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The relative hydrolytic reactivities of pyrophosphites and pyrophosphates†

Dharmit Mistry and Nicholas Powles*

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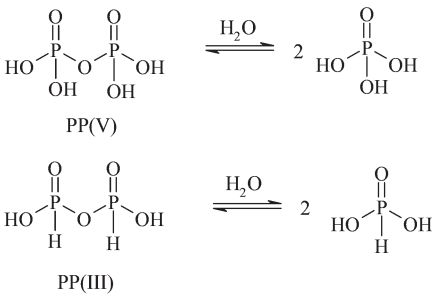
The pH-rate profiles for the hydrolysis of pyrophosphate (PP(v)) and pyrophosphite (PP(iii), pyro-di-H-phosphonate) are a complex function of pH, reflecting the different ionic species and their relative reactivities. PP(iii) is more reactive than PP(v) at all pHs and only PP(iii) shows a hydroxide-ion reaction at high pH, so it is 10^{10}-fold more reactive than PP(v) in 0.1 M NaOH. The pK_{a2} of PP(iii) ~0.44, so the dominant species at pH's > 1 is the di-anion PP(iii)$^{2-}$. Although there is no observable (NMR or ITC) binding of Mg^{2+} to the PP(iii) di-anion there is a modest increase in the rate of hydrolysis of PP(iii) by Mg^{2+}. PP(iii) is neither a substrate nor an inhibitor of pyrophosphatase, the enzyme that efficiently catalyses the hydrolysis of PP(v).

Introduction

The reactions and stabilities of phosphate mono- and di-esters underpin most life processes such as the storage and manifestation of genetic information, energy transduction, signalling, regulation, differentiation, compartmentalisation, substrate modification to facilitate chemical reactions, and as structural components.[1,2] One of the amazing contrasts within this list is the remarkable variation from extreme stability to high reactivity: from half-lives of million of years for the spontaneous hydrolyses of some phosphate esters to milli-second turnovers of their enzyme catalysed reactions.[3] A fundamental property of phosphate mono- and di-esters is their negative charge over the normal pH range encountered in living systems, which in turn contributes to their versatility.[1] Phosphate mono- and di-esters show remarkable resistance to spontaneous hydrolysis under normal physiological conditions, hence their contribution to the stability of genes. By contrast enzyme-catalysed phosphoryl group transfer reactions are highly efficient and show some of the largest enzymatic rate enhancements[4] – up to 10^{20}. The key to understanding enzyme catalysis is the charge and geometric complementarity between substrate and enzyme expressed in the transition state, so a major feature of enzymes catalysing phosphoryl transfer is the neutralisation of the substrate negative charge.[5,6]

Pyrophosphate (PP(v)) is the anhydride of two phosphate ions (Scheme 1) and is important in intermediary metabolism and cell growth.[7] The control of intracellular levels of PP(v) during energy metabolism is an essential part of the synthesis of proteins, DNA and RNA.[8] The group of enzymes controlling levels of PP(v) are pyrophosphatases (PPases).[9] There are two classes of PPases which have been identified – Type I PPases are found in eukaryotes and *Escherichia coli* and differ from the Type II PPases found in some bacteria.[10] These enzymes have remarkably similar active sites but Type I PPases require three Mg^{2+} ions for activity,[11] whereas Type II PPases require four Mn^{2+} ions.[12]



Scheme 1

The P(iii) pyrophosphite analogue of pyrophosphate exists as pyro-di-H-phosphonate (PP(iii))[13] but has only two ionisable protons compared with the four in PP(v) (Scheme 1). We have been interested in H-phosphonates as phosphorylating agents, in particular diethyl pyro-di-H-phosphonate (**1**).[14] Pyrophosphite has also been suggested as a phosphorylating agent in pre-biotic chemistry.[15] Herein we report on a comparison of the relative reactivities of the P(iii) pyrophosphite (pyro-di-H-phosphonate) and P(v) pyrophosphates towards hydrolysis (Scheme 1) over a wide range of pH and their relative interactions with PPases. The main factor controlling reactivity is the state of ionisation of both species at different pHs.

IPOS, The Page Laboratories, Department of Chemical and Biological Sciences, The University of Huddersfield, Queensgate, Huddersfield, HD1 3DH, UK. E-mail: n.t.powles@hud.ac.uk

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Results and discussion

pH-rate profiles for the hydrolysis of PP(v) and PP(iii)

The rates of hydrolysis of pyrophosphate and pyro-di-H-phosphonate were determined by $^{31}P\{H\}$ NMR as a function of pH at 25 °C and ionic strength I = 1.0 M and by auto-titration. The observed first order rate constants were determined at constant pH with different buffer concentrations and the buffer independent rate constant obtained by extrapolation to zero buffer concentration (Fig. 1).

