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Stereochemistry of phosphoryl transfer

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A general method has been developed for the synthesis of chiral [^{16}O,^{17}O,^{18}O]phosphate monoesters of known absolute configuration. An analytic method for determining the absolute configuration of chiral phosphate esters has also been developed, which is based on the isotope effects of ^{17}O and ^{18}O at phosphorus in the ^{31}P nuclear magnetic resonance spectrum. These methods have shown that phosphoryl transfer catalysed by hexokinase, phosphofructokinase and pyruvate kinase occurs with inversion of configuration. This is most simply interpreted as an 'in-line' transfer of the phosphoryl group between substrates in the enzyme–substrate ternary complex.

INTRODUCTION

The enzymes of glycolysis include an impressive array of phosphoryl transferases. These fall into two subgroups, namely the phosphokinases, which include hexokinase, phosphofructokinase, phosphoglycerate kinase and pyruvate kinase, and the phosphomutases, phosphoglucomutase and phosphoglycerate mutase. Both of these subgroups of phosphoryl transferases present mechanistic problems which cannot be solved by kinetic methods.

In general, the phosphokinases follow a random sequential pathway (figure 1), i.e. substrate binding to the enzyme and product dissociation from the enzymes are not ordered (although one binding and one dissociation route may predominate) but phosphoryl transfer only takes place when both substrates are bound. The kinetic criteria that support this pathway provide no information about the mechanism of the interconversion of the ternary complexes, which could be any one of the four mechanisms shown in figure 2. The simplest of these is the associative 'in-line' transfer of the phosphoryl group (figure 2*a*). The dissociative 'in-line' transfer of the phosphoryl group (figure 2*b*) involves the formation of a metaphosphate intermediate; the 'in-line' course of this reaction, however, seems likely to be assured by the necessity for the enzyme to bind tightly such a highly reactive intermediate. If the three peripheral oxygens of the phosphoryl group were distinguished as shown in figure 2, these two mechanisms would be expected to occur with inversion of configuration at phosphorus, although the possibility that the dissociative mechanism might lead to racemization by rotation about one of the P–O bonds of the transient metaphosphate ion should not be overlooked. The third mechanism involves 'adjacent' attack on the phosphoryl residue, but since leaving groups only depart from apical positions of trigonal bipyramidal phosphoranes, a pseudorotation (or turnstile rotation) must occur before product formation (figure 2*c*) which would lead to retention of configuration at phosphorus. The fourth mechanism involves the formation of a phosphoryl-enzyme intermediate (which may occur by any one of the first three mechanisms). By making the reasonable assumption that the two phosphoryl transfer steps follow the same stereochemical course, the overall effect will be retention of configuration at phosphorus. If, therefore, the stereochemical course of a phosphoryl transfer reaction can be established, two of the four possible mechanisms could

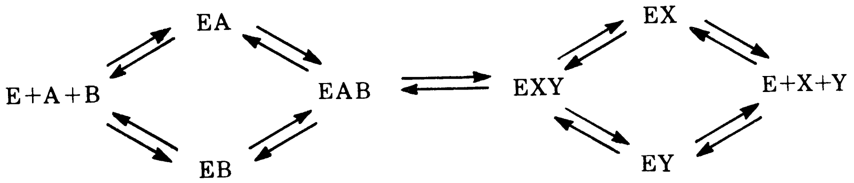


FIGURE 1. The random sequential pathway followed in general by phosphokinases. A and B are substrates; X and Y are products; E is the enzyme.

