Bacterial Alkaline Phosphatase. Part III.[1] Kinetic Studies with Substituted Phenyl Phosphates and Structurally Related Inhibitors

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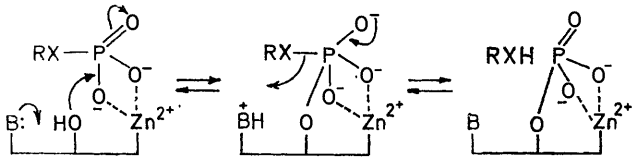
Michaelis–Menten parameters have been measured for a series of substituted phenyl phosphate substrates of alkaline phosphatase (from *Escherichia coli*) at pH 8·00, 0·1 M ionic strength, 0·1 M-trishydroxymethylaminomethane buffer and 25 °C. Values for k_0 are constant but k_0/K_m varies with substituent with a Hammett sensitivity of +0·43 (r = 0·975). The results are interpreted in terms of electrophilic assistance, probably by zinc(II), during phosphorylation or binding steps of the enzyme. Substituted phenyl- and benzyl-phosphonates (structural analogues of the substrates) competitively inhibit the enzyme but the inhibition constants (K_i) do not show a regular variation with substituent. Incursion of binding modes equivalent to non-productive binding of the substrate probably cause the random binding ability of these inhibitors. Amino-substituted phenylphosphonate inhibitors exhibit ' mixed ' inhibition at pH 8. Increase of K_i for phenylphosphonic acid with concentration of trishydroxymethylaminomethane buffer is supposed to result from chelation of the amine in the co-ordination sphere of the zinc chelate at the active site of the enzyme. Trishydroxymethylaminomethane acts not merely as an acceptor of the phosphate from the phospho-enzyme as was previously supposed.

WE argued[1] that phosphorylation of alkaline phosphatase from *Escherichia coli* (E.C.3.1.3.1) by monophosphate esters involves an $S_N2(P)$ rather than an $S_N1(P)$ mechanism. It was suggested that the enzyme



SCHEME 1

effectively neutralises the phospho-dianion facilitating bimolecular substitution (Scheme 1) by the serine hydroxy rather than the more usual $S_N1(P)$ mechanism observed in related model systems. Since the sensitivity has been measured[2] for a number of substituted phenyl phosphate esters to cleavage of the aryl–oxygen bond by a variety of nucleophiles it is reasonable that a study of the kinetics of hydrolysis of substituted phenyl monophosphate esters should give an indication of the electronic requirements in phosphorylation and binding.

Little work has been reported concerning alkaline phosphatase-catalysed hydrolysis of substituted phenyl

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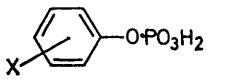
[1] A. Williams and R. A. Naylor, *J. Chem. Soc.* (*B*), 1971, 1973 is considered to be Part II; Part I, A. Williams, *Chem. Comm.*, 1966, 676.

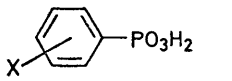
[2] (*a*) R. H. Bromilow and A. J. Kirby, *J.C.S. Perkin II* 1972, 149; (*b*) A. Williams and R. A. Naylor, *J. Chem. Soc.* (*B*), 1971, 1967; (*c*) S. A. Khan and A. J. Kirby, *ibid.*, 1970, 1172; (*d*) T. R. Fukuto and R. L. Metcalf, *J. Agric. Food Chem.*, 1956, **4**, 930; (*e*) D. F. Heath, ' Organo-phosphorus Poisons,' Pergamon, Oxford, 1961, p. 79; (*f*) A. J. Kirby and M. Younas, *J. Chem. Soc.* (*B*), 1970, 510; (*g*) p. 1165; (*h*) S. A. Khan, A. J. Kirby, M. Wakselman, D. P. Horning, and J. M. Lawlor, *ibid.*, p. 1182; (*i*) A. Williams and K. T. Douglas, *J.C.S. Perkin II*, 1972, 1454.

phosphates.[1,3] Barrett *et al.*[4] reported work on an extensive series of aromatic phosphates in connection with partitioning of the phospho-enzyme between trishydroxymethylaminomethane and water but no Michaelis–Menten parameter is quoted. We now extend the data for three phenyl phosphates from a previous study [1] using the substrates (I).

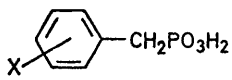
The measurement of simple Michaelis–Menten parameters does not allow the separation of binding factors from reactivity arising from electronic effects. Reversible inhibitors of the enzyme which are structural analogues of the substrates (II) and (III) should be subject to the same binding influences as are the substrates; thus the variation in K_i (binding constant for inhibitor) for these inhibitors should be a measure of the variation in K_s (inaccessible binding constant for substrate) for the corresponding substrates. It is recognised that K_i is not necessarily equivalent to K_s.



(I)



(II)



(III)

X		X	
a;	4-NO_2	f;	3-NH_2
b;	3-NO_2	g;	4-Me
c;	3-Cl	h;	4-OMe
d;	4-Cl	i;	4-NH_2
e;	H	j;	4-CHO
		k;	4-Ac

EXPERIMENTAL

Materials.—4-Nitrophenylphosphonic acid was prepared from 4-nitroaniline *via* 4-nitrobenzenediazonium tetrafluoroborate. 4-Nitroaniline (0·25 mol) was dissolved in a solution of HCl (5N, 0·375 mol) and cooled to *ca.* 0 °C. A cold solution of sodium nitrite (0·25 mol, 50% in water) was added slowly with stirring. Stirring was continued for 5 min after addition was complete and the mixture filtered through a sintered glass funnel. Sodium tetrafluoroborate (0·34 mol) in water (120 ml) was then added to the filtrate with vigorous stirring which was continued for 30 min after addition was complete. A precipitate of the diazonium tetrafluoroborate was filtered off, washed with ice-cold sodium tetrafluoroborate solution (25 ml, 5%), ice-cold methanol (30 ml), and several portions of diethyl ether (3 × 50 ml). The crystals (65% yield) decomposed at 145 °C (lit.,[5] 156 °C). The diazonium salt (0·2 mol) [6] was suspended in ethyl acetate (250 ml) in a three-necked, round-bottomed flask with a sealed stirrer, large gas outlet (connected to a water trap), and thermometer (dipping below the surface of the liquid). Phosphorus trichloride (0·2 mol) and cuprous bromide (4 g, freshly prepared) were added with stirring and after a slight rise in temperature a 1 h lag was observed before a vigorous reaction occurred needing cooling to avoid violence. Water (50 ml) was then added *via* a dropping funnel slowly with cooling as considerable heat was liberated. The mixture was steam-distilled and after 1 l of distillate came over was evaporated under reduced pressure to *ca.* 100 ml and the first precipitate (diarylphosphinic acid) was removed. Further evaporation gave a second precipitate which was freed from copper by raising the pH of a suspension in water to 8 with sodium hydroxide solution (20%), filtering, boiling with charcoal, filtering, and returning to pH 4. The free acid was obtained by evaporating the solution, taking up in 5N-HCl, and extracting the dry material in a Soxhlet apparatus with diethyl ether. The acid was recrystallised from ethyl acetate (17% overall yield). Less than 0·005% of free inorganic phosphate was shown to be present.

