Nucleophilic Catalysis of Glycoside Hydrolysis. The Hydrolysis of 4-Nitrophenyl α- and β-D-glucopyranoside Tetraphosphates

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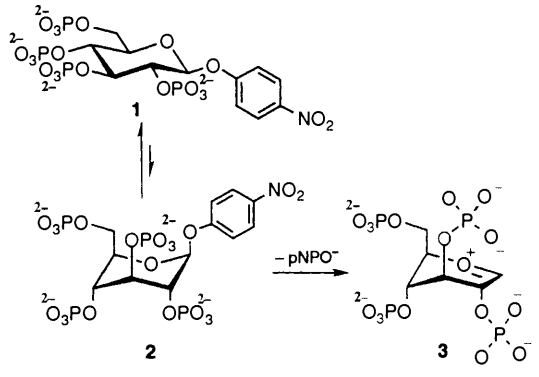
The octaanion of the tetraphosphate of 4-nitrophenyl β-D-glucopyranoside is hydrolysed up to 100 times faster than model compounds, in a reaction which is pH-independent above pH 9. The mechanism involves intramolecular nucleophilic catalysis by the phosphate group in the 2-position: the intermediate five-membered 1,2-cyclic phosphate can be observed directly by ^{31}P NMR spectroscopy under suitable conditions. In base this gives α-D-glucopyranose 1,3,4,6-tetraphosphate and inorganic phosphate: it appears that glucose 2,3,4,6-tetraphosphate is not stable under basic conditions, but is converted into the triphosphate of a saccharinic acid. The tetraphosphate of 4-nitrophenyl α-D-glucopyranoside shows no significant acceleration.

The study of enzyme mechanism is a two-way process. Informed discussion is necessarily based on the known chemistry of the functional groups involved in the particular enzyme reaction. This often leads to more focussed work on the basic mechanism, and in the most interesting cases what we learn about the enzyme reaction teaches us new chemistry. A classic case is the lysozyme reaction: the first enzyme crystal structure[1] clearly defined the reaction involved, as glycoside cleavage catalysed by two carboxy groups; but neither carboxy nor carboxylate was known at the time to catalyse the hydrolysis of acetals. The enzyme reaction thus set the agenda for many years of mechanistic work on this area of chemistry.

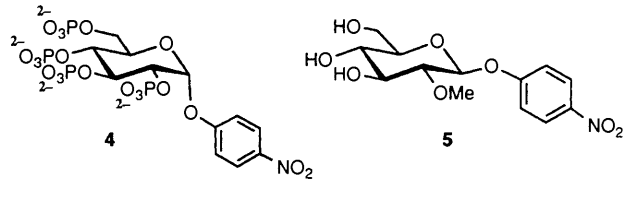
We now have a fairly detailed understanding of how a COOH group can act as a general acid to catalyse acetal hydrolysis,[2] and a growing understanding of how this can be achieved with high efficiency.[3] The role of the second, COO^- group, has been more difficult to establish,[2] but there is a growing consensus that it is involved as a nucleophile† in the reactions of retaining glucosides,[4] which in effect carry out a double-displacement reaction at the glycosidic centre. On the one hand, Withers and his co-workers have identified[5] a glycosyl-enzyme in the hydrolysis of a 2-fluorinated substrate by β-glucosidase from *Agrobacterium*, while the work of Jencks and his group[6,7] has shown that the non-enzymic hydrolysis of glycosides is an enforced-concerted process. Indeed, intermolecular nucleophilic catalysis of acetal hydrolysis is now a well-established mechanism.[8,9] The known chemistry has thus caught up with the enzyme reaction, in the sense that the pieces of the mechanism are now in place: however, we cannot claim to understand the enzyme in detail because we still cannot put the pieces together to achieve efficient non-enzymic glycoside hydrolysis under physiological conditions.

We have prepared 4-nitrophenyl β-D-glucopyranoside tetraphosphate **1**, partly because of a report[10] that *myo*-inositol hexaphosphate, which has one axial and five equatorial phosphate groups, is converted at high pH into the inverted chair structure. This must have five axial phosphate groups, all presumably in the dianion form. If the same process were to occur to a significant extent with a glycoside tetraphosphate (the extreme case is represented by **1** ⟷ **2**) departure of the good 4-nitrophenoxide leaving group would generate an oxocarbenium ion (*cf.*, the extreme case represented by **3**) sandwiched between stabilising negative charges. An acceleration of hydrolysis would result, as predicted in one model for the lysozyme mechanism.[2] We have measured the accelerating effects of phosphorylation on 4-nitrophenyl β-D-glucoside, and report results with this and various related glucosides, including the α-anomer **4** of **1**, which allow us to identify their origin unambiguously as nucleophilic catalysis by the 2-phosphate group.



Results

4-Nitrophenyl α- and β-D-glucopyranosides were phosphorylated using an excess of dibenzyl *N,N*-diisopropylphosphorochloridite, followed by oxidation to the tetraphosphate. The eight benzyl groups were removed selectively using trimethylsilyl bromide. 4-Nitrophenyl 2-*O*-methyl β-D-glucopyranoside **5**



† Convincing but indirect evidence for intramolecular nucleophilic catalysis by the amide group of 4-nitrophenyl 2-acetamido-2-deoxy-β-D-glucopyranoside was presented many years ago by D. Piszkiewicz and T. C. Bruice, *J. Am. Chem. Soc.*, 1968, **90**, 2156; other related work in the area is described in ref. 2.

