Synthesis and Hydrolysis Studies of Phosphonopyruvate

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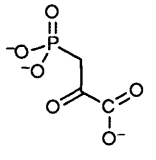
Phosphonopyruvate (**1**) is prepared in 56% yield from triethyl phosphonopyruvate (**3**) and pK_a values of 1.63, 2.40 and 7.41 determined. The stability of phosphonopyruvate is monitored at 75 °C in aqueous buffers over the pH range 0.6 to 8.3. The only products detected are pyruvate and inorganic phosphate. The pH–rate profile shows that the C–P bond is hydrolysed fastest in the monoanion and dianion of phosphonopyruvate, with rate constants of 1.94×10^{-4} s^{-1} for the monoanion and 1.05×10^{-4} s^{-1} for the dianion. For the reaction at pH 4.75, where the dianion predominates, $\Delta H^{\ddagger}$ is 114.0 kJ mol^{-1} and $\Delta S^{\ddagger}$ is 5.4 J mol^{-1} K^{-1}, the deuterium isotope effect, k_H/k_D is 1.08 ± 0.04, and there is non-selective phosphorylation of methanol and H_2O in an equimolar solution of these solvents. These results are consistent with a mechanism of hydrolysis of both the monoanion and dianion that involves a very largely dissociative transition state with monomeric metaphosphate character.

In 1968 Warren proposed[1] that the C–P bond of naturally occurring phosphonates[2] was biosynthesised by the rearrangement of phosphoenolpyruvate (**2**; PEP) to phosphonopyruvate (**1**). This proposal has recently been confirmed with the isolation of the enzyme phosphoenolpyruvate mutase[3–5] [E.C. 6.4.2.9]. This proof took 20 years to emerge because phosphonopyruvate could not be isolated from cell-free extracts. The early failures were attributed to the presumed chemical instability of phosphonopyruvate; however, results reported here do not support this assumption. When the enzyme was characterised, the rearrangement was shown to have an unexpected equilibrium constant, being a factor of at least 100 in favour of PEP. This surprising result, which explains the difficulty in detecting phosphonopyruvate in cells, prompted us to examine the stability of phosphonopyruvate. Details of the crystal structure of phosphonopyruvate and calculations on its equilibrium with PEP have been reported.[6]



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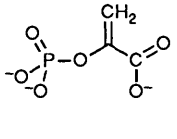

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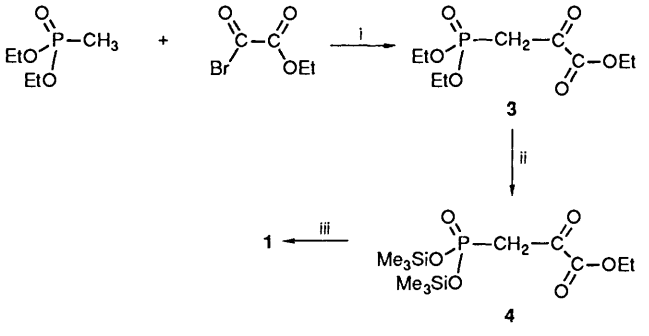
Results and Discussion

