Analysis of the Chirality of [^{16}O,^{17}O,^{18}O] Phosphate Esters by ^{31}P Nuclear Magnetic Resonance Spectroscopy

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A stereospecific method for cyclising D-glucose 6-[^{16}O,^{17}O,^{18}O]phosphate and adenosine 5′-[^{16}O,^{17}O,^{18}O]-phosphate to their conformationally locked six-membered cyclic phosphate diesters has been developed. Using [^{16}O,^{17}O,^{18}O]phosphate esters of known absolute configuration it is shown by ^{31}P n.m.r. spectroscopy, after esterification to the axial and equatorial triesters, that the cyclisation occurs with inversion of configuration. It is now possible, therefore, to determine the chirality at phosphorus of D-glucose 6-[^{16}O,^{17}O,^{18}O]phosphate and adenosine 5′-[^{16}O,^{17}O,^{18}O]phosphate by this procedure.

IN ORDER to delineate the stereochemical course of a chemical or enzyme catalysed phosphoryl transfer reaction, the *pro-pro*-chiral phosphate monoester must be made chiral at phosphorus, ideally by isotopic substitution. Fortunately this is possible since oxygen exists as three stable isotopes, namely ^{16}O, ^{17}O, and ^{18}O, and we have recently reported a general method for the synthesis of chiral [^{16}O,^{17}O,^{18}O]phosphate esters of known absolute configuration.[1] We now report a method for the stereochemical analysis of chiral [^{16}O,^{17}O,^{18}O]phosphate esters using ^{31}P n.m.r. spectroscopy.

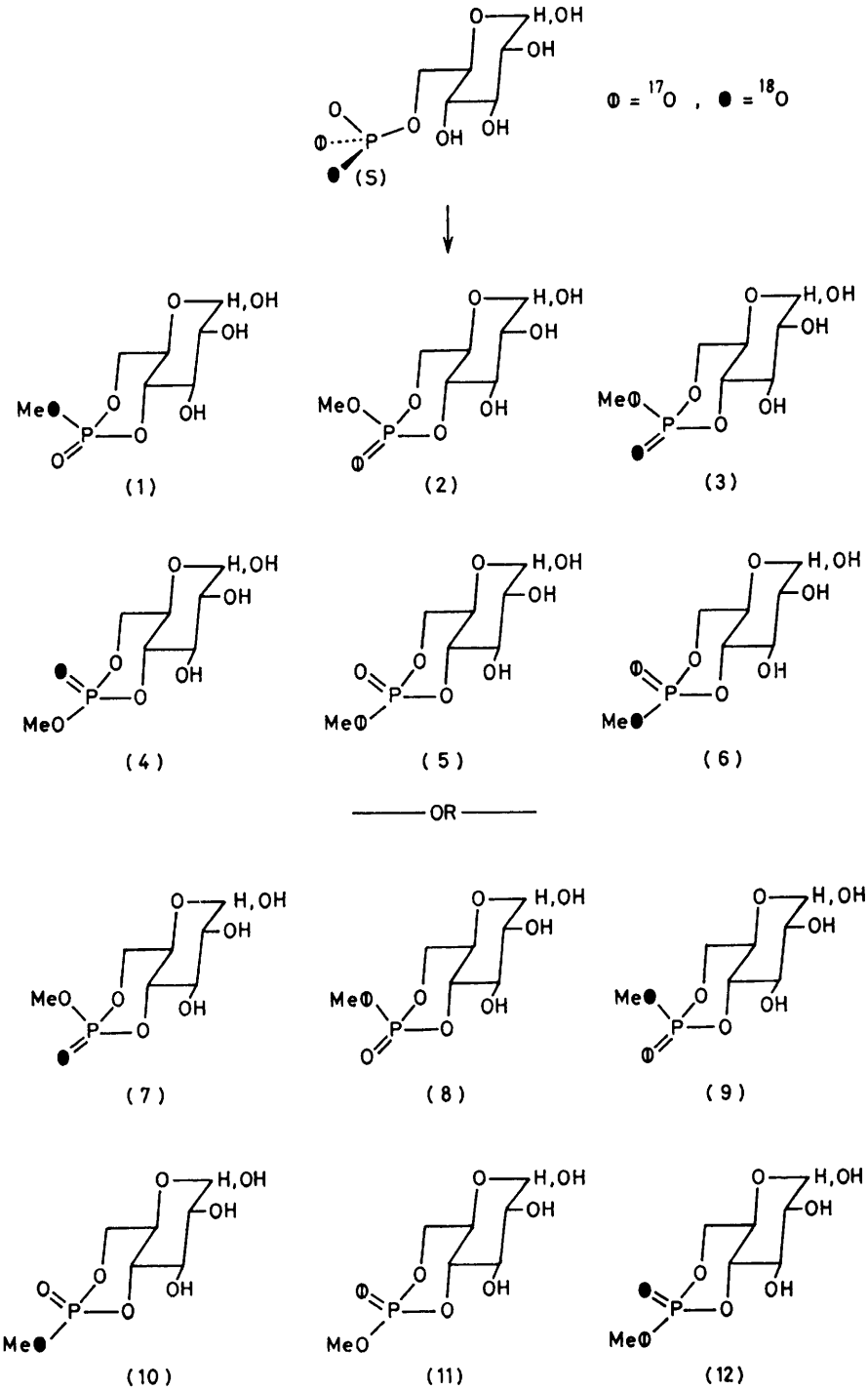
The analysis depends on the stereospecific cyclisation of the chiral [^{16}O,^{17}O,^{18}O]phosphate monoester into a conformationally locked six-membered cyclic phosphate diester, followed by esterification to give the axial and equatorial triesters. In the cyclisation step, any one of the peripheral oxygen isotopes will be lost with equal probability (the kinetic isotope effect being negligible) and the residual oxygen isotopes will occupy diastereotopic sites. D-Glucose 6-[^{16}O,^{17}O,^{18}O]phosphate and adenosine 5′-[^{16}O,^{17}O,^{18}O]phosphate were selected for this analysis since in addition to having the required structural features for making the conformationally locked six-membered cyclic phosphate diesters, they are products from a variety of enzymic reactions.

It will be appreciated that D-glucose 6-[(*S*)-^{16}O,^{17}O,-^{18}O]phosphate after cyclisation and esterification would give species (1)—(6) or species (7)—(12) depending on whether the cyclisation occurs with retention or inversion of configuration at phosphorus (Scheme 1). Owing to the nuclear electric quadrupole moment of ^{17}O, the ^{31}P n.m.r. signal of phosphorus directly bonded to ^{17}O is broadened and virtually obliterated,[2,3] so that only those species not containing ^{17}O bonded to phosphorus will be seen in the ^{31}P n.m.r. spectrum, namely (1) and (4), or (7) and (10). Since ^{18}O when singly bonded to phosphorus causes a smaller isotope shift than when doubly bonded to phosphorus,[2] the isotope shift on the ^{31}P resonance of the axial ester (1) will be smaller than that for the axial ester (7). Similarly, the isotope shift on the ^{31}P resonance of the equatorial ester (10) will be smaller than that of the equatorial ester (4). The analysis outlined in Scheme 1 however, is based on the assumption that all the labelled sites are fully enriched. In practice the sites labelled as ^{18}O will be about 99 atom % ^{18}O, but since ^{17}O is currently available at only about 50 atom % enrichment, species will also be present where sites labelled as ^{17}O will be ^{16}O or ^{18}O. This means that four axial and four equatorial triesters will be observed in the ^{31}P n.m.r. spectrum, *viz.* [$^{16}O_2$]-, [$^{16}O_{ax}$, $^{18}O_{eq}$]-, [$^{18}O_{ax}$, $^{16}O_{eq}$]- and [$^{18}O_2$]-axial and equatorial triesters. This, however, is an advantage since the [$^{16}O_2$]- and [$^{18}O_2$]-triesters will provide the necessary reference signals for determining the isotope shifts, and the ratio of the [$^{16}O_{ax}$, $^{18}O_{eq}$]- and [$^{18}O_{ax}$, $^{16}O_{eq}$]-triesters will provide the stereochemical information; thus species (1) and (4), or (7) and (10) will *predominate* in the ^{31}P n.m.r. spectrum, but will not be the exclusive resonances.