The pK_a's of pyrophosphate are known[16] but not those for the two ionisable protons of pyro-di-H-phosphonate, which are presumed to be low. However the $^{31}P\{H\}$ NMR chemical shift is unchanged from pH 2 to 13.5, suggesting that both pK_a's are <1.0 and that the dominant species over this pH range is the di-anion PP(iii)$^{2-}$. However, rapid scanning of $^{31}P\{H\}$ NMR spectra at different pH's shows that the chemical shift of PP(iii)$^{2-}$ at δ −5.08 ppm does change below pH 2.0 (Fig. 2). Unfortunately PP(iii) undergoes acid catalysed hydrolysis at low pH and it is not possible to measure the chemical shift below pH 0.6. Nonetheless, if it is assumed that the observed data is part of a normal sigmoidal titration, it can be fitted to a pK_a = 0.44 ± 0.1 as indicated by the solid line (Fig. 2). This value is compatible with analogous systems, which show that P(iii) derivatives are generally more acidic than the corresponding P(v) compound. For example, a similar $^{31}P\{H\}$ NMR titration with pH for ethyl H-phosphonate (**3**) yields a pK_a = 0.47 which may be compared with that for ethyl phosphate = 1.60.[17] The first pK_a of P(iii) phosphorous acid is 1.07[16] and a calculated value is quoted as 0.9[18] compared with 2.0 for P(v) phosphoric acid.[16] The first pK_a of isohypophosphoric acid which contains a P(iii) and a P(v) phosphorous atom bridged by an oxygen has been estimated to be 0.6.[19] A value <0.5 for the pK_a of PP(iii) is also consistent with the kinetic pH-rate profile reported later.

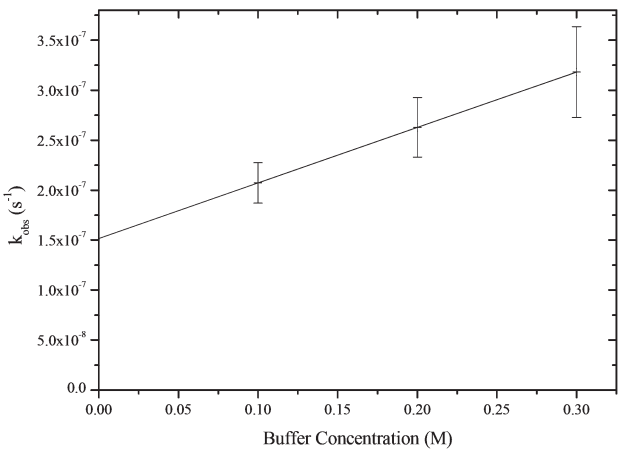


Fig. 1 Plot of the observed first-order rate constants for the hydrolysis of PP(iii) as a function of MOPS buffer concentration at pH 8.0 at I = 1.0 M (KCl) and 25 °C.

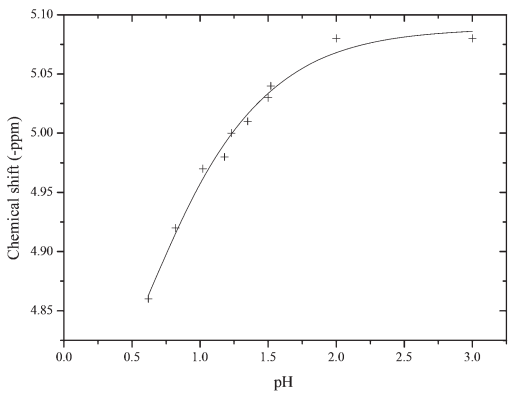


Fig. 2 $^{31}P\{H\}$ NMR chemical shift of PP(iii) against pH with external reference to diphenyl phosphate at I = 1.0 M, 25 °C.

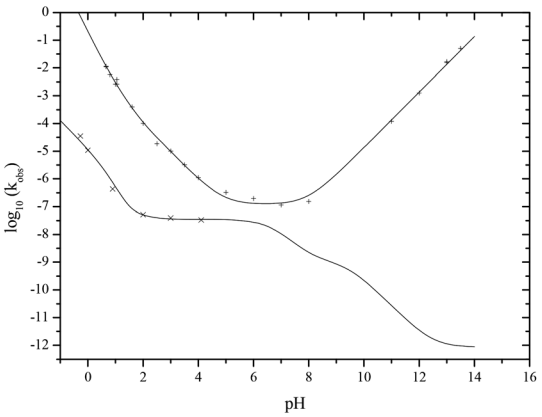


Fig. 3 pH rate-profile for the hydrolysis of PP(iii) (+) and PP(v) (×) at 25 °C.

