. B. Sigler, D. M. Blow, B. W. Matthews, and R. Henderson, *J. Mol. Biol.*, 1968, **35**, 143.

acting as a general base.[1] A feature implicated in these processes is that of electrophilic assistance at the carbonyl oxygen of the substrate.[2] It has been suggested that the peptide NH groups of the reactive serine and the preceding amino-acid residues provide a polar environment to activate the carbonyl oxygen of the ester in the ground state and stabilise the incipient oxyanion of the transition state, which resembles a tetrahedral intermediate. *X*-Ray evidence provides support in the chymotrypsin,[3] subtilisin,[4] and trypsin[5] cases where transition-state analogues have been studied; it is suggested that the carbonyl oxygen of the acyl protease fits into a ' pocket ' surrounded by the peptide backbone NH groups of serine and the preceding residues, and that this ' oxyanion ' pocket[4] is distinct from a similar space lined with hydrophobic groups which accepts a non-polar side chain of the substrate. The stereochemistry around the tetrahedral intermediate may be close to that for the transition-state analogue for the hydrolysis of 4-toluoylchymotrypsin, namely tosylchymotrypsin (1).

$$ArCO_2{\cdot}CHR^1{\cdot}CO{\cdot}NR^2R^3 \quad (2)$$



(3)

This work involves the study of simple models to elucidate the proposed electrophilic interaction between the oxyanion pocket and the incipient oxyanion of the transition state. Data have been obtained for the alkaline hydrolysis of a series of carboxylic esters (2) where there is a possibility of amide interaction with

[1] (*a*) M. L. Bender and F. J. Kezdy, *Ann. Rev. Biochem.*, 1965, **34**, 49; (*b*) S. G. Waley, *Quart. Rev.*, 1967, **21**, 379; (*c*) A. Williams, *ibid.*, 1969, **23**, 1.

[2] (*a*) A. Williams, *Biochemistry*, 1970, **9**, 3383; (*b*) A. Williams and G. Woolford, *J.C.S. Perkin II*, 1972, 272; (*c*) A. Williams and G. Salvadori, *J. Chem. Soc.* (*B*), 1971, 2401.

[3] (*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 Symposia on Quantitative Biology*, 1971, **36**, 63.

[4] (*a*) J. D. Robertus, J. Kraut, R. A. Alden, and J. J. Birktoft, *Biochemistry*, 1972, **11**, 4293; (*b*) D. A. Matthews, R. A. Alden, J. J. Birktoft, S. T. Freer, and J. Kraut, *J. Biol. Chem.*, 1975, **250**, 7120.

[5] (*a*) R. Huber, D. Kukla, W. Steigemann, J. Deisenhofer, and A. Jones, Bayer Symposium (V) ' Proteinase Inhibitors,' 1974, p. 497; (*b*) D. M. Blow, *ibid.*, p. 473; (*c*) R. Huber, 6th Harden Conference, Wye, 1974; (*d*) D. M. Blow, J. Janin, and R. M. Sweet, *Nature*, 1974, **249**, 54; (*e*) R. M. Sweet, H. T. Wright, J. Janin, C. H. Chothia, and D. M. Blow, *Biochemistry*, 1974, **13**, 4212.

TABLE 1

Second-order rate constants for hydrolysis of the model esters in alkaline solution [a]