Phosphonopyruvate has recently been prepared from phosphonoalanine by two methods.[7,8] Here an alternative route has been used (Scheme 1), the key intermediate being the triethyl ester of phosphonopyruvate (**3**).[9] Treatment of this ester with trimethylsilyl bromide,[10] followed by hydrolysis of the product ethyl bis(trimethylsilyl)phosphonopyruvate (**4**) gave phosphonopyruvate which was isolated either by crystallisation of its tri(cyclohexylammonium) salt, which was analytically pure, or by anion- then cation-exchange chromatography to give the free acid. The ^1H NMR spectrum of the tri(cyclohexylammonium) salt of phosphonopyruvate gave a pyruvate methylene doublet at 2.99 ppm with a large J_{PH} coupling constant of 20.3 Hz, and the ^{31}P NMR spectrum gave a triplet with the same coupling at 12.15 ppm. Although a tribasic acid, only two pK_a values of 2.35 and 6.60 were previously quoted,[7] and we also observed only two clear end-points on titration of the free acid with 0.1 mol dm^{-3} KOH at 21 °C. These occurred after the addition of two and three equivalents of base with no indication of the first end-point. It has been shown that for dibasic acids, inflection points in a titration curve are not discernible if $K_1 < 16K_2$. This is equivalent to a difference of about 1.2 between the respective pK_a values.[11] Indeed, when the values are closer than 2.7 units the ionisation processes overlap and corrections must be made to allow determination of the individual dissociation constants.[12] Calculation of these data using a BASIC translation of the program listed for this purpose by Albert and Serjeant[12] provided estimates of 1.82 and 2.58 for the two lower pK_a values. However, a wide variability, particularly for pK_{a1}, was evident and it may be that this analysis is rather sensitive to small errors when the pK_a values are very close. The concentration of phosphonopyruvate (0.0344 mol dm^{-3}) was sufficient to eliminate false constants in this range and to enable the reliable estimation of pK_a values of 1.5 or above.[12]



Scheme 1 Synthesis of phosphonopyruvate. *Reagents:* i, BuLi; ii, Me_3SiBr, 2 equivs.; iii, H_2O.

In an attempt to separate the two lower pK_a values a non-linear fit of the titration data was undertaken by calculation of the hydrogen ion concentration according to eqn. (1), where K_1

$$H^4 + H^3(K_1 + B) + H^2(K_1K_2 + K_1B - K_w - K_1[HA]_T) + H(K_1K_2B - K_1K_w - 2K_1K_2[HA]_T) - K_1K_2K_w = 0 \quad (1)$$

and K_2 represent the first and second dissociation constants, H is the hydrogen ion concentration, B is the concentration of base added during the titration and $[HA]_T$ is the total concentration of phosphonopyruvate in the titration cell. Both B and $[HA]_T$ were corrected for dilution during titration. Solutions for the dissociation constants, corrected for activity effects, were obtained using the Newton–Raphson method

Table 1 Rates of hydrolysis of phosphonopyruvate at 75 °C at various pH values measured from the rate of formation of pyruvate, k(Py) and inorganic phosphate, $k(P_i)$

pH	k(Py)/10^{-4} s^{-1}	$r[k(Py)]^a$	$k(P_i)/10^{-4}$ s^{-1}	$r[k(P_i)]^b$
0.59	0.398	0.9994	0.420	0.9952
0.68	0.374	0.9998	—	—
1.03	0.868	0.9993	—	—
1.16	0.839	0.9999	0.874	0.9987
1.55	1.314	0.9999	—	—
1.56	1.323	0.9996	1.290	0.9941
1.91	1.583	0.9997	—	—
1.95	1.477	0.9999	1.466	0.9988
2.65	1.378	0.9987	—	—
2.69	1.292	0.9999	1.338	0.9989
3.28	1.170	0.9992	1.150	0.9958
3.87	1.014	0.9993	1.114	0.9985
3.88	1.029	0.9996	—	—
4.75	1.060	0.9994	1.138	0.9971
4.75	1.066	0.9997	—	—
5.79	0.910	0.9977	—	—
5.80	0.895	0.9990	0.949	0.9971
6.90	0.433	0.9999	0.392	0.9984
7.00	0.395	0.9997	0.441	0.9992
8.32	0.065	0.9945	0.061	0.9985

[a] Correlation coefficient, from plots of ln $\{[Py]_{inf} - [Py]_t\}$ *vs.* t. [b] Correlation coefficient, from plots of ln $\{[P_i]_{inf} - [P_i]_t\}$ *vs.* t.