3-Nitrophenylphosphonic acid was prepared by adding phenylphosphonic acid (50 g) to stirred fuming nitric acid (280 ml) during 15 min. The stirring was continued for a further 40 min then the mixture was poured on ice (500 g). The product was steam-distilled until 1·5 l of distillate had collected. The residue was evaporated to dryness and the product recrystallised from ether–benzene to give material possessing less than 0·005% of free inorganic phosphate.

3-Aminophenylphosphonic acid was prepared by basifying the residue from the nitration of 50 g of phenylphosphonic acid to pH 9 with 20% NaOH and then heated under gentle reflux for 2 h with sodium sulphide ($Na_2S,9H_2O$, 160 g) and water (350 ml). The dark solution was acidified with concentrated HCl, cooled, filtered, and concentrated to *ca.* 400 ml. The solution was then taken to pH 4 with 20% NaOH and allowed to stand, whereupon the phosphonic acid precipitated as a greyish microcrystalline powder with inorganic phosphate content of less than 0·01%.

3-Chlorophenylphosphonic acid was prepared by dissolving 3-aminophenylphosphonic acid (2·5 g) in 5N-HCl (25 ml) and diazotising at 0 °C with sodium nitrite (1 g). The solution was treated with cuprous chloride (2·9 g, freshly prepared) in concentrated HCl, and, when the reaction had subsided, heated at 95 °C for 0·5 h. The mixture was diluted to 75 ml and the bulk of the copper removed with hydrogen sulphide gas. The solution was evaporated, cooled, and the crystalline precipitate collected. Recrystallisation from 5N-HCl gave material with less than 0·01% inorganic phosphate.

4-Bromophenylphosphonic acid was prepared by the methods used for the 4-nitro-compound and was used as an intermediate in the preparation of the 4-amino-derivative. The intermediate 4-bromobenzenediazonium tetrafluoroborate prepared by Roe's method [7a] decomposed at 127—130 °C (lit.,[7b] 133 °C). A 20% yield of the bromophosphonic acid was obtained, m.p. 185—190 °C (lit.,[7c] 198—199 °C).

4-Chlorophenylphosphonic acid was obtained by refluxing

[3] (*a*) D. R. Trentham and H. Gutfreund, *Biochem. J.*, 1968, **106**, 455; (*b*) W. K. Fife, *Biochem. Biophys. Res. Comm.*, 1967, **28**, 309; (*c*) S. H. D. Ko and F. J. Kézdy, *J. Amer. Chem. Soc.*, 1967, **89**, 7139; (*d*) D. Levine, T. W. Reid, and I. B. Wilson, *Biochemistry*, 1969, **8**, 2374; (*e*) C. Lazdunski and M. Lazdunski, *Eur. J. Biochem.*, 1969, **7**, 294.

[4] H. Barrett, R. Butler, and I. B. Wilson, *Biochemistry*, 1969, **8**, 1042.

[5] (*a*) E. B. Starkey, *Org. Synth.*, 1943, Coll. Vol. 2, 225; (*b*) E. B. Starkey, *J. Amer. Chem. Soc.*, 1937, **59**, 1479; (*c*) G. P. Hager and E. B. Starkey, *J. Amer. Pharm. Soc.*, 1941, **30**, 65.

[6] G. O. Doak and L. D. Freedman, *J. Amer. Chem. Soc.*, 1952, **74**, 753; (*b*) 1951, **73**, 5658; (*c*) 1953, **75**, 4905.

[7] (*a*) A. Roe, *Org. Reactions*, 1949, **5**, 203; (*b*) G. Schiemann and R. Pillarsky, *Ber.*, 1931, **64**, 1340; (*c*) G. M. Kosolapoff, *J. Amer. Chem. Soc.*, 1948, **70**, 3465.

a mixture of chlorobenzene (1 mol), phosphorus trichloride (3 mol), and aluminium trichloride (1 mol) for 4 h. Excess of phosphorus trichloride was removed under reduced pressure and the residual mass diluted with 2 volumes of tetrachloroethane and then treated with dry chlorine gas (with good agitation and cooling) until absorption was complete (*ca.* 2 h). Excess of ethanol (5 mol) was then added to the stirred and cooled (<15 °C) mixture and the hydrogen chloride removed under vacuum. The mixture was treated with ice and dilute HCl, and the organic layer was separated, washed three times with water, and dried. The solvent was evaporated and the ester distilled under reduced pressure. The ester was hydrolysed by refluxing with excess of concentrated HCl (12 h) and the acid which crystallised on cooling was recrystallised from ethyl acetate. Phosphate analysis showed a content of less than 0·005% inorganic phosphate.

Phenylphosphonic acid was prepared by refluxing iodobenzene (0·22 mol), copper powder (0·22 mol), and triethyl phosphite (1 mol) at 150 °C for 24 h.[8] The residual copper was filtered off and the product distilled under reduced pressure. The fraction of b.p. 88—93 °C at 0·01 mmHg was collected and redistilled to give the diethyl ester (31% yield), b.p. 89—91 °C at 0·01 mmHg. The ester was hydrolysed by refluxing for 12 h with a six-fold excess of concentrated HCl. The acid crystallised on cooling of the hydrolysate and was recrystallised from water to give a 58% yield from the ester. Inorganic phosphate content was less than 0·005%.

4-Methylphenylphosphonic acid was prepared as for the phenylphosphonic acid by use of 4-iodotoluene. The diethyl ester distilled over the range 110—112 °C at 0·1 mmHg (lit.,[8] b.p. 110 °C at 0·1 mmHg) and was obtained in 62% yield. Hydrolysis to acid gave a 45% yield of material with less than 0·005% of inorganic phosphate.

4-Methoxyphenylphosphonic acid was prepared from 4-anisidine *via* 4-methoxybenzenediazonium tetrafluoroborate by the method employed for 4-nitrophenylphosphonic acid. The diazonium salt was converted into the acid by Kosolapoff's modification [9a] of Doak and Freedman's method.[6a] The acid was recrystallised from water and had less than 0·005% of free inorganic phosphate.