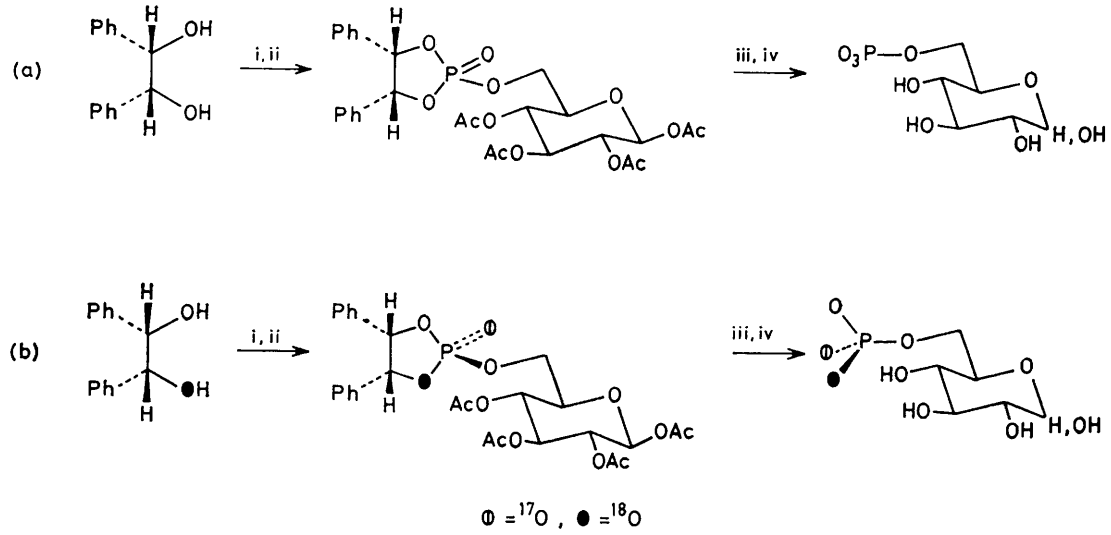
RESULTS AND DISCUSSION

In order to determine the stereochemical course of the cyclisation step it is necessary to know the absolute configuration of the chiral [^{16}O,^{17}O,^{18}O]phosphate. We now know that our general method of synthesis gives [(*S*)-^{16}O,^{17}O,^{18}O]phosphate esters,[1] but in the preliminary report of this work, they had been assigned the (*R*)-configuration.[4]

Preparation of D-*Glucose* 6-[^{18}O]*Phosphate and* D-*Glucose* 6-[(S)-^{16}O,^{17}O,^{18}O]*Phosphate.*—Ukita *et al.* described a synthesis of D-glucose 6-phosphate by the route outlined in Scheme 2a, albeit in an overall yield of 7%; catalytic hydrogenolysis of the intermediate cyclic phosphate (whose stereochemistry at phosphorus was not defined) had, however, been performed in methanolic solution.[5] Since this solvent opens five-membered cyclic phosphate triesters at room temperature,[1] it seemed that this was a likely cause of the low overall yield. Indeed we showed in a preliminary ^{31}P n.m.r. experiment that the intermediate cyclic phosphate triester (in Scheme 2a) is cleaved in methanol at room temperature. The cyclic phosphate triester was, however, completely stable in dry ethyl acetate and this proved to be an excellent solvent for catalytic hydrogenolysis. The product formed in this way gave, after deacetylation, D-glucose 6-phosphate in an overall yield of 55%. By using phosphorus [^{18}O]oxychloride (prepared from phosphorus pentachloride and [^{18}O]water) in place of phos-



SCHEME 1 D-Glucose 6-[(*S*)-^{16}O,^{17}O,^{18}O]phosphate, after cyclisation and esterification, would give species (1) to (6) or (7) to (12) depending on whether the cyclisation occurs with retention or inversion of configuration at phosphorus

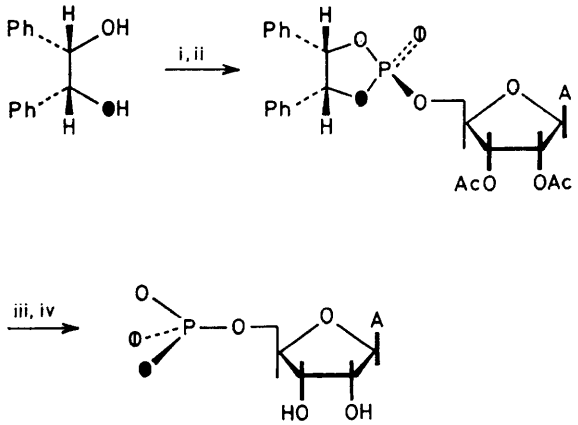


SCHEME 2 *Reagents*: (i) C_5H_5N, (a) $POCl_3$ or (b) $P^{17}OCl_3$; (ii) 1,2,3,4-tetra-acetyl-β-D-glucose, C_5H_5N; (iii) H_2, Pd; (iv) KOMe, MeOH

phorus oxychloride in Scheme 2a, D-glucose 6-[^{18}O]-phosphate was made in similar yield.

The synthesis of D-glucose 6-[(*S*)-^{16}O,^{17}O,^{18}O]-phosphate is outlined in Scheme 2b. The chemistry is essentially the same as outlined in Scheme 2a, but of course *meso*-hydrobenzoin is replaced by (1*R*, 2*S*)-1,2-[1-^{18}O]-dihydroxy-1,2-diphenylethane,[1] and phosphorus oxychloride by phosphorus [^{17}O]oxychloride.

Preparation of Adenosine 5′-[^{18}O] *Phosphate and Adenosine* 5′-[(S)-^{16}O,^{17}O,^{18}O]*Phosphate*.—Adenosine 5′-[^{18}O]-phosphate was made by the hydrolysis of adenosine 3′,-5′-phosphate catalysed by beef heart cyclic AMP phosphodiesterase in [^{18}O]water. Adenosine 5′-[(*S*)-^{16}O,^{17}O, ^{18}O]phosphate was prepared by our general method of synthesis in 52% overall yield as outlined in Scheme 3.



SCHEME 3 *Reagents*: (i) $P^{17}OCl_3$, C_5H_5N; (ii) 2′,3′-diacetyl-adenosine, C_5H_5N; (iii) H_2, Pd; (iv) NH_3, EtOH

The Stereospecific Cyclisation of D-*Glucose* 6-[(S)-^{16}O,-^{17}O,^{18}O]*Phosphate*.—In order to analyse the chirality at phosphorus of D-glucose 6-[^{16}O,^{17}O,^{18}O]phosphate as outlined in Scheme 1 a stereospecific method of cyclisation is required. The only literature procedure for converting D-glucose 6-phosphate into D-glucose 4,6-phosphate employs dicyclohexylcarbodi-imide in aqueous pyridine at room temperature for 3 days.[6] We confirmed that this method indeed gives D-glucose 4,6-phosphate, which could be converted into a mixture of the axial and equatorial phosphate triesters with diazomethane. However when the cyclisation was repeated with D-glucose 6-[^{18}O]phosphate, and the mixture of phosphate triesters investigated by ^{31}P n.m.r. spectroscopy, it was apparent from the relative intensity of the [$^{16}O_2$]-peak to those of the [$^{16}O_{ax}$, $^{18}O_{eq}$]- and [$^{18}O_{ax}$, $^{16}O_{eq}$]-peaks that approximately 20% loss of isotope had occurred.

A more convenient and efficient cyclisation procedure was investigated in which the pyridine-soluble mono-4-morpholino-*N*,*N*-dicyclohexylcarboxamidinium (MDCA) salt of D-glucose 6-phosphate in anhydrous pyridine was added dropwise to a refluxing solution of dicyclohexylcarbodi-imide (1 equiv.) in anhydrous pyridine, to give the MDCA salt of D-glucose 4,6-phosphate almost quantitatively in less than 15 min. The ^{31}P n.m.r. spectrum of the product after esterification is shown in Figure 1. The resonances at −4.1 and −6.1 p.p.m. are assigned to the equatorial and axial triesters respectively, since it has been found that axial esters of 2-substituted-2-oxo-1,3,2-dioxaphosphorinanes resonate at higher field than the equatorial derivatives by *ca.* 1—3 p.p.m.[7,8] Thus the diastereoisomeric phosphate triesters do not have to be physically separated, which is an important advantage of the ^{31}P n.m.r. method of analysis over the mass-spectrometric method of analysis.[9] Each of these resonances appears as a doublet because the triesters exist as anomers.