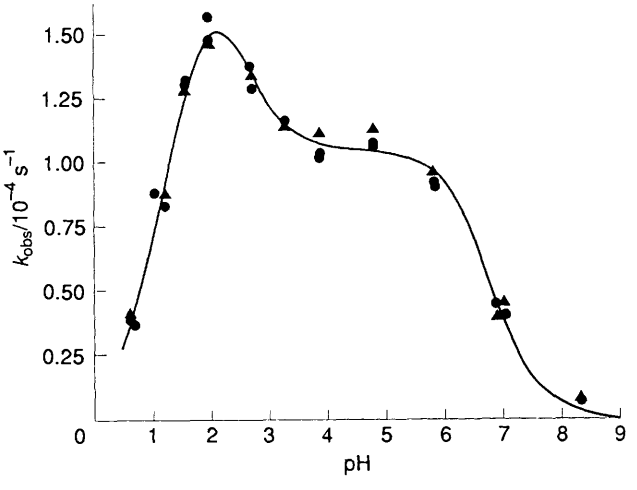


Fig. 1 pH–Rate profile for the hydrolysis of phosphonopyruvate. Rate data for the formation of inorganic phosphate (▲) and pyruvate (●). Solid line calculated according to eqn. (3).

incorporated into the program NONREG.[13] This routine was tested with literature data[12] and gave estimates for succinic acid of 4.19 and 5.50 compared with the quoted values of 4.20 and 5.63. For phosphonopyruvate iteration rapidly converged to yield final values of 1.63 and 2.40 for the first two pK_a values. A similar method has recently been reported but uses volume, rather than pH, as the dependent variable.[14] This precludes the use of a volume correction for dilution during the titration which is essential in our case.

The calculation of the third pK_a value of phosphonopyruvate, from the data between the first and second end-points, gave an estimate of 7.41. Although this is significantly higher than the literature value[7] of 6.60, it seems more consistent with the literature values of 7.74 for the second pK_a of methylphosphonate[12] and 8.60 for the third pK_a of phosphonoacetate.[15]

In our earlier publication,[6] the co-ordinates of phosphonopyruvate and the available ones of PEP, were used for structural optimisation with MNDO. Energies of the optimised forms were obtained by *ab initio* calculations using the STO-3G basis set. These gave an equilibrium constant of at least 2500 in favour of PEP, consistent with the inability to detect phosphonopyruvate *in vivo*. With the equilibrium greatly in favour of PEP, it was possible that the 1,3-phospho group transfer from C to O could proceed in the absence of enzyme.[16] Indeed, a phosphonate to phosphate rearrangement of dimethyl 2,2,2-trichloro-1-hydroxyethylphosphonate occurs in the presence of base.[17] Although we cannot detect the formation of PEP from phosphonopyruvate, our attempts have led us to characterise the hydrolysis of phosphonopyruvate in detail.

The C–P bond of phosphonates is usually resistant to vigorous acid and base hydrolytic conditions, as well as to the action of phosphatase.[2] However, some phosphonates are unstable towards hydrolytic C–P bond cleavage giving either inorganic phosphite or phosphate. The former product arises from compounds having either a carbonyl or a hydroxy group on the α-carbon, for example phosphonoformic acid.[18] Inorganic phosphate is formed when there is an electron-accepting group on the β-carbon,[19] for example a halogen or a carbonyl substituent. The naturally occurring phosphonate, phosphonoacetaldehyde, is hydrolysed to acetaldehyde and inorganic phosphate upon heating for 8 h at 90 °C at pH 5.[20] It was therefore anticipated that phosphonopyruvate, which has a β-carbonyl group, would decompose to inorganic phosphate and pyruvate. These proved to be the only products, as established by a quantitative yield for pyruvate using the lactate dehydrogenase/NADH assay[21] and the observation of only inorganic phosphate formation by ^{31}P NMR spectroscopy. Benkovic and Schray have studied the hydrolysis of PEP in detail.[22] The hydrolysis of phosphonopyruvate was studied under similar conditions, at 75 °C over a pH range of 0.6 to 8.3, so that a direct comparison could be made. The appearance of pyruvate and inorganic phosphate were monitored over ten half-lives and the rate data are given in Table 1. From the pH–rate profile in Fig. 1, the hydrolyses of PEP[22] and phosphonopyruvate are very similar between pH 2 and 8.3. This could be explained by a rearrangement of phosphonopyruvate to PEP, with subsequent rate-limiting hydrolysis of PEP. However, this is unlikely because PEP could not be detected by either ^{31}P NMR spectroscopy or enzyme assay, even though the rates of hydrolysis of phosphonopyruvate proved comparable with those reported for PEP. Below pH 1, where the free acid forms dominate, phosphonopyruvate is much more stable than PEP. Unlike phosphonopyruvate, the neutral form of PEP undergoes both a solvent and an acid-catalysed reaction.[22]