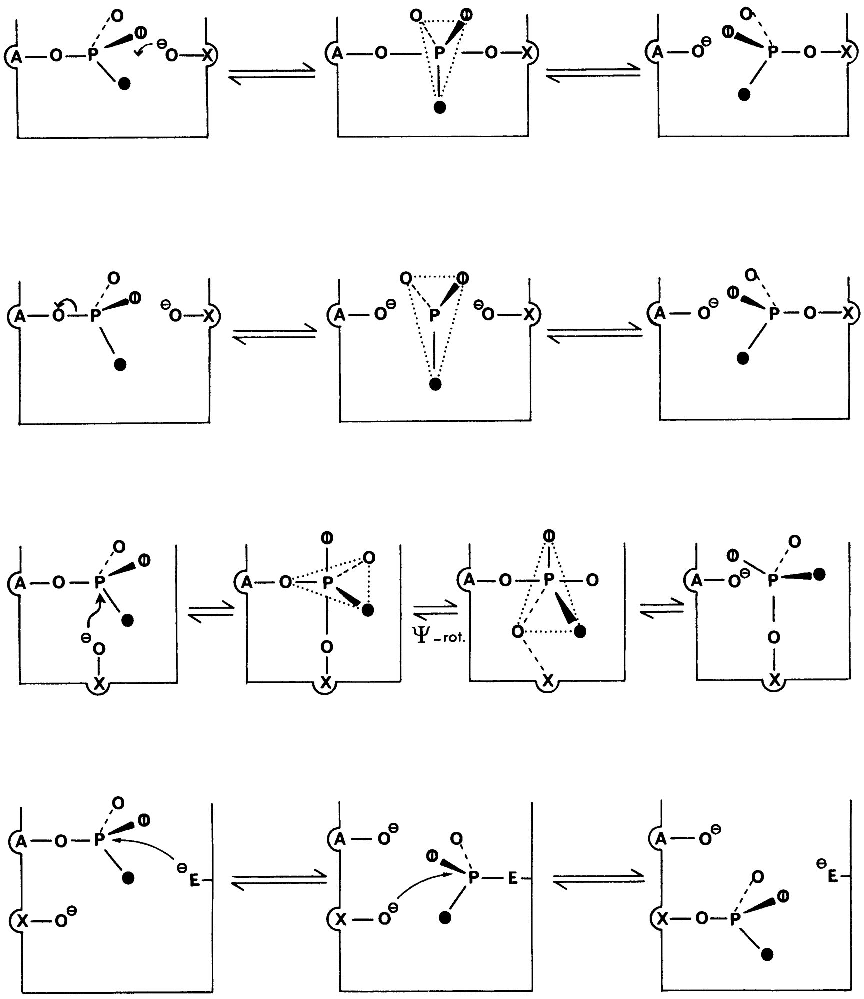


FIGURE 2. The four mechanisms of phosphoryl transfer that phosphokinases could follow. The three peripheral oxygens of the phosphoryl group are distinguished so that the stereochemical consequence of each mechanism is evident.

be eliminated. A distinction between the remaining two possibilities could be achieved by a positional isotope exchange experiment (Lowe & Sproat 1978*b*, 1980; Rose 1980).

During the past few years there has been intense interest in determining the stereochemical course of phosphokinases by using chiral [^{18}O] phosphorothioate analogues of the natural substrates, but phosphorothioates are invariably much poorer substrates than the natural phosphate esters and in some cases they are not substrates at all (Eckstein 1975, 1979; Knowles 1980). It has been widely assumed, however, that such analogues will follow the same stereochemical course as the natural substrate, although concern that they may not has occasionally been expressed.

It has been shown that glycerol kinase catalyses the transfer of both the thiophosphoryl group from adenosine-5′-[γ-^{18}O, γ-thiol]triphosphate and the γ-phosphoryl group of adenosine-5′-[(*S*)-^{16}O,^{17}O,^{18}O]triphosphate with inversion of configuration (Pliura *et al.* 1980; Blättler & Knowles 1979), and three of the four phosphokinases in the glycolytic pathway have been investigated by using phosphorothioate analogues (Orr *et al.* 1978; Webb & Trentham 1980). However, since hexokinase was to be a key enzyme for the stereochemical analysis of all phosphokinases, it was imperative that this enzyme should be investigated by means of a chiral [^{16}O,^{17}O,^{18}O]phosphate.