	λ_i/nm [b]	λ_k/nm [c]	M.p. (°C)	N [d]	k_{OH}/l mol^{-1} s^{-1}	k_{OH}/k_{OD} [e]
O-Benzoyl-2-hydroxymalonamides						
4′-Nitro	278	300	256—258	4	115	
3′-Chloro	227	240	216—218.5	3	14.8	1.00
4′-Chloro	235	255	239—241	3	6.03	1.22
3′-Methoxy	288, 263, 230	246	204—205.5	5	5.03	
Parent	224	245	223—225	6	2.63	
4′-Methoxy	252, 223	275	244—245	2	2.33	2.31
4′-Methyl	237	255	222—224	3	1.51	
4′-Hydroxy	285, 234, 227	310	279—281	5	0.40	
O-Benzoyl-lactamides						
Parent	222	240	117—118	6	0.13	
3′-Methoxy	288, 260	303	134—136.5	3	0.15	
3′-Hydroxy	319, 270	350	171—172	3	0.013	
4′-Methoxy	249	268	137—139	4	0.031	
4′-Hydroxy	284, 239	313	220—221	2	0.001 7	
O-Benzoylglycolamides						
4′-Nitro	277	295	169—172	3	9.12	
3′-Chloro	223	245	119—122	3	1.82	0.74
4′-Chloro	229	258	140—144	4	0.93	
3′-Methoxy	288, 262	305	123—124	4	0.72	
Parent	223	245	121—122	4	0.5	
4′-Methyl	233	258	154.5—156.5	4	0.22	
4′-Methoxy	249	275	143.5—146	4	0.13	0.75
3′-Hydroxy	320, 274	340	151—153	3	0.072	
4′-Hydroxy	287, 238	310	253—259	3	0.004 4	
4′-Dimethylamino	299, 245	325	187—189	3	0.031	
O-Benzoyl-2-phenylglycolamides [f]						
4′-Nitro	279	305	182—184	3	4.96	
3′-Chloro	225	245	163—165	5	1.88	
3′-Methoxy	289, 264	250	110—113	6	0.46	
Parent	220	245	166—167	6	0.41	
4′-Dimethylamino	300, 245	340	227.5—230.5	3	0.021	
O-Benzoyl-*N*-ethylglycolamides						
3′-Chloro	222	240	80—84	4	1.40	0.83
4′-Chloro	231	255	133—135	6	0.75	0.79 [g]
3′-Methoxy	287, 262	305	83—85	6	0.41	
Parent	222	243	73—74	5	0.35	
4′-Methyl	233	255	120—125	4	0.17	
4′-Dimethylamino	299, 244	325	123—127	5	0.019	
O-Benzoyl-*NN*-diethylglycolamides						
4′-Nitro	278	295	133—135	3	2.40	
3′-Chloro	221	240	46—47	3	0.35	
4′-Chloro	232	257	111—114	3	0.22	
3′-Methoxy	289, 260	305	40	3	0.2	
Parent	223	246	110—112	3	0.12	
4′-Methyl	227	258	82.5—84.5	3	0.041	
4′-Methoxy	248, 222	278	74—77	3	0.039	
3′-Hydroxy	319, 273	340	164—164.5	3	0.020	
4′-Hydroxy	285, 237	310	123—126	2	0.000 60	
4′-Dimethylamino	297, 244	330	148—150	3	0.004 8	
O-Benzoyl-*N*-phenylglycolamides						
4′-Nitro	Complex kinetics [h]		170—171			
3′-Chloro	220	245	129—130	4	2.14	
4′-Chloro	285	250	148.5—150.5	3	1.0	
3′-Methoxy	289, 272	305	127—129	4	0.72	
Parent	225	245	151—152	6	0.59	
4′-Methyl	235	255	146—148	4	0.25	
4′-Methoxy	250, 221	314	128—131	5	0.18	
3′-Hydroxy	318, 279	340	154—156.5	4	0.098	
4′-Hydroxy	285, 240	314	179—181	4	0.004 1	
4′-Dimethylamino	297, 242	325	160—162	2	0.028	

[a] 25 °C; ionic strength made up to 0.1M with NaCl. [b] Isosbestic wavelength. [c] Wavelength for kinetic studies. [d] The number of duplicate runs between pH 10 and 13. [e] Determined with 0.005M- and 0.08M-NaOH(D). [f] Rate constants determined from pseudo-first-order rate constants (0.02M-NaOH). [g] Determined with 0.02M-NaOH(D), ionic strength 0.02M. [h] This reaction will be covered in depth in a subsequent report.

the ester grouping; this is illustrated by structure (3) for one of the models.

