

Zirconium-hydroxide-mediated Alkaline Hydrolysis of Phosphate Esters

By John F. Kennedy,* S. Alan Barker, and John D. Humphreys, Department of Chemistry, The University of Birmingham, Edgbaston, Birmingham B15 2TT

Under mildly alkaline conditions (pH 7—9) the hydrolysis of phosphate monoesters such as 4-nitrophenyl phosphate, glucose 1-phosphate, and glycerol 1-phosphate proceeds slowly, if at all. However, in the presence of gelatinous zirconium hydroxide the rate of hydrolysis of 4-nitrophenyl phosphate is increased by a factor of *ca.* 30, although the rates of hydrolysis of a range of phosphate esters and related compounds such as adenosine mono- and triphosphates, nitrophenyl glycosides, and nitrophenyl sulphates and acetates are not altered. The effects of pH, zirconium hydroxide concentration, phosphate ion, and zirconium phosphate upon this catalysis are described. Titanium(IV) hydroxide exhibited only a slight catalytic effect. A mechanism for the selective catalytic reaction of the zirconium hydroxide is suggested.

THE catalysis of liquid phase reactions by dissolved metal ions is not uncommon; more unusual however, is the catalysis of such reactions by an insoluble metal compound. Such an effect was observed when insoluble zirconium hydroxide suspensions were added to alkaline solutions containing 4-nitrophenyl phosphate, a substrate for the colourimetric determination of alkaline phosphatase [orthophosphoric-monoester phosphohydrolase (alkaline optimum), E.C.3.1.3.1.] activity.¹ The extent of this catalytic effect on 4-nitrophenyl phosphate and other phosphates has been investigated in some detail and the results are now reported.

DISCUSSION

The effect of zirconium(IV) hydroxide on the hydrolysis of 4-nitrophenyl phosphate was first noticed when attempts were being made to measure the enzymic activity of an alkaline phosphatase immobilised on zirconium hydroxide (*cf.* ref. 2*a*). From studies at a series of pH values, it was evident that in each case the

presence of zirconium(IV) hydroxide had an effect upon the hydrolysis of 4-nitrophenyl phosphate anion. The maximum effect in the pH range 7.0—9.0 was exerted at pH 7.0 (Table 1).

The next series of studies was directed towards discovering how general this effect might be; first by testing the related titanium(IV) hydroxide, secondly by testing other phosphate esters, and thirdly by testing other nitrophenyl derivatives. The results of the investigation with titanium(IV) hydroxide showed that this was not nearly as active (Table 2) as zirconium hydroxide. This may be attributable to differences in the molecular structures of the hydroxides (see refs. 2*b* and *c*) and/or their affinities, if any, for the substrate. However the effect was again greater at the lower pH value. The hydrolysis of the other substrates, with the

¹ A. Torriani, *Biochim. Biophys. Acta*, 1960, **38**, 460.

² (a) J. F. Kennedy, S. A. Barker, and J. D. Humphreys, *J.C.S. Perkin I*, 1976, 962; (b) J. F. Kennedy and I. M. Kay, *ibid.*, p. 329; (c) J. F. Kennedy, S. A. Barker, and J. D. Humphreys, *Nature*, 1976, **261**, 242.

exception of glucose 1-phosphate, which occurred to a very small extent in the absence of zirconium(IV) hydroxide, was not influenced to any detectable amount by the addition of zirconium(IV) hydroxide. This was surprising in the case of adenosine triphosphate, since

TABLE 1

Effect of zirconium(IV) hydroxide upon the alkaline hydrolysis of 4-nitrophenyl phosphate as a function of pH

pH	Zr ^{IV} hydroxide added (μmol)	Reaction time (min)	Resultant 4-nitrophenol concentration (μmol cm ⁻³)	Hydrolysis (%)
7.0	0.0	35	0.006	0.07
7.0	2.6	5	0.053	0.66
7.0	2.6	15	0.105	1.31
7.0	2.6	25	0.116	1.46
7.0	2.6	35	0.190	2.38
7.5	0.0	35	0.005	0.06
7.5	2.6	5	0.046	0.58
7.5	2.6	15	0.091	1.15
7.5	2.6	25	0.144	1.80
7.5	2.6	35	0.167	2.08
8.0	0.0	35	0.004	0.05
8.0	2.6	5	0.045	0.56
8.0	2.6	15	0.075	0.94
8.0	2.6	25	0.105	1.31
8.0	2.6	35	0.116	1.45
8.5	0.0	35	0.004	0.04
8.5	2.6	5	0.038	0.48
8.5	2.6	15	0.059	0.74
8.5	2.6	25	0.079	0.98
8.5	2.6	35	0.093	1.16
9.0	0.0	35	0.005	0.07
9.0	2.6	5	0.030	0.37
9.0	2.6	15	0.052	0.65
9.0	2.6	25	0.058	0.73
9.0	2.6	35	0.062	0.78