SYNTHESIS OF CHIRAL [^{16}O,^{17}O,^{18}O]PHOSPHATE ESTERS

Oxygen is the lightest element to possess three stable isotopes, namely ^{16}O, ^{17}O and ^{18}O. Moreover, in recent years, very high enrichments of ^{17}O and ^{18}O have been achieved, so that ^{18}O-water is available in excess of 99 at.% ^{18}O, and ^{17}O-water is available at about 50 at.% ^{17}O. It has therefore become feasible to synthesize chiral [^{16}O,^{17}O,^{18}O]phosphate monoesters. This has been achieved by a route that leaves no doubt about the absolute configuration of the resulting [^{16}O,^{17}O,^{18}O]phosphate monoester.

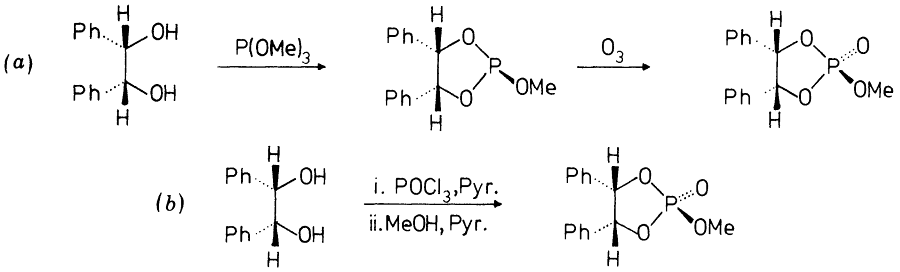
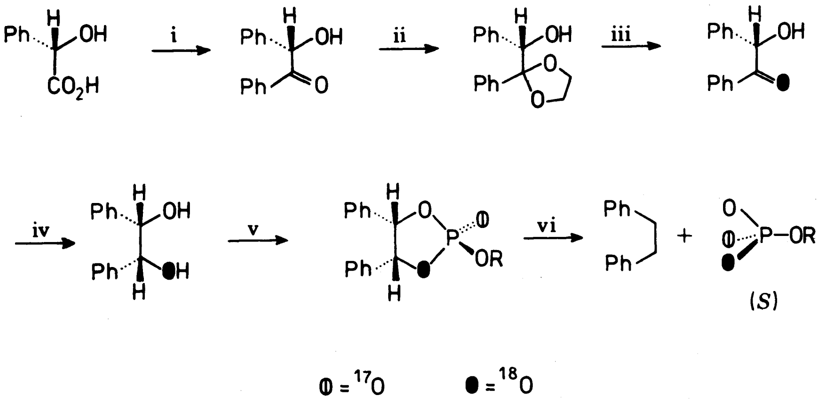


FIGURE 3. Chemical syntheses of *trans*-2-methoxy-2-oxo-4,5-diphenyl-1,3,2-dioxaphospholan.

Transesterification of trimethyl phosphite with *meso*-hydrobenzoin gives a single cyclic diastereoisomer, which on treatment with ozone gives exclusively *trans*-2-methoxy-2-oxo-4,5-diphenyl-1,3,2-dioxaphospholan (figure 3*a*); both structures were established by X-ray crystallography (Newton & Campbell 1974). The *trans*-diastereoisomer is also obtained by treating *meso*-hydrobenzoin with phosphorus oxychloride in pyridine, which gives a single crystalline cyclic phosphorochloridate, followed by treatment with methanol (figure 3*b*) (Ukita *et al.* 1961; Ukita 1961; Cullis *et al.* 1981). This ester can be converted to phosphate monoesters by hydrogenolysis of the benzylic bonds without perturbing the phosphorus–oxygen bonds. Ukita's route to the *trans*-diastereoisomer allows the introduction of ^{17}O and ^{18}O from isotopically labelled water and has been adapted to give a general synthesis of chiral [^{16}O,^{17}O,^{18}O]phosphate monoesters (Cullis & Lowe 1978; Cullis *et al.* 1981) (figure 4).