The pH-rate profile for the hydrolysis of pyrophosphate and pyro-di-H-phosphonate is a complex function of pH (Fig. 3) reflecting the different ionic species and their relative reactivities. PP(iii) is more reactive than PP(v) at all pHs and only the former shows a hydroxide-ion reaction at high pH. The rate of hydrolysis of pyrophosphate PP(v) decreases with increasing pH and the rate constants for the tri- and tetra-anion (Fig. 3) at higher pH are calculated from a recent report.[20] The results reported here for the neutral, mono- and di-anionic forms of PP(v) are from our observations using ^{31}P NMR.

The observed pseudo first order rate constant for the hydrolysis of PP(iii) shows a first order dependence on H^+ concentration between pH 2 and 4, where the dominant species in solution is the di-anion. However, below pH 2 this changes to a second order dependence on H^+ concentration, best seen by the enlarged Fig. 4 and changes in values of $k_{obs}/[H^+]$ as a function of pH (ESI†). In addition there is a pH independent term for hydrolysis of the PP(iii) di-anion between about pH 5 and 8 followed by a rate term that is dependent on hydroxide-ion concentration at higher pH. The observed rate law for the hydrolysis of PP(iii) is thus given by eqn (1), where k_1, k_2, k_0 and k_{OH} all refer to the rate constants for the hydrolysis of PP(iii) dianion and k_2 is that which is first order in H^+ concentration, k_0 is the pH independent hydrolysis and k_{OH} is the

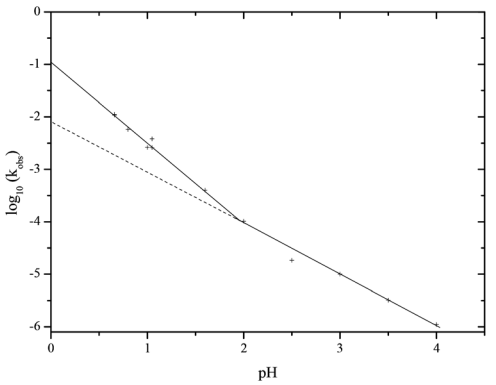


Fig. 4 pH rate profile enlarged from Fig. 3 to show the change from first order dependence on H^+ (dashed line is a continuation of a first order dependence on H^+) to second order dependence on H^+.

Table 1 Rate constants (eqn (1)) for the hydrolysis of $PP(III)^{2-}$ at 25 °C

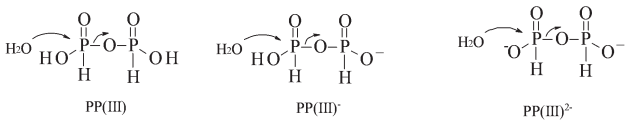
Rate constants (eqn (1))	
k_1	2.00×10^{-1} (M^{-2} s^{-1})
k_2	9.40×10^{-3} (M^{-1} s^{-1})
k_0	1.20×10^{-7} (s^{-1})
k_{OH}	1.35×10^{-1} (M^{-1} s^{-1})

hydroxide-ion catalysed hydrolysis of $PP(III)^{2-}$. Experimentally, there is no substantial levelling off of the observed pseudo first-order rate constants at low pH or a reversion to a first-order dependence on $[H^+]$ which may be expected if the PP(III) mono-anion or undissociated acid become significant species. This is compatible with the $^{31}P\{H\}$ NMR data indicating a pK_a = 0.44. Consequently, it appears that the k_1 term (eqn (1)) refers to a reaction involving PP(III) dianion and $[H^+]^2$ or its kinetic equivalent.

$$\text{Rate}/[PP(III)_{tot}] = k_{obs} = k_1[H^+]^2 + k_2[H^+] + k_0 + k_{OH}[OH^-] \quad (1)$$

The line in Fig. 3 is generated from the values of the rate constants given in Table 1. The first two terms in the rate law, k_1 and k_2 (eqn (1)) have kinetically equivalent expressions, and so, for example, the k_1 term could represent the spontaneous hydrolysis of neutral PP(III), and the k_2 term could actually reflect either the hydrolysis of the mono-anionic species $PP(III)^-$ or even hydroxide-ion attack upon neutral PP(III), although the calculated rate constant for the latter $k_2K_{a1}K_{a2}/K_w$ is greater than the diffusion controlled rate and so can be excluded.

