Nucleophilic *versus* General Base Catalysis in Phosphyl (P^V) Group transfer: Application to α-Chymotrypsin Action

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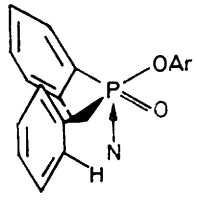
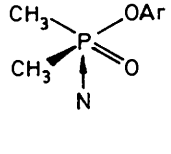
The hydrolysis of aryl dimethylphosphinates is shown to be catalysed *via* a nucleophilic pathway by a series of bases including imidazole which is less efficient than phosphate dianion. The more reactive nucleophilic pathway is allowed because dimethylphosphinate is less sterically hindered than diphenyl phosphinate where general base-catalysis predominates. Acylation of α-chymotrypsin by 4-nitrophenyl diphenylphosphinate, a *bona fide* general base mechanism, has a low solvent deuterium oxide isotope effect not characteristic of such a mechanism.

STUDY of the factors controlling the choice between nucleophilic and general base routes in catalysed solvolyses of P^V esters is, as yet, at an embryonic stage compared with the situation for carboxylic esters. Important contributions have been suggested to include the nature of the leaving group,[1] the nucleophile,[1,2] strain in the substrate,[3] and steric effects.[3,4]

Recently, it was proposed that the imidazole-catalysed hydrolysis of aryl diphenylphosphinates (I) involved general base activated attack of water because of the high steric constraints which would be imposed on a transition-state for the nucleophilic mechanism (I).[3] We report a study of the hydrolysis of a series of ten aryl substituted dimethylphosphinates (II) in which much of the steric hindrance is lifted and show that this ' steric relief ' does



(I)



(II)

alter the mechanism from general base to nucleophilic attack. We also use the effect of large steric requirements which can ' force ' the reaction to take a general

[1] S. A. Khan and A. J. Kirby, *J. Chem. Soc.* (*B*), 1970, 1172.
[2] G. O. Dudek and F. H. Westheimer, *J. Amer. Chem. Soc.*, 1959, **81**, 2641.
[3] A. Williams and R. A. Naylor, *J. Chem. Soc.* (*B*), 1971, 1967.
[4] H. J. Brass and M. L. Bender, *J. Amer. Chem. Soc.*, 1972, **94**, 7421.

base pathway to investigate the still vexing nucleophilic–general base ambiguity in chymotrypsin catalysis.

EXPERIMENTAL

Materials.—The dimethylphosphinate esters, $(CH_3)_2P(O)OAr$, were prepared by non-aqueous Schotten–Bauman procedures from the phenols and dimethylphosphinoyl chloride; the route to the latter common precursor involved steps detailed in the literature [reaction (1)]. Bisdimethyl-

$$CH_3MgBr \xrightarrow{PSCl_3} \underset{(III)}{[(CH_3)_2PS]_2} \xrightarrow{SOCl_2} \underset{(IV)}{(CH_3)_2POCl} \quad (1)$$

phosphinyl disulphide (III) was prepared by the method of Parshall [5] and was recrystallised from ethanol as white needles, m.p. 230° (lit.,[5] 227°). Dimethylphosphinoyl chloride (IV) was prepared by the method of Pollart and Harwood [6] as a yellow solid, m.p. 66°, b.p. 128—132° at 45 Torr (lit.,[6] m.p. 66°, b.p. 110—113° at 35 Torr).

Aryl dimethylphosphinates were prepared by the following general procedure (applied to the 4-nitrophenyl ester): equimolar quantities of 4-nitrophenol, pyridine, and dimethylphosphinoyl chloride were stirred together overnight at ambient temperature using dichloromethane as solvent. The white precipitate of pyridinium chloride was removed and the filtrate dried ($MgSO_4$). Evaporation of solvent gave a red mobile oil which on trituration with dry ether gave a solid. Purification was effected after three recrystallisations from dichloromethane. Liquid esters were purified by distillation under reduced pressure. The 4-methoxyphenyl ester gave a dark brown intractable oil which gave crystals after chromatography in dichloromethane through a column of alumina (Camag MFC; 100—200 mesh; Hopkins and Williams). All the esters readily decomposed as is reflected in the elemental analyses (carried out by Mr. G. M. Powell of this laboratory with a Hewlett–Packard 185-CHN analyser). The solid species were recrystallised just before use, liquid esters were used directly after distillation.

Physical properties of the esters are given in Table 1 and analytical details in Supplementary Publication No. SUP 21624 (3 pp.).* Good ^1H n.m.r. spectra and integrations were obtained in all cases using deuterio-acetone or -chloroform as solvent; they exhibited 1 : 1 doublets at τ *ca.* 8 for PCH_3 groups (split by ^{31}P) and complicated aromatic multiplets. The ^{31}P n.m.r. spectrum of the 4-nitrophenyl ester in acetone had an unresolved envelope at −54 p.p.m. relative to H_3PO_4, shifting to −57 p.p.m. on addition of water. I.r. data are recorded in SUP 21624.