When this cyclisation procedure was applied to D-glucose 6-[^{18}O]phosphate and the product esterified with diazomethane, the reaction product had the ^{31}P n.m.r. spectrum shown in Figure 2. The six high-field lines are due to the [$^{16}O_2$]-, [$^{16}O_{ax}$, $^{18}O_{eq}$]- and [$^{18}O_{ax}$, $^{16}O_{eq}$]-axial triesters each of which exists as a pair of anomers. The

three low-field lines are due to the [$^{16}O_2$]-, [$^{16}O_{ax}$, $^{18}O_{eq}$]- and [$^{18}O_{ax}$, $^{16}O_{eq}$]-equatorial triesters, the anomer resonances not being separated. Convincing proof that the anomers were present, however, was provided by running the same sample in [$^2H_4$]MeOH containing dimethyl sulphoxide which separated the anomer resonances (not

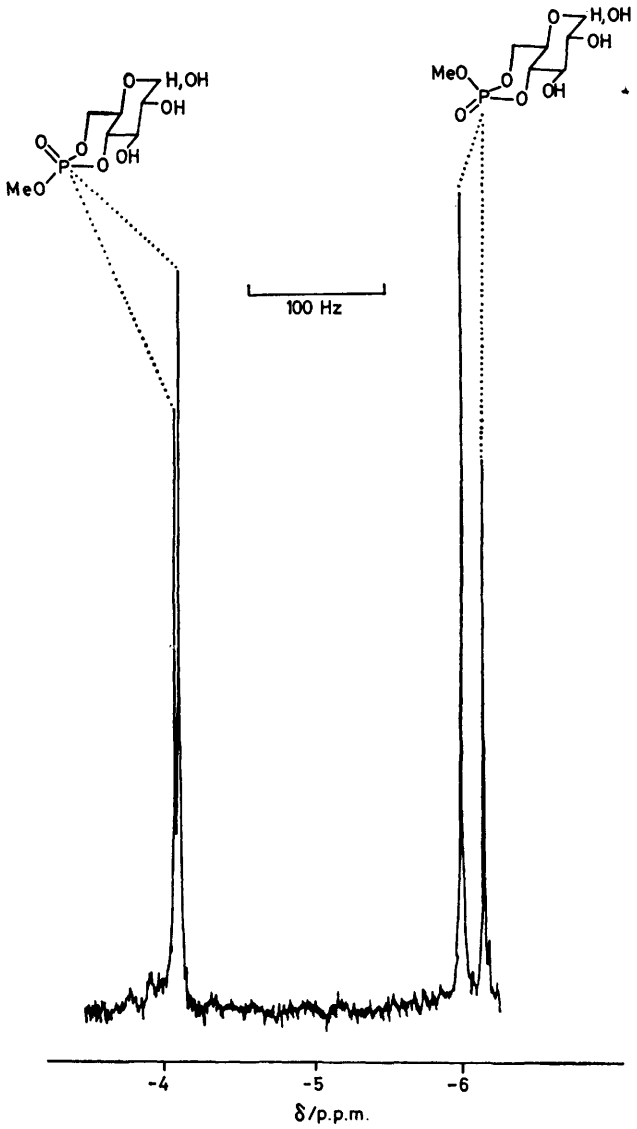


FIGURE 1 The ^{31}P n.m.r. spectrum (121.5 MHz) of the α- and β-anomers of the axial and equatorial methyl esters of D-glucose 4,6-phosphate in 25% CD_3OD, 75% MeOH containing 8-hydroxyquinoline. Parameters: Offset 2 240 Hz, sweep width 2 000 Hz, acquisition time 2.05 s, pulse width 16 μs, broadband proton decoupling, line broadening 0.2 Hz in 8 K and Fourier transform in 32 K

shown). Since the isotopic enrichment of the D-glucose 6-[^{18}O]phosphate used was 95 atom %, it could be estimated that essentially no isotope was lost in the cyclisation and esterification steps.

D-Glucose 6-[(*S*)-^{16}O,^{17}O,^{18}O]phosphate was now cyclised and esterified by the above procedure. The high-field axial resonances in the ^{31}P n.m.r. spectrum consisted of two sets of four lines due to the anomers of the [$^{16}O_2$]-, [$^{16}O_{ax}$, $^{18}O_{eq}$]-, [$^{18}O_{ax}$, $^{16}O_{eq}$]-, and [$^{18}O_2$]-triesters as expected. However the two central lines of each anomer group were of equal intensity indicating that the cyclisation had proceeded with racemisation at phosphorus. Although the anomer resonances of the equatorial triesters were somewhat overlapping they too were consistent with this conclusion. Clearly therefore, cyclisation by dicyclohexylcarbodi-imide is of no use for analysing the chirality at phosphorus of D-glucose 6-[^{16}O,^{17}O,^{18}O]phosphate although this stereochemical observation does have implications for the mechanism of cyclisation by this reagent.[10]

Diphenyl phosphorochloridate was next investigated as an activating agent for D-glucose 6-phosphate. This reagent has been shown to react quantitatively with the mono-trioctylammonium salt of adenosine 5′-phosphate in dry dioxan to give a stable mixed anhydride,[11] which could be isolated and converted into adenosine 3′,5′-phosphate with potassium t-butoxide in dimethyl sulphoxide.[12] When the mono-trioctylammonium salt of D-glucose 6-phosphate was treated with diphenyl phosphorochloridate in dry dioxan a virtually quantitative yield of the mixed pyrophosphate was obtained. The base-catalysed cyclisation to D-glucose 4,6-phosphate could not be effected in dioxan, but after addition of dry dimethylformamide to the reaction mixture to give dimethylformamide to dioxan in a ratio of approximately 3 : 1, treatment with potassium t-butoxide led to an immediate precipitation of the potassium salt of D-glucose 4,6-phosphate, the diphenylphosphate remaining in solution. It proved possible and convenient to alkylate this salt with methyl iodide in dimethyl sulphoxide in the presence of 18-crown-6, to give the axial and equatorial phosphate triesters in the ratio of approximately 2 : 1. The ratio does vary however, and appears to be critically dependent on the precise reaction conditions. The cyclisation and esterification procedure is summarised in Scheme 4.

This procedure was now applied to D-glucose 6-[^{18}O]-phosphate and the ^{31}P n.m.r. spectrum is shown in Figure 3a. In order to separate all the resonances, methanol was added to the reaction mixture. The assignments are shown on the spectrum, and the integration shows that about 4% loss of label occurred during the chemical transformation. D-Glucose 6-[(*S*)-^{16}O,^{17}O,-^{18}O]phosphate was now cyclised and then methylated by this procedure, and the ^{31}P n.m.r. spectrum of the product is shown in Figure 3b. All the resonances shown in Figure 3a are present, together with those of the anomers of the [$^{18}O_2$]-axial and equatorial triesters. It is easy to see that the [$^{16}O_{ax}$, $^{18}O_{eq}$]- and [$^{18}O_{ax}$, $^{16}O_{eq}$]-triesters are not of equal intensity, the most intense resonances corresponding to (7) and (10) in Scheme 1. Cyclisation has occurred therefore with *inversion of configuration at phosphorus*.