TABLE 2

Effect of titanium(IV) hydroxide upon the alkaline hydrolysis of 4-nitrophenyl phosphate as a function of pH

pH	Ti ^{IV} hydroxide added (μmol)	Reaction time (min)	Resultant 4-nitrophenol concentration (μmol cm ⁻³)	Hydrolysis (%)
7.0	0.0	35	0.009	0.11
7.0	2.6	5	0.017	0.20
7.0	2.6	15	0.017	0.20
7.0	2.6	25	0.022	0.28
7.0	2.6	35	0.022	0.28
8.0	0.0	35	0.004	0.05
8.0	2.6	5	0.009	0.11
8.0	2.6	15	0.012	0.15
8.0	2.6	25	0.014	0.18
8.0	2.6	35	0.015	0.19
9.0	0.0	35	0.003	0.04
9.0	2.6	5	0.009	0.11
9.0	2.6	15	0.006	0.08
9.0	2.6	25	0.009	0.11
9.0	2.6	35	0.010	0.12

metal ions (Mg²⁺ or Mn²⁺) are a requirement for adenosine triphosphate-dependent reactions, their function being to form chelates with the adenosine triphosphate molecule, thereby increasing its electrophilicity and,

hence, increasing its susceptibility to attack by nucleophilic reagents. It seemed likely that zirconium hydroxide would form a similar chelate and thus promote the hydrolytic reaction, but this was apparently not the case.

Conventionally, 4-nitrophenyl phosphate is hydrolysed in a manner similar to alkyl phosphates,³⁻⁷ and considerable amounts of work on its hydrolysis and on the hydrolysis of other phenyl phosphates have been reported. The hydrolysis in solution is undoubtedly unimolecular.⁸ Our findings could therefore be explained in terms of an acceleration by zirconium(IV) hydroxide of the unimolecular breakdown of the 4-nitrophenyl substrate. However zirconium(IV) hydroxide easily forms complexes with a variety of molecules. Furthermore, it was found that the liberation of 4-nitrophenol from 4-nitrophenyl phosphate was not linear with respect to time in the initial stages of the reaction (0-60 min), but after this time, which depended on the amount of zirconium(IV) hydroxide used, linearity was established (Table 3). The reaction may be interpreted as occurring

TABLE 3

Effect of zirconium(IV) hydroxide upon the alkaline hydrolysis of 4-nitrophenyl phosphate as a function of zirconium(IV) hydroxide concentration

Zr ^{IV} hydroxide added (μmol)	Reaction time (min)	Resultant 4-nitrophenol concentration (μmol cm ⁻³)	Hydrolysis (%)
0	35	0.005	0.06
2.6	5	0.048	0.60
2.6	15	0.073	0.91
2.6	25	0.106	1.32
2.6	35	0.175	2.19
2.6	65	0.204	2.60
2.6	95	0.253	3.16
2.6	125	0.310	3.92
0	35	0.005	0.06
1.3	5	0.024	0.30
1.3	15	0.045	0.56
1.3	25	0.052	0.65
1.3	35	0.075	0.94
1.3	65	0.088	1.10
1.3	95	0.106	1.33
1.3	125	0.121	1.51
0	35	0.005	0.06
0.65	5	0.018	0.22
0.65	15	0.028	0.35
0.65	25	0.040	0.50
0.65	35	0.047	0.59
0.65	65	0.060	0.75
0.65	95	0.076	0.95
0.65	125	0.091	1.04
0	35	0.005	0.06
0.33	5	0.011	0.14
0.33	15	0.014	0.18
0.33	25	0.016	0.20
0.33	35	0.019	0.24
0.33	65	0.027	0.34
0.33	95	0.038	0.48
0.33	125	0.049	0.61

in two distinct stages. Initially the substrate forms a complex with the zirconium(IV) hydroxide, the strength

³ P. W. C. Barnard, C. A. Bunton, D. Kellerman, M. M. Mhala, B. Silver, C. A. Vernon, and V. A. Welch, *J. Chem. Soc. (B)*, 1966, 227.