TABLE 1

Physical properties of substrates

Dimethylphosphinate	λ/nm [d]	M.p. or b.p. (°C)
3,5-Dinitrophenyl	410	72—78 [a]
4-Nitrophenyl	400	97—101 [a]
3-Nitrophenyl	300 (340) [c]	81—84
4-Ethoxycarbonylphenyl	300	210—220 (15) [b]
4-Chlorophenyl	300 (281) [c]	182—184 (27) [b]
3-Chlorophenyl	295	158—164 (17) [b]
4-Acetylphenyl	345 (300) [c]	219—228 (30) [b]
Phenyl	290 (300) [c]	164—166 (27) [b]
4-Methylphenyl	298	160—170 (15) [b]
4-Methoxyphenyl	300	69—71

[a] With decomposition. [b] Pressure in Torr. [c] Wavelength for imidazole buffers. [d] Wavelength for hydroxide buffers.

4-Nitrophenyl diphenylphosphinate and benzoate were from another study [3,7] and the dimethylphosphate ester was a gift from Dr. R. C. Woodcock. Buffer constituents were of analytical quality or were recrystallised or redistilled from reagent grade materials. Dioxan was purged of peroxides by passage of the AnalaR material through a column of alumina. Reagent grade imidazole was dissolved in methanol, decolourised with charcoal, and recrystallised twice from dry benzene. Thionyl chloride, methyl bromide, and thiophosphoryl chloride were commercial products and used without further purification. Double distilled water was used throughout and α-chymotrypsin was purchased from Boehringer. Deuterium oxide was from Ryvan Chemical Company and ^{18}O-enriched water from Prochem.

Methods.—Reactions were followed spectrophotometrically using Beckman-DBG or Unicam SP 800 instruments. A typical procedure for the non-enzymatic hydrolyses involved adding a portion (10—50 μl) of a stock solution of substrate (*ca.* 10^{-3}M) in dioxan to 2.5 ml of buffer equilibrated to the correct temperature in a quartz cell in the thermostatted cell compartment of the instrument. The progress curve of the absorbance *versus* time at a fixed wavelength was recorded on a Servoscribe potentiometric recorder. A similar procedure was employed for the enzyme reactions except that the substrate was run with the buffer to check background hydrolysis before the portion of enzyme was added.

Repetitive scanning of the spectrum during reaction for each substrate in the appropriate buffer was used to determine the best wavelength for kinetic study (Table 1). First-order rate constants were determined from logarithmic plots using infinity values of absorbance measured after at least six half lives.

Mass spectral studies on the product 3-nitrophenol were carried out by Dr. R. B. Turner on an AEI-MS902 high resolution mass spectrograph. N.m.r. spectra were recorded with a Perkin-Elmer R10 machine.

RESULTS

Repetitive scanning of the spectrum of the hydrolysing esters gave good isosbestic wavelengths indicative of clean 1 : 1 stoicheiometry and these are given in SUP 21624. In the case of the 4-nitrophenyl esters the quantity of phenol released is estimated using the absorption coefficient at 400 nm and confirmed the 1 : 1 stoicheiometry. The rates for buffer and lyate catalysed reactions exhibited first-order kinetics up to *ca.* 90% of the reaction.

Hydroxide Ion Catalysis.—The pseudo-first-order rate constants for release of 4-nitrophenol from the corresponding dimethylphosphinate ester were proportional to hydroxide ion concentration up to 10^{-3}M (see SUP 21624). The bimolecular rate constants for hydroxide attack at the other phenyl esters were obtained using 10^{-3}M-NaOH (see Table 2).

* For details of Supplementary Publications see Notice to Authors No. 7 in *J.C.S. Perkin II*, 1975, Index issue. Items less than 10 pp. are supplied as full-size copies.

[5] G. W. Parshall, *Org. Synth.*, 1965, **45**, 102.
[6] K. A. Pollart and H. J. Harwood, *J. Org. Chem.*, 1962, **27**, 4446.
[7] A. Williams and G. Salvadori, *J. Chem. Soc.* (*B*), 1971, 2401.

Imidazole-catalysed Hydrolyses.—Plots of pseudo-first-order rate constants for release of 4-nitrophenol from the corresponding dimethylphosphinate ester in imidazole buffers *versus* total buffer concentration are linear. The effect of concentration change at different pH values

TABLE 2

Alkaline hydrolysis of substituted phenyl dimethylphosphinates [a]

Phenyl substituent	σ^- [b]	k_{OH}/l mol^{-1} s^{-1}
3,5-Dinitro	1.42	18.8
4-Nitro	1.24	16.0
3-Nitro	0.71	6.77
4-Acetyl	0.87	6.53
4-Ethoxycarbonyl	0.68	6.3
4-Chloro	0.23	2.20
3-Chloro	0.37	2.48
H	0.00	0.80
4-Methyl	−0.17	0.61
4-Methoxy	−0.27	0.73

[a] 25°, ionic strength made up to 0.1M with NaCl, 10% dioxan, tolerance in the rate constants is ±5%. [b] From G. B. Barlin and D. D. Perrin, *Quart. Rev.*, 1966, **20**, 75.