Table 1 First-order rate constants for the hydrolysis of 4-nitrophenyl α- and β-D-glucopyranoside tetraphosphates and related compounds, at 80 °C and ionic strength 0.2 mol dm^{-3} (KCl)

Conditions		Observed rate constants				
Buffer (% free base)	pH	k_{obs}/GP4β[a]	k_{obs}/GP4α[a]	k_{obs}/G2-OMe[a]	k_{obs}/G2-OH[a]	k_{obs}/PNA[a]
MES[a] (50)	5.82	1.42×10^{-7}				
Imidazole (50)	6.23	2.56×10^{-7}		1.16×10^{-7}	1.02×10^{-7}	
TRIS[a] (50)	6.93	5.34×10^{-7}		1.20×10^{-7}	1.12×10^{-7}	
TRIS[a] (80)	7.65	1.31×10^{-6}	3.68×10^{-7}	1.28×10^{-7}	1.88×10^{-7}	
Borate (20)	8.05	2.06×10^{-6}	2.93×10^{-7}	1.28×10^{-7}	5.80×10^{-7}	1.14×10^{-9}
Borate (50)	8.82	6.17×10^{-6}	3.80×10^{-7}		2.70×10^{-6}	8.86×10^{-9}
CAPS[a] (50)	9.25	7.83×10^{-6}		1.32×10^{-7}	5.70×10^{-6}	
CAPS[a] (80)	9.90	8.09×10^{-6}		2.00×10^{-7}	2.20×10^{-5}	
0.01 mol dm^{-3} NaOH	10.35	7.99×10^{-6}	3.48×10^{-7}	3.18×10^{-7}	2.90×10^{-5}	2.23×10^{-7}
0.05 mol dm^{-3} NaOH	11.05			1.73×10^{-6}		1.42×10^{-6}
0.1 mol dm^{-3} NaOH	11.35	8.27×10^{-6}	4.30×10^{-7}	3.55×10^{-6}	8.87×10^{-4}[b]	2.86×10^{-6}
0.15 mol dm^{-3} NaOH	11.53			5.04×10^{-6}		6.21×10^{-6}
0.2 mol dm^{-3} NaOH	11.65			7.04×10^{-6}		

[a] Abbreviations used: GP4α and β are the glucoside tetraphosphates **1** and **4**, G2-OH and G2-OMe are 4-nitrophenyl β-D-glucopyranoside and its 2-*O*-methyl derivative (**5**), and PNA is 4-nitroanisole. Buffers: MES is morpholine *N*-ethanesulfonate, TRIS is tris(hydroxymethylamino)methane and CAPS is 3-cyclohexylaminopropane-1-sulfonic acid. [b] Similar to the rate constant measured under similar conditions by D. Piszkiewicz and T. C. Bruice, *J. Am. Chem. Soc.*, 1967, **89**, 6237.

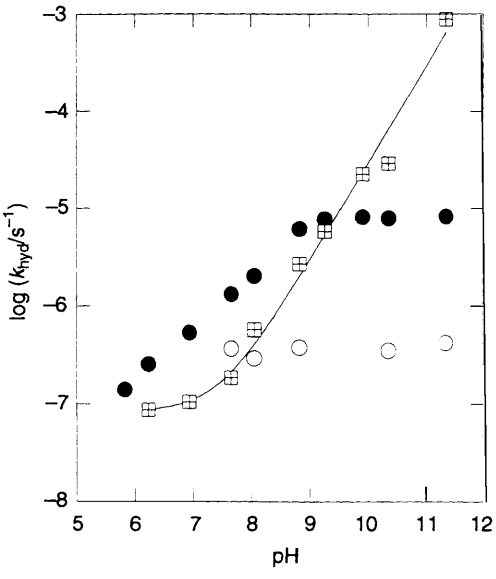


Fig. 1 pH–rate profile for hydrolysis of 4-nitrophenyl β-D-glucopyranoside tetraphosphate **1** (●) compared with those for the α-anomer **4** (○), and for 4-nitrophenyl β-D-glucopyranoside itself (⊞). Data measured in water at 80 °C and ionic strength 0.2 mol dm^{-3}.

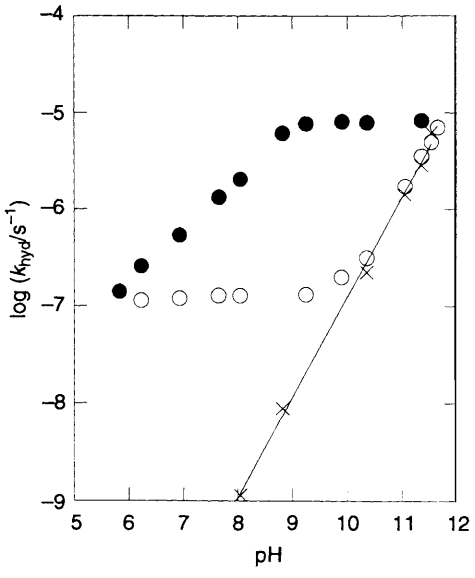


Fig. 2 pH–rate profile for hydrolysis of 4-nitrophenyl β-D-glucopyranoside tetraphosphate **1** (●) compared with those for 4-nitrophenyl-2-*O*-methyl-β-D-glucopyranoside **5** (○), and for 4-nitroanisole (×). Data measured in water at 80 °C and ionic strength 0.2 mol dm^{-3}.

was prepared by methylation of 4-nitrophenyl 4,6-*O*-benzylidene-β-D-glucopyranoside with methyl iodide, removal of the benzylidene group and separation of the 2- and 3-*O*-methyl derivatives.