The pH–rate profile is consistent with a model involving reaction only of the monoanion (H_2A^-) and the dianion (HA^{2-}), with the free acid and triionic forms showing negligible rates of hydrolysis. The observed rate constant is given by eqn. (2), where k_1 and k_2 are the reaction rate constants involving

$$k_{obs} = k_1 \alpha_{H_2A^-} + k_2 \alpha_{HA^{2-}} \quad (2)$$

the monoanion and dianion respectively, $\alpha_{H_2A^-}$ is the fractional amount of phosphonopyruvate in the monoionic form and $\alpha_{HA^{2-}}$ is that of the dianion. Incorporating this relationship into models relating the anion composition to pH provides eqn. (3).

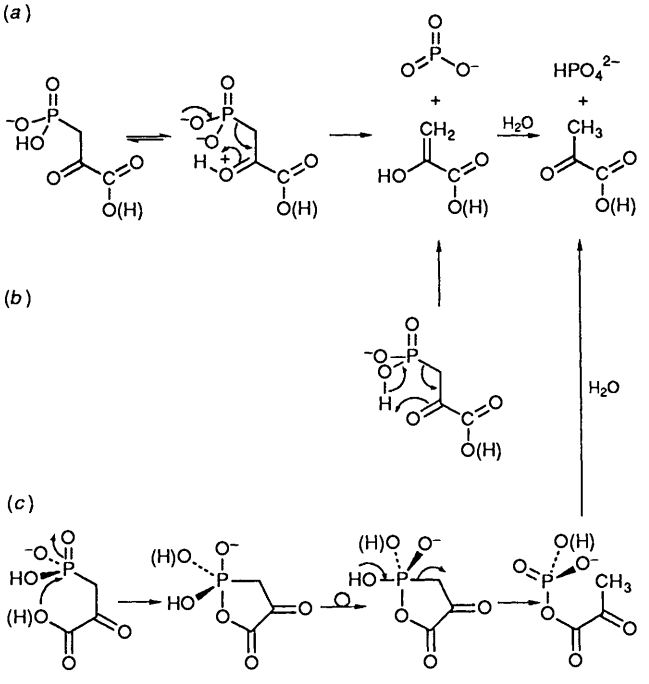
$$k_{obs} = \frac{k_1K_1H^2 + k_2K_1K_2H}{H^3 + K_1H^2 + K_1K_2H + K_1K_2K_3} \quad (3)$$

A non-linear fit of the pH–rate data to this model, formulated for use in program NONREG with activity correction,[23] provided estimates of all variables under the experimental conditions. Values of 1.53, 2.69 and 7.06 were obtained for the

Table 2 Rates of hydrolysis of phosphonopyruvate at pH 4.75–4.79 at different temperatures (T/°C), measured by the rate of formation of pyruvate, k(Py)

pH	T/°C	k(Py)/10^{-6} s^{-1}	$r[k(Py)]^a$
4.79	35	0.563	0.9995
4.75	55	8.78	0.9986
4.75	75	106.0	0.9994
4.75	75	106.6	0.9997

[a] Correlation coefficient, from plots of ln $\{[Py]_{inf} - [Py]_t\}$ *vs.* t.



Scheme 2 Possible mechanisms for the hydrolysis of the monoanion and dianion of phosphonopyruvate

pK_a values of phosphonopyruvate which are gratifyingly similar to those obtained by titration, especially as the temperature and ionic strength are very different. The rate constants k_1 and k_2 are 1.94×10^{-4} and 1.05×10^{-4} s^{-1}, indicating that the monoanion is almost twice as reactive as the dianion. The corresponding rate constants for PEP are 1.47×10^{-4} and 1.03×10^{-4} s^{-1}.