(slope/total buffer concentration *versus* fraction of base species) indicates that catalysis is *via* the base species (Figure 1). Values of $k_{imidazole}$ (k_{im}) for other aryl esters were obtained from experiments using 0.9M-imidazole buffers (fr. base = 0.9) and the data are collected in Table 3.

TABLE 3

Imidazole-catalysed hydrolysis of substituted phenyl dimethylphosphinates [a]

Phenyl substituents	$10^4 k_{im}$/l mol^{-1} s^{-1} [c]
4-Nitro	32.2 [b]
3-Nitro	6.72
4-Acetyl	5.78
4-Ethoxycarbonyl	4.09
4-Chloro	0.947
3-Chloro	1.36
H	0.232

[a] 59.8°, ionic strength made up to 0.1M with NaCl, 10% dioxan. [b] Arrhenius parameters $\Delta H_{25}^{\ddagger}$ 9.3 kcal mol^{-1} and $\Delta S_{25}^{\ddagger}$ −40 cal mol^{-1} K^{-1} may be calculated from values at 25 and 39.9° (4.94×10^{-4} and 1.46×10^{-3} l mol^{-1} s^{-1} respectively). [c] Tolerance in rate constants is ±5%.

Other Buffer Species.—Other buffers (phosphate dianion, carbonate, Tris, pyridine, and collidine) catalyse the release of 4-nitrophenol from the dimethylphosphinate ester to a greater or lesser extent; the parameters (Table 4) are derived from concentration studies at a single pH assuming only the basic species to be effective. The effect of water as a 'catalyst' is determined from intercepts of rate constant *versus* buffer concentration at constant pH and allowing for the small contribution of hydroxide catalysis (water concentration is taken as 55.5M to derive the bimolecular constant).

Analysis of phosphate catalysis at different pH values (as in Figure 1) indicates that the $H_2PO_4^-$ species has little catalytic power compared with the HPO_4^{2-} species. The intercept at fr. base = 0 has a value equal to its error: $k_{HPO_4^{2-}} = 3.55 \times 10^{-2}$ l mol^{-1} s^{-1} and $k_{H_2PO_4^-} < 0.68 \times 10^{-3}$ l mol^{-1} s^{-1} (at 39.9°, ionic strength = 0.1).[4]

Deuterium Oxide Solvent Isotope Effect.—For imidazole catalysis of 4-nitrophenyl dimethylphosphinate hydrolysis $k_{im}^{H}/k_{im}^{D} = 1.7$ (see SUP 21624).

Site of Fission.—When 3-nitrophenyl dimethylphosphinate ester is hydrolysed in enriched water there was no incorporation of the enriched oxygen into the product 3-nitrophenol as compared with the appropriate control experiments (SUP 21624) indicating P–O cleavage as the major reaction. This experiment eliminates reactions occurring *via* attack on nucleophiles on the aromatic nucleus.

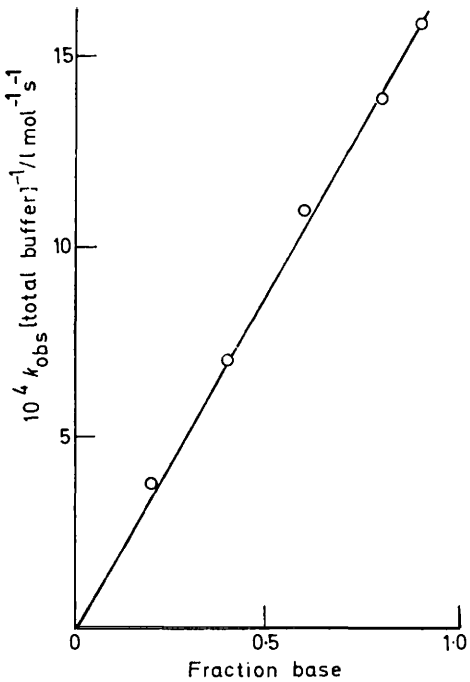


FIGURE 1 Imidazole-catalysed hydrolysis of 4-nitrophenyl dimethylphosphinate at different pH. Intercept on fraction base = 1.0 axis is 1.77×10^{-3}. Conditions: 39.9°; ionic strength 1.0M

In any case the reactivity is higher than would be expected for such a mechanism.[8]

TABLE 4

Bimolecular rate constants for catalytic hydrolysis of 4-nitrophenyl dimethylphosphinate [a,b]

Catalyst	pK_a [d]	k_{cat}/l mol^{-1} s^{-1}
Hydroxide	15.7	16
Carbonate	10.33	0.143
Tris(hydroxymethyl)aminomethane	8.10	⩾100
Imidazole	6.95	4.94×10^{-4}
Phosphate dianion	7.21	1.06×10^{-2}
sym-Collidine	7.48	1.28×10^{-6}
Pyridine	5.17	$<10^{-6}$
Water	−1.7	1.6×10^{-6}
Phosphate monoanion	2.14	$<0.68 \times 10^{-3}$ [c]