4-Aminophenylphosphonic acid was prepared by mixing 4-bromophenylphosphonic acid (6 g crude), cuprous oxide (3·6 g), and 35% aqueous ammonia (60 ml). Reaction commenced immediately and the mixture was refluxed for 2 h until all the red colour had faded and was replaced by a blue solution. Copper was precipitated with hydrogen sulphide and the crude acid product obtained as a precipitate by adjusting the pH to 4 with concentrated HCl. The precipitate was dissolved in acid, treated with animal charcoal, filtered, and precipitated as before. The acid obtained in 23% yield had a free inorganic phosphate content of less than 0·05%.

4-Nitrobenzylphosphonic acid was prepared by the general method of Kreutzkamp and Cordes.[9b] 4-Nitrobenzyl bromide (0·05 mol) and triethyl phosphite (0·1 mol) were heated until reaction started and then at 150 °C for 2 h. The yellow solution was distilled *in vacuo* and a forerun of triethyl phosphite obtained (b.p. 24—60 °C at 0·2 mmHg). The product diester distilled from 183—187 °C at 0·5 mmHg (lit.,[10] b.p. 148—153 °C at 0·1 mmHg). The diester was hydrolysed with concentrated HCl (30 ml) for 12 h at reflux. Crystals of the acid deposited on cooling and were recrystallised from boiling water.

3-Nitrobenzylphosphonic acid was prepared from the alcohol. 3-Nitrobenzyl alcohol (0·1 mol) was dissolved in toluene (30 ml) cooled in an ice–salt bath. Phosphorus tribromide (1·12 mol) was added slowly with stirring, the solution kept for 30 min at 0 °C, and finally refluxed for 4 h. On cooling, an orange precipitate appeared and the supernatant solution was poured on ice (100 g). The toluene layer was separated, washed with dilute H_2SO_4 (2 × 10 ml), water, 10% aqueous $NaHCO_3$, and water, and dried ($MgSO_4$). The solution was concentrated to 20 ml, mixed with light petroleum (b.p. 40—60 °C) (60 ml), and cooled in ice. The bromide crystallised and had m.p. 57—58 °C (lit.,[11] 57—58°). The diester prepared as for the 4-nitrobenzylphosphonic acid was an orange oil, b.p. 179—180 °C at 0·1 mmHg. The phosphonic acid obtained from the diester by hydrolysis with 5N-HCl was recrystallised from ethyl alcohol–benzene.

4-Chlorobenzylphosphonic acid was prepared from 4-chlorobenzyl chloride as for the 4-nitro-derivative. The diester was an oil, b.p. 137—138 °C at 0·01 mmHg. The phosphonic acid was obtained in 95% overall yield from hydrolysis in concentrated HCl and was recrystallised from water.

Benzylphosphonic acid was prepared from benzyl bromide as for the 4-nitro-derivative. The diester was an oil, b.p. 154 °C at 144 mmHg. The phosphonic acid from acid hydrolysis of the diester was recrystallised from ethyl acetate.

4-Methylbenzylphosphonic acid was prepared *via* the bromide from the alcohol.[12] The diester was an oil, b.p. 120—122 °C at 0·1 mmHg (lit.,[13] 130—132 °C at 2 mmHg). The phosphonic acid obtained by acid hydrolysis of the diester was recrystallised from boiling water.

4-Methoxybenzylphosphonic acid was obtained from the alcohol *via* the bromide [14] as for the 3-nitro-derivative. The diester, an oil, had b.p. 151 °C at 0·1 mmHg and the acid obtained by acid hydrolysis was recrystallised from boiling water.

4-Nitrophenyl phosphate was prepared as described previously.[1]

4-Acetylphenyl phosphate was prepared from 4-hydroxyacetophenone as described for the 4-nitrophenyl ester.[1] The ester was recrystallised as white needles from acetone–benzene.

4-Methylphenyl phosphate prepared from 4-cresol by the above method was recrystallised from chloroform.

4-Formylphenyl phosphate prepared from 4-hydroxybenzaldehyde by the above method was recrystallised as pale yellow needles from ethyl acetate–light petroleum (b.p. 40—60 °C).

4-Chlorophenyl phosphate was obtained as the dicyclohexylammonium salt from a preparation based on that for the 4-nitro-derivative.[1] The reaction mixture, instead of being neutralised with sodium carbonate, was treated with

[8] P. Tavs and F. Korte, *Tetrahedron*, 1967, **23**, 4677.
[9] (*a*) G. M. Kosolapoff, *J. Amer. Chem. Soc.*, 1953, **75**, 4901; (*b*) N. Kreutzkamp and G. Cordes, *Arch. Pharm.*, 1961, **294**, 49.
[10] G. M. Kosolapoff, *J. Amer. Chem. Soc.*, 1949, **71**, 1876.
[11] J. F. Norris, M. Watt, and R. Thomas, *J. Amer. Chem. Soc.*, 1916, **38**, 1077.
[12] J. I. G. Cadogan, V. Gold, and D. P. N. Satchell, *J. Chem. Soc.*, 1955, 561.
[13] B. P. Ludovkin and B. A. Arbuzov, *Izvest. Akad. Nauk S.S.S.R., Otdel. kihm. Nauk*, 1950, 56 (*Chem. Abs.*, 1951, **45**, 7002).
[14] W. Q. Board and C. R. Hauser, *J. Org. Chem.*, 1960, **25**, 334.

cyclohexylamine (to pH 9). The product was recrystallised from hot water.

Structures of substrates and inhibitors were confirmed by i.r. and n.m.r. spectroscopy (we thank Mr. P. Simmonds for running these spectra on a Perkin-Elmer model R-10 machine). Analytical and physical data are in Table 1.

Trishydroxymethylaminomethane was obtained from the Sigma Chemical Co. as Trizma Base. Other materials were of analytical reagent grade and glass-distilled water was used throughout.