Treatment of (*S*)-mandelic acid with phenyllithium gives (*S*)-benzoin. Although it was possible to catalyse ^{18}O exchange from ^{18}O-water into the carbonyl group, some dilution of isotope would have been encountered. Instead, the (*S*)-benzoin was first converted to its ketal;

this was then hydrolysed with ^{18}O-water. Reduction of benzoin with lithium aluminium hydride at 0 °C gives virtually exclusively *meso*-hydrobenzoin (Pohoryles *et al.* 1959). Reduction of (2*S*)-[1-^{18}O]benzoin with lithium aluminium hydride gives (1*R*,2*S*)-1,2-[1-^{18}O]dihydroxy-1,2-diphenylethane, which owes its chirality solely to isotopic substitution, and which possesses a measurable circular dichroism (Cullis *et al.* 1978). Treatment with phosphorus [^{17}O]oxychloride (derived from phosphorus pentachloride and one equivalent of ^{17}O-water) in pyridine, followed by methanol in pyridine, gave (2*R*,4*S*,5*R*)-2-methoxy-2-[^{17}O]oxo-4,5-diphenyl-1,3,2-[1-^{18}O]-dioxaphospholan as a single crystalline isomer. On catalytic hydrogenolysis, methyl-[(*S*)-^{16}O,^{17}O,^{18}O]phosphate (and 1,2-diphenylethane) was obtained (Cullis & Lowe 1978; Cullis *et al.* 1981). The absolute configuration follows from the use of (*S*)-mandelic acid and the known relative configuration of the *trans*-substituted 1,3,2-dioxaphospholan. Since methanol can be replaced by any alcohol, a general method for the synthesis of chiral [^{16}O,^{17}O,^{18}O]phosphate monoesters of known absolute configuration is now available.



i. PhLi
ii. HO.CH_2.CH_2.OH, *p*-Me.C_6H_4.SO_3H
iii. H_2●, dioxan, *p*-Me.C_6H_4.SO_3H
vi. $LiAlH_4$
v. P◐Cl_3, C_5H_5N; ROH, C_5H_5N
vi. H_2, Pd/C

FIGURE 4. A general synthesis of chiral [(*S*)-^{16}O,^{17}O,^{18}O]phosphate monoesters.

ANALYSIS OF CHIRAL [^{16}O,^{17}O,^{18}O] PHOSPHATE ESTERS

The next requirement is to develop a method for the analysis of [^{16}O,^{17}O,^{18}O]phosphate monoesters of unknown chirality. The method we have developed is based on ^{31}P nuclear magnetic resonance spectroscopy and depends first on the fact that ^{17}O with a nuclear spin quantum number of $\frac{5}{2}$ has a nuclear electric quadrupole moment and so rapidly relaxes the ^{31}P resonance when directly bonded to phosphorus; this in effect means that it is not observed in the nuclear magnetic resonance spectrum (Lowe *et al.* 1979; Tsai 1979; Tsai *et al.* 1980). Secondly, ^{18}O causes an isotope shift to higher field when attached directly to phosphorus (Lowe & Sproat 1978*a*; Cohn & Hu 1978). This was very simply demonstrated by hydrolysing phosphorus pentachloride with 50 at.% ^{18}O-water. The five isotopically labelled species are resolved in the ^{31}P nuclear magnetic resonance spectrum and are in the expected binomial ratio of 1:4:6:4:1 (figure 5). Addition of unlabelled phosphate enhanced the low field signal, so demonstrating that the isotope shift was to higher field, and in this case 0.020×10^{-6} per ^{18}O atom. The isotope shift, however, depends on the nature of the bond to phosphorus, and one factor that influences this is the bond order, as shown by the data in table 1 (Lowe *et al.* 1979).