⁴ A. Désjobert, *Bull. Soc. chim. France*, 1963, 683.

⁵ C. A. Bunton, D. R. Llewellyn, K. G. Oldham, and C. A. Vernon, *J. Chem. Soc.*, 1958, 3574, 3588.

⁶ A. Désjobert, *Bull. Soc. chim. France*, 1947, 14, 809.

⁷ A. J. Kirby and A. G. Varvoglis, *J. Amer. Chem. Soc.*, 1967, 89, 415.

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

of the P-O bond is thereby lowered, and hydrolysis takes place liberating 4-nitrophenol and leaving phosphate ions attached to the zirconium(IV) hydroxide to give a mixed hydroxy-phosphate complex. That this is feasible is demonstrated by the experiment in which inorganic phosphate was added to zirconium(IV) hydroxide (Table 4): 2.6 μmol of zirconium(IV) hydroxide (the amount

TABLE 4

Removal of phosphate ions from solution by zirconium(IV) hydroxide

Phosphate added (μmol)	Phosphate remaining in solution	
	μmol	%
0.114	0.003	2.6
0.228	0.004	1.8
0.456	0.015	3.3
0.912	0.118	12.9

routinely used) was able to combine with *ca.* 0.8 μmol of added phosphate. The reaction slows down as phosphate ions occupy more and more 'sites' on the hydrox-

good 'leaving group' properties of the 4-nitrophenolate ion. Although amine nucleophiles accelerate the hydrolysis of 4-nitrophenyl phosphates, it has been shown by Kirby and Jencks⁸ (and by our own controls) that Tris is not effective in this way. However, as has been pointed out to us, the alternative metaphosphate species may be involved, as is the case for 2,4-dinitrophenyl phosphate.⁹

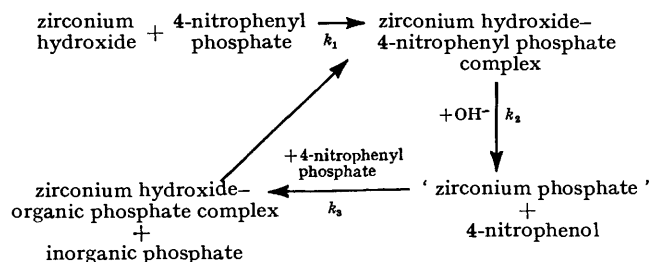
The hydrolysis of phosphate esters may also be catalysed by certain rare-earth hydroxides present in the form of gels.^{10,11} For example, in the region pH 7–10, the rate of hydrolysis of glycerol 1-phosphate is increased by a factor of 10^3 by chelation of an ion such as La^{III} between two oxygen atoms of the α - and β -substituents. This reaction bears a superficial resemblance to the effect of zirconium(IV) hydroxide, but it has been shown that the rate of alkaline hydrolysis of glycerol 1-phosphate is unaffected by the presence of zirconium(IV) hydroxide, and there are further dissimilarities. Although it is probable that the glycerol 1-phosphate is adsorbed on the

TABLE 5

Effect of zirconium(IV) phosphate on the alkaline hydrolysis of 4-nitrophenyl phosphate

Zr ^{IV} hydroxide added (μmol)	Reaction time (min)	Zr ^{IV} phosphate from Zr ^{IV} hydroxide		Zr ^{IV} phosphate from ZrCl_4	
		Resultant 4-nitrophenol concentration ($\mu\text{mol cm}^{-3}$)	Hydrolysis (%)	Resultant 4-nitrophenol concentration ($\mu\text{mol cm}^{-3}$)	Hydrolysis (%)
0	35	0.005	0.06	0.005	0.06
2.6	5	0.014	0.18	0.006	0.08
2.6	15	0.024	0.30	0.009	0.11
2.6	25	0.032	0.40	0.010	0.13
2.6	35	0.040	0.50	0.011	0.14

ide surface, until all available sites are filled. The second mechanism now predominates; inorganic phosphate ligands are replaced by organic phosphate molecules in what is presumably the rate-determining step, and then undergo relatively rapid hydrolysis, liberating 4-nitrophenol and leaving the phosphate attached to the metal hydroxide surface as before. The use of prepared zirconium hydroxy-phosphates demonstrates that these are still capable of catalytic activity (Table 5), especially if prepared from the hydroxide, thus substantiating this proposed mechanism. The mechanism may be summarised as in the Scheme; k_1 and k_2 must both be