TABLE 1

Analytical and physical properties of substrates and inhibitors [a]

Compound	M.p. (lit. m.p.) (°C)	Found (%) C [g]	H	N	Formula	Calc.(%) C	H	N
Phenylphosphonic acid								
(IIa)	193—195 (d)	35·2	3·4	6·6	$C_6H_6NO_5P$	35·5	3·0	6·9
(IIb)	141—143	35·6	3·1	6·8	$C_6H_6NO_5P$	35·5	3·0	6·9
(IIc)	133—134 (136—137) [b]	37·5	3·3		$C_6H_6ClO_3P$	37·4	3·1	
(IId)	193—194 (187—188) [b,c]	37·6	3·3		$C_6H_6ClO_3P$	37·4	3·1	
(IIe)	159—160 (158—159) [c,d]	45·7	4·6		$C_6H_7O_3P$	45·6	4·4	
(IIf)	288 (d) (290, d) [b]	41·0	4·5	7·9	$C_6H_8NO_3P$	41·6	4·6	8·1
(IIg)	188—189 (189) [e]	48·3	5·4		$C_7H_9O_3P$	48·8	5·2	
(IIh)	173—174 (179) [d]	45·0	5·2		$C_7H_9O_4P$	44·7	4·8	
(IIi)	254—256 (245) [e]	40·5	4·7	7·8	$C_6H_8NO_3P$	41·6	4·6	8·1
Benzylphosphonic acid								
(IIIa)	225—226 (d) (226) [e]	39·0	4·2	6·4	$C_7H_8NO_5P$	38·7	3·7	6·5
(IIIb)	177—179 (179) [f]	38·6	3·8	6·3	$C_7H_8NO_5P$	38·7	3·7	6·5
(IIId)	168—171	40·8	4·1		$C_7H_8ClO_3P$	40·7	3·9	
(IIIe)	168—170 (169—170) [d]	49·1	5·6		$C_7H_9O_3P$	48·8	5·2	
(IIIg)	185—186 (185—186) [g]	51·7	6·4		$C_8H_{11}O_3P$	51·6	5·9	
(IIIh)	204—206	47·6	5·7		$C_8H_{11}O_4P$	47·5	5·4	
Phenyl phosphate								
(Ik)	126—128	44·4	4·0		$C_8H_9O_5P$	44·4	4·2	
(Ig)	114—115	43·7	4·9		$C_7H_9O_4P$	44·7	4·8	
(Ij)	140—142	40·5	3·9		$C_7H_7O_5P$	41·6	3·5	
(Id)	200—202 [h]	52·7	7·3	6·8	$C_6H_6ClO_4P$	53·1	7·9	6·9

[a] Analyses were by M. G. Powell of this laboratory using a Hewlett–Packard-185 Analyser. M.p.s were determined on a Kofler 'Thermospan' instrument. [b] G. M. Kosolapoff and W. F. Huber, *J. Amer. Chem. Soc.*, 1947, **69**, 202. [c] H. Bauer, *J. Amer. Chem. Soc.*, 1941, **63**, 2137. [d] Ref. 6*a*. [e] Ref. 10. [f] Ref. 9*b*. [g] B. P. Ludovkin and B. A. Arbuzov, *Izvest. Akad. Nauk S.S.S.R., Otdel khim. Nauk*, 1950, 56 (*Chem. Abs.*, 1951, **45**, 7002). [h] Isolated as dicyclohexylammonium salt. [i] We have continuing difficulties with carbon analyses for phosphorus(v) compounds in our CHN analyser (see footnote *a*) and are seeking a reliable phosphorus(v) standard. Where carbon is in doubt we have mass-spectral, n.m.r., t.l.c., and m.p. evidence for purity and identity.

Alkaline phosphatase was isolated from cultures of *Escherichia coli* as described.[1]

Kinetics.—The kinetic techniques were as before.[1] All rate constants were determined by spectroscopic measurements (Unicam SP 800 or Beckman DBG) and the appropriate wavelengths and extinction coefficients are in

TABLE 2

Spectral data for substrate hydrolyses [a]

Substrate	Wavelength/nm	Extinction change
4-Acetyl	325	11,200
4-Formyl	330	19,400
4-Chloro	290	756
4-Methyl	285	1140

[a] pH 8·00, 0·1M ionic strength, 25 °C 0·1M-trishydroxymethylaminomethane buffer.

Table 2. Michaelis–Menten parameters were obtained from rate data by Wilkinson's method [15] and a programme written in 'Basic'.* The same programme was used to obtain parameters for data depending on concentration according to a kinetic law similar to that of Henri. Inhibitor constants were measured at varying inhibitor concentrations by use of 4-nitrophenyl phosphate as substrate and taking the average value for K_i.

A phosphate test by the Lowry–Lopez method [16] indicated the presence of less than 0·015% of free inorganic phosphate in each of the phenyl phosphate esters. This represents for the most concentrated solutions employed (*ca.* 5×10^{-5}M substrate) a concentration of $7·5 \times 10^{-9}$M-inorganic phosphate. This is far below the inhibition constant of phosphate [*ca.* $(2—6) \times 10^{-6}$ mol l^{-1}] [1,17,18a] and thus has no effect on the kinetic results.

RESULTS

Hydrolysis of phenyl phosphates catalysed by alkaline phosphatase obeyed Michaelis–Menten kinetics [equation (1)]. A typical plot of reciprocal velocity against re-

$$\text{Rate} = [E][S]k_0/([S] + K_m) \qquad (1)$$

ciprocal concentration of substrate (4-formylphenyl phosphate) is shown in Figure 1. Repeated u.v. spectrum scan experiments for the hydrolysis of the phenyl phosphates all showed good isosbestic wavelengths indicating beyond reasonable doubt a 1 : 1 stoicheiometry. Values for k_0 and K_m are in Table 3.

* Copies of this programme are available from the senior author.

[15] G. N. Wilkinson, *Biochem. J.*, 1961, **80**, 324.

[16] O. H. Lowry and J. A. Lopez, *J. Biol. Chem.*, 1946, **87**, 421.

[17] M. L. Schlesinger and K. Barrett, *J. Biol. Chem.*, 1965, **240**, 4284.

[18] (*a*) T. W. Reid and I. B. Wilson, *Biochemistry*, 1971, **10**, 380; (*b*) W. N. Aldridge, T. E. Barman, and H. Gutfreund, *Biochem. J.*, 1964, **92**, 23c.

Substituted benzylphosphonic acids were all inhibitors of the enzyme possessing inhibition constants varying from 10^{-2} to 10^{-3} mol l^{-1} (Table 3). Competitive inhibition

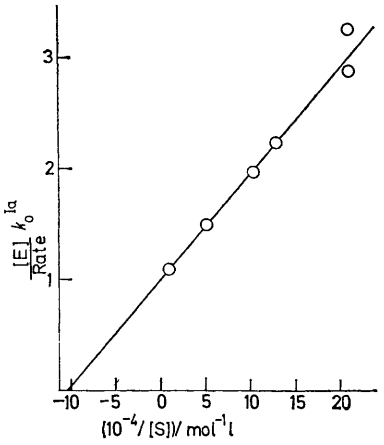


FIGURE 1 Typical kinetic results for substrates: 4-formylphenyl phosphate, pH 8·00, 0·1M-trishydroxymethylaminomethane, 0·1M ionic strength, 25 °C

TABLE 3

Michaelis–Menten parameters for some substrates of alkaline phosphatase and inhibition constants of some inhibitors [a]