In order to determine the stereoselectivity of the cyclisation, a number of factors have to be taken into consideration. First, the isotopic composition of the

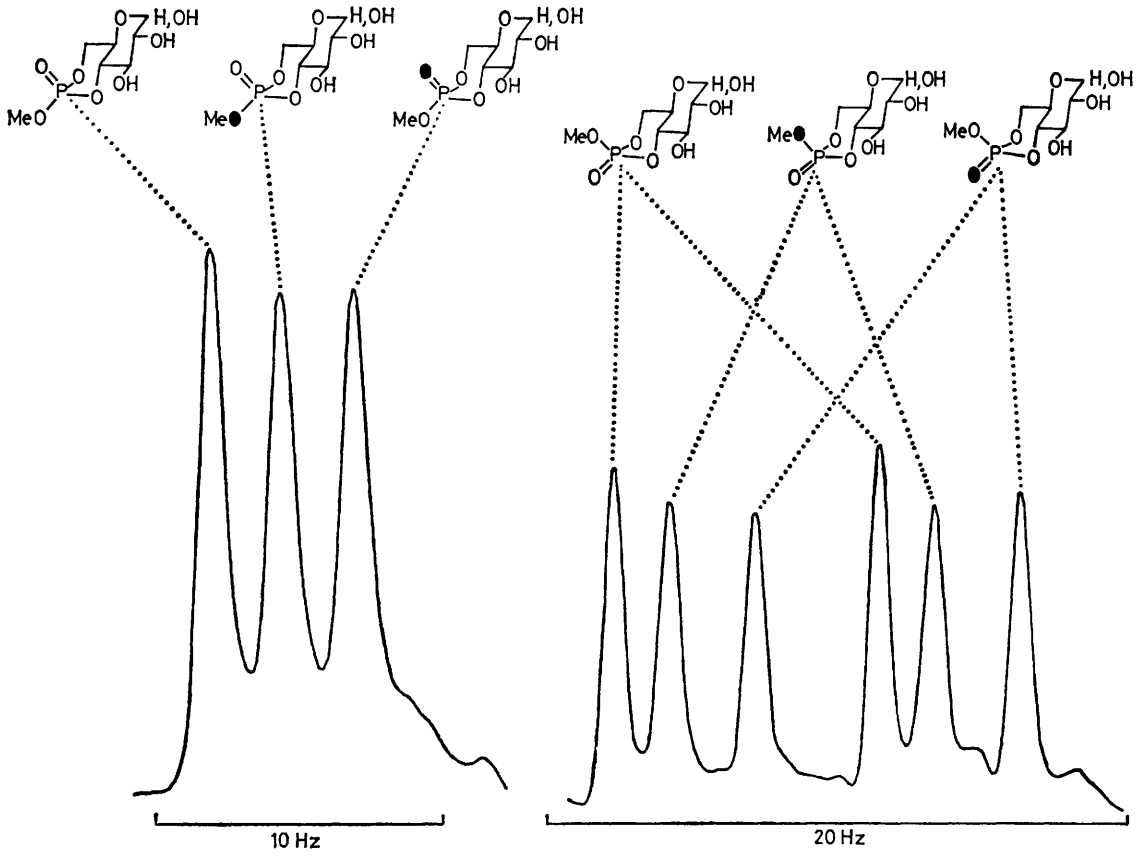
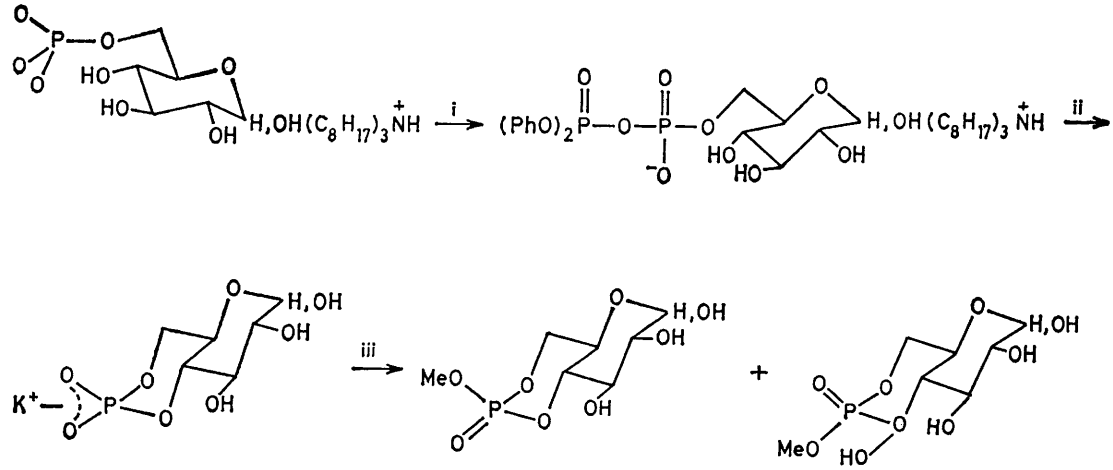


FIGURE 2 The ^{31}P n.m.r. spectrum (121.5 MHz) of the axial and equatorial triesters (in CD_3OD) obtained by cyclisation and esterification of D-glucose 6-[^{18}O]phosphate using dicyclohexylcarbodi-imide in pyridine. Parameters: offset 2 649 Hz, sweep width 1 202 Hz, acquisition time 2.05 s, pulse width 16 μs, broadband proton decoupling, gaussian multiplication (line broadening −1.0 Hz, gaussian broadening 0.1) in 4 K and Fourier transform in 32 K



SCHEME 4 *Reagents*: (i) $(PhO)_2POCl$, Bu^n_3N in dioxan; (ii) Bu^tOK in $Me_2N{\cdot}CHO$; (iii) 18-crown-6, MeI in Me_2SO