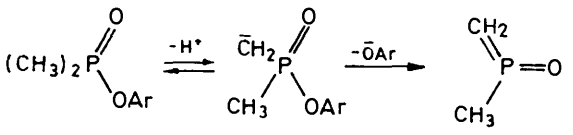
[a] 25°, ionic strength made up to 0.1M with NaCl, 10% dioxan, tolerance in the rate constants ±5%. [b] Brønsted law holds for the oxygen nucleophiles: $\log_{10} k_{cat} = 0.41 pK_a - 5.09$ (correlation coefficient r 0.99). [c] 39.9°. [d] pK_a values from W. P. Jencks and J. Regenstein in 'Handbook of Biochemistry', ed. H. A. Sober, The Chemical Rubber Co., Cleveland, 1970, 2nd edn., section J-187.

Solvolysis in Dimethyl Sulphoxide.—4-Nitrophenyl dimethylphosphinate spontaneously lost 4-nitrophenol in

[8] A. J. Kirby and W. P. Jencks, *J. Amer. Chem. Soc.*, 1965, **87**, 3209.

DMSO (Koch–Light; puriss) at 25° with $k_{obs} = 6.28 \times 10^{-3}$ s^{-1} ($\lambda_{kinetic}$ 436, $\lambda_{isosbestic}$ 300 nm). The corresponding diphenylphosphinate ester was unreactive even at 70°.

Exchange of α-Hydrogen Atoms.—The lability of the hydrogen atoms on the phosphinoyl methyl groups was shown to be negligible by measuring the ^{1}H n.m.r. spectrum of the 4-nitrophenyl ester in D_2O in the presence and absence of NaOD. The aromatic envelope integrated with respect to the phosphinoyl methyl doublet gave the ratio 0.67 (expected for no exchange, 0.67). If an elimination–addition (EA) type reaction (2) occurs then at least 1/6 of the methyl protons would be exchanged and the minimum value for the ratio would be 0.8. The error limits on this technique



(2)

allow us to exclude as a major path the EA mechanism which can therefore be omitted from the discussion.

DISCUSSION

Nucleophilic Catalysis.—Although a high correlation coefficient is not expected for a wide variety of bases in a general base process [4] the high degree of dispersion exhibited by the Brønsted type plot (Figure 2) is incompatible with such a route and more representative of a nucleophilic mechanism.[1,4,9-12] Where a continuity of

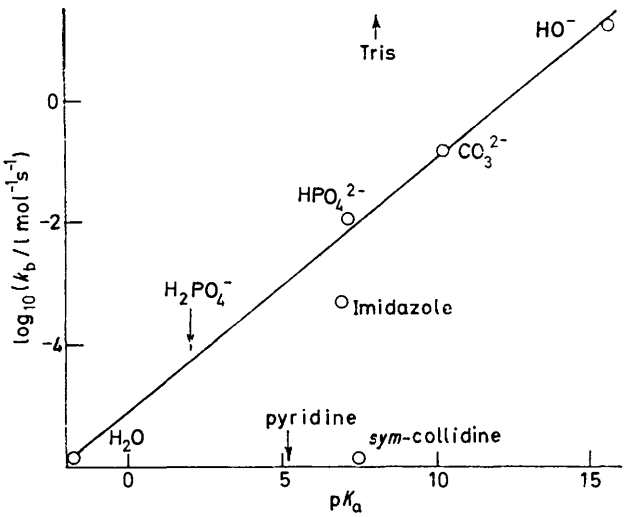


FIGURE 2 Brønsted type plot of reactivity of basic reagents with 4-nitrophenyl dimethylphosphinate; line is theoretical (see Table 4); data from Table 4; arrows refer to upper and lower limits

structural type exists, as in the attack of oxygen species (HO^-, CO_3^{2-}, HPO_4^{2-}, H_2O), a good correlation holds (see Table 4 and Figure 2). The rate constant for Tris catalysis is greater than that of carbonate by *ca.* 10^3 although the latter is the stronger base by *ca.* 2 pK_a units. Imidazole and Tris are respectively some 10^3- and 10^6-fold more efficient than collidine although the pK_a values are similar. Such comparisons show either a change in mechanism from, presumably, general base catalysis for collidine to nucleophilic catalysis or constancy of mechanism with high steric demand in the transition state, a situation more marked in nucleophilic than in general base catalysis.[10,13,14] The higher reactivity of collidine compared with pyridine, although the former is the more sterically hindered, implies that general base catalysis is the predominant mechanism for these species.