EXPERIMENTAL

Materials.—2-Halogenoacyl halides were prepared by the method of Anwers and Bernhardi; [6] the crude products were distilled once at atmospheric pressure and the pure material was obtained normally in 60—70% yield. 2-Bromo-2-phenylacetyl bromide was prepared as follows and used directly to synthesise the corresponding amide. Phenylacetic acid (44 g, 0.33 mol) was stirred with benzene (150 ml) and red phosphorus (3.4 g). Bromine (110 g, 1.4 mol) was added dropwise with stirring and cooling. The mixture was then warmed until evolution of HBr fumes ceased, and the solution was decanted from the phosphoric acid formed. The benzene was evaporated off *in vacuo*.

2-Halogenoamides were prepared by adding the acid chloride dropwise to a cooled, stirred solution of ammonia or the appropriate amine. The precipitate was filtered off, washed, and recrystallised from ethanol–water. Chloroacetamide and 2-chloro-*N*-ethylacetamide were purchased from Koch-Light Ltd. and Schuchardt, respectively.*

O-Acylglycolamides † were prepared by mixing the appropriate acid (50 mmol) with triethylamine (50 mmol) in ethyl acetate. The halogenoacetamide (50 mmol) in ethyl acetate, ethanol, or dimethyl sulphoxide was added dropwise to the solution, which was warmed and stirred and then gently refluxed for 12 h. Two work-up procedures were employed: (*a*) the mixture was cooled, an excess of water added, and the precipitate washed and recrystallised three times; (*b*) the mixture was cooled and on addition of water an oil was formed. The latter was induced to crystallise by diluting with a large excess of water, cooling, and scratching, or by extracting repeatedly with portions of ether, drying the ether layer, and evaporating to give an oil which yielded crystals on scratching. The most satisfactory recrystallisation solvents for the various series of esters were: malonamides, dimethylformamide; lactamides and glycolamides, methanol; 2-phenylglycolamides and *N*-phenylglycolamides, methanol–dimethylformamide; *NN*-diethyl- and *N*-ethyl-glycolamides, methanol–water.

We were unable to prepare a series of amides of structure (4). Treatment of the appropriate acids with 2-bromoiso-

$$ArCO_2 \cdot CMe_2 \cdot CO \cdot NH_2$$

(4)

butyramide in the presence of triethylamine yielded only the starting acid on work-up. Use of sodium methoxide gave the same result, which was attributed to steric hindrance to access of the carboxylate ion to the α-carbon centre.

Other materials were of analytical reagent grade or were redistilled or recrystallised from standard laboratory reagents before use. Acetonitrile was purified by the method of Lewis and Smyth [7] and dioxan by passage over alumina to free it from peroxides.

Methods.—Hydrolyses were carried out by using aqueous buffer maintained at 1M ionic strength (except where stated) with NaCl, and were followed spectrophotometrically.

* I.r. spectra confirmed the structures of these materials; m.p. and literature m.p. data are available as Supplementary Publication No. SUP 22053 (4 pp.). For details see Notice to Authors No. 7, *J.C.S. Perkin II*, 1976, Index issue.

RESULTS

The hydrolyses all exhibited good first-order kinetics up to 90% completion of reaction. In all cases clean isosbestic points were observed in the repetitive scanning experiments, indicating a 1 : 1 stoicheiometry. The rate constants were proportional to hydroxide ion concentration and the derived second-order rate constants together with the isosbestic wavelengths are recorded in Table 1. The hydrolysis rate constants of selected substrates exhibit only a small deuterium oxide solvent isotope effect (Table 1); no buffer effects were observed throughout the investigation but the substrates were less reactive in media containing high proportions of dioxan.

TABLE 2

Effect of dioxan on the alkaline hydrolysis of *O*-(3-anisoyl)glycolamide [a]

Dioxan % (v/v)	0	50	65
Parent:[a]	5.1	2.6	2.5
N-Ethyl:[a]	5.1	2.5	
NN-Diethyl:[a]	0.94	0.33	0.26
Ratio of parent to *NN*-diethyl:	5.4	7.9	9.6

[a] Rate constants ($10^2 k/s^{-1}$) in 0.1M-NaOH at 25 °C.