Compound	k_0(rel) [b] s^{-1}	10^3K_i or 10^5K_m mol l^{-1}	$10^{-3}k_0/K_m$ l mol^{-1} s^{-1} [c]
Phenyl phosphate			
(Ia) [d]	1·0	1·2	89
(Ib) [d]	1·01	1·87	54
(Ik)	1·02 ± 0·05	1·46 ± 0·18	69·9 ± 1·1
(Ij)	1·00 ± 0·02	0·906 ± 0·047	110·5 ± 7·7
(Id)	1·05 ± 0·02	2·86 ± 0·21	36·7 ± 2·4
(Ie) [d]	1·02	2·09	35·2
(Ig)	1·06 ± 0·03	4·76 ± 0·41	22·2 ± 1·8
Phenylphosphonic acid			
(IIa)		549 ± 99	
(IIb)		329 ± 100	
(IIc)		1530 ± 220	
(IId)		322 ± 149	
(IIe)		1230 ± 240 [h]	
(IIf) [g]		286 ± 30(K_i)	3580 ± 500(K_i')
(IIi) [g]		408 ± 40(K_i)	1990 ± 200(K_i')
(IIg)		1020 ± 260	
(IIh)		1720 ± 180	
Benzylphosphonic acid			
(IIIa)		1350 ± 40	
(IIIb)		80·4 ± 5·4	
(IIId)		1760 ± 260	
(IIIe)		1210 ± 90	
(IIIg)		3430 ± 330	
(IIIh)		1220 ± 250	

[a] pH 8·00, 0·1M ionic strength, 25 °C, 0·1M-trishydroxymethylaminomethane buffer. [b] An error is noted in Table 5 of ref. 1. [c] Value of k_0 is relative to that for 4-nitrophenyl phosphate; for the enzyme preparation used here the 'absolute' value for k_0 is 16·3 ± 0·4 s^{-1} on the assumption of molecular weight of 86,000; the enzyme thus showed about a third of the specific activity of other preparations. [d] Results from ref. 1. [e] Relative to k_0 = 1 s^{-1} (arbitrary) for 4-nitrophenyl phosphate (footnote *c*). [f] Concentration of aryl phosphates 10^{-5} to 10^{-4}M; enzyme concentration at 0·00562 mg ml^{-1} for all experiments. [g] Interpretation of K_i and K_i' is discussed in the Results section. [h] The value given in Table 5 of ref. 1 is in slight error.

[equation (2)] was observed for these inhibitors and a typical double-reciprocal plot is illustrated in Figure 2 for

$$\text{Rate} = [E][S]k_0/\{[S] + K_m^0([I]/K_i + 1)\} \quad (2)$$

4-methylbenzylphosphonic acid. The value of K_m [equation (1)] at an inhibitor concentration [I] of zero is K_m^0; K_i is defined as [I][E]/[IE].

Substituted phenylphosphonates were also found to be competitive [equation (2)] inhibitors of the enzyme (Figure 2) and inhibition constants varied from 10^{-3} to 10^{-2} mol l^{-1}.

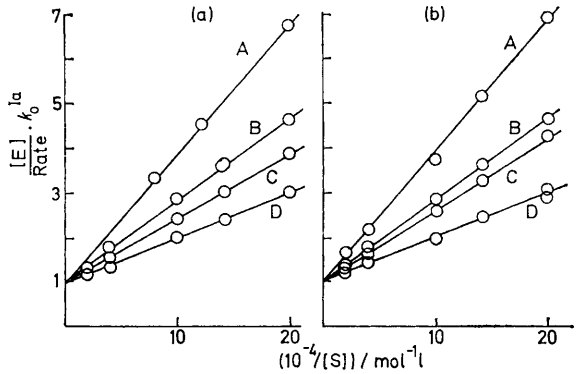


FIGURE 2 Typical kinetic results for inhibitors at pH 8·00, 0·1M-trishydroxymethylaminomethane, 0·1M ionic strength, 25 °C, 4-nitrophenyl phosphate (Ia) used as assay substrate: (a) 4-methylphenylphosphinic acid (IIg); [inhibitor] = 5 × 10^{-2}M, 3 × 10^{-2}M, 1·25 × 10^{-2}M, and 0 from top to bottom; (b) 4-methylbenzylphosphonic acid (IIIg); inhibitor concentrations 2 × 10^{-2}M, 10^{-2}M, 5 × 10^{-3}M, and 0 from top to bottom

The 3- and 4-amino-substituted phenylphosphonates exhibited *mixed* inhibition and the apparent Michaelis–Menten parameter K_m obeyed equation (3) [19] which is

$$K_m = K_m^0([I]/K_i + 1)/([I]/K_i' + 1) \quad (3)$$

derived from the simplest scheme involving the equilibria (4). Equation (3) can be rearranged to give equation (5)

$$E + I \rightleftharpoons EI \qquad K_i = [E][I]/[EI] \quad (4a)$$

$$ES + I \rightleftharpoons ESI \qquad K_i' = [ES][I]/[ESI] \quad (4b)$$

$$E + S \rightleftharpoons ES \qquad K_s = [E][S]/[ES] \quad (4c)$$

which enables values of K_i and K_i' to be obtained from data for K_m^0, K_m, and [I]. The fit of the data to equation (5) is illustrated for 3-aminophenylphosphonic acid in Figure 3.

$$(K_m - K_m^0)/(K_m \cdot [I]) = K_m^0/(K_m \cdot K_i) - 1/K_i \quad (5)$$

Hammett plots of k_0/K_m for substituted phenylphosphates, and K_i for substituted benzylphosphonates and for substituted phenylphosphonates (the competitive components, K_i, being used for the aminophenyl inhibitors) are illustrated in Figures 4—6. The correlation for substrates was good provided σ^- was used ($\rho = +0{\cdot}43$, $r = 0{\cdot}975$); with σ a poorer correlation exists ($\rho = 0{\cdot}56$, $r = 0{\cdot}803$). A poor correlation was observed between K_i and σ (or σ^-) for the inhibitors.

The enzyme-catalysed hydrolysis of 4-nitrophenyl phosphate was found to be strongly dependent on trishydroxymethylaminomethane buffer concentration in agreement

[19] A. Williams, 'Introduction to the Chemistry of Enzyme Action,' McGraw-Hill, London, 1969.

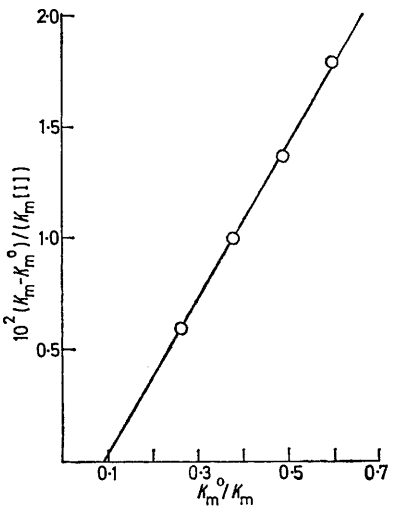


FIGURE 3 Kinetic results for inhibition by the inhibitor 3-aminophenylphosphonic acid (IIf), pH 8·00, 0·1M-trishydroxymethylaminomethane, 0·1M ionic strength, 25 °C, 4-nitrophenyl phosphate (Ia) as substrate.