The calculation of these kinetically equivalent mechanisms requires a knowledge of the two pK_a's of PP(III). The estimated value of pK_{a2} = 0.44 is subject to error and there is no simple way to estimate pK_{a1}. Nonetheless it is a worthwhile exercise to enable a comparison with the PP(V) analogues. The third order rate constant k_1 is kinetically equivalent to the spontaneous hydrolysis of the undissociated acid with a first order rate constant equal to $k_1K_{a1}K_{a2}$. Based on the pH dependence of the ^{31}P NMR chemical shifts (Fig. 2) and the pH-rate profile (Fig. 3) the product $K_{a1}K_{a2}$ is $>10^{-1}$. The calculated rate



Scheme 2

constant for the hydrolysis of the undissociated PP(III) is >0.073 s^{-1}. Presumably, the undissociated PP(III) is much more reactive than its mono- and di-anions so that between pH 1 and 2 it is the reactive hydrolytic species even though its concentration is small and falls off with a second order dependence on $[H^+]$. Similarly, between about pH 2 and 5 where the rate of hydrolysis shows a first order dependence on $[H^+]$ and the rate law is dominated by the k_2 term in eqn (1), hydrolysis is actually occurring through the mono-anion of PP(III) even though, again, the dominant species in solution is the di-anion. The calculated first order rate constant for the hydrolysis of the mono-anion $PP(III)^-$ is given by $k_2K_{a1} = 3.41 \times 10^{-3}$ s^{-1}. The rate constant for the spontaneous hydrolysis of the PP(III) di-anion is 1.20×10^{-7} s^{-1}, so the relative hydrolytic reactivities of the neutral, anionic and di-anionic PP(III) are approximately: $6 \times 10^5 : 3 \times 10^4 : 1.0$, respectively. The rates of hydrolysis of the neutral and mono-anionic PP(III) differ by a factor of 20 and presumably both involve nucleophilic attack by water on the more electrophilic neutral P centre although they require the expulsion of different leaving groups, the phosphite mono- and di-anions, respectively (Scheme 2). These relative reactivities are compared later with the hydrolysis of ethyl-H-phosphonate (**3**) to generate a simple linear free-energy relationship. The much reduced activity of the di-anion of PP(III) reflects the less favourable ease of nucleophilic attack by water on the relatively negatively charged P centre and expulsion of the di-anionic phosphite.

In contrast to that for the hydrolysis of PP(III), the observed pseudo first order rate constant for the hydrolysis of PP(V) continues to decrease with increasing pH (Fig. 3) as the state of ionisation increases and forms anions of increasingly lower reactivity. The rate law for the hydrolysis of PP(V) is given by eqn (2). The constants for the tri- and tetra-anions of PP(V) are taken from the recent literature obtained by extrapolation from determinations at elevated temperatures[20] and are compatible with other reports.[21] The known pK_a values (0.79 (I = 1.0 M), 1.72 (I = 1.0 M), 6.6 and 9.4) of PP(V)[16] and the rate constants obtained in this work at lower pH (Table 2) have been used to generate the overall pH-rate-profile (Fig. 3).

$$\text{Rate} = k_H[PP(V)][H^+] + k_0[PP(V)] + k_{1-}[PP(V)^-] + k_{2-}[PP(V)^{2-}] + k_{3-}[PP(V)^{3-}] + k_{4-}[PP(V)^{4-}] \quad (2)$$

There is no base-catalysed hydrolysis observed in the hydrolysis of the PP(V) anions in alkaline solutions. This is unlike the behaviour of di-anionic PP(III) which, despite its negative charge, does undergo a hydroxide-ion catalysed hydrolysis and, consequently PP(III) is much more reactive than PP(V) at

Table 2 Rate constants (eqn (2)) for the hydrolysis of PP(v) at 25 °C

Rate constants (eqn (2))	
k_H	1.30×10^{-5} (M^{-1} s^{-1})
k_0 (I = 1.0 M)	1.00×10^{-7} (s^{-1})
k_{1-} (I = 1.0 M)	7.00×10^{-8} (s^{-1})
k_{2-} (I = 1.0 M)	3.50×10^{-8} (s^{-1})
k_{3-}	1.07×10^{-9} (s^{-1})[a]
k_{4-}	8.59×10^{-13} (s^{-1})[a]

[a] From ref. 20.

high pH (Fig. 3) *e.g.* it is 10^{10}-fold more reactive in 0.1 M NaOH.