Rates of hydrolysis were compared under standard conditions [aqueous solution, 80° and ionic strength 0.2 mol dm^{-3} (NaCl)] for the tetraphosphates of 4-nitrophenyl β- and α-D-glucopyranosides (**1** and **4**, respectively), and, for comparison, for the parent 4-nitrophenyl β-D-glucopyranoside, its 2-*O*-methyl derivative **5**, and for 4-nitrophenylanisole. The data are shown in Table 1.

The pH–rate profile for the hydrolysis of the tetraphosphate of 4-nitrophenyl β-D-glucopyranoside (Fig. 1) shows that the rate of release of 4-nitrophenolate increases as the pH rises from 6 to 9, in the region of the successive second dissociations of the four phosphate groups. (Potentiometric titrations of the tetraphosphates show that the four second dissociations occur in this region, though they cannot of course be separated.) The pH-independent reaction above pH 9 thus represents the hydrolysis of the octaanion **1**. The hydrolysis of the corresponding α-D-glucopyranoside **4** (Fig. 1) is pH-independent down to about pH 7, and 23 times slower.

By contrast, the reaction of the parent 4-nitrophenyl β-D-glucopyranoside is base-catalysed down almost to pH 7 (see Fig. 1). This reaction can be eliminated by protecting the 2-hydroxy group: we have used the methyl ether. The pH–rate profile for the hydrolysis of 4-nitrophenyl 2-*O*-methyl-β-D-glucopyranoside **5**, shown in Fig. 2, shows a pH-independent reaction up to pH 9–10, at which point base-catalysed hydrolysis sets in. This latter reaction is readily identified as nucleophilic aromatic substitution by hydroxide, because the second-order rate constant for the release of 4-nitrophenolate is almost identical with that for the same reaction of 4-nitroanisole (see Fig. 2).

Product Studies.—Product studies were carried out by ^{1}H and ^{31}P NMR spectroscopy. When a reaction mixture near pH 9 was monitored by ^{31}P NMR the signal corresponding to the

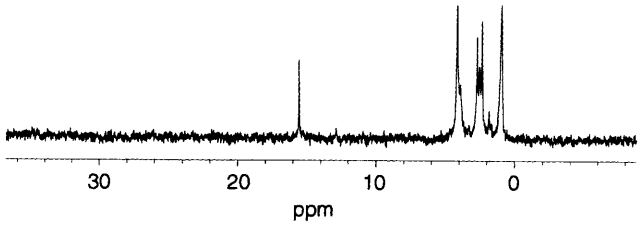
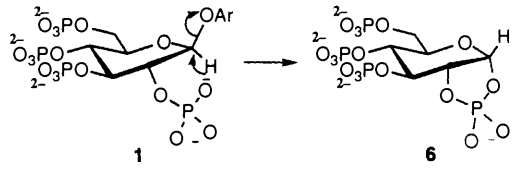


Fig. 3 ^{31}P NMR spectrum of the reaction mixture after keeping 4-nitrophenyl β-D-glucopyranoside 2,3,4,6-tetraphosphate **1** in 0.04 mol dm^{-3} borate buffer (50% free base, pH = 8.82, 0.2 mol dm^{-1} buffer diluted with 4 parts of D_2O) at 80 °C for 40 h. The signal at 15.5 ppm is not present in either starting material or product, and is identified as the cyclic phosphate **6**. Broad signals at lower field are combination peaks, but the three central, sharp signals at 2.25, 2.53 and 2.62 ppm represent the 2-phosphate group of the starting material, the 1-phosphate group of product glucose 1,3,4,6-tetraphosphate and inorganic phosphate, respectively.

2-phosphate group of **1** disappeared and a new signal at 15.54 ppm appeared and subsequently disappeared (Fig. 3). This intermediate, identified as the 1,2-cyclic phosphate **6**, has a half-life of many hours under these conditions. Other new signals, from inorganic phosphate and β-D-glucopyranose 1,3,4,6-tetraphosphate, accumulate at the end of the reaction. Integration shows that disappearance of the 2-phosphate signal of **1** occurs with a rate (6×10^{-6} s^{-1}) consistent with that observed for the release of nitrophenoxide at the same pH. At higher pH (0.1 mol dm^{-3} NaOH) the same products were formed but no intermediate could be observed.