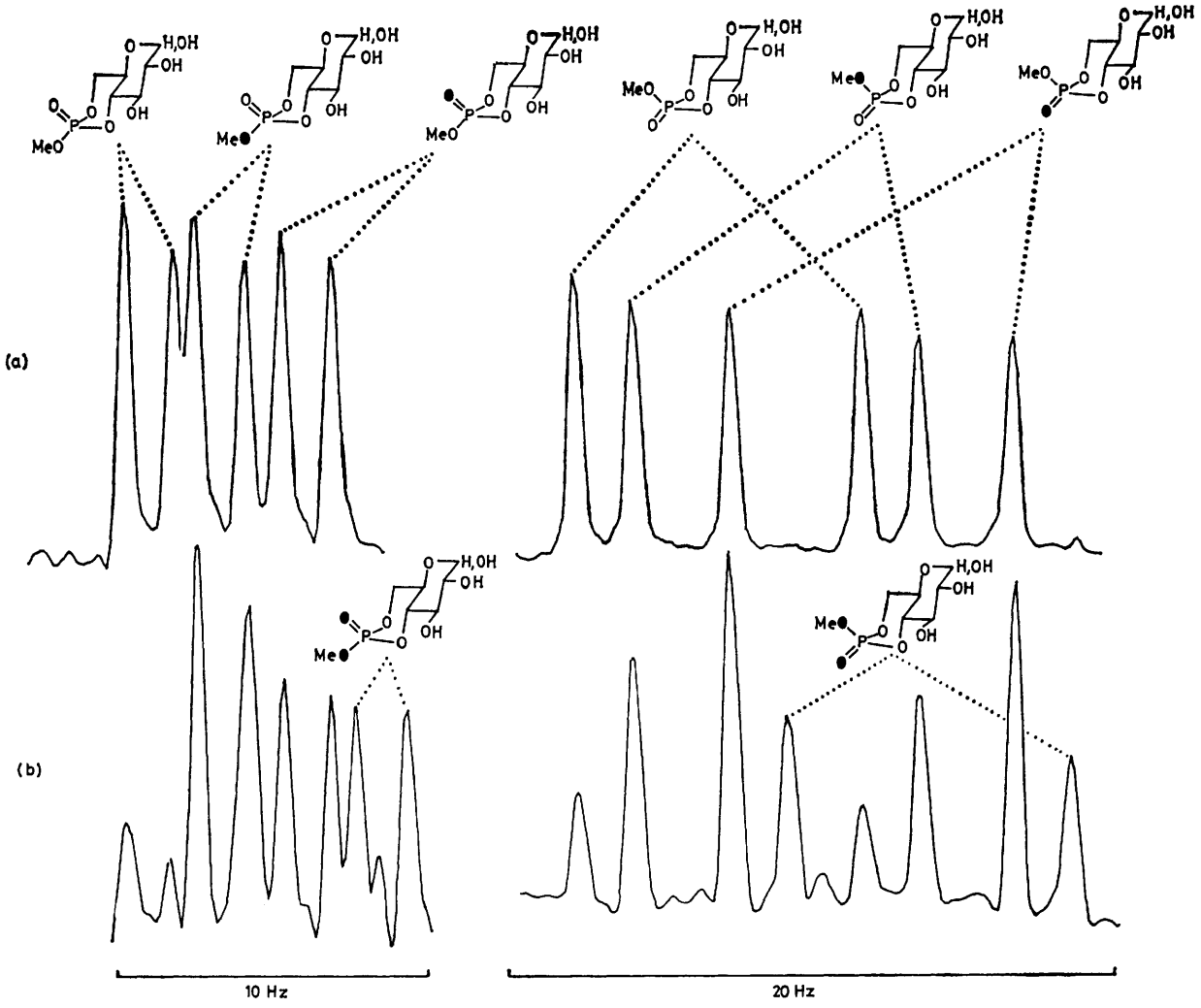


FIGURE 3 The ^{31}P n.m.r. spectra (121.5 MHz) of the axial and equatorial triesters (in 25% CD_3OD, 25% MeOH, 50% Me_2SO) obtained by cyclisation and esterification of (a) D-glucose 6-[^{18}O]phosphate and (b) D-glucose 6-[(*S*)-^{16}O,^{17}O,^{18}O]phosphate as shown in Scheme 4. Parameters: offset 2 240 Hz, sweep width 2 000 Hz, acquisition time 2.05 s, pulse width 16 μs, broadband proton decoupling, gaussian multiplication (line broadening −0.6 Hz, gaussion broadening 0.4) in 8 K, and Fourier transform in 32 K. The gain control for the equatorial triester is four times that for the axial ester in (a) and sixteen times that for the axial triester in (b)

phosphorus [^{17}O]oxychloride (determined by mass spectrometry after conversion into trimethyl phosphate) used in the synthesis was 3.3 atom % ^{16}O, 43.5 atom % ^{17}O, and 53.2 atom % ^{18}O. Secondly the (1*R*, 2*S*)-[1-^{18}O]-1,2-dihydroxy-1,2-diphenylethane used in the synthesis was derived by reduction of (2*S*)- [1-^{18}O]benzoin which was 91.8% (*S*) and 8.2% (*R*), was labelled with 97 atom % ^{18}O and was contaminated with 6% (1*S*, 2*S*)-[1-^{18}O]-1,2-dihydroxy-1,2-diphenylethane.* A small loss of isotope was also detected during the hydrogenolysis of the triester (Scheme 2b) even though the catalyst was extensively dried (over P_2O_5). From an independent experiment this was estimated to be about 5% exchange of the phosphoryl oxygen and about 10% ring opening. Finally, the previously estimated 4% loss of isotope during the cyclisation step was allowed for. Taking all of these factors into consideration the expected relative intensities of the peaks in the ^{31}P n.m.r. spectrum were calculated for inversion and retention of configuration at phosphorus and are compared in the Table with the observed intensities from Figure 3b. Clearly the cyclisation has occurred with inversion of configuration at phosphorus, and with a stereoselectivity in excess of 94%.

The Stereospecific Cyclisation of Adenosine 5′-[(S)-^{16}O,-^{17}O,^{18}O]*Phosphate*.—Adenosine 5′-phosphate was converted into adenosine 3′,5′-phosphate in excellent yield by triphenylphosphine–2,2′-dipyridyl disulphide,[13] but the reported esterification of adenosine 3′,5′-phosphate with diazomethane in hexamethylphosphoramide takes seven days,[8] which was not convenient for our purpose. By contrast, the potassium salt of adenosine 3′,5′-phosphate was esterified readily by methyl iodide in dimethyl sulphoxide in the presence of one equivalent of 18-crown-6. The ^{31}P n.m.r. spectrum showed peaks at −4.0 and −5.3 p.p.m. in a ratio of approximately 1 : 2 which were

* This contamination can however be avoided.[1]

assigned to the equatorial and axial triesters respectively.[7,8] The products were also found to be methylated on the adenine ring, presumably at N-1.[14] When the cyclisation and alkylation procedure was applied to adenosine 5′-[^{18}O]phosphate, the ^{31}P n.m.r. spectrum of the product showed that about 40% loss of ^{18}O had occurred; this may have arisen by reaction of the reagent with adenosine 3′,5′-phosphate. Clearly this cyclisation procedure was unsatisfactory for our purpose.

TABLE

The observed relative peak intensities of the ^{31}P resonances (from Figure 3b average of both anomers, and Figure 4) of the ^{18}O-labelled diastereoisomeric triesters derived by cyclisation and esterification of D-glucose 6-[(*S*)-^{16}O,^{17}O,^{18}O]phosphate (G-6P) and adenosine 5′[(*S*)-^{16}O,^{17}O,^{18}O]phosphate (A-5′P), are compared with the expected values for cyclisation with retention and inversion of configuration at phosphorus. The stereoselectivity of the cyclisation is determined by the ratio of the intensities of the two mono-^{18}O triesters, compared with the calculated values, ● = ^{18}O

	Equatorial triester			
	Observed		Calculated	
	G-6P	A-5′P	Retention	Inversion
MeO—P = O	Too inaccurate	0.35	0.28	0.28
Me●—P = O	1.00	1.00	0.74	1.00
MeO—P = ●	0.72	0.75	1.00	0.74
Me●—P = ●	0.65	0.55	0.49	0.49
	Axial triester			
	Observed		Calculated	
	G-6P	A-5′P	Retention	Inversion
MeO—P = O	0.41	0.34	0.28	0.28
Me●—P = O	0.76	0.75	1.00	0.74
MeO—P = ●	1.00	1.00	0.74	1.00
Me●—P = ●	0.62	0.59	0.49	0.49

The cyclisation procedure which had been successfully developed for D-glucose 6-[(*S*)-^{16}O,^{17}O,^{18}O]phosphate had originally been applied to nucleoside 5′-phosphates.[11] When the mono-tri-n-octylammonium salt of adenosine 5′-phosphate in dioxan–dimethylformamide (1 : 1) was treated with diphenyl phosphorochloridate followed by potassium t-butoxide, the potassium salt of adenosine 3′,5′-phosphate was precipitated from solution. After ion-exchange chromatography it was esterified with methyl iodide (as above) to give a mixture of the axial and equatorial phosphate triesters.

Adenosine 5′-[(*S*)-^{16}O,^{17}O,^{18}O]phosphate was cyclised and esterified by this procedure. The ^{31}P n.m.r. spectrum of the reaction product is shown in Figure 4; the absence of anomers of course simplifies the spectrum. The four high-field resonances are assigned to the axial triesters as before;[7,8] the configuration of the axial ethyl ester of adenosine 3′,5′-phosphate has been confirmed crystallographically,[15] and correlated with the ^{31}P n.m.r. chemical shift.[8] The relative intensities of the [$^{16}O_{ax}$, $^{18}O_{eq}$]- to [$^{18}O_{ax}$, $^{16}O_{eq}$]-triesters indicate that the cyclisation has occurred with inversion of configuration. Taking into consideration the same factors as with the cyclisation of D-glucose 6-[(*S*)-^{16}O,^{17}O,^{18}O]phosphate, the expected relative intensities of the peaks in the ^{31}P n.m.r. spectrum were calculated for retention and inversion of configuration at phosphorus and are compared in the Table with the observed intensities from Figure 4. From these we can say that the cyclisation of adenosine 5′-[(*S*)-^{16}O,^{17}O,^{18}O]phosphate has occurred with *inversion of configuration at phosphorus* with a stereoselectivity in excess of 94%.