Of course, there are kinetically equivalent processes for the hydrolysis of PP(v). For example, if the reported rate constant for the spontaneous hydrolysis of PP(v) tri-anion[20] actually reflects the kinetically equivalent hydroxide-ion catalysed hydrolysis of the di-anion, then the corresponding second-order rate constant would be $K_{a3}k_{3-}/K_w$ = 1.7×10^{-3} M^{-1} s^{-1} which can be compared with k_{OH} for PP(III)$^{2-}$ = 1.35×10^{-1} M^{-2} s^{-1}. This 79-fold higher rate for the pyro-di-H-phosphonate compared with pyrophosphate is of the order expected, so the hydrolysis of the PP(v) tri-anion probably does occur through a mechanism involving hydroxide-ion attack on the di-anion.

Mg^{2+} catalysed hydrolysis of PP(III)

Many enzymes that catalyse phosphate ester hydrolysis and transesterification require one or more metal-ions as cofactors. It is interesting to note that using $^{31}P\{H\}$ NMR or ITC techniques there is no observable binding of Mg^{2+} to the PP(III) di-anion. Nonetheless, there is a modest increase in the rate of hydrolysis of PP(III) by Mg^{2+} (Fig. 5) giving a second order rate constant for this process = 2.20×10^{-5} M^{-1} s^{-1}. If it is assumed that Mg^{2+} catalysis is due to initial complexation of Mg^{2+} to PP(III) di-anion followed by nucleophilic attack by water and that metal-ion binding has a weak association constant of $<10^{-2}$, then the calculated rate constant for water attack on this complex (**2**) is $>2 \times 10^{-3}$ s^{-1} which is 4-orders of magnitude greater than that for the uncomplexed PP(III) di-anion.

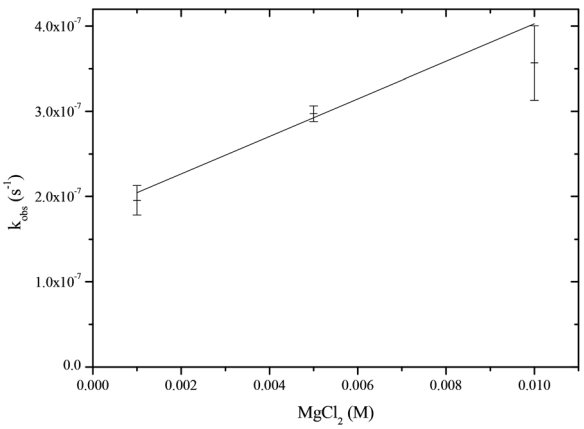
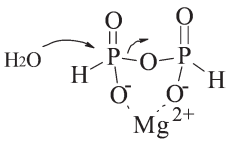


Fig. 5 Pseudo first-order rate *versus* $MgCl_2$ concentration of PP(III) at fixed buffer concentration (0.8 M), pH 7, I = 1.0 M at 25 °C.

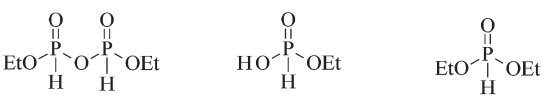
The Mg^{2+} catalysed hydrolysis of PP(v) at neutral pH is assumed[20] to be that of the tetra-anion, *i.e.* the overall di-anionic $MgPP^{2-}$ is the reactive species with an estimated rate constant at 25 °C of 2.8×10^{-10} s^{-1}, three orders of magnitude greater than that of PP(v)$^{4-}$. The dissociative or associative nature of phosphoryl transfer reactions is defined by the extent of bond formation between phosphorus and the incoming nucleophile and that between phosphorus and the leaving group in the transition state.[3] The processes of phosphorylation and dephosphorylation that occur by associative type mechanisms usually have a penta-coordinate trigonal-bipyramidal geometry in the transition state, whereas in the case of the dissociative pathway it effectively involves a trigonal planar metaphosphate anionic species, PO_3^-. The 3 orders of magnitude rate enhancement of the hydrolysis of PP(v) by Mg^{2+} is presumably due to the metal-ion binding to the tetra-anion and so neutralising some of the negative charge and facilitating nucleophilic attack by water. However, the hydrolysis of phosphate monoesters proceeding through the dissociative pathway is *not* sensitive to the presence of Mg(II) ions in aqueous solution.[22] Although metal-ion coordination may enhance the electrophilicity of the phosphorus it will disfavour electron-donation from the non-bridging oxygens thus reducing their ability to assist in expelling the leaving group, which is essential in the dissociative pathway. Similarly, Mg^{2+} only enhances the rate of hydrolysis of ATP^{4-} by 3-fold and binds preferentially between the β and γ phosphates, making the di-anionic γ phosphate less effective at expelling the ADP leaving group, but does not facilitate an alternative associative pathway.[23] An associative transition state can be sensitive to stabilization by Mg^{2+} ions if coordination occurs to the leaving group.[24]