The cyclic phosphate **6** was not expected to be hydrolysed exclusively to the 1,3,4,6-tetraphosphate, so additional experiments were run in 50% methanol–water (containing 0.1 mol dm^{-3} NaOH) to identify the methyl phosphate diesters expected to be formed by the opening of **6** by methanol. Major products under these conditions were inorganic phosphate and β-D-glucopyranose 1,3,4,6-tetraphosphate, as before, and methyl phosphate monoester. No methyl phosphate diesters were detected: evidently β-D-glucopyranose 2,3,4,6-tetraphosphate, and its 2-methyl phosphate diester, are not stable under the conditions of our experiments. A presumed decomposition product, thought to be a saccharinic acid derivative, was identified by a signal at 1.3 ppm (d, J 7.0 Hz) in the ^1H NMR spectrum of the product solutions in D_2O.

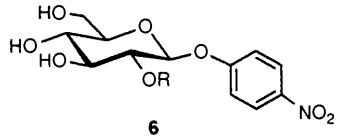


Discussion

The hydrolysis of the glycosidic group of 4-nitrophenyl β-D-glucopyranoside tetraphosphate **1** is clearly accelerated when all four phosphate groups are fully ionised (Fig. 1). The reaction of interest for this work is the pH-independent hydrolysis of the octa-anion at pH > 9. The rate constant for this reaction is 8.1×10^{-6} s^{-1}, and we need to estimate the extent of the acceleration which can be attributed to the presence of the four phosphate groups, and to identify its origin. The reaction is 23 times faster than the reaction of the α-anomer, which is also independent of pH in this region. However, the reaction of the α-anomer could involve nucleophilic attack on the nitrophenyl ring by the 2-phosphate dianion, as suggested for the anion derived from the 2-hydroxy group of 4-nitrophenyl α-D-glucopyranoside by Horton and his co-workers.[11] An alternative comparison is with the parent compound, 4-nitrophenyl β-D-glucopyranoside, but the hydrolysis of this compound is base-catalysed in the pH-region of interest (Fig. 1). The base-catalysed reaction has been ascribed to intramolecular nucleophilic displacement of 4-nitrophenoxide by the anion of the 2-hydroxy group, and is thus not a typical acetal hydrolysis.

We concluded that the best estimate of the rate of the pH-independent cleavage of a 4-nitrophenyl β-D-glucopyranoside near pH 9 would be obtained by protecting this 2-hydroxy group, so we measured also the pH–rate profile (Fig. 2) for the hydrolysis of 4-nitrophenyl 2-*O*-methyl-β-D-glucopyranoside. This shows a pH-independent reaction up to pH 9, though at higher pH this compound also shows base-catalysed hydrolysis. This latter process involves aryl–oxygen cleavage [4-nitroanisole reacts at the same rate (Fig. 2)], and it is possible for all 4-nitrophenoxy-compounds: if no faster reaction is available aromatic nucleophilic substitution by hydroxide gives 4-nitrophenolate.

Two comparisons are thus available to allow us to estimate the acceleration occasioned by the presence of the four phosphate groups of **1**—the pH-independent rates of hydrolysis of 4-nitrophenyl β-D-glucopyranoside below pH 7, and of its 2-*O*-methyl ether below pH 9. The rate constants are similar: 8.28×10^{-8} and 1.16×10^{-7} dm^3 mol^{-1} s^{-1} for the 2-OH and the 2-OMe compounds, respectively, as expected if the same reaction (**7**) is being measured. The rate acceleration attributable to phosphorylation is thus some two orders of magnitude, based on rate ratios of about 100 and 70 for **6**, R = H and Me, respectively.



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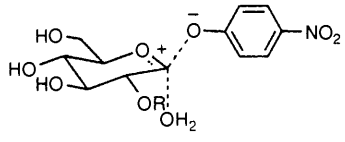

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The origin of this rate enhancement is nucleophilic participation by the phosphate group in the 2-position of **1**. Product studies by ^{31}P NMR spectroscopy establish unambiguously that this phosphate group is involved as a nucleophile, and the 1,2-cyclic phosphate can be observed under appropriate conditions as an intermediate.* The remaining ambiguity is the possibility that the neighbouring nucleophile traps a pre-formed oxocarbenium ion, but there is now a great deal of evidence that such a species has no significant lifetime in the presence of water,[4,7] still less in close proximity to a good nucleophile. The conclusion seems inescapable that this glycoside hydrolysis shows true nucleophilic catalysis.

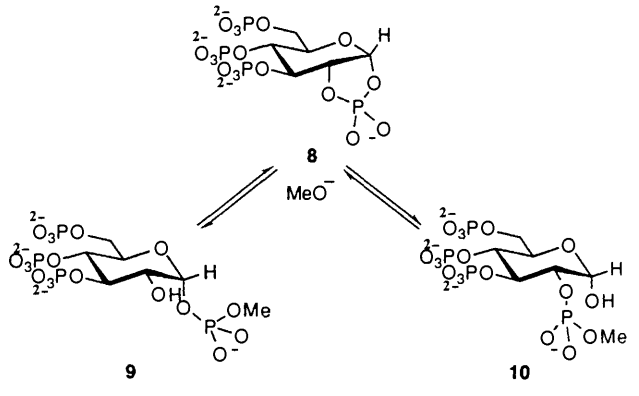
Reactions of the cyclic phosphate **8**.—The presence of the cyclic phosphate **8** as an intermediate (see Fig. 3) on the reaction pathway is established directly by the observation (by ^{31}P NMR) that it is formed at the same rate as 4-nitrophenolate near pH 9. It has a half-life of many hours under the conditions