Hydroxide attack exhibiting a σ^- dependence (Table 2, Figure 3) is a unique observation because only a σ

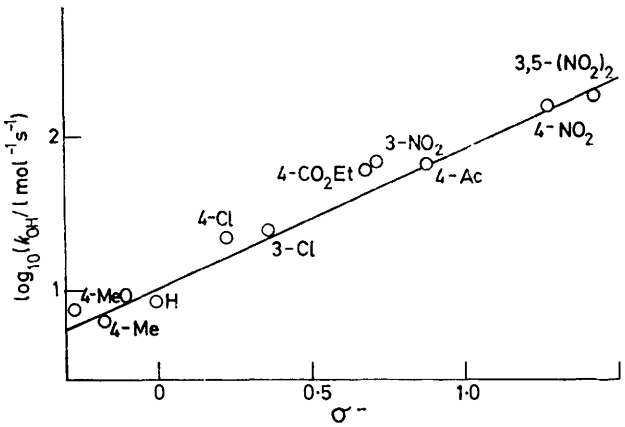


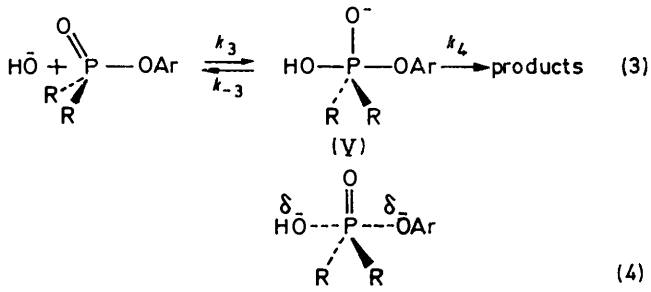
FIGURE 3 Hammett σ^- dependence of reactivity of hydroxide ion with substituted phenyl dimethylphosphinates; values of σ^- from G. B. Barlin and D. D. Perrin, *Quart. Rev.*, 1966, **20**, 75. Line is theoretical [$\log_{10}k = 0.93\sigma^- + 0.05$ ($r = 0.984$)]; data from Table 2

correlation is observed in all the recorded cases of hydroxide attack at P^V.[3,15] The data are consistent with considerable bond breaking (ArO–P) in the transition state of the rate-limiting step. Either a stepwise mechanism with the breakdown of an intermediate rate limiting or a synchronous process occurs. The absence of oxygen-18 incorporation into unchanged ester in the partial hydrolysis of P^V esters [16] indicates that the oxygens of the intermediate [reaction (3)] are not equivalent or that the process is synchronous [reaction (4)].

Support for a non-synchronous mechanism in phosphinate hydrolysis has been claimed in a recent report by Haake *et al.*[17] who observed an induction period in the

[9] F. Covitz and F. H. Westheimer, *J. Amer. Chem. Soc.*, 1963, **85**, 1173.
[10] M. L. Bender, 'Mechanisms of Homogeneous Catalysis—From Protons to Proteins,' Wiley, New York, 1971, pp. 176 *et seq.*
[11] A. J. Kirby and S. G. Warren, 'Organic Chemistry of Phosphorus,' Elsevier, Amsterdam, 1967, pp. 317—322.
[12] H. J. Brass, J. O. Edwards, and M. Biallas, *J. Amer. Chem. Soc.*, 1970, **92**, 4675.
[13] L. W. Deady and J. A. Zoltiewicz, *J. Org. Chem.*, 1972, **37**, 603.
[14] S. L. Johnson, *Adv. Phys. Org. Chem.*, 1967, **5**, 237.
[15] A. Williams and K. T. Douglas, *J.C.S. Perkin II*, 1972, 1454 and references therein.
[16] (*a*) D. Samuel and B. L. Silver, *Adv. Phys. Org. Chem.*, 1965, **3**, 177; (*b*) P. C. Haake, C. E. Diebert, and R. S. Marmor, *Tetrahedron Letters*, 1968, 5247.
[17] (*a*) P. C. Haake, R. D. Cook, P. C. Turley, and A. H. Fierman, *J. Amer. Chem. Soc.*, 1972, **94**, 9260; (*b*) R. D. Cook, C. E. Deibert, W. Schwarz, P. C. Turley, and P. C. Haake, *ibid.*, 1973, **95**, 8088.

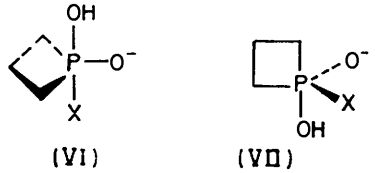
base hydrolysis of methyl di-isopropylphosphinate and a very high dependence of the rate constant for alkaline hydrolysis on σ^* (ρ^* *ca.* 8).



(V)

(4)

Species (V; R = CH_3) corresponds to the most efficient mode of attack for the esters because (a) nucleophilic attack directly produces the permutational isomer of minimal energy; any isomerisation will be disfavoured since it must produce a higher energy isomer; (b) the isomer with the aryloxy group apical preparatory to departure (as demanded by microscopic reversibility) is directly formed; (c) the face attacked might be expected to provide the least stereoelectronic repulsion to the entering ligand. A consequence of this route of attack is that inversion should occur at phosphorus and this is the case.[18] Attack at phosphorus in cyclic phosphinates in the same manner as in acyclic ones would yield a ' diequatorial ' ring (VI) and an equatorial-axial ring (VII) is preferred; permutational isomerisation of this species to put the leaving group axial leads to retention of configuration.