2

Hydrolysis of other P(III) derivatives

The mechanisms of hydrolysis of pyrophosphate mono- and di-esters resemble those for phosphate monoesters,[25] except that a phosphate ester or salt is expelled rather than an alcohol. For comparison, the hydrolytic activity of diethyl pyro-di-H-phosphonate (**1**) H-phosphonate mono- and di-ethyl esters (**3** and **4**) were investigated.



1 **3** **4**

The rate of hydrolysis of diethyl pyro-di-H-phosphonate (**1**) is too fast to measure by $^{31}P\{H\}$ NMR and has a rate constant at pH 7 > 0.1 s^{-1}.

Diethyl-H-phosphonate (**4**) undergoes hydrolysis to give ethyl-H-phosphonate (**3**) and shows an acid catalysed hydrolysis with a second order rate constant $k_{H^+} = 1.21 \times 10^{-4}$ M^{-1} s^{-1} at 25 °C (I = 1.0 M). It also undergoes a hydroxide-ion catalysed hydrolysis with a second order rate constant k_{OH} = 81.3 M^{-1} s^{-1} at 25 °C (I = 1.0 M), obtained from observed pseudo first-order rate constants at constant pH with different buffer concentrations and the buffer independent rate constant obtained by extrapolation to zero buffer concentration.

Ethyl-H-phosphonate (**3**) is a strong acid and exists predominantly as its mono-anion above pH 1. Its hydrolysis to give phosphorous acid and ethanol at 25 °C (I = 1.0 M) shows a much slower hydroxide-ion catalysed reaction than the di-ester (**4**) with a second order rate constant $k_{OH} = 1.55 \times 10^{-5}$ M^{-1} s^{-1}. This corresponds to a rate decrease of 5×10^6 fold due to the additional negative charge and repulsion against the negatively charged nucleophile.

The acid catalysed hydrolysis of the mono-anion shows a second order rate constant $k_{H^+} = 6.90 \times 10^{-6}$ M^{-1} s^{-1}. The latter reaction is kinetically equivalent to the hydrolysis of the neutral (**3**) and if it is assumed that the pK_a of (**3**) is ~0, then the corresponding first order rate constant would be 6.90×10^{-6} s^{-1}. This can then be combined with the data reported earlier for the hydrolysis of other derivatives involving water attack on the neutral H-phosphonates expelling phosphites and ethanol to generate a simple Brønsted plot (Fig. 6) as a function of the pK_a of the leaving group XH (**5**) using the data summarised in Table 3.

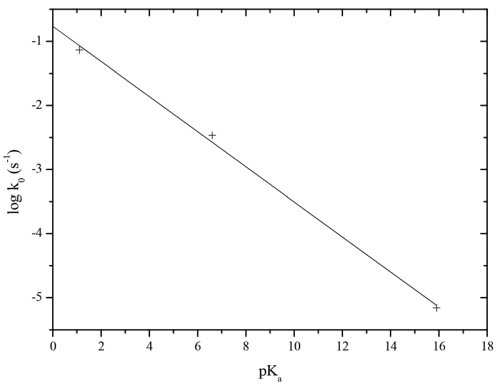
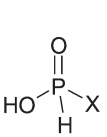


Fig. 6 Brønsted plot for the spontaneous pH independent hydrolysis of H-phosphonate derivatives (HO)PH-X at 25 °C as a function of the pK_a of the leaving group XH.

Table 3 Summary kinetic data calculated for the spontaneous pH independent hydrolysis of H-phosphonate derivatives (HO)PH-X at 25 °C

X	pK_a XH	k_0 (s^{-1})
H_2PO_3	1.1	0.073
HPO_3^-	6.6	3.41×10^{-3}
EtO	15.9	6.90×10^{-6}



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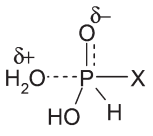

6

The phosphite mono-anion is a better leaving group with a pK_a of its conjugate acid being 1.1 compared with 6.6 for that of the di-anion.[16] Perhaps surprisingly, the expulsion of the poor leaving group ethoxide fits well on the Brønsted plot, which, although only based on three points, does cover a pK_a range of 14 to generate a Brønsted $\beta_{lg} = -0.27$. This relatively small value is indicative of little or no P–O bond cleavage in the transition state with the development of only a small change in the effective charge on oxygen, relative to that in the reactant.[26] This is consistent with rate limiting formation of a trigonal-bipyramidal intermediate (**6**).