Conclusions.—With the above procedures we now have an experimentally simple and stereochemically rigorous method for determining the absolute configuration at phosphorus of D-glucose 6-[^{16}O,^{17}O,^{18}O]phosphate and adenosine 5′-[^{16}O,^{17}O,^{18}O]phosphate or the enantiomeric excess of the (*R*)- or (*S*)-stereoisomers. A preliminary communication of similar methodology for the analysis of the chirality at phosphorus of 1-[^{16}O,^{17}O,-^{18}O]phospho-(*S*)-propane-1,2-diol has recently been reported.[16]

EXPERIMENTAL

Routine ^{1}H and ^{31}P n.m.r. spectra were determined on Perkin-Elmer R32 (90 MHz) and Bruker WH90 (36.43 MHz) spectrometers respectively. The ^{31}P isotope shift measurements however were measured on a Bruker WH300 (121.5 MHz) spectrometer, with a Bruker data system BNC12 computer interfaced with the spectrometer. Solutions in 10 mm tubes were thermally controlled and either [$^{2}H_6$]dimethyl sulphoxide or [$^{2}H_4$]methanol used as the lock signal. In all cases the chemical shifts (δ) are reported as positive when the resonances are downfield of the reference signal at 0 p.p.m. Chemical shifts are referred to internal tetramethylsilane (non-aqueous) for ^{1}H n.m.r. spectra and external trimethyl phosphate in D_2O for ^{31}P n.m.r. spectra. All ^{31}P n.m.r. spectra are broad band proton decoupled. Mass spectra were performed on a Varian CH7 spectrometer.

[^{18}O]Water (99 atom %) was obtained from Prochem Ltd, and [^{17}O]water [44 atom % ^{17}O, 2 atom % ^{16}O and 54 atom % ^{18}O] from Monsanto Research Corporation, Miamisburg, Ohio, U.S.A.

Phosphorus [^{17}O]*Oxychloride*.—This compound was prepared by the method of Laulicht *et al.*[17] [^{17}O]Water (*ca.* 44 atom % ^{17}O, 91 μl) was added dropwise by microsyringe to solid phosphorus pentachloride (1.07 g, 5.1 mmol) cooled in an ice-bath. The flask was allowed to warm up to room temperature with stirring after the last addition and the clear solution heated under reflux for 30 min and subsequently distilled to give phosphorus [^{17}O]oxychloride as a colourless liquid (0.57 g, 73%), b.p. 105—106 °C (lit.,[17] b.p. 105—106 °C).

Trimethyl [^{17}O-phosphoryl]*Phosphate*.—Phosphorus [^{17}O]-oxychloride was added dropwise to rapidly stirred methanol (5 ml) at 0 °C. After 30 min the flask was warmed to room temperature and the solvent removed *in vacuo* to give a liquid which was dissolved in benzene and evaporated down (×3) to remove hydrogen chloride; δ_P ($CDCl_3$) 0.0 [s, ^{18}OP(OMe)$_3$], [sextet, ^{17}OP(OMe)$_3$, $\Delta\nu$ *ca.* 90 Hz, $J_{^{17}O,^{31}P}$ *ca.* 156 Hz); *m/z* 140 (6) [^{16}OP(OMe)$_3^+$], 141 (79), [^{17}OP(OMe)$_3^+$], and 142 (100) [^{18}OP(OMe)$_3^+$].

Phosphorus [^{18}O]*Oxychloride*.—This compound was prepared as for phosphorus [^{17}O]oxychloride except that [^{18}O]-water (99 atom % ^{18}O) replaced [^{17}O]water.

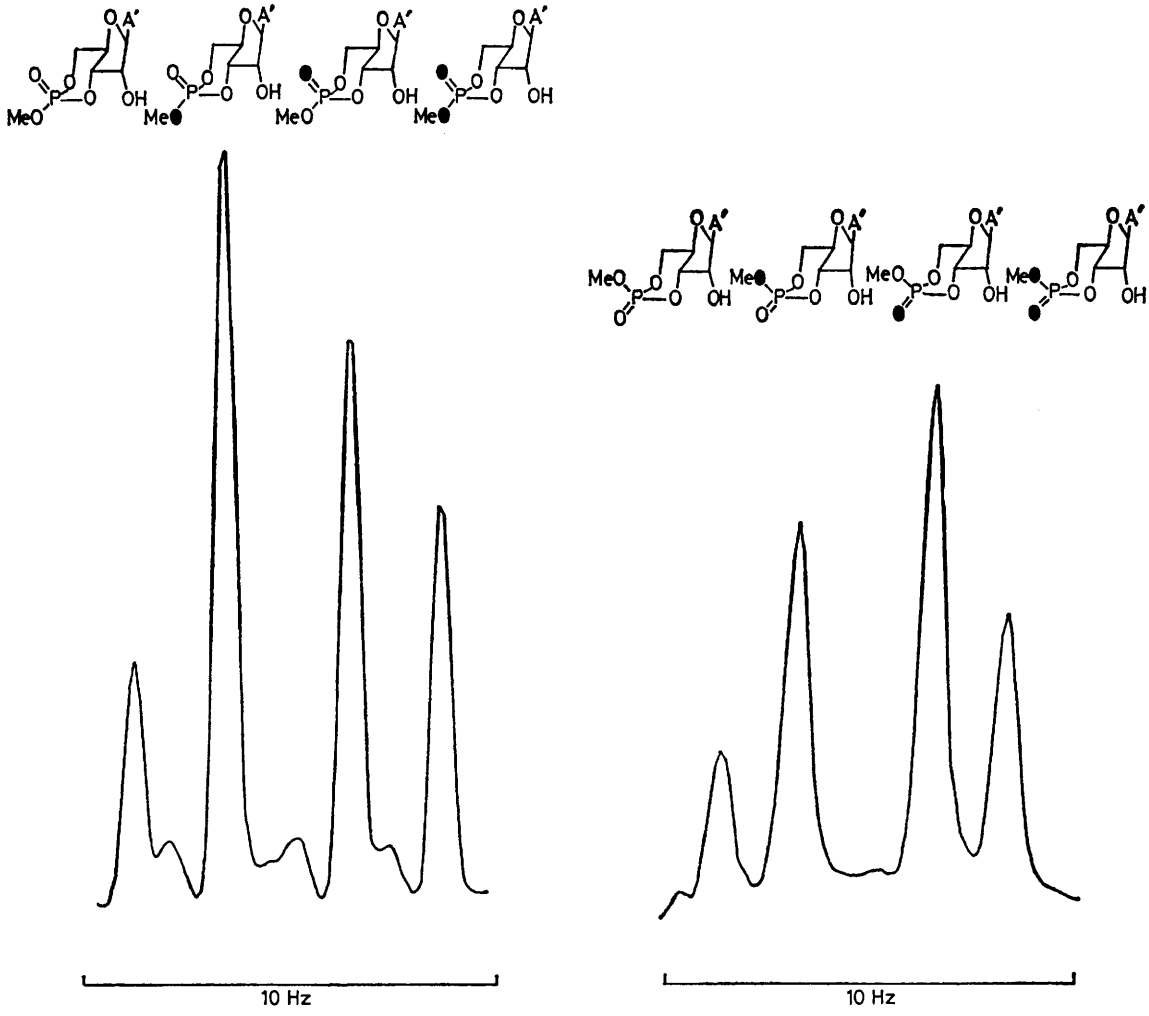


FIGURE 4 The ^{31}P n.m.r. spectrum (121.5 MHz) of the axial and equatorial triesters (in 1:1 Me_2SO–$(CD_3)_2SO$) derived by cyclisation (with diphenyl phosphorochloridate followed by potassium t-butoxide) and esterification of adenosine 5′-[(*S*)-^{16}O,^{17}O-^{18}O]phosphate. Parameters are similar to those in Figure 3. The gain control for the equatorial triester is twice that of the axial triester. A′ = *N*-1-methyladenine

trans-2-(1,2,3,4-*Tetra-acetyl*-6-β-D-*glucosyl*)-2-*oxo*-4,5-*diphenyl*-1,3,2-*dioxaphospholan*.—A solution of β-D-glucose-1,2,3,4-tetra-acetate [18] (384 mg, 1.0 mmol) in pyridine (3 ml) was added dropwise during 20 min to an ice-cooled stirred solution of *trans*-2-chloro-2-oxo-4,5-diphenyl-1,3,2-dioxaphospholan [1] [prepared *in situ* from *meso*-hydrobenzoin (214 mg, 1.0 mmol) and phosphorus oxychloride (94 μl, 1.0 mmol) in pyridine (2 ml)]. The mixture was stirred overnight and the pyridine evaporated to give a foam to which was added dry ethyl acetate (25 ml). The pyridinium hydrochloride was filtered off under nitrogen and the filtrate evaporated to give the product as a white crystalline solid which was pumped exhaustively for 2 h (530 mg, 88%), δ_P (EtOAc) D_2O lock, 11.4; δ_H ($CDCl_3$) 2.0 (broad s, 12 H, Me), 3.95 (m, 1 H, 5-H), 4.38 (m, 2 H, 6-H), 5.2 (m, 3 H, 2-, 3-, 4-H), 5.78 (d, 1 H, 1-H $^3J_{HH}$ 8 Hz), 5.82 (d, 2 H, –CH– in 5-ring, $^3J_{PH}$ 7.5 Hz), and 7.06 (broad s, 10 H, Ar).