At pH 4.75, where the dianion exists almost exclusively, the hydrolysis of phosphonopyruvate was additionally monitored at 35 and 55 °C and the rate constants from the appearance of pyruvate are given in Table 2. Using the Arrhenius equation $k = A \exp(-E_a/RT)$, the energy of activation (E_a) is 116.9 ± 0.7 kJ mol^{-1} and A (pre-exponential factor) is 3.75×10^{13} s^{-1}, which are comparable to those given for the hydrolysis of phosphate monoanions.[24] On substitution of these values into the equations $\Delta H^{\ddagger} = E_a - RT$ and $\Delta S^{\ddagger} = (\Delta H^{\ddagger} - \Delta G^{\ddagger})/T$, the enthalpy of activation ($\Delta H^{\ddagger}$) was calculated to be 114.0 kJ mol^{-1} and the entropy of activation ($\Delta S^{\ddagger}$) was +5.4 ± 1.9 J mol^{-1} K^{-1} at 75 °C. Similarly, the hydrolyses of phosphate monoesters have positive or near-zero entropies of activation, consistent with a dissociative mechanism, proceeding *via* monomeric metaphosphate rather than an associative, bimolecular pathway consistent with large negative entropies of activation.[25] The rate of hydrolysis of phosphonopyruvate is not significantly changed in deuterium oxide, with the reaction just 1.08 ± 0.04 times faster in H_2O than in D_2O. The presence of only a small isotope effect suggests that a proton transfer is not involved in the rate limiting step.

For a mechanistic insight into the hydrolyses of phosphate monoesters, the product composition of the reaction in various water–alcohol mixtures has been extensively studied[26] with non-selective phosphorylation providing good evidence for the highly reactive intermediate, monomeric metaphosphate. When phosphonopyruvate was allowed to react in the presence of an equimolar mixture of methanol and water, ^{31}P NMR spectroscopy showed that the products were 53% inorganic phosphate and 47% methyl phosphate. All of the information is consistent with the mechanism of hydrolysis of phosphonopyruvate being related to that for phosphate monoesters, which react by a pre-equilibrium proton transfer from a terminal phosphate oxygen to the bridge oxygen followed by the rate-limiting formation of monomeric metaphosphate.[24,26]

Phosphonopyruvate cannot strictly proceed by this pathway because the CH_2 group cannot protonate before the rate-limiting cleavage of the P–C bond. Therefore a pre-equilibrium proton transfer to the oxygen of the oxo group could be envisaged [Scheme 2(*a*)]. For the hydrolysis of the monoanion of acetyl phosphate, a concerted proton transfer to the carbonyl group with the formation of monomeric metaphosphate and acetic acid has been suggested.[25] Jencks claims that this mechanism is consistent with the observed negligible deuterium isotope effect because of the 'apparent absence of a deuterium isotope effect in certain reactions which almost certainly involve a proton transfer as part of the rate-determining step.'[25] This mechanism can be adopted for the monoanion and the dianion of phosphonopyruvate, with the hydrolysis facilitated by a concerted proton transfer to the oxo group to give monomeric metaphosphate and enolpyruvate [Scheme 2(*b*)]. Whatever the precise details, monomeric metaphosphate is likely to be the intermediate which is known to react readily with water to give inorganic phosphate.[27] It has also been suggested in the C–P bond cleavage reactions of other phosphonates, examples being the reaction of aminomethylphosphonic acid with ninhydrin,[28] the reaction of 2-amino-3-phosphonopropionic acid with pyridoxal[29] and the base-catalysed reaction of β-halophosphonates.[30] The intermediate enolpyruvate, which has a half-life of 3.6 min at 20 °C,[31] readily undergoes tautomerism to pyruvate. It is also possible that the carboxylate group of phosphonopyruvate could participate in the hydrolysis reaction. Bis(2-carboxyphenyl) phosphate is hydrolysed 10^{10} times faster than diphenyl phosphate and this rate enhancement is accounted for by intramolecular nucleophilic catalysis by the carboxylate group with the formation of a cyclic acyl phosphate intermediate.[32] A similar mechanism has been proposed to account for the rapid hydrolyses of the *P,P*-dimethyl and *P,P*-dibenzyl esters of PEP to PEP, which are thought to proceed *via* a 5-membered cyclic acyl phosphate.[33,34] Although the carboxylate group of PEP is thought not to be of major kinetic importance for its hydrolysis to pyruvate and inorganic phosphate, observation of ^{18}O exchange from the water into PEP, suggests that the cyclic acyl phosphate could play some role.[34] Thus, hydrolysis of phosphonopyruvate could proceed by the cyclic acyl phosphonate with the carboxylate group axial and the CH_2 group equatorial. Pseudorotation to place the CH_2 leaving group into an axial position with subsequent departure would give the acyclic acyl phosphate which can hydrolyse to phosphate and pyruvate [Scheme 2(*c*)]. Although our results cannot rule out this possibility, the simpler mechanisms proceeding by monomeric metaphosphate seem more attractive for the hydrolysis of phosphonopyruvate.