Pyrophosphatase catalysed hydrolysis of PP(III)

Inorganic pyrophosphate (PP(V)) or pyrophosphite (PP(III)) may have acted as a phosphoryl group donor in primitive biological systems.[15,27] In modern organisms, the enzyme-catalysed hydrolysis of PP(V) is an important part of the metabolism to remove PP(V) that is generated by biosynthetic reactions.[7,28] There are two classes of inorganic pyrophosphatase (PPase) that catalyse the hydrolysis of PP(V), both of which have an unusual activity dependence on metal ions but similar active sites.[10] Type I PPases (found in eukaryotes and *E. coli*) require three Mg^{2+} ions for activity[11] and differ significantly from those of Type II PPases (found in some bacteria) which require four Mn^{2+} ions.[12] In both enzymes PP(V) tetra-anion is co-ordinated to metal-ions so that the extremely polar active site neutralizes the negative charges on substrate to facilitate nucleophilic attack by water.[29] The metal ions probably also coordinate to the attacking water so decreasing its pK_a and providing an activated nucleophile if the Brønsted β_{nuc} for attack by nucleophiles is <1.0.[30]

It is difficult to follow the pyrophosphatase catalysed hydrolysis of PP(V) by $^{31}P\{H\}$ NMR because of limited solubility of the substrate. However, the use of isothermal titration calorimetry (ITC) provides a suitably sensitive continuous assay. Experiments were conducted at pH 8.4 and 7.55 as hydrolysis of PP(V) is the slowest step at the higher pH, rather than substrate binding or product release.[31] ITC can be used to follow the kinetics of an enzyme catalysed reaction because the amount of heat released is directly proportional to the amount of substrate that has reacted. The instrument output reflects the energy required to balance the heat generated by the reaction to maintain constant temperature (Fig. 7). The initial positive spike represents the endothermic heat of injection/dilution. The exothermic hydrolysis reaction gives a negative displacement from the baseline and as substrate is consumed, the rate at which heat is generated decreases and the output trace returns to the baseline.

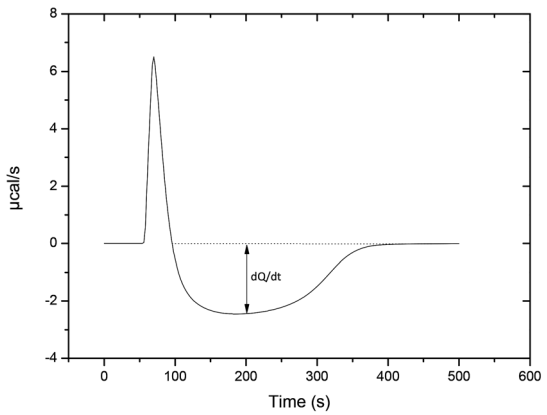

Fig. 7 ITC trace of the enzymatic hydrolysis of pyrophosphate (PP(v)) with 1×10^{-9} M *E. coli* PPase, 3 mM $MgCl_2$, initiated with PP(v) to give a final concentration of 4.11×10^{-5} M at 25 °C. The endothermic peak represents the heat of dilution followed by PP(v) consumption until the heat generated with respect to time (d*Q*/d*t*) returns to the baseline.

The total heat released by hydrolysis is represented by the well area under the dashed line and d*Q*/d*t* is the difference between the baseline and the heat released at any time *t* (Fig. 7). The concentration of PP(v) at any time *t* can be used to give the Michaelis–Menten kinetic parameters k_{cat} and K_m as previously described.[20]