* Glycopyranoside 1,2-cyclic phosphates are known compounds, usually made by the (P–O bond-forming) cyclisation of sugar 1-phosphates (J. V. O'Connor, H. A. Nunez and R. Barker, *Biochemistry*, 1979, **18**, 500).

of this experiment (borate buffer, pH 8.82, conditions as for the kinetic experiments), but cannot be observed at high pH, no doubt because of its rapid base-catalysed hydrolysis. However, it is still an intermediate under these conditions, as shown by the products of hydrolysis. In 0.1 mol dm^{-3} NaOH major phosphorus-containing products are inorganic phosphate and β-D-glucopyranose 1,3,4,6-tetraphosphate: the 2,3,4,6-tetraphosphate does not appear to be present. When the same experiment was carried out in 50% methanol–water the same products were observed, with the addition of methyl phosphate monoester. Under these conditions no methyl phosphate diesters could be detected.

These observations can be rationalised as shown (Scheme 1) for solvolysis by methanol, though some of the reactions concerned are initially surprising. The 1,2-cyclic phosphate is expected to be opened to a mixture of 1- and 2-phosphates, with the former likely to predominate (the hemiacetal oxygen will be the better leaving group). Since we see no 2-phosphate among the final products of the reaction this must have reacted further. Furthermore, neither methyl phosphate diester (**9** or **10**) is observed, only methyl phosphate monoester. The fact that methanol has been phosphorylated requires that the cyclic phosphate **8** is an intermediate, but both **9** and **10**, like the 2-phosphate produced by attack by hydroxide ion, must react further under the conditions.

This is not surprising for the diester **9** and **10**, because their formation will be reversible, and the thermodynamic products will be the monoesters formed by hydroxide attack on the cyclic ester (**8**) But if the 2-phosphate monoester reacts further then the corresponding diester must react in the same way, but faster, by a pathway not involving recyclisation. The unexpected conclusion is thus that the 'escape route' from the reversible system shown in Scheme 1 is not the C–O cleavage of the 1-phosphate, but the disappearance of the 2-phosphate.



Scheme 1

A possible explanation is suggested by the work of Horton and Luetzow,[11] who suggested that the 4-nitrophenyl ether formed by intramolecular nucleophilic attack on the aromatic ring by the 2-hydroxy group of 4-nitrophenyl α-D-glucopyranoside reacts further to form a saccharinic acid. We are investigating this reaction in a model system. Our results so far are broadly consistent with this explanation,* though not with the mechanism suggested by the authors.[11,12]

Experimental

Materials and Methods.—Acetonitrile was distilled from calcium hydride and stored over molecular sieves (3 Å). 4-Nitrophenyl 4,6-*O*-benzylidene-β-D-glucopyranoside was prepared from 4-nitrophenyl β-D-glucopyranoside, by a standard procedure using benzaldehyde–formic acid (1:2). Dibenzyl *N*,*N*-diisopropylphosphoramidite was prepared according to Yu and Fraser-Reid.[13] All other compounds used for synthesis were of standard commercial grade. All buffer substances were the best available commercial grades. ^{31}P NMR spectroscopy was performed at 109.4 MHz (resolution 1.2 Hz) and chemical shifts are reported relative to 2% H_3PO_4 in D_2O. ^1H NMR spectroscopy was performed at 269.7 MHz (except for the 4-nitrophenyl octabenzyl-α- and -β-D-glucopyranoside 2,3,4,6-tetraphosphates for which the spectra were recorded at 400 MHz), and chemical shifts were measured relative to the solvents used (HDO in D_2O: 4.73 ppm at 25 °C and 4.51 ppm at 50 °C; CHD_2CN in CD_3CN: 1.90 ppm; $CHCl_3$ in $CDCl_3$: 7.27 ppm).

Kinetic Measurements.—Standard conditions were 80 °C and ionic strength 0.2 mol dm^{-3}, maintained with sodium chloride. pH measurements were made under the conditions of the kinetic experiments using a Radiometer PHM82 pH meter equipped with a Russell CTWL electrode. All kinetic experiments (except for the reaction of 4-nitrophenyl β-D-glucopyranoside in 0.1 mol dm^{-3} NaOH, which was followed over three half-lives) were performed as initial rate studies up to a maximum of 4% of reaction. Reactions were followed in the thermostatted cell compartment of a Varian Cary 3 or Gilford 2600 UV–VIS spectrophotometer, in solutions about 0.5 mmol dm^{-3} in substrate, by monitoring the absorbance (408 nm = λ_{max} at 80 °C) of released nitrophenoxide as a function of time. End-points were determined by hydrolysing the respective compounds in 0.1 mol dm^{-3} sodium hydroxide (or alternatively in 0.2 mol dm^{-3} HCl followed by a pH-jump to 0.1 mol dm^{-3} sodium hydroxide) until a constant value for the absorption (408 nm at 80 °C) of formed nitrophenoxide was obtained. Values presented in Table 1 represent extrapolations to zero buffer concentration (no significant buffer catalysis was in fact observed).