DISCUSSION

The main conclusion of this work is that intramolecular electrophilic assistance in the form of hydrogen-bond donation to the ester from the amide NH does not operate in either ground or transition state during alkaline hydrolysis of the models. If the amide were acting as an electrophile in these cases, causing polarisation of the ester carbonyl bond, this might be reflected in a low ρ value in comparison with species without the possibility of this type of assistance. This result might be observed if the amide NH were a better donor than water (the normal solvating species), and since this is unlikely the lower observed ρ values in comparison with that for hydroxide attack on ethyl benzoates (Table 3) cannot be

TABLE 3

Hammett ρ values and k_{OH} for the substituted benzoates

	ρ [a]	k_{OH}/l mol^{-1} s^{-1} [b]
Ethyl benzoates	2.45 [c]	0.002 89
Phenyl benzoates	2.02	0.056 [d]
O-(Substituted benzoyl)glycolamides	1.52	0.5 [e]
O-(Substituted benzoyl)-*N*-ethylglycolamides	1.55	0.35 [e]
O-(Substituted benzoyl)-*NN*-diethylglycolamides	1.68	0.12 [e]
O-(Substituted benzoyl)-2-hydroxymalonamides	1.28	2.63 [e]

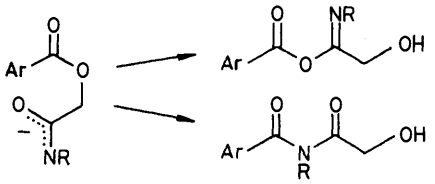
[a] σ Values from G. B. Barlin and D. D. Perrin, *Quart. Rev.*, 1966, **20**, 75. [b] For parent benzoic acid. [c] E. Tommila and C. N. Hinshelwood, *J. Chem. Soc.*, 1938, 1801. [d] J. F. Kirsch, W. Clewell, and A. Simon, *J. Org. Chem.*, 1968, **33**, 127. [e] From this study.

† The structures of the *O*-acylglycolamides were confirmed by i.r. and n.m.r. [$(CD_3)_2SO$ or CCl_4], analytical, and physical data, available in the Supplementary Publication.

[6] K. Anwers and R. Bernhardi, *Ber.*, 1891, **24**, 2219.
[7] G. L. Lewis and C. P. Smyth, *J. Chem. Phys.*, 1939, **7**, 1085.

used as evidence for electrophilic assistance. This is especially so since one of the ester series (*NN*-diethylglycolamides) has a low ρ value but these reactions cannot involve electrophilic assistance from the amide moiety. Table 3 indicates that Hammett selectivity decreases in the same order as hydrolysis reactivity increases, and this appears to be consistent with Hammond's postulate.[8a] The absence of a change in selectivity such as observed by Kirsch *et al.*[8b] for benzoate ester hydrolysis is probably due to the smaller overall change in reactivity (from phenyl benzoate to 4-nitrophenyl benzoate involves only a 20-fold increase).

The absence of a considerable deuterium oxide solvent isotope effect (Table 1) is consistent with the absence of electrophilic interaction and with hydrogen bonding where the proton is not transferred to any great extent from the nitrogen. This result is not due to the absence of proton exchange on the nitrogen of the amide; this is known to be catalysed by base and the rate constants

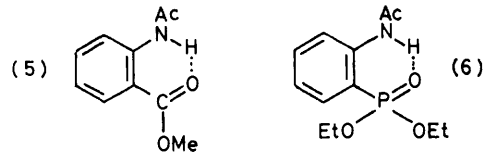


are larger than the values for the present hydrolytic reactions.[9]

Increasing the dioxan concentration (Table 2) decreases the rate of alkaline hydrolysis of a particular ester, but the ratio of rate constants for those modes where amide participation could occur to those of esters where no interaction is possible only changes by about two-fold up to a high dioxan content. This technique has been used in previous studies:[10] the effect of increasing dioxan content on the alkaline hydrolysis of esters which are able to provide their own solvation shell is not as marked as in those where internal solvation is impossible. The present results therefore point to the absence of internal solvation.

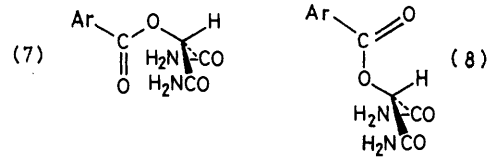
The reactivity of the glycolamides towards hydroxide ion does not suggest any special electrophilic assistance by NH, since increasing substitution at nitrogen has little effect. Intramolecular nucleophilic catalysis in the series [equation (i)] is not a possibility; if it were there should also be a large discrepancy in k_{OH} among the *N*-substituted glycolamides, and the five-fold change observed (parent up to *NN*-diethyl) is not consistent with this mechanism.