(VI) (VII)

The intermediate (V) would be unlikely to give rise to a σ^- relationship (breakdown rate limiting) because hydroxide ion is presumably a much poorer leaving group than aryl oxide. Thus the evidence at present points to a synchronous process for the aryl dimethylphosphinates. Attack of the heavily solvated hydroxide ion would have stringent steric requirements and this is presumably the reason for the large difference in character between the reaction of diphenyl- and dimethyl-phosphinates with this ion.

The σ^- relationship (Figure 4) for imidazole-catalysed hydrolysis of dimethylphosphinates is consistent with either a stepwise or a synchronous mechanism analogous to (3) and (4) respectively involving nucleophilic attack by imidazole at P^V. The deuterium oxide solvent isotope effect of 1.7 on the 4-nitrophenyl ester reaction is rather high for nucleophilic attack; Johnson [14] suggests a value >2 for general base catalysis and 0.8—1.9 for nucleophilic routes. There is a precedent for a high value of k_H/k_D (*ca.* 1.8) in the imidazole catalysed hydrolysis of 4-nitrophenyl diethylphosphate.[19] It was suggested that

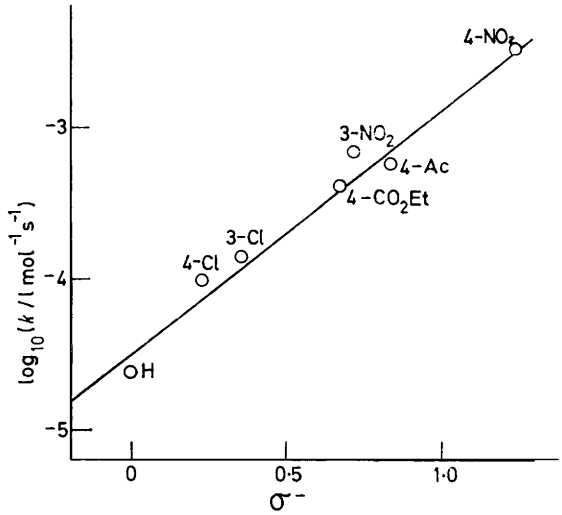
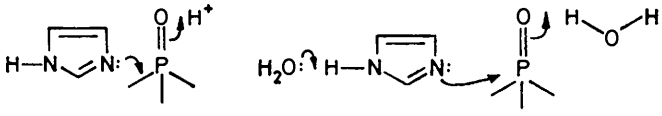


FIGURE 4 Hammett σ^- dependence of reactivity of imidazole with substituted phenyl dimethylphosphinates; σ^- values as for Figure 3; line is theoretical [$\log_{10}k = 1.60\sigma^- - 4.49$ (r = 0.985)]; data from Table 3

this might be due to concerted proton transfer (VIII) but this cannot be so for the dimethylphosphinates because the rate law implied by (VIII) involves [H^+] and [imidazole] and there is no conjugate acid term (Figure 1). A similar scheme (IX) involving considerable water participation could give the higher ratio and this is supported by the known high tendency of the phosphinoyl oxygen atom to act as a hydrogen-bond acceptor.[20] Such an ordered transition state could explain the very high negative entropy of activation for imidazole catalysis ($\Delta S^\ddagger = -40$ cal mol^{-1} K^{-1}). In



(VIII) (IX)

general, bimolecular nucleophilic processes on P^V have $\Delta S^\ddagger$ between −10 and −30 cal mol^{-1} K^{-1}.[21]

Although *N*-dimethylphosphinoylimidazole was not directly observed, its probable reactivity, by comparison with the known activity of phosphorylimidazoles to solvolysis [22] and the higher inherent reactivity of

[18] (*a*) M. Green and R. F. Hudson, *J. Chem. Soc.*, 1963, 3883; (*b*) M. J. Gallagher and I. D. Jenkins, *Topics Stereochem.*, 1968, **3**, 31, 82.

[19] M. L. Bender and F. C. Wedler, *J. Amer. Chem. Soc.*, 1972, **94**, 2101.

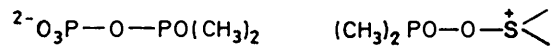
[20] G. Aksnes, *Acta Chem. Scand.*, 1960, **14**, 1475, 1526.

[21] (*a*) L. Ginjaar and S. Blasse-Vel, *Rec. Trav. chim.*, 1966, **85**, 694; (*b*) ref. 11, p. 302.

[22] (*a*) J. Baddiley, J. G. Buchanan, and R. Letters, *J. Chem. Soc.*, 1956, 2812; (*b*) T. Wagner-Jauregg and B. E. Hackley, *J. Amer. Chem. Soc.*, 1953, **75**, 2125; (*c*) F. Cramer, H. Schaller, and H. A. Staab, *Chem. Ber.*, 1961, **94**, 1612; (*d*) L. N. Nikolenko and E. V. Degterev, *Zhur. obshchei Khim.*, 1967, **37**, 1350; (*e*) B. Atkinson and A. L. Green, *Trans. Faraday Soc.*, 1957, **53**, 1334; (*f*) R. L. Blakeley, F. Kerst, and F. H. Westheimer, *J. Amer. Chem. Soc.*, 1966, **88**, 112; (*g*) B. S. Cooperman and G. L. Lloyd, *ibid.*, 1971, **93**, 4883, 4889; (*h*) W. P. Jencks and M. Gilchrist, *ibid.* 1965, **87**, 3199.

phosphinates compared with phosphates,[23] is consistent with its being an intermediate.