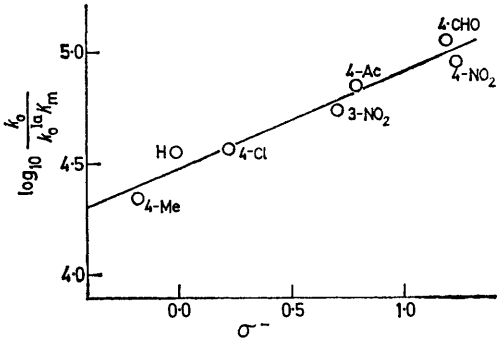


FIGURE 4 Dependence on σ^- of $k_0/(K_m \cdot k_0^{Ia})$ for phenyl phosphates (I). Data from Table 3, σ values from G. B. Barlin and D. D. Perrin, *Quart. Rev.*, 1966, **20**, 75 [4-formyl group from J. J. Ryan and A. A. Humffray, *J. Chem. Soc.* (*B*), 1966, 842]. Line is theoretical (see Results section). The ratio k_0/k_0^{Ia} is the measured quantity; k_0^{Ia} does not affect sensitivity to σ

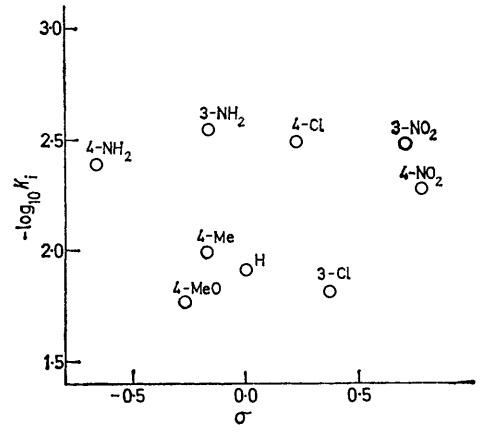


FIGURE 5 Dependence on σ of K_i for phenylphosphonates (II). Data from Table 3 and σ values as for Figure 4

with the work of Wilson and his colleagues.[20] The catalytic rate constant k_0 was found to increase towards a saturation level (Figure 7). The value of k_0 was determined from reaction at $1{\cdot}06 \times 10^{-3}$M-substrate concentration and halving the concentration did not affect the rate; thus

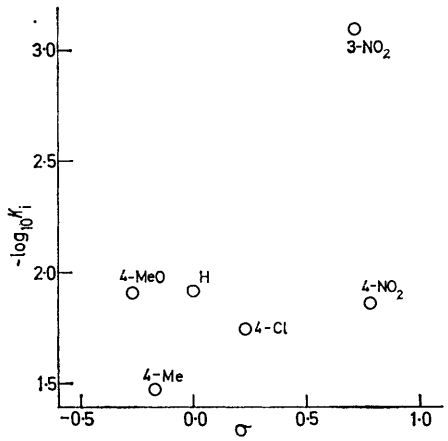


FIGURE 6 Dependence on σ of K_i for benzylphosphonic acids (III). Data from Table 3 and σ values as in Figure 4

division of the velocity of the reaction by enzyme concentration gave k_0. The dependence of k_0 on trishydroxymethylaminomethane concentration fitted a Michaelis–Menten type equation with maximal $k_0 = 5{\cdot}2 \pm 0{\cdot}11$ (relative to k_0 for the substrate at a trishydroxymethylaminomethane concentration of 0·1M; see footnote *c* of Table 3). The concentration of trishydroxymethylaminomethane for half maximal k_0 is $0{\cdot}88 \pm 0{\cdot}03$M.

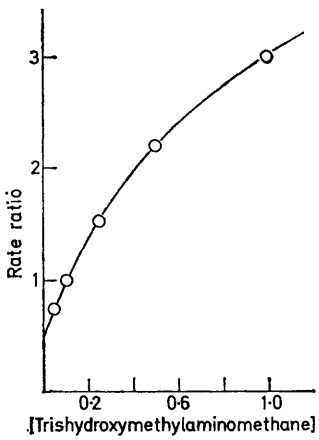


FIGURE 7 Dependence on trishydroxymethylaminomethane concentration of the ratio of k_0 to k_0^{Ia} with 0·1M-trishydroxymethylaminomethane at ionic strength 1·0M, 25 °C, and pH 8·00. Line is theoretical from parameters given in the Results section

In order to check whether a variation in trishydroxymethylaminomethane concentration affected K_s, the binding constant for substrate with enzyme, we determined the inhibition constant (K_i) for phenylphosphonic acid at a variety of trishydroxymethylaminomethane buffer concentrations. K_m for a substrate is a much more complicated function than is an inhibition constant. The value of K_i rose as trishydroxymethylaminomethane buffer concentration was increased (Figure 8) and the variation obeyed a

Michaelis–Menten type law with $K_{i(max.)} = 5{\cdot}12 \pm 0{\cdot}67 \times 10^{-2}$ mol l^{-1} and trishydroxymethylaminomethane concentration for half maximal $0{\cdot}47 \pm 0{\cdot}13$M.

DISCUSSION

A problem common to most hydrolytic enzymes where a nucleophile acts in concert with a base is whether the enzyme acts as a zwitterion ($H\overset{+}{B}EO^-$) or as a neutral species (BEOH). This problem has been solved satisfactorily for papain and for chymotrypsin (which act as neutral species) by the use of ' diffusion ' arguments.[21] Alkaline phosphatase is phosphorylated by 4-nitrophenyl phosphate with a bimolecular rate constant (see Table 3) greater than 10^6 l mol^{-1} s^{-1} at 25 °C and pH 8. Thus the rate of phosphorylation is greater than $10^6[BEOH][S]$ which is equivalent to $10^6 K_a'/K_a''[H\overset{+}{B}EO^-][S]$. Thus if the dissociation constants of $H\overset{+}{B}E$ and EOH are respectively 10^{-7} (imidazolyl) and 10^{-14} (alcohol) for K_a'