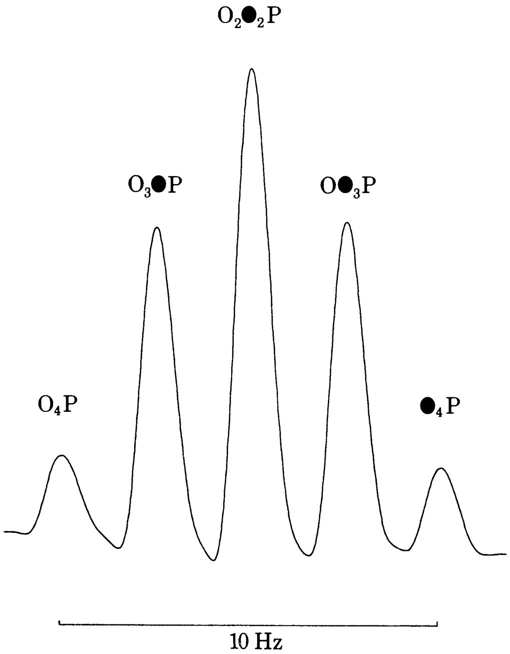


FIGURE 5. The ^{31}P nuclear magnetic resonance spectrum (121.5 MHz) of the five isotopically labelled species of inorganic phosphate in the ratio 1:4:6:4:1 derived by hydrolysing phosphorus pentachloride with 50 at.% ^{18}O-water. Addition of unlabelled inorganic phosphate enhances the low field signal, showing that each ^{18}O atom changes the chemical shift of the ^{31}P resonance to higher field by 0.020×10^{-6}.

TABLE 1. ^{18}O ISOTOPE SHIFTS IN ^{31}P NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY AT 162 MHz

$(MeO)_3P^{18}O$	5.83 Hz	0.036×10^{-6}
$(MeO)_2PO^{18}O^-$	4.74	0.029
$(MeO)PO_2{}^{18}O^{2-}$	3.72	0.023
O_3P-^{18}O-(ADP)	3.38	0.021

The analysis depends on converting a chiral[^{16}O,^{17}O,^{18}O]phosphate monoester into two diastereoisomeric conformationally locked six-membered cyclic phosphate triesters (figure 6) (Jarvest *et al.* 1980, 1981; Cullis *et al.* 1981). In the cyclization 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 take up axial and equatorial positions. Methylation of the isotopically labelled cyclic phosphate diester should give the diastereoisomeric axial and equatorial triesters. D-Glucose-6-[(*S*)-^{16}O,^{17}O,^{18}O]phosphate is used here to illustrate the method, since it will feature in our analysis used for the glycolytic enzymes, but adenosine-5′-[(*S*)-^{16}O,^{17}O,^{18}O]phosphate can also be analysed by essentially the same procedure (Jarvest *et al.* 1980, 1981).

The species **1**–**6** (figure 6) would be formed if cyclization occurred with retention of configuration at phosphorus, whereas species **7**–**12** would be formed if cyclization occurred with inversion of configuration. If the sites were fully enriched with isotope, the only species that would be observed in the ^{31}P nuclear magnetic resonance spectrum would be **1** and **4**, or **7** and **10**, as all the others contain ^{17}O directly bound to phosphorus. Since ^{18}O, when singly