SCHEME

greater than k_3 for the initial fast reaction to be observed. That this is so is presumably due to the high affinity of the insoluble zirconium species for phosphate and the

gel, it is the La^{III} in solution and not the La^{III} ion in the gel that is chelated; this is shown by the increase in the rate of hydrolysis observed upon dilution of the gel with water. The reverse is true for zirconium(IV) hydroxide: the rate of hydrolysis of 4-nitrophenyl phosphate decreases as the gel is diluted (Table 3), suggesting that it is the insoluble hydroxide that is responsible, rather than a soluble ionic species as in the lanthanum case. Two maxima in the pH-rate profile for the La^{III} -catalysed reaction were found, one at pH 8.6 and the other at pH 10.4; it is possible that the two species $\text{La}(\text{OH})_2^{2+}$ and $\text{La}(\text{OH})_3^+$ are involved at these two pH values.¹² That intermediate chelate formation occurs is further confirmed by the presence of a substituent containing a nitrogen or oxygen donor atom in the β -position of the ester. Thus, the rate of alkaline hydrolysis of ethyl phosphate is not affected by the presence of lanthanum hydroxide gel, but the rates of alkaline hydrolysis of β -aminoethyl, β -hydroxyethyl, and β -methoxyethyl phosphates are considerably increased.¹³

In view of the foregoing, the effect of zirconium(IV) hydroxide on the hydrolysis of 4-nitrophenyl phosphate appears to represent an entirely separate type of reaction

¹¹ E. Bamann and E. Nowotny, *Chem. Ber.*, 1948, **81**, 451, 455, 463.

¹² E. Bamann and W. D. Mutterlein, *Chem. Ber.*, 1958, **91**, 471.

¹³ W. W. Butcher and F. H. Westheimer, *J. Amer. Chem. Soc.*, 1955, **77**, 2420.

⁹ D. G. Gorenstein, *J. Amer. Chem. Soc.*, 1972, **94**, 2523.

¹⁰ E. Bamann and M. Meisenheimer, *Ber.*, 1938, **71**, 1711.

from that catalysed by rare-earth ions, involving the formation of an insoluble hydroxide complex and not taking place in solution. The structure of the complex formed is also probably different. Zirconium species have a high affinity for phosphate ions and hence it is likely that the complex would involve two rather than just one of the oxygen atoms of the phosphate group.

This reaction, therefore, appears to involve an initial interaction between the insoluble hydroxide and inorganic phosphate ions produced by hydrolysis, followed by heterogeneous catalysis of the hydrolytic reaction by the mixed hydroxy-phosphate complex so produced. The reaction thus seems to be a specific one between the metal hydroxide and 4-nitrophenyl phosphate and in this respect could be regarded as pseudoenzymic. Specific alkaline phosphatases which are active only against 4-nitrophenyl phosphate have recently been described.¹⁴ It is possible therefore that a study of this type of interaction could lead to a better understanding of enzymic reaction mechanisms.

EXPERIMENTAL

Preparation of Zirconium(IV) Hydroxide.—Zirconium(IV) hydroxide was prepared from aqueous 0.65M-zirconium tetrachloride (2.0 cm³; 1.3 mmol) by slowly adding 2.0M-ammonia solution with stirring to give a pH of 7.0. Distilled water was subsequently added to give a total volume of 10.0 cm³ and, after homogenisation, samples of the gelatinous suspension were removed and tested as below.