It is of interest to determine if PP(III) is a substrate of PPase and, if not, whether it binds sufficiently to act as an inhibitor of the enzyme. Using the PPase from *E. coli* (2×10^{-7} M) there was no discernible rate difference from the background hydrolysis of PP(III) at pH 7.55 (300 mM buffer, 50 mM Mg^{2+}, I = 1.0 M), indicating that PP(III) is not a substrate and that k_{cat}/K_m is $<6\ M^{-1}\ s^{-1}$. This may be due to PP(III) showing no significant binding to Mg^{2+} and/or having insufficient negative charge to bind to the positively charged active site of PPase. To investigate whether PP(III) binds sufficiently to act as an inhibitor of PPase, the rates of the enzyme catalysed hydrolysis of PP(v) were measured in the absence and presence of PP(III). Above saturation, the k_{cat} values measured for PP(v) at 25 °C at pH 8.40 and 7.55 were 208 s^{-1} and 82 s^{-1}, respectively. This is in good agreement with a value of k_{cat} = 290 s^{-1} at pH 8.4 which has been reported.[31] The second order rate constant for the enzyme catalysed hydrolysis $k_{cat}/K_m = 3.68 \times 10^7\ M^{-1}\ s^{-1}$ and $2.89 \times 10^7\ M^{-1}\ s^{-1}$ again at 25 °C, pH 8.40 and 7.55, respectively. These parameters decreased by less than 27% with 1×10^{-3} M PP(III) indicative that the binding of PP(III) to PPase is weak and K_i must be $>1 \times 10^{-3}$ M.

Conclusions

The pH-rate profiles for the hydrolysis of pyrophosphate (PP(v)) and pyrophosphite (PP(III), pyro-di-H-phosphonate) are a complex function of pH. Increasing ionisation with increasing pH steadily decreases the rate of hydrolysis of PP(v). PP(III) is more reactive than PP(v) at all pHs. The pK_{a2} of PP(III) = 0.44, so the dominant species at pH's > 1 is the di-anion $PP(III)^{2-}$ which shows a hydroxide-ion reaction at high pH, so it is 10^{10}-fold more reactive than PP(v) in 0.1 M NaOH. Although there is no observable (NMR or ITC) binding of Mg^{2+} to the PP(III) di-anion there is a modest increase in the rate of hydrolysis of PP(III) by Mg^{2+}. PP(III) is neither a substrate nor an inhibitor of pyrophosphatase, the enzyme that efficiently catalyses the hydrolysis of PP(v).

Experimental

Materials

PP(III) – samples of PP(III) were initially and kindly provided by T. Kee (University of Leeds) and prepared by dissolving phosphorous acid (16.4 g) and sodium hydroxide (8.0 g) in 100 ml water. The water was removed under reduced pressure. The resulting mixture was heated in a tube furnace with N_2 flowing through at 200 °C for three days.

Diethyl pyro-di-H-phosphonate (**1**) and H-phosphonate monoethyl esters (**3**) were prepared as previously described.[14]

H-phosphonate di-ethyl ester (>99%) (**4**) and *E. coli* pyrophosphatase (lyophilized powder ≥90%, ≥800 units per mg protein) were purchased from Sigma.

Kinetics

Ionic strength of solutions were maintained using KCl. Reactions monitored by $^{31}P\{1H\}$ NMR at 25 °C used a 500 MHz Bruker Avance I, and a D_2O insert was used for a lock signal and diphenylphosphate as an internal standard. Where auto-titration was used to follow reactions these were carried out using a Metrohm 859.

Auto-titration: a jacketed titration vessel aspirated with N_2 was used to maintain a constant temperature. The vessel was charged with 50 ml HCl or NaOH at I = 1.0 M and titrated with an appropriate solution to maintain the pH. The titration was initiated by addition of 1.0 mmole PP(III) to the vessel.

Effect of Mg^{2+} on the hydrolysis of PP(III): samples were run in 0.8 M MOPS buffer at pH 7 and 1.0 M ionic strength containing 10% D_2O for a D_2O lock and 20 mM diphenylphosphate as an internal standard. Magnesium concentrations were varied by the addition of 0.001 M, 0.005 M and 0.01 M magnesium chloride ($MgCl_2$), to 37.6 mM PP(III).

E. coli Pyrophosphatase catalysed reactions using VP- ITC: the enzyme (1×10^{-9} M) was prepared in 20 mM HEPES buffer pH 8.40 or 20 mM MOPS buffer pH 7.55, with 3 mM magnesium chloride. 10 mM PP(v) in the same buffer, excluding PPase and magnesium chloride was used to initiate the reaction to give a final concentration of 0.0411 mM or 0.0211 mM, I = 0.1 M at 25 °C. Both solutions were degassed for 15 minutes to prevent bubble formation during the experiment. The same experiment was repeated but with the addition of 1 mM PP(III) as a potential inhibitor.

The pK_a of PP(III) was monitored by $^{31}P\{H\}$ NMR which contained an insert with diphenylphosphate in MOPS buffer, pH 7, I = 1.0 M as an external standard.

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