Detection of the Intermediate 1,2-*Cyclic Phosphate* **6** *by NMR Spectroscopy.*—4-Nitrophenyl-β-D-glucopyranoside 2,3,4,6-tetraphosphate was dissolved in 0.04 mol dm^{-3} borate buffer (50% free base, pH = 8.82, 0.2 mol dm^{-3} buffer diluted with 4 parts of D_2O). The reaction was followed at 80 °C by recording ^{31}P NMR spectra at different times. Under these conditions a resonance at 2.25 ppm (d, J = 9.8 Hz) disappeared with time and an intermediate at 15.54 ppm appeared and subsequently disappeared as the reaction proceeded. Furthermore the intensities of signals at 2.53 ppm (d, J = 8.6 Hz) and 2.62 ppm (sharp singlet) increased with time. The signals were assigned to the disappearing 2-phosphate of the starting material (2.25 ppm), the intermediate cyclic phosphate (15.54 ppm: the pronounced downfield shift is characteristic of five-membered ring phosphates[14]), the 1-phosphate (2.53 ppm) formed, and inorganic phosphate (2.62 ppm). Integration of these resonances showed that the fraction of the 2-phosphate signal (relative to the sum of the above assigned signals) disappeared with a rate (6×10^{-6} s^{-1}) consistent with that observed for the release of nitrophenoxide at the same pH.

Product analysis at higher pH was done for reactions carried out in sealed ampoules for 70 h at 80 °C, in either H_2O or H_2O–MeOH (50:50) 0.1 mol dm^{-3} in sodium hydroxide. The resulting samples were neutralised with HCl and the solution extracted with dichloromethane. The aqueous phase was lyophilised, dissolved in D_2O, lyophilised again and finally dissolved in D_2O for ^{31}P and ^1H NMR analysis. The ^{31}P NMR spectrum of the reaction products in H_2O showed a dominant

* A referee suggests that the 3-phosphate group is being lost, by an El_{CB} mechanism. This is not obviously consistent with the evidence, but cannot rigorously be ruled out at this stage.

sharp singlet which coincided with added inorganic phosphate (as dipotassium monohydrogen phosphate) whereas reaction in MeOH–H_2O gave the same resonance and also a quartet (4.8 ppm, $^3J_{PH}$ = 10.1 Hz) which coincided with added methyl phosphate (the dicyclohexylammonium salt, prepared according to Kirby[15]). For both reactions ^{1}H NMR showed only one signal (5.47 ppm) in the anomeric proton region. This was a double doublet, with one coupling constant ($^3J_{HH}$ = 3.3 Hz) characteristic for coupling between *cis*-1- and -2-protons in pyranosides;[16] the larger ($^3J_{PH}$ = 7 Hz) represents coupling to neighbouring phosphorus ($^3J_{PH}$ from the ^{1}H NMR experiment corresponds to that found in the ^{31}P NMR, within the limits of the resolution). It seems clear that this proton resonance arises from a product with an anomeric phosphate group (*i.e.*, α-D-glucopyranose 1,3,4,6-tetraphosphate). Homodecoupling experiments identified a coupling between this resonance and a signal at 3.72 ppm (thus the 2-H). The 2-H signal, in contrast with the signal for the anomeric proton, showed no coupling to phosphorus.

The ^{1}H NMR spectrum of the reaction products in MeOH–H_2O showed an additional resonance at 3.5 ppm (d, $^3J_{PH}$ = 10.3 Hz) coincident with that of added methyl phosphate (as above). For both reactions the ^{1}H NMR spectrum also showed a doublet (1.3 ppm, J = 7.0 Hz) coupled to a signal at 4.07 ppm. This suggests that these are resonances from the 2- and 3-H in the saccharinic acid derivative that is formed when methyl phosphate or inorganic phosphate is released.

4-Nitrophenyl Octabenzyl-α- and -β-D-glucopyranoside 2,3,4,6-Tetraphosphates.—The phosphorylation procedure is based upon that published by Yu and Fraser-Reid.[13] Acetonitrile (15 cm^3) was added to tetrazole (466 mg, 6.66 mmol) and evaporated under reduced pressure and the procedure repeated. After the addition of 4-nitrophenyl β-D-glucopyranoside (168 mg, 0.554 mmol), 10 cm^3 of ethyl acetate were added then evaporated, and the procedure repeated with dichloromethane (10 cm^3). The residue was dissolved in dry dichloromethane (20 cm^3) and the solution cooled on an ice–water bath. Dibenzyl *N,N*-diisopropylphosphoramidite (1.15 g, 3.32 mmol) was then added and the mixture stirred at room temperature for 2.5 h. The reaction mixture was cooled in an acetonitrile–dry ice bath and 3-chloroperbenzoic acid (956 mg, 4.44 mmol) was added. The solution was moved to an ice–water bath on which it was kept stirring for 1 h. The solution was kept at room temperature for 5 min and 80 cm^3 of dichloromethane were added. The solution was washed with aqueous solutions of sodium sulfite (10%, 2 × 30 cm^3) and saturated sodium hydrogen carbonate (2 × 25 cm^3), then with water and finally with 5% aqueous sodium chloride. The organic phase was dried with sodium sulfate for 5 min, then the salt was removed by filtration and the filtrate evaporated under reduced pressure. The solid residue was triturated twice with hexane then purified on a short silica gel column (flash mode, eluent toluene–ethyl acetate 1 : 1). The collected fractions were evaporated under reduced pressure and the solid recrystallised from diethyl ether to give 685 mg (0.51 mmol, 92%) of *4-nitrophenyl octabenzyl-β-D-glucopyranoside 2,3,4,6-tetraphosphate* as white needles, m.p. (uncorrected) 96–97 °C. The same procedure using the 4-nitrophenyl α-glucoside gave 560 mg (0.42 mmol, 75%) of *4-nitrophenyl octabenzyl-α-D-glucopyranoside 2,3,4,6-tetraphosphate.*