Experimental

The m.p.s were determined on a Gallenkamp capillary melting point apparatus and are uncorrected. Elemental analysis was

performed by Butterworth Laboratories Ltd, Middlesex, UK. NMR spectra were recorded on a Bruker AC-300 spectrometer operating at 300 MHz for ^1H and 121.5 MHz for ^{31}P. Chemical shifts are reported in ppm from tetramethylsilane (TMS) in ^1H NMR and from 85% H_3PO_4 in ^{31}P NMR both as external standards. Positive chemical shifts are at low field with respect to the standard. *J* values are given in Hz. The ^{31}P NMR spectra were recorded in H_2O in a 10 mm tube, with the D_2O lock in a 5 mm tube inserted into the 10 mm tube. Spectrophotometric assays were performed on a Cecil CE 594 spectrophotometer. The pH was measured using a WPA CD 660 digital pH meter with a Gallenkamp electrode, type PHM-110-070N. The conductivity was measured with a Jenway 4010 conductivity meter.

Materials.—All reagents were obtained from Aldrich Chemical Company unless otherwise stated. Lactate dehydrogenase (from bovine heart), pyruvate kinase (from rabbit muscle), ADP and NADH were obtained from Sigma. The D_2O was 99.9% isotope enriched. The AG 1-X8 anion-exchange resin (formate form, 200–400 mesh) was from Bio-Rad and the Dowex 50-X8 cation-exchange resin (Na form) was from BDH Chemicals Ltd.

Synthesis of Phosphonopyruvate.—Trimethylsilyl bromide (1.29 g, 8.45 mmol, 3 equiv.) was added to triethyl phosphonopyruvate[9] (0.71 g, 2.82 mmol) at 0 °C. After 2 h, the excess of trimethylsilyl bromide and ethyl bromide was removed by rotary evaporation. The ethyl bis(trimethylsilyl)phosphonopyruvate was hydrolysed by the addition of dioxane (2 cm^3), H_2O (2 cm^3) and cyclohexylamine (0.84 g, 8.45 mmol, 3 equiv.). After 16 h, the solution was concentrated to dryness and then dissolved in H_2O (10 cm^3). Acetone (90 cm^3) was added portionwise upon which the tri(cyclohexylammonium) salt of phosphonopyruvate precipitated (0.731 g, 3.01 mmol, 56%), ^1H NMR (D_2O, ppm) 2.99 (2 H, d, J_{PH} 20.3, P–CH_2), and for the cyclohexylammonium cations 2.95 (3 H, m), 1.77 (6 H, br s), 1.59 (6 H, br d, *J* 5.1), 1.44 (3 H, br d, *J* 12.5), 1.13 (9 H, quintet, *J* 11) and 1.00 (3 H, m); ^{31}P NMR $\delta_P(D_2O)$ 12.15 (s, ^1H decoupled), (t, J_{PH} 20.3, ^1H coupled).