Effect of Zirconium(IV) Hydroxide on the Alkaline Hydrolysis of 4-Nitrophenyl Phosphate as a Function of pH.—To aqueous 0.4M-solutions of 4-nitrophenyl phosphate (0.5 cm³) in 0.1M-Tris [2-amino-2-(hydroxymethyl)propane-1,3-diol] buffer (2.0 cm³) at pH values between 7 and 9 (achieved by addition of 0.5M-sodium hydroxide before final dilution) and 37 °C, were added samples (0.02 cm³) of zirconium(IV) hydroxide suspension (pH 7.0), and each solution was made up to 10 cm³ with distilled water. The solutions were stirred for varying lengths of time, then centrifuged, and their absorbances measured at 420 nm. The extent of hydrolysis was calculated (Table 1) by using calibration curves constructed for 4-nitrophenol at the pH of measurement of its release in order to obtain data independent of the variation of extinction coefficient of 4-nitrophenol with pH. Samples of a solution of 4-nitrophenol in water (280 mg⁻¹) were diluted in duplicate with 0.1M-Tris buffer previously adjusted to the required pH value, and the absorbance was measured at 420 nm [absorbance (as % of that at pH 9.0 after 40 min), after 5, 40, and 90 min respectively: pH 6.0 8.1, 10.3, and 13.4; pH 7.0 50.4, 50.9, and 50.9; pH 8.0 93.8, 93.8, and 93.8; pH 9.0 100.0, 105.0, and 103.0]. A sample with no added zirconium(IV) hydroxide was used as a control (Table 1). Further controls to ascertain the effect of Tris upon 4-nitrophenyl phosphate were conducted by using the above solutions without the additions of zirconium(IV) hydroxide and without the dilution to 10 cm³. At pH 6–9 the solutions showed no change in absorbance at 420 nm for times up to at least 1 h at 20 °C (the small absorbance noted has been incorporated in the calculations as a background). Simi-

larly a solution of the 4-nitrophenyl phosphate in water at pH 8.0 showed no change with time.

Effect of Titanium(IV) Hydroxide on the Alkaline Hydrolysis of 4-Nitrophenyl Phosphate.—The same procedure as above was adopted, with equivalent amounts of titanium(IV) hydroxide prepared from 15% w/v titanium(IV) chloride in 15% w/v hydrochloric acid, in place of zirconium(IV) hydroxide. The results were obtained in a similar manner (Table 2).

Effect of Zirconium(IV) Hydroxide on the Alkaline Hydrolysis of 4-Nitrophenyl Phosphate as a Function of Zirconium(IV) Hydroxide Concentration.—From a sample of zirconium(IV) hydroxide suspension, samples (1.0 cm³) were taken and diluted two, four, and eight times. Portions (0.02 cm³) of these samples were taken and tested against solutions of 4-nitrophenyl phosphate in Tris buffer (pH 8.0) as above. The results are given in Table 3.

Effect of Zirconium(IV) Hydroxide on the Alkaline Hydrolysis of D-Glucose 1-Phosphate.—Solutions (1 cm³) of D-glucose 1-phosphate (20 mM) were added to Tris buffer (2.0 cm³; 0.1M; pH 8.0) at 37 °C. Samples of zirconium(IV) hydroxide suspension (0.02 cm³) were added as above and the supernatants of the samples were assayed for D-glucose at various time intervals by the method of Werner *et al.*¹⁵ (oxidation of ammonium 3,3'-diethyl 2,2'-azinodibenzothiazoline-6-sulphonate). The amount of D-glucose produced in 35 min in the presence of zirconium(IV) hydroxide (0.0032 μmol) was not significantly different from the amount produced in the same time in its absence (0.0028 μmol).

Effect of Zirconium(IV) Hydroxide on the Alkaline Hydrolysis of Glycerol 1-Phosphate.—Solutions (10 cm³) of glycerol 1-phosphate (20 mM) were added to potassium tetraborate buffer (2.0 cm³; 0.1M; pH 8.0) at 37 °C. Samples of zirconium(IV) hydroxide (0.02 cm³) suspension were added as above, and the supernatants of the samples were examined along with appropriate standards and controls for glycerol by periodate oxidation and detection of liberated formaldehyde by complex formation with acetylacetone¹⁶ (1 mol of glycerol 1-phosphate should produce 1 mol of formaldehyde whereas 1 mol of glycerol should produce 2 mol of formaldehyde); 0.90 mol of formaldehyde per mol of substrate were detected in both the hydroxide-treated samples and the untreated controls.

Effect of Zirconium(IV) Hydroxide on the Alkaline Hydrolysis of Adenosine Mono- and Tri-phosphates.—Solutions (1.0 cm³) of adenosine monophosphate and triphosphate (both 60mM) were added to Tris buffer (2.0 cm³; 0.1M; pH 8.0) at 37 °C. Samples of zirconium(IV) hydroxide suspension (0.02 cm³) were added as usual and the supernatants were examined for evidence of hydrolysis after a series of times by t.l.c. on cellulose (MN 300 G, Macherey, Nagel, and Co.) plates. Control solutions of the phosphate esters were treated similarly.