The pyrophosphate (X), would have a high solvolysis rate [2,24] compatible with its existence as an intermediate in phosphate catalysis. For general base processes



(X) (XI)

imidazole and phosphate dianion should have a similar activity but as a nucleophile the former is often 10^3-fold more reactive than the latter.[14] Imidazole and phosphate catalysed hydrolysis of 4-nitrophenyl diphenylphosphinate hydrolyses have similar reactivities in accord with a general base pathway.[3] The high reactivity of phosphate compared with imidazole for hydrolysis of the dimethylphosphinate (Table 4) is probably not due to bifunctional action since phosphate lies on the same Brønsted line as hydroxide and water. The hydrolysis of 4-nitrophenyl dimethylphosphate is catalysed to an equal extent by phosphate and imidazole (Table 5) but the deuterium oxide solvent isotope effect (1.4) [3] indicates nucleophilic attack. These observations cast doubt on the use, alone, of the imidazole : phosphate reactivity ratio as a simple mechanistic tool.

Steric Hindrance and Catalytic Mechanism.—This work provides direct evidence that lowering steric demand of the substrate leads to a transition from general base to nucleophilic catalysis. The results for methyl bis-(4-nitrophenyl) phosphate also argue this view [4] and the higher steric requirements of the diphenyl- compared with the dimethyl-phosphinate are confirmed by solvolysis of the latter ester in moist DMSO presumably *via* a mechanism similar to that proposed by Ratz and others [25] for phosphodichloridate reactions [*via* (XI)].

Steric inhibition of nucleophilic catalysis at the four-co-ordinate phosphyl centre does not have an analogue in carboxy chemistry due to the lower steric demands of the three-co-ordinate carboxy carbon. For example, even 4-nitrophenyl pivalate hydrolysis is catalysed by imidazole *via* a nucleophilic pathway. This does not mean that the steric effect will be unimportant in enzyme mechanisms since the effective co-ordination number of a bound acyl group may be raised increasing its stereoselectivity. For example the imidazolyl of histidine-57 acts as a general base in the ageing of α-chymotrypsin inhibited by bis-4-nitrophenyl carbonate [19] whereas imidazole acts as a nucleophile in model systems.[19,26]

The only direct evidence presently available to distinguish general base from nucleophilic catalysis in acylation of chymotrypsin is the deuterium oxide solvent isotope effect which is <2 for a number of acylations.[27] The observation of an isotope effect (1.6) for acylation by 4-nitrophenyl diphenylphosphinate which is normally constrained to hydrolyse *via* a general base mechanism indicates that the isotope data are not as conclusive as was previously thought.[27]

TABLE 5

Comparison of 4-nitrophenyl phosphinate and phosphate hydrolyses [a]

Substrate	k_{OH}	k_{Im}	$k_{HPO_4^{2-}}$	k_{H_2O} [b]
Diphenylphosphinate [f]	7.3	1.03×10^{-3} (4.88×10^{-3} [c])	2.05×10^{-4}	
Dimethylphosphinate	16	4.94×10^{-4}	1.06×10^{-2}	8.88×10^{-5}
Dimethylphosphate	8.8×10^{-2} [c]	3.22×10^{-3} [d]	3.47×10^{-4} [d]	1.16×10^{-6} [d]

[a] Unless otherwise stated the temperature is 25°; units in l mol^{-1} s^{-1}. [b] Units in s^{-1}. [c] 55°. [d] 50°. [e] L. Ginjaar and S. Blasse-Vel, *Rec. Trav. chim.*, 1958, **77**, 956. [f] Values from ref. 3.

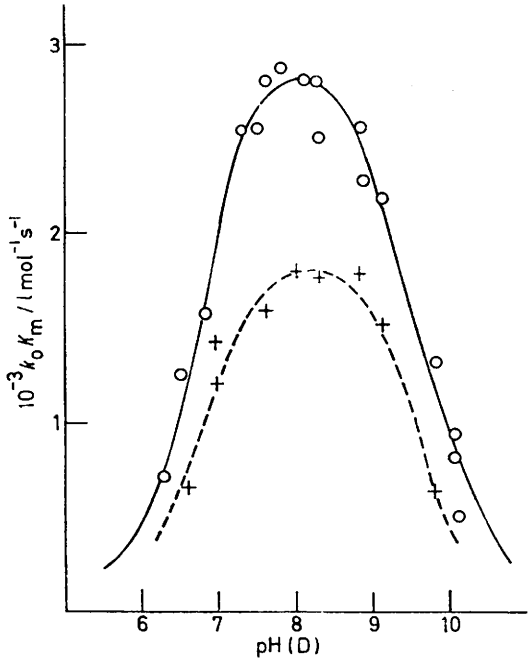


FIGURE 5 pH(D)-Dependence of k_0/k_m for 4-nitrophenyl diphenylphosphinate and α-chymotrypsin. Conditions as in Table 6; ×, deuterium oxide solvent; ○, water solvent

Binding of Substrates to Chymotrypsin.—The bell-shaped pH profiles (k_0/K_m) for the non-specific substrates

[23] R. F. Hudson and G. E. Moss, *J. Chem. Soc.*, 1964, 1040.