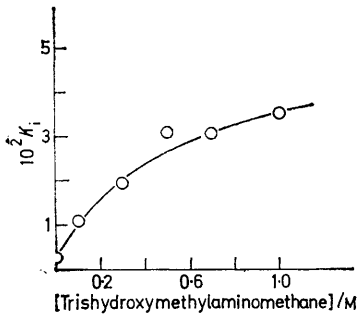


FIGURE 8 Dependence of K_i for phenylphosphonic acid on trishydroxymethylaminomethane concentration at ionic strength 1·0M, 25 °C, and pH 8·00. Line is theoretical from parameters given in the Results section

and K_a'' then zwitterion and substrate react together with a rate constant greater than 10^{13} l mol^{-1} s^{-1} which is higher than the maximal rate constant attainable for enzyme systems (*ca.* 10^9 l mol^{-1} s^{-1}) owing to diffusion becoming rate-limiting.[22] A similar argument can be applied to the hydrolysis of the phospho-enzyme where a number of studies have indicated a rate constant of the order of 10^2 s^{-1} at pH 8.[18] If the water component were acting as hydroxide ion (this is the microscopic reverse of the forward zwitterion mechanism) then the protonated enzyme species ($H\overset{+}{B}E$–O–PO_3^{2-}) will react with hydroxide with a rate constant *ca.* 10^9 l mol^{-1} s^{-1}. This is close to the diffusion-controlled rate constant.

In concert with other studies the value of the Michaelis–Menten parameter (k_0) for substituted phenyl phosphates is substantially constant.[1,3a,23] This constancy has been ascribed to (*a*) rate-determining decomposition of a phospho-enzyme intermediate or (*b*) a rate-determining isomerisation between two forms of enzyme. Our previous results [1] are in accord with either of these explanations as substrates which should possess lower phosphorylation rate constants than 4-nitrophenyl phosphate such as anilido, ethyl, methyl, isopropyl, n-butyl, and benzyl phosphates which show a smaller k_0 consistent with a changeover in a rate-limiting step. In case (*a*) it is dephosphorylation which becomes rate-limiting as leaving-group ability increases and in (*b*) it is the isomerisation.

We have shown in a previous paper that k_0/K_m can be regarded as the bimolecular rate constant for phosphorylation of free enzyme by free substrate.[1] Bender and Kézdy [21d] also show that this parameter does not possess any contribution due to non-productive binding modes. Thus the variation of k_0/K_m (k_2/K_s) with substituent should provide an idea of the electronic requirements during both binding and phosphorylation in the enzyme. The Hammett correlation for k_0/K_m is better with σ^- than with σ, pointing to considerable phenolate-ion character in the transition-state of the rate-limiting step. Reaction of hydroxide ion at the phosphorus in a number of phenyl phosphorus esters [2b–i] has a ρ value of *ca.* 1·2—1·6 to change in the substituent. The sensitivity to substituent for phosphorylation of alkaline phosphatase should be higher than for hydroxide since the enzyme reacts in its neutral form analogous to water. Reaction of water with substituted phenyl diphenylphosphinates (catalysed as a general base by imidazole) has a ρ value of 2·88.[2b] Reaction of water with aryl formates has a ρ value of 1·58 compared with that of 1·09 for reaction of hydroxide ion.[20b] Thus the sensitivity of phosphorylation to change in substituent on the phenyl nucleus (+0·43) is considerably lower than expected for the addition–elimination mechanism. We interpret the low Hammett sensitivity for k_0/K_m to the incursion of an electrophilic component in either the phosphorylation step or in binding of substrate to enzyme. Previous hypotheses concerning binding of the monophosphate as a ligand in the co-ordination sphere of a zinc(II) chelate in the enzyme are in accord with the low Hammett sensitivity.

Bunton [24] and Kirby [25] and their co-workers find that the elimination–addition mechanism for hydrolysis of the monoanion of aryl monophosphates has a Brønsted β of *ca.* +0·3 when compared with the pK_a of the departing phenol leaving group. This corresponds to a ρ = *ca.* 0·6 by use of the ρ for phenol ionisation (*ca.* 2·1) [26] and is in excess of the value for the addition–elimination

[20] (*a*) J. Dayan and I. B. Wilson, *Biochem. Biophys. Acta*, 1964, **81**, 620; (*b*) I. B. Wilson, J. Dayan, and K. Cyr, *J. Biol. Chem.*, 1964, 239, 4182; (*c*) H. Neumann, *Eur. J. Biochem.*, 1969, **8**, 164.

[21] (*a*) E. C. Lucas and A. Williams, *Biochemistry*, 1969, **8**, 5125; (*b*) A. Williams, E. C. Lucas, and K. T. Douglas, *J.C.S. Perkin II*, 1972, 1493; (*c*) M. L. Bender and F. J. Kézdy, *J. Amer. Chem. Soc.*, 1964, **86**, 3704; (*d*) *Ann. Rev. Biochem.*, 1965, **34**, 49.

[22] M. Eigen and G. G. Hammes, *Adv. Enzymol.*, 1963, **25**, 1.

[23] (*a*) L. A. Heppel, D. R. Harkness, and R. J. Hilmoe, *J. Biol. Chem.*, 1962, **237**, 841; (*b*) H. Neumann, L. Boross, and E. Katchalski, *J. Biol. Chem.*, 1967, **242**, 3142.

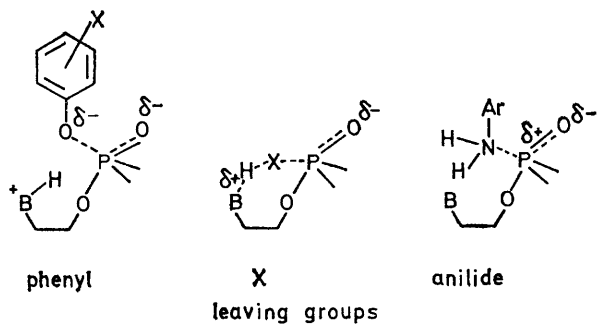
[24] C. A. Bunton, E. J. Fendler, E. Humeres, and Y-U. Yang, *J. Org. Chem.*, 1967, **32**, 2806.

[25] (*a*) A. J. Kirby and A. G. Varvoglis, *J. Amer. Chem. Soc.*, 1967, **89**, 415; (*b*) A. J. Kirby and A. G. Varvoglis, *ibid.*, 1966, **88**, 1823; (*c*) A. J. Kirby and W. P. Jencks, *ibid.*, 1965, **87**, 3209.