4-Nitrophenyl octabenzyl-β-D-glucopyranoside 2,3,4,6-tetraphosphate (Found: C, 60.7; H, 4.95; N, 1.05; P, 9.45. $C_{68}H_{67}NO_{20}P_4$ requires: C, 60.85; H, 5.03; N, 1.04; P, 9.23%).

4-Nitrophenyl octabenzyl-α-D-glucopyranoside 2,3,4,6-tetraphosphate (Found: C, 60.55; H, 4.9; N, 1.3; P, 9.2%).

4-Nitrophenyl octabenzyl-β-D-glucopyranoside 2,3,4,6-tetraphosphate. δ_P(25 °C; $CDCl_3$) −2.4, −2.6, −2.8 and −3.6; δ_H(25 °C, $CDCl_3$) 7.88 (d, 2 H, J = 9.2, NO_2Ph-H), 7.10–7.28 (m, 40 H, Ar-H), 6.82 (d, 2 H, J = 9.2, NO_2Ph-H), 4.85–5.09 (m, 17 H, Bn-H and H-1), 4.80 (q, 1 H, J 9.0, H-2), 4.66 (q, 1 H, J 8.0, H-4), 4.52 (ddd, 1 H, J = 11.7, 6.4 and 2.2, H-6), 4.43 (q, 1 H, J 9.8, H-3), 4.13 (m, 1 H, H-6) and 3.82 (dt, 1 H, J = 7.2 and 2.0, H-5).

4-Nitrophenyl octabenzyl-α-D-glucopyranoside 2,3,4,6-tetraphosphate. δ_P(25 °C, $CDCl_3$) −2.1, −2.4, −3.0 and −3.1; δ_H(25 °C, $CDCl_3$) 8.0 (d, 2 H, J = 8.9, NO_2Ph-H), 7.11–7.30 (m, 40 H, Ar-H), 6.97 (d, 2 H, J = 9.0, NO_2Ph-H), 5.88 (d, 1 H, J = 3.3, H-1), 4.84–5.21 (m, 17 H, Bn-H and H-2) 4.54 (q, 1 H, J = 9.6, H^{-3}), 4.32–4.42 (m, 3 H, H-4 and H-6) and 3.95 (d, 1 H, J = 7.7, H-5).

4-Nitrophenyl α- and β-D-glucopyranoside 2,3,4,6-tetraphosphates.—4-Nitrophenyl octabenzyl-β-D-glucopyranoside 2,3,4,-6-tetraphosphate (335 mg, 0.25 mmol) was rendered anhydrous by evaporating added acetonitrile twice. The compound was then dissolved in 2.5 cm^3 of dry acetonitrile, trimethylsilyl bromide (405 cm^3, 3 mmol) was added, and the mixture kept at room temperature for 1 h. After this time 12.5 cm^3 of acetonitrile and 0.5 cm^3 of water were added. More water (15 cm^3) was added and the solution was then washed with dichloromethane (3 × 30 cm^3). The aqueous layer was evaporated under reduced pressure to remove remaining organic solvents and then diluted to 30 cm^3 with more water. The pH was adjusted to 5–5.5 with sodium hydroxide and the solution lyophilised. The resulting solid was treated with boiling methanol for 5 min, filtered and washed several times with methanol then diethyl ether, and finally dried in a desiccator overnight to give *β-D-glucopyranoside 2,3,4,6-tetraphosphate* (168 mg, equivalent to a yield of 95% for the Na_4-salt or 99% for the Na_3-salt). The *4-nitrophenyl α-glucopyranoside 2,3,4,6-tetraphosphate* was prepared in the same way.

MS [FAB (+1)]: the strongest molecular ion peaks were at m/z = 688 and 710 (Na_3 and Na_4 salts), but other peaks ±22n (*e.g.*, 666, 732) corresponding to different sodium adducts were also observed. After treatment of the sample with Dowex (H^+) resin the dominant molecular ion was m/z = 622 (corresponding to the fully protonated compound) with only traces of signals at m/z 644 and 666. Both compounds gave similar results.

4-Nitrophenyl β-D-glucopyranoside 2,3,4,6-tetraphosphate. δ_P(25 °C, D_2O) 2.0, 0.8, −0.3 and −0.5; δ_H(25 °C, D_2O) (resonances assigned by homonuclear decoupling experiments) 8.26 (d, 2 H, J = 9.3, NO_2Ph-H), 7.30 (d, 2 H, J = 9.3, NO_2Ph-H), 5.42 (d, 1 H, J = 7.7, H-1), 4.15–4.45 (m, 4 H, H-2 to H-5) and 3.92–4.09 (m, 2 H, H-6).