D-*Glucose* 6-*Phosphate*.—*trans*-2-(1,2,3,4-Tetra-acetyl-6-β-D-glucosyl)-2-oxo-4,5-diphenyl-1,3,2-dioxaphospholan (530 mg) dissolved in anhydrous ethyl acetate (25 ml) was hydrogenolysed over 10% Pd/C catalyst (500 mg) which had been thoroughly dried *in vacuo* over phosphorus pentoxide. When uptake of hydrogen ceased (*ca.* 1 h) the mixture was carefully filtered through glass fibre paper and the catalyst washed thoroughly with dry ethyl acetate and dry methanol. The filtrate and washings were evaporated under reduced pressure to give a mixture of 1,2,3,4-tetra-acetyl-β-D-glucose-6-phosphate, free acid, and diphenylethane as a gummy solid which was dissolved in dry methanol (10 ml). A freshly prepared solution of potassium methoxide in methanol (1.0M; 2 ml) was then added to it. Within a few minutes the di-potassium salt of D-glucose 6-phosphate was precipitated, but the mixture was kept at −20 °C overnight. The solid was centrifuged down, washed thoroughly with cold dry methanol, dissolved in water, filtered, and lyophilised to give di-potassium D-glucose 6-phosphate as a white crystalline solid (185 mg, 55%), δ_P (D_2O) 2.2 (s, G-6-P).

D-*Glucose* 6-[^{18}O]*Phosphate*.—This compound was prepared in the same way as D-glucose-6-phosphate except that *trans*-2-chloro-2-[^{18}O]oxo-4,5-diphenyl-1,3,2-dioxaphospholan (prepared *in situ* from *meso*-hydrobenzoin and phosphorus [^{18}O]oxychloride) was used in the preparation of the protected intermediate.

D-*Glucose* 6-[(S)-^{16}O,^{17}O,^{18}O]*Phosphate*.—This compound was prepared in the same way as D-glucose 6-phosphate except that (2*R*,4*S*,5*R*)-2-chloro-2-[^{17}O]oxo-4,5-diphenyl-1-[^{18}O]-1,3,2-dioxaphospholan (prepared *in situ* from (1*R*,-2*S*)-[1-^{18}O]dihydroxy-1,2-diphenylethane [1] and phosphorus [^{17}O]oxychloride) was used in the preparation of the protected intermediate.

Adenosine 5′-[^{18}O]*Phosphate*.—Glycylglycine (2.8 mg, 21.5 μmol), magnesium acetate tetrahydrate (1.0 mg, 4.7 μmol), and cAMP hemihydrate (40 mg, 120 μmol) were dissolved in water (0.4 ml) and sodium hydroxide added (1.0M; 0.1 ml). The solution was lyophilised and the residue dissolved in [^{18}O]water (99 atom %, 0.5 ml). cAMP phosphodiestrase (from beef heart, 4 mg, 0.81 units) was added at 4 °C and the mixture was incubated at 37 °C, the pH of the solution being adjusted periodically by the addition of solid Tris to maintain pH 7.7. After 3.5 h the solution was frozen and the [^{18}O]water recovered by lyophilisation on a vacuum line. This procedure was repeated and the two residues combined, dissolved in water, and vigorously shaken with chloroform to denature the protein prior to application to a column of DEAE Sephadex A-25. A gradient of triethylammonium hydrogencarbonate buffer pH 8.0 was run from 50mM—200mM over 16 h at a rate of 22 ml h^{-1}, fractions being collected every 30 min. Fractions 14—22 were evaporated *in vacuo* to give adenosine 5′-[^{18}O]phosphate bistriethylammonium salt as a glassy solid (106 mg, 82%), δ_P (D_2O) + 1.1 (96 atom % ^{18}O).

Adenosine 5′-*Phosphate*.—2′,3′-Diacetyladenosine (703 mg, 2.0 mmol) in pyridine was added dropwise to an ice-cooled stirred solution of *trans*-2-chloro-2-oxo-4,5-diphenyl-1,3,2-dioxaphospholan [1] (prepared *in situ* from *meso*-hydrobenzoin (428 mg, 2.0 mmol) and phosphorus oxychloride (188 μl, 2.0 mmol) in pyridine (4 ml), and the solution stirred overnight. The solution [δ_P (pyridine) D_2O lock, +11.8 (s, 5-ring P)] was separated *in vacuo*, dissolved in dry dimethylformamide and evaporated (2 × 20 ml). The residue was dissolved in dry dimethylformamide (10 ml) catalyst added (10% Pd/C, 1 g; Pd black, 90 mg), and the mixture stirred in an atmosphere of dry hydrogen. After 3 h the mixture was filtered and the catalyst washed thoroughly with 300mM-ammonia in 50% aqueous ethanol (*ca.* 800 ml) and the filtrate monitored by the optical density at 260 nm. The filtrate was evaporated to dryness and the residue dissolved in methanol and added to methanol which had been saturated with ammonia at 0 °C. Ammonia was passed through the solution for a further few minutes and the resulting suspension stirred at room temperature for 0.5 h. The solvent was removed and the residue dissolved in water, brought to pH 8.0 and the solution applied to a column (100 ml) of DEAE Sephadex A-25 and eluted with a gradient of 50—200mM triethylammonium hydrogencarbonate run at 82 ml h^{-1} for 2 h. Fractions were collected every 15 min and the AMP eluted in fractions 37—57 (*ca.* 105—135 mM). After evaporation under reduced pressure adenosine 5′-phosphate bistriethylammonium salt was obtained in 52% yield on starting materials, 77% on recovered nucleotide; δ_P (D_2O) + 1.1.

Adenosine 5′-[(S)-^{16}O,^{17}O,^{18}O]*Phosphate*.—This compound was prepared in the same way as adenosine 5′-phosphate except that (2*R*,4*S*,5*R*)-2-chloro-2-[^{17}O]oxo-4,5-diphenyl-1-[^{18}O]-1,3,2-dioxaphospholan (prepared *in situ* from (1*R*,2*S*)-[1-^{18}O]dihydroxy-1,2-diphenylethane [1] and phosphorus [^{17}O]oxychloride) was used in the preparation of the protected intermediate.

D-*Glucose* 4,6-*Phosphate Methyl Esters*.—Three methods of preparation were considered.

(a) The cyclisation step is a modification of the method of Khorana *et al.*[6] D-Glucose 6-phosphate dipotassium salt (100 mg, 0.36 mmol) was converted into the mono-pyridinium salt using Dowex 50W (pyridinium form) and the gum after evaporation of water was dissolved in water (0.5 ml). To the solution was added an excess of dicyclohexylcarbodiimide (0.42 g) in pyridine (5 ml) and the resulting mixture stirred for three days at room temperature, when water (15 ml) was added and the crystalline dicyclohexylurea filtered off. The aqueous filtrate was extracted three times with ether and evaporated down under reduced pressure to yield pyridinium D-glucose 4,6-cyclic phosphate as a gum, δ_P (D_2O) — 5.4.

This salt in water was stirred with Dowex 50W (H^+ form) for 15 min. After evaporation under reduced pressure the gummy free acid was dissolved in methanol and a freshly distilled ethereal solution of diazomethane added until the yellow colour persisted. Excess of diazomethane was purged with nitrogen and the solution evaporated to give the axial and equatorial methyl esters in quantitative yield.