Recrystallisation from acetone–water (3:1) gave needle-shaped crystals, m.p. 180–185 °C (with decomposition). An increase in absorbance at λ 253 nm was observed when the compound was added to a solution containing 1% semicarbazide–HCl and 2% sodium acetate, consistent with the formation of a semicarbazone.[7] (Found: C, 54.33; H, 9.63; N, 8.96. $C_{15}H_{31}N_3O_6$ requires C, 54.17; H, 9.53; N, 9.03%).

For the kinetic studies, phosphonopyruvate was purified by ion-exchange chromatography. An aqueous solution of potassium hydroxide (3 equiv.) was added to ethyl bis(trimethylsilyl)phosphonopyruvate, prepared as described above. After 0.5 h, the solution was diluted with H_2O and loaded onto an AG 1-X8 anion-exchange column equilibrated with 10^{-2} mol dm^{-3} triethylammonium hydrogen carbonate (TEAB) buffer at pH 7.4. The column was eluted with a linear gradient of 10–500 × 10^{-3} mol dm^{-3} TEAB. Fractions having both an absorbance at λ = 255 nm and an increased extinction at λ = 253 nm in the presence of semicarbazide were combined and concentrated. The triethylammonium salt of phosphonopyruvate was adjusted to pH 7 and diluted to 100 cm^3.

A portion of this solution (12 cm^3) was concentrated and the excess TEAB was removed by repeated addition and removal of isopropylamine (3 × 5 cm^3). The residue was dissolved in H_2O (20 cm^3) and applied to a column of DOWEX-50 (15 cm^3, H^+ form). The column was eluted with H_2O (100 cm^3) and the eluent was concentrated to 10 cm^3. From hydrolysis studies and the semicarbazide assay, this solution has a concentration of 34.4 mmol dm^{-3}, confirmed also by potentiometric titration.

pK_a *Measurement of Phosphonopyruvate.*—The pH of the 34.4 mmol dm^{-3} solution of the free acid of phosphonopyruvate (1 cm^3) was measured at 21 °C. Portions (20 mm^3) of 0.1 mol dm^{-3} KOH were added, and after each addition, the pH was measured, and the pK_a calculated as detailed by Albert and Serjeant.[12]

Rates of Hydrolysis of Phosphonopyruvate in H_2O.—The buffers used in the hydrolysis studies were HCl (pH 0.5, 1, 1.5 and 2), formic acid (0.2 mol dm^{-3}, pH 3), acetic acid (0.2 mol dm^{-3}, pH 4, 4.35 and 5), morpholinoethanesulphonic acid (MES, 0.2 mol dm^{-3}, pH 6), tris(hydroxymethyl)aminomethane (TRIS, 0.2 mol dm^{-3}, pH 7) and potassium carbonate (0.2 mol dm^{-3}, pH 9). The buffers contained 1.7 × 10^{-3} mol dm^{-3} ethylenediaminetetraacetic acid (EDTA). The pH electrode was calibrated at pH 7 and 4 or pH 7 and 10 at 75 °C using temperature calibrated buffers. The pH of the buffer was adjusted with either HCl or KOH at 75 °C. Their conductivities were adjusted to 0.2 S with KCl, except for the HCl buffer at pH 0.5 which had a conductivity of 0.38 S before the addition of KCl.