[26] J. E. Leffler and E. Grunwald, ' Rates and Equilibria of Organic Reactions,' Wiley, New York, 1963, p. 178.

mechanism shown to predominate in phosphorylation of the enzyme.[1] The fit of k_0/K_m to σ is not so much poorer than to σ^- so that mechanistic conclusions from this observation (but not from the sensitivity) are not too firm. The fit to σ^- might not seem to agree with the main conclusion that electrophilic catalysis is involved. However, the latter effect is suggested to come from the action (of zinc) on the two oxyanions and not from the phenol part of the substrate molecule. The phenol leaving ability can act as a probe for the electron density at the phosphorus but still depart as a phenolate anion in the transition state. If we can rely on the σ^- dependence being meaningful then the phenolate-ion character of the transition state (deduced from this) predicts that the rate-limiting step in phosphorylation is *either* breakdown of the quinquecovalent intermediate *or* addition of nucleophile concerted with elimination of phenolate anion (in an earlier paper we eliminated the elimination–addition mechanism[1]); of these two possibilities the literature favours the former for ester hydrolysis.[27] There is some precedent for the observation of a σ^- dependence in aryl phosphate hydrolysis; Kirby and Younas[28] report that the hydrolysis of diaryl phosphate monoanion by water involves an addition–elimination mechanism and exhibits a σ^- Hammett relationship. The hydrolysis of aryl monophosphate dianions also exhibits a σ^- dependence[25] but this is due to an elimination–addition mechanism.

The negative ρ value observed for phosphorylation by substituted phenyl phosphoramidates[1] probably does not arise from electrophilic assistance by the zinc and general acid catalysis of the breakdown of a quinquecovalent intermediate is a more likely explanation. General acid catalysis of this step for the phenyl phosphate substrates is not likely owing to the exceptionally good leaving ability of the phenolate anion.



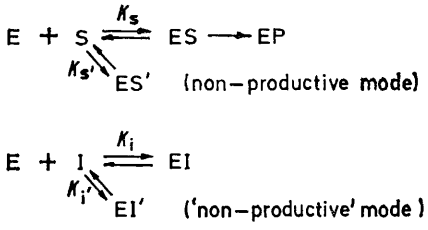
SCHEME 2

Arguments similar to these have been presented by us for a number of hydrolases.[29]

[27] (*a*) C. A. Bunton, *Accounts Chem. Res.*, 1970, **3**, 257; (*b*) F. H. Westheimer, *ibid.*, 1968, **1**, 70.
[28] A. J. Kirby and M. Younas, *J. Chem. Soc.* (*B*), 1970, 510.
[29] (*a*) A. Williams and G. Woolford, *J.C.S. Perkin II*, 1972, 272; (*b*) A. Williams, E. C. Lucas, and A. R. Rimmer, *ibid.*, p. 621; (*c*) A. Williams, *Biochemistry*, 1970, **9**, 3383.
[30] (*a*) H. N. Fernley and P. G. Walker, *Nature*, 1966, **212**, 1435; (*b*) *Biochem. J.*, 1969, **111**, 187.

We suggest that a gradation in mechanism exists from acid-catalysed departure of the poor amine (anion) leaving group to the non-catalysed departure of the phenolate anion. Scheme 2 illustrates the decomposition of the quinquecovalent intermediate for a range of leaving groups. As the leaving-group ability decreases (from phenolate through alcoholate to amine) so the proton from the conjugate acid (BH^+) is progressively more bound to the leaving group in the transition state.

It is not possible to assign the electrophilic component to either phosphorylation (k_2) or binding (K_s) steps except by the use of special kinetic techniques to measure these parameters individually. The value of K_i was measured for a series of inhibitors (II) and (III) which are substrate analogues. Figures 5 and 6 indicate that there is no simple relationship between structure



SCHEME 3

and inhibition constant. Data for the benzyl series are on the whole less scattered presumably because the structural variation is more distant from the site of binding. The ratio $k_2 : K_s$ does not possess a contribution from non-productive binding modes but K_i can include a contribution from binding analogous to both productive *and* non-productive binding of the substrate (Scheme 3). Thus it is not possible to equate variations in K_i with variations in K_s as the former may occur in the ' non-productive ' modes.

Effect of Trishydroxymethylaminomethane on the Enzyme Parameters.—Wilson and his co-workers[20] observed that the rate of release of 4-nitrophenol from its phosphate ester was accelerated by increasing the concentration of trishydroxymethylaminomethane; the concomitant formation of *O*-phosphoryltrishydroxymethylaminomethane was also observed. These results were interpreted as rate-determining decomposition of a phosphoenzyme intermediate which was accelerated by trishydroxymethylaminomethane acting as a nucleophile. Fernley and Walker[30] indicated that at least for umbelliferyl phosphate dephosphorylation was *not* rate-limiting at pH 8. Wilson's evidence could thus point in a simple scheme to an effect of trishydroxymethylaminomethane on other steps in the mechanism and this is acceptable since this amine is known to complex quite strongly with metal ions similar to zinc(II).[31] The

[31] (*a*) D. E. Allen, D. J. Baker, and R. D. Gillard, *Nature*, 1967, **214**, 906; (*b*) K. S. Bai and A. E. Martell, *J. Inorg. Nuclear Chem.*, 1969, **31**, 1697; (*c*) P. D. W. Boyd, R. J. Pilbrow, and T. D. Smith, *Austral. J. Chem.*, 1971, **24**, 59; (*d*) J. L. Hall, J. A. Swisher, D. G. Brannon, and T. H. Liden, *Inorg. Chem.*, 1962, **1**, 409; (*e*) W. J. O'Sullivan and D. D. Perrin, *Biochemistry*, 1964, **3**, 18.

chelation of trishydroxymethylaminomethane with the zinc ion at the active site on the enzyme should affect the binding of the substrate (K_s) but this parameter is not easily measured; K_i values can be utilised provided we accept the ambiguity over ' non-productive ' binding modes. K_i for phenylphosphonic acid increases with trishydroxymethylaminomethane concentration, obeying a Michaelis–Menten type law with a concentration, presumably equivalent to a dissociation constant for a ligand, of 0·47M at half-maximal K_i. If these results can be transferred to the substrate then K_s, k_2, and k_0 will all vary with amine concentration. Thus Wilson's observations of the effect of trishydroxymethylaminomethane could be reconciled with a rate-limiting phosphorylation step which is modified by chelated trishydroxymethylaminomethane. Our results, however, need not be in contradiction with the main conclusion of a rate-controlling isomerisation of free enzyme.[18a]

Since it is likely that trishydroxymethylaminomethane will bind with different affinities with different metal ions it is possible that this is an explanation of the absence of *trans*-phosphorylation (to the amine) observed by Vallee [32] and Lazdunski [3e] in reactions of the cobalt(II) enzyme.

We thank the S.R.C. for a studentship (to S. G. C.) and the M.R.C. for financial support.

[2/1298 *Received*, 8*th June*, 1972]

[32] (*a*) G. Tait and B. Vallee, *Proc. Nat. Acad. Sci. U.S.*, 1966, **56**, 1247; (*b*) M. Gottesman, R. Simpson, and B. Vallee, *Biochemistry*, 1969, **8**, 3776.