4-Nitrophenyl α-D-glucopyranoside 2,3,4,6-tetraphosphate. δ_P(25 °C, D_2O) 1.0, 0.6, −0.1 and −0.6, δ_H(25 °C; D_2O) (resonances assigned by homonuclear decoupling experiments) 8.28 (d, 2 H, J = 9.5, NO_2Ph-H), 7.37 (d, 2 H, J = 9.3, NO_2Ph-H), 5.98 (d, 1 H, J = 3.7, H-1), 4.74 (q, 1 H, J = 9.3, H-3), 4.35 (dt, 1 H, J = 3.7 and 9.5, H-2), 4.31 (q, 1 H, J = 9.3, H-4) and 3.98–4.07 (m, 3 H, H-5 and H-6).

4-Nitrophenyl 2-O-Methyl-β-D-glucopyranoside.—4-Nitrophenyl 4,6-*O*-benzylidene-β-D-glucopyranoside (312 mg, 0.8 mmol) was dissolved in acetonitrile together with methyl iodide (64 cm^3, 1.2 mmol). Silver oxide (139 mg, 0.6 mmol) was added and the solution was kept at 50–60 °C. Equal portions of MeI and Ag_2O were added again after 24 h and the reaction mixture was stirred for another 24 h. The condenser was then removed and the mixture left for 30 min to remove excess MeI. The mixture was then filtered through Celite and the filtrate evaporated under reduced pressure. The crude mixture of 4-nitrophenyl 4,6-*O*-benzylidene-2- and -3-*O*-methyl-β-D-glucopyranoside was deprotected by dissolving it in 80% acetic acid (aq.). The mixture was kept for 2 h at room temperature then the acetic acid evaporated off under reduced pressure.

Acetonitrile was added to the residue and then evaporated under reduced pressure. The mixture of 4-nitrophenyl 2- and 3-*O*-methyl-β-D-glucopyranoside was separated using silica gel column chromatography (flash mode, gradient from chloroform to chloroform–methanol, 9:1). Both compounds were crystallised from diethyl ether to give 98 mg (0.31 mmol, 39%) of the 3-OMe sugar and 109 mg (0.35 mmol, 43%) of the 2-OMe compound.

4-*Nitrophenyl* 2-O-*methyl*-β-D-*glucopyranoside* (Found: C, 49.3; H, 5.3; N, 4.45. $C_{13}H_{17}NO_8$ requires: C, 49.52; H, 5.43; N, 4.44%); NMR: signals were assigned using homonuclear decoupling experiments and the position of the methyl group was also confirmed by reacting free OH groups with trichloroacetyl isocyanate (TAI) and then recording additional spectra.

4-*Nitrophenyl* 2-O-*methyl*-β-D-*glucopyranoside*. δ_H(25 °C, CD_3CN) = 8.17 (d, 2 H, J = 9.2, NO_2Ph-H), 7.15 (d, 2 H, J = 9.5, NO_2Ph-H), 5.07 (d, 1 H, J = 7.7, H-1), 3.74 (d, 1 H, J = 11.7, H-6), 3.57 (m, 1 H, H-6), 3.56 (s, 3 H, MeO), 3.28–3.45 (m, 3 H, H-3 to H-5) and 3.12 (t, 1 H, J = 8.1, H-2). (After addition of TAI): 8.19 (d, 2 H, J = 9.2, NO_2Ph-H), 7.21 (d, 2 H, J = 9.2, NO_2Ph-H), 5.39 (d, 1 H, J = 7.7, H-1), 5.29 (t, 1 H, J = 9.5, H-3), 5.13 (t, 1 H, J = 9.7, H-4), 4.43 (dd, 1 H, ^2J = 12.1, ^3J = 2.8, H-6), 4.36 (dd, 1 H, ^2J = 12.1, ^3J = 5.1, H-6), 4.16 (m, 1 H, H-5), 3.57 (t, 1 H, J = H-2) and 3.57 (s, 3 H, MeO).

4-*Nitrophenyl* 3-O-*methyl*-β-D-*glucopyranoside*. δ_H(25 °C, CD_3CN) 8.17 (d, 2 H, J = 9.5, NO_2Ph-H), 7.14 (d, 2 H, J = 9.5, NO_2Ph-H), 5.02 (d, 1 H, J = 8.1, H-1), 3.73 (d, 1 H, J = 12.1, H-6), 3.57 (m, 1 H, H-6), 3.56 (s, 3 H, MeO), 3.34–3.51 (m, 3 H, H-2, H-4 and H-5) and 3.12 (t, 1 H, J = 8.8, H-3. (After addition of TAI): 8.16 (d, 2 H, J = 9.5, NO_2Ph-H), 7.17 (d, 2 H, J = 9.5, NO_2Ph-H), 5.42 (d, 1 H, J = 7.7, H-1), 5.18 (t, 1 H, J = 9.5, H-4), 5.08 (dd, 1 H, J = 9.5 and 8.1, H-2), 4.44 (dd, 1 H, ^2J = 12.1, ^3J = 2.2, H-6), 4.33 (dd, 1 H, ^2J = 12.1, ^3J = 4.8, H-6), 4.15 (m, 1 H, H-5), 3.77 (t, 1 H, J = 9.5, H-3) nd 3.49 (s, 3 H, MeO).

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