The adenosine monophosphate-type samples were run in butan-1-ol-acetone-acetic acid-aqueous 5% ammonia-water (7 : 5 : 3 : 3 : 2 v/v), with adenosine monophosphate and adenosine (3 mM) solutions as standards and examining the plates in u.v. light. No adenosine was detected in any of the samples, which all gave single spots corresponding to adenosine monophosphate, even after treatment with zirconium(IV) hydroxide for 6 h at 37 °C. The adenosine

¹⁴ J. Attias and H. Durand, *Biochim. Biophys. Acta*, 1973, **321**, 561.

¹⁵ W. Werner, H. G. Reg, and H. Wielinger, *Z. analyt. Chem.*, 1970, **252**, 224.

¹⁶ T. Nash, *Biochem. J.*, 1953, **55**, 416.

triphosphate samples were run in 2-methylbutan-2-ol-formic acid-water (3 : 2 : 1 v/v) with adenosine tri-, di-, and mono-phosphate as standards. No hydrolysis products were detected in any of the samples, which had been treated with zirconium(IV) hydroxide for up to 9 h at 37 °C.

Effect of Zirconium(IV) Hydroxide on Other Nitrophenyl-containing Compounds.—(a) *Nitrophenyl derivatives of monosaccharides.* Solutions (0.5 cm³) of 4-nitrophenyl 2-acetamido-2-deoxy-β-D-glucopyranoside and 2-nitrophenyl β-D-galactopyranoside (each 1 mg ml⁻¹) were added to Tris buffer (2.0 cm³; 0.1M; pH 8.0) at 37 °C. Zirconium(IV) hydroxide suspension (0.02 cm³) was added and the mixtures were stirred for 60 min. After this time the absorbances of the supernatants were measured and found to be identical with those of controls from which the metal hydroxide had been omitted.

(b) *Nitrophenyl sulphate and acetate esters.* Solutions (1.0 cm³) of 4-nitrophenyl sulphate (20mM) and 4-nitrophenyl acetate (10mM) were added to Tris buffer (2.0 cm³; 0.1M; pH 8.0) at 37 °C. Zirconium(IV) hydroxide suspension (0.02 cm³) was added to each and the mixtures were stirred for various lengths of time, after which the absorbances of the supernatants were determined along with those of controls, incubated under the same conditions, but without the insoluble hydroxide. Neither the samples nor the controls containing 4-nitrophenyl sulphate were hydrolysed to any appreciable extent. The samples and controls containing 4-nitrophenyl acetate were hydrolysed rapidly but with no significant difference between the rates for the presence or absence of zirconium(IV) hydroxide.

Complexing of Phosphate Ions by Zirconium(IV) Hydroxide.—To samples (0.02 cm³) or zirconium(IV) hydroxide sus-

pension were added portions of Tris buffer (2.0 cm³; 0.1M; pH 8.0) and three aqueous solutions of disodium hydrogen phosphate (0.5 cm³; 0–1.0 μmol). These mixtures were stirred for 30 min at 37 °C and centrifuged, and the amounts of phosphate remaining in solution were determined spectrophotometrically by the molybdate complex method¹⁷ (Table 4).

Effect of Zirconium(IV) Phosphate on the Alkaline Hydrolysis of 4-Nitrophenyl Phosphate.—(a) *Zirconium(IV) phosphate prepared from zirconium(IV) hydroxide.* To a sample (1.0 cm³) of zirconium(IV) hydroxide suspension was added disodium hydrogen phosphate solution (9.0 cm³; 0.1M; pH 9.2), and the mixture was stirred at 37 °C for 2 h, then centrifuged; the supernatant was discarded. Distilled water was then added to make the volume up to 10.0 cm³ and portions (0.02 cm³) of the suspension were tested against 4-nitrophenyl phosphate as above (Table 5).

(b) *Zirconium(IV) phosphate prepared from zirconium(IV) tetrachloride solution.* To zirconium tetrachloride solution (2.0 cm³; 0.65M) were added disodium hydrogen phosphate (as above) and distilled water to give a pH of 7.0 and a total volume of 10.0 cm³. Portions (0.02 cm³) of this suspension were then tested against 4-nitrophenyl phosphate solution as above (Table 5).

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¹⁷ J. F. Kennedy and D. A. Weetman, *Analyt. Chim. Acta*, 1971, **55**, 448.