[24] (*a*) T. C. Bruice and S. J. Benkovic, *Bio-org. Chem.*, 1966, **2**, 158; (*b*) D. M. Brown and N. K. Hamer, *J. Chem. Soc.*, 1960, 1155; (*c*) D. S. Samuel and B. Silver, *ibid.*, 1961, 4321.

[25] (*a*) R. Rätz and O. J. Sweeting, *J. Org. Chem.*, 1963, **28**, 1612; (*b*) M. A. Ruveda, E. N. Zerba, and E. M. de Moutier Aldao, *Tetrahedron*, 1972, **28**, 5011.

[26] (*a*) J. R. Corfield, N. J. De'Ath, and S. Trippett, *Chem. Comm.*, 1970, 1502; (*b*) S. E. Cremer and B. C. Trivedi, *J. Amer. Chem. Soc.*, 1969, **91**, 7200; (*c*) R. F. Hudson and C. Brown, *Accounts Chem. Res.*, 1972, **5**, 204.

[27] C. D. Hubbard and J. F. Kirsch, *Biochemistry*, 1972, **11**, 2483.

(Table 6 and Figure 5) agree with those for other non-specific substrates.[28] It is proposed that the reactivity of

TABLE 6

Limiting values for kinetic parameters of substrates of α-chymotrypsin [a]

Substrate	$k_0^{-1}K_m$/ l mol^{-1} s^{-1}	pK_1	pK_2
4-Nitrophenyl diphenylphosphinate	2 800 (H_2O)	6.7	9.6
4-Nitrophenyl diphenylphosphinate	1 800 (D_2O)	6.8	9.6
4-Nitrophenyl dimethylphosphinate	91	6.8	
4-Nitrophenyl benzoate	603	7.1	8.9 [b]

[a] 25°, ionic strength made up to 0.1M with NaCl, 10% acetonitrile. [b] The tolerance in this value is rather high (±0.5) because only one point was measured in the high pH region. As we are only interested in $k_0/K_{m(lim)}$ this is of little consequence.

acylation of chymotrypsin involves a considerable *binding* component; presumably the aromatic functions bind in the ' tosyl-hole ' which accepts aromatic side chains and dioxan.[29] The greater number of binding possibilities for the diphenyl as opposed to the dimethyl substrate (three instead of one aromatic group) is reflected by the higher reactivity of the former substrate in contrast to the normal order (Table 5).

Model building studies with 4-nitrophenyl acetate and a model of chymotrypsin built with Kendrew models (Cambridge Repetition Engineers) using crystallographic co-ordinates, supplied by Dr. D. M. Blow before publication,[30] indicate that if the 4-nitrophenyl group is placed in the ' tosyl-hole ' the nucleophilic participation of histidine-57 is impossible. The situation is not so simple however because 4-nitrophenyl pivalate and acetate acylate elastase, where the ' tosyl-hole ' is blocked for aromatic binding by a side chain of valine-216 and of threonine-226,[31] react at approximately the same rate as for chymotrypsin.[32]

K. T. D. thanks the Government of Northern Ireland for a Scholarship.

[5/1571 *Received*, 8*th August*, 1975]

[28] (*a*) M. L. Bender, G. E. Clement, F. J. Kezdy, and H. d'A. Heck, *J. Amer. Chem. Soc.*, 1964, **86**, 3688; (*b*) M. L. Bender and F. J. Kezdy, *Biochemistry*, 1962, **1**, 1097.

[29] (*a*) T. A. Steitz, R. Henderson, and D. M. Blow, *J. Mol. Biol.*, 1969, **46**, 337; (*b*) R. P. Bell, J. E. Critchlow, and M. I. Page, *J.C.S. Perkin II*, 1974, 66.

[30] J. J. Birktoft, B. W. Matthews, and D. M. Blow, *Biochem. Biophys. Res. Comm.*, 1969, **36**, 1.

[31] B. S. Hartley and D. M. Shotton, ' The Enzymes,' eds. P. D. Boyer, H. A. Lardy, and K. Myrbäck, Academic Press, New York, 1971, 3rd edn., p. 323.

[32] (*a*) M. L. Bender and T. H. Marshall, *J. Amer. Chem. Soc.*, 1968, **90**, 201; (*b*) M. L. Bender and K. Nakamura, *ibid.*, 1962, **84**, 2577.