(b) D-Glucose 6-phosphate dipotassium salt (100 mg, 0.36 mmol) was converted into the mono-pyridinium salt as above. Addition of 4-morpholine *N*,*N*′-dicyclohexylcarboxamidine (106 mg, 0.36 mmol) to a solution of this salt in 50 : 50 pyridine–water followed by evaporation under reduced pressure and successive evaporation of several volumes of dry pyridine gave the D-glucose 6-phosphate mono-MDCA salt as a glassy solid. This salt, in dry pyridine, was added dropwise to a boiling solution of dicyclohexylcarbodi-imide (61.9 mg, 0.3 mmol) in dry pyridine (20 ml) under anhydrous conditions. After 30 min the mixture was cooled, evaporated to small bulk under reduced pressure and water added to precipitate the dicyclohexylurea which was filtered off and washed with water. The filtrate and washings were evaporated to give D-glucose 4,6-cyclic phosphate mono-MDCA salt as a gummy solid.

This salt was soluble in methanol and the ion-exchange with Dowex 50W (H^+ form) was therefore performed directly in this solvent, the rest of the procedure being as under (a).

(c) D-Glucose 6-phosphate dipotassium salt (100 mg, 0.36 mmol) was converted into the mono-tri-n-octylammonium salt as follows. The salt was stirred with Dowex 50W (H^+ form) and the filtrate and washings evaporated to dryness. Several volumes of dry dioxan (in which the free acid is insoluble) were evaporated off and a solution of tri-n-octylamine (126 mg, 0.36 mmol) in dry dioxan (2 ml) added with vigorous shaking. Methanol was also added to assist formation of the salt. After evaporation of solvent and the evaporation of several volumes of dry methanol followed by dry dioxan, the gum was dissolved in dry dioxan (2 ml) and to this stirred solution under anhydrous conditions were added (freshly distilled) diphenyl phosphorochloridate (97 mg, 0.36 mmol) in dry dioxan (0.5 ml) followed immediately by dry tri-n-butylamine (67 mg, 0.36 mmol) in dry dioxan (0.85 ml). A precipitate of amine hydrochloride appeared immediately and the solution was stirred for a further 5 min to allow complete conversion into P_1-(6-D-glucosyl)-P_2-diphenyl pyrophosphate, δ_P (dioxan) D_2O lock, —13.4, —13.5 (2 d, CH_2OP, α and β anomers, $^2J_{PP}$ 18 Hz), —26.2 (d, $(PhO)_2$ P–O, $^2J_{PP}$ 18 Hz). To this solution was added with vigorous stirring dry dimethylformamide (15 ml) followed immediately by a freshly prepared solution of potassium t-butoxide (1.0M in t-butyl alcohol, 2 ml). The

immediately precipitated material was centrifuged down, washed four times with dry dimethylformamide, dissolved in water, and the pH adjusted to 8.0 with hydrochloric acid to yield the potassium salt of D-glucose 4,6-cyclic phosphate in *ca.* 60% yield, δ_P (D_2O) − 5.8 (s, 6-ring P), δ_H (D_2O) 3.2—4.2 (m, all protons except at C-1) 4.55 (d, 1-H (β), $^3J_{HH}$ 8.0 Hz), 5.07 (d, 1-H (α), $^3J_{HH}$ (4.00 Hz).

To this salt, dissolved in a minimum of water, was added 18-crown-6. Several volumes of dry dimethylformamide were removed to ensure the absence of water and the material dissolved in dry dimethyl sulphoxide (0.75 ml) (some potassium chloride does not dissolve). Methyl iodide was added (100 μl) and the mixture stirred overnight; a 50 : 50 mixture of dry methanol–[$^2H_4$]methanol was then added (1.35 ml). The material was carefully filtered and examined by ^{31}P n.m.r. spectroscopy.

N-1-*Methyladenosine* 3′,5′-*Phosphate Methyl Esters*.—(a) This cyclisation procedure is similar to that of Mukaiyama and Hashimoto.[13] Adenosine 5′-monophosphate (55 mg, 0.1 mmol) was converted into the free acid using Dowex 50W (H^+) and dissolved in pyridine (5 ml) and water (1 ml). Morpholine *N*,*N*′-dicyclohexylmorpholine-4-carboxamidine (30 mg, 0.1 mmol) was added with slight warming, the solvents removed *in vacuo*, and the salt dried by repeated evaporation of dry pyridine. The resulting gum was dissolved in pyridine (15 ml). To the refluxing solution were added in one portion triphenylphosphine (131 mg, 0.5 mmol) and 2,2′-dipyridyl disulphide (110 mg, 0.5 mmol). The mixture was refluxed for 3 h, water (25 ml) then added, and the mixture kept for a further 2 h. The mixture was evaporated *in vacuo* and the residue partitioned between water (20 ml) and dichloromethane (20 ml). The aqueous layer was exhaustively extracted with dichloromethane (5 × 50 ml) and evaporated to yield adenosine 3′,5′-phosphate MDCA salt, δ_P (D_2O) −4.7.

The MDCA salt was dissolved in water and stirred with Dowex 50W (K^+ form). The filtrate was evaporated to give the potassium salt of cyclic AMP which was dissolved in a minimum of water and 18-crown-6 added (26 mg, 0.1 mmol). The mixture was evaporated thoroughly under reduced pressure to give a gum which was dissolved in dry [$^2H_6$]dimethyl sulphoxide (1 ml) and methyl iodide (100 μl, 1.5 mmol) added. The mixture was stirred overnight for 20 h, after which the methyl iodide was thoroughly removed under reduced pressure to give a solution containing the *N*-1-methyladenosine 3′,5′-phosphate methyl esters, δ_P [$(CD_3)_2$SO] −4.0 (equatorial ester) and −5.3 (axial ester). (b) Adenosine 5′-phosphate bistriethylammonium salt (0.1 mmol) was converted into the monopyridinium salt by stirring with Dowex 50W (pyridinium form) in methanol–water (1 : 1) and filtering. The solvent was removed, the dry glass suspended in methanol to which was added tri-n-octylamine (4.7 μl, 0.11 mmol), and the mixture heated until everything dissolved. The solvent was removed and the salt dried by repeated evaporation of dry dimethylformamide (×3). The residue was dissolved in dimethylformamide (0.75 ml) and dioxan (0.75 ml) and tributylamine (19 μl, 0.1 mmol) and diphenyl phosphorochloridate (20 μl, 0.1 mmol) were added. The solution was stirred for 20 min and dimethylformamide (7.5 ml) followed by potassium t-butoxide (1.0M in t-butyl alcohol; 1.0 ml) were added. After 5 min the solution was centrifuged and the precipitate washed twice with dimethylformamide and then dissolved in water. The aqueous solution was brought to pH 8.0 with carbon dioxide. Ion exchange chromatography was carried out on DEAE-Sephadex A25 (8 ml) with a linear gradient of triethylammonium hydrogencarbonate (pH 8.0, 0—150-mM) over 24 h with a flow rate of 22 ml/h and the collection of 3 fractions per hour. Adenosine 3′,5′-phosphate (0.035 mmol) was eluted at 35—40mM buffer, 35% yield based on AMP and 69% on recovered nucleotide.

The triethylammonium salt of adenosine 3′,5′-phosphate was converted into the potassium salt by stirring an aqueous solution with Dowex 50 W (K^+ form) and filtering. 18-Crown-6 (14 mg) was added and the solvent removed. The salt was dried by addition and evaporation of dimethylformamide (×3). The residue was dissolved in dimethyl sulphoxide (0.5 ml), methyl iodide (100 μl) was added, and the solution was stirred for 20 h. Excess of methyl iodide was removed under reduced pressure to give a solution of the axial and equatorial *N*-1-methyladenosine 3′,5′-phosphate methyl esters.

The authors thank the S.R.C. for the award of research studentships (to R. L. J. and B. V. L. P.) and financial support. This is a contribution from the Oxford Enzyme Group supported by the S.R.C.

[1/687 *Received* 30*th April*, 1981]

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