Earlier studies[10] showed that amides are able to participate as electrophiles in the alkaline hydrolysis of alkyl esters and phosphonates [(5) and (6)]; the interaction is particularly effective when the solvent is less able to solvate the transition state. The absence of an intramolecular effect in the present study is interesting because the malonamide derivatives possess most of the constituent groups of an oxyanion pocket [see (1) and (3)]. Dreiding-type models are not very informative, but space-filling Corey, Pauling, and Koltun models indicate that the conformation (7) of a malonamide having a favourable amide interaction with the ester group is excessively crowded in comparison with (8), where no such interaction occurs. The system with C–O antiperiplanar to the C–H bond (7) is less stable



than the synperiplanar form (8). We can draw the same conclusions from models for the monoamide esters.

The Function of the Oxyanion Pocket in Serine Protease Action.—The enzymic acylation and deacylation steps are essentially solid-phase reactions where the substrate moiety is in a cleft in a surface; the bound substrate is close packed into the active site, which is essentially a vacancy in a close-packed array of atoms.[11,*] If the oxyanion of a tetrahedral intermediate is to be generated in a close-packed system the provision of internal solvation is important because the oxyanion will not be open to solvent. The latter appears to be the case with the serine proteases so far studied by *X*-ray crystallography, and since the enzyme acts as an acyl transfer agent the acyl-enzyme will transfer to a nucleophilic acceptor, which must approach from the solvent; the other groupings of the substrate ought therefore to be directed into the bulk of the enzyme rather than out into the solvent where they would hinder the approach of the nucleophile. The only grouping capable of accepting and thus stabilising an oxyanion in a close-packed arrangement is a hydrogen-bond donor, or better three such donors arranged roughly at the three corners of a tetrahedron with oxyanion central. This and other work[10] has shown that the effective enhancement which might be



* *X*-Ray studies of enzyme inhibitor complexes indicate that the inhibitor bound in the active site is rigid, and model building readily shows that substrate is close-packed.

[8] (*a*) G. S. Hammond, *J. Amer. Chem. Soc.*, 1955, **77**, 334; (*b*) J. F. Kirsch, W. Clewell, and A. Simon, *J. Org. Chem.*, 1967, **33**, 127.

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[10] (*a*) A. Williams and G. Salvadori, *J.C.S. Perkin II*, 1972, 883; (*b*) R. A. Naylor and A. Williams, *ibid.*, 1976, 1908.

[11] (*a*) M. H. Klapper, *Biochim. Biophys. Acta*, 1971, **229**, 557; (*b*) W. Kauzman, *Nature*, 1974, **248**, 447; (*c*) F. M. Richards *J. Mol. Biol.*, 1974, **82**, 1; (*d*) J. C. Kendrew, *Science*, 1963, **139**, 1259.

gained from internal solvation is not large, and if the hydrogen-bonding ability is close to that of water the effective polarisation will be negligible in comparison with the case where water is the only solvator. An entropic advantage is gained however with internal solvation, because entropy is lost in solvating a species with three molecules of water as compared with solvation by one species,[12] although this is modified to some extent by entropy gained by loss of solvation of the ground state.

The absence of internally solvating groups will block the formation of the tetrahedral intermediate in acylation and deacylation because the oxyanion would have to be formed in an apolar or non-polar medium and would therefore be a particularly unstable structure. It is possible that serine proteases exist where the oxyanion is solvated by external solvent; but this is unlikely as the binding interactions necessary between enzyme and substrate and the external solvation shell would probably cause excessive steric requirements at the acyl carbon atom for nucleophilic attack by the donor nucleophile.

The same arguments apply to the mechanistically similar cysteine proteases,[13] which also possess the features of the oxyanion pocket.[14]

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[14] H. C. Hawkins and A. Williams, *J.C.S. Perkin II*, 1976, 723.