For each hydrolysis study in either H_2O or D_2O, buffer (3 cm^3) was placed in a 5 cm^3 screw top tube sealed with a Teflon rubber septum. The tube was placed in a circulating water bath set at the appropriate temperature (35, 55 or 75 °C). After 15 min, phosphonopyruvate solution (1 cm^3) was added to give a *ca.* 0.86 mmol dm^{-3} solution. After mixing, a sample (0.3 cm^3) was removed by syringe. The sample was returned to the water bath and 6–9 samples of 0.3 cm^3 were removed at time intervals over 10 half-lives. The pH of the reaction mixture was measured at 75 °C during the course of the reaction. The samples were stored at −20 °C until the final sample had been taken. The samples were assayed spectrophotometrically at λ = 340 nm for pyruvate[21] using lactate dehydrogenase (LDH) with NADH in a triethanolamine buffer at pH 7.4. The samples taken after 10 half-lives were shown to have a pyruvate concentration $[Py]_{inf}$ between 7.915 and 8.84 × 10^{-3} mol dm^{-3}. The reaction followed first-order kinetics and the rate constants and their correlation coefficients, *r* from the plots of $\ln\{[Py]_{inf} - [Py]_t\}$ against time *t* are given in Tables 1 and 2. The samples were also analysed for phosphoenolpyruvate by the addition of ADP and pyruvate kinase to the LDH assay.[21] The presence of PEP was not detected in any sample. The samples from the hydrolysis studies at 75 °C were also analysed spectrophotometrically for inorganic phosphate using an ammonium molybdate/ammonium vanadate assay[35] at λ = 350 nm. The samples taken after 10 half-lives were shown to have an inorganic phosphate concentration $[P_i]_{inf}$ between 7.54 and 8.95 × 10^{-3} mol dm^{-3}. The rate constants and the correlation coefficients, *r*, for plots of $\ln\{[P_i]_{inf} - [P_i]_t\}$ *vs.* *t* are given in Table 1.

Rates of Hydrolysis of Phosphonopyruvate in D_2O.—The phosphonopyruvate solution (1 cm^3) was concentrated to dryness at 20 °C. D_2O was added and the reaction mixture was stored at 0 °C for 3 days. The D_2O was removed and further D_2O (1 cm^3) was added. The pD of a 0.2 mol dm^{-3} solution of acetic acid in D_2O containing 1.7 × 10^{-3} mol dm^{-3} EDTA was adjusted to 5 at 75 °C using a solution of KOD. The pD was calculated from the pH meter reading by the method of Fife and Bruice.[36] The rates of reaction of phosphonopyruvate in D_2O and H_2O were measured simultaneously as described for the reactions in H_2O. The results are given in Table 3.

Products of Hydrolysis of Phosphonopyruvate.—A volume of the phosphonopyruvate solution (0.5 cm^3) was added to the acetate buffer (1.5 cm^3) at pH 5. The solution was heated for 110 min (1 half-life) at 75 °C. The solution was examined by ^{31}P

Table 3 Rates of hydrolysis of phosphonopyruvate at 75 °C in H_2O and D_2O measured by the rate of formation of pyruvate, k(Py)

pH	pD[a]	k(Py)/10^{-4} s^{-1}	$r[k(Py)]$[b]
—	4.70	0.956	0.9974
—	4.70	1.013	0.9992
4.75	—	1.060	0.9994
4.75	—	1.066	0.9997

[a] For measurements[36] in D_2O, pD = pH meter reading + (429/T/K) − 1.04. [b] Correlation coefficient, from plots of $\ln\{[Py]_{inf} - [Py]_t\}$ *vs.* t.

NMR spectroscopy which showed that half of the phosphonopyruvate (δ_P 14.1) had decomposed, the only product being inorganic phosphate (δ_P 2.3). The identity of this peak was confirmed when addition of K_2HPO_4 to the sample caused the phosphate peak to increase relative to that for phosphonopyruvate.

Reaction of Phosphonopyruvate with MeOH–H_2O.—The free acid solution of phosphonopyruvate (1 cm^3) was concentrated to dryness. The residue was dissolved in a mixture of MeOH (1.385 cm^3, 34.2 mmol) and acetate buffer at pH 5 (0.615 cm^3, 34.2 mmol). The solution was heated for 2 h at 75 °C after which time a third of the phosphonopyruvate remained [δ_P 14.0 (s, ^1H decoupled; t, J_{PH} 21.2, ^1H coupled)]. Two products were formed: inorganic phosphate [53%; δ_P 3.07 (s, ^1H decoupled or coupled)] and methyl phosphate [47%; δ_P 4.28 (s, ^1H decoupled; q, J_{PH} 10.5, ^1H coupled)].

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