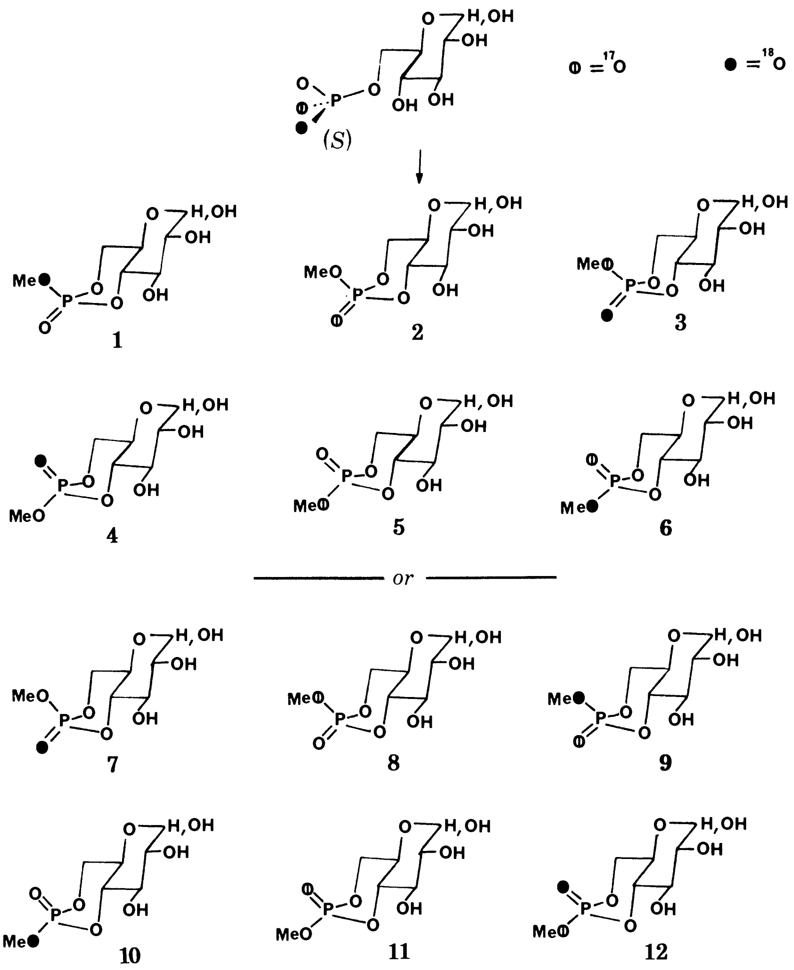


FIGURE 6. The consequence of cyclizing D-glucose-6-[(S)-^{16}O,^{17}O,^{18}O]phosphate with retention of configuration at phosphorus followed by methylation should give species **1**–**6**. If the cyclization occurs with inversion of configuration at phosphorus, species **7**–**12** would be obtained after methylation.

bonded to ^{31}P, causes a smaller isotope shift than when doubly bonded to ^{31}P, the isotope shift on **1** will be smaller than on **7**. Similarly, the isotope shift on **10** will be smaller than that on **4**. The ^{31}P resonances of the axial and equatorial esters have substantially different chemical shifts (about 2×10^{-6}), which means that they do not have to be separated physically. If, however, the sites indicated by ^{17}O and ^{18}O were fully enriched then it would be necessary to add unlabelled material as an internal reference to determine the isotope shift. The fact that the '^{17}O site' contains some ^{16}O and some ^{18}O means that additional species of lower intensity will be formed, which provide the necessary internal references. The presence of both diastereoisomeric triesters provides a useful double check in the analysis.

The reagents necessary to bring about the chemical transformation were first established with D-glucose-6-phosphate and are outlined in figure 7. The ^{31}P nuclear magnetic resonance spectrum showed the axial and equatorial esters to be separated by about 2×10^{-6}. The axial ester is assigned to the high field resonance as is invariably found for 1,3,2-dioxaphosphorinan-2-ones (Cooper *et al.* 1974; Engels & Schlaeger 1977). Both esters exist as α- and β-anomers

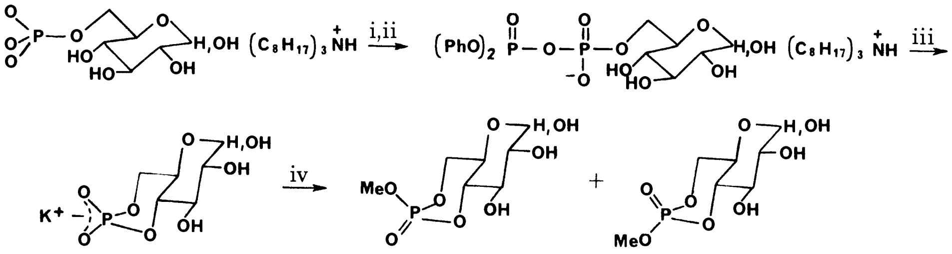


FIGURE 7. The chemical steps involved in converting D-glucose-6-phosphate into the diastereoisomeric cyclic phosphate triesters. Reagents: i, $(PhO)_2POCl$ in dioxan; ii, Bu^n_3N in dioxan; iii, Bu^tOK in $Me_2N.CHO$; iv, 18 crown 6 – MeI in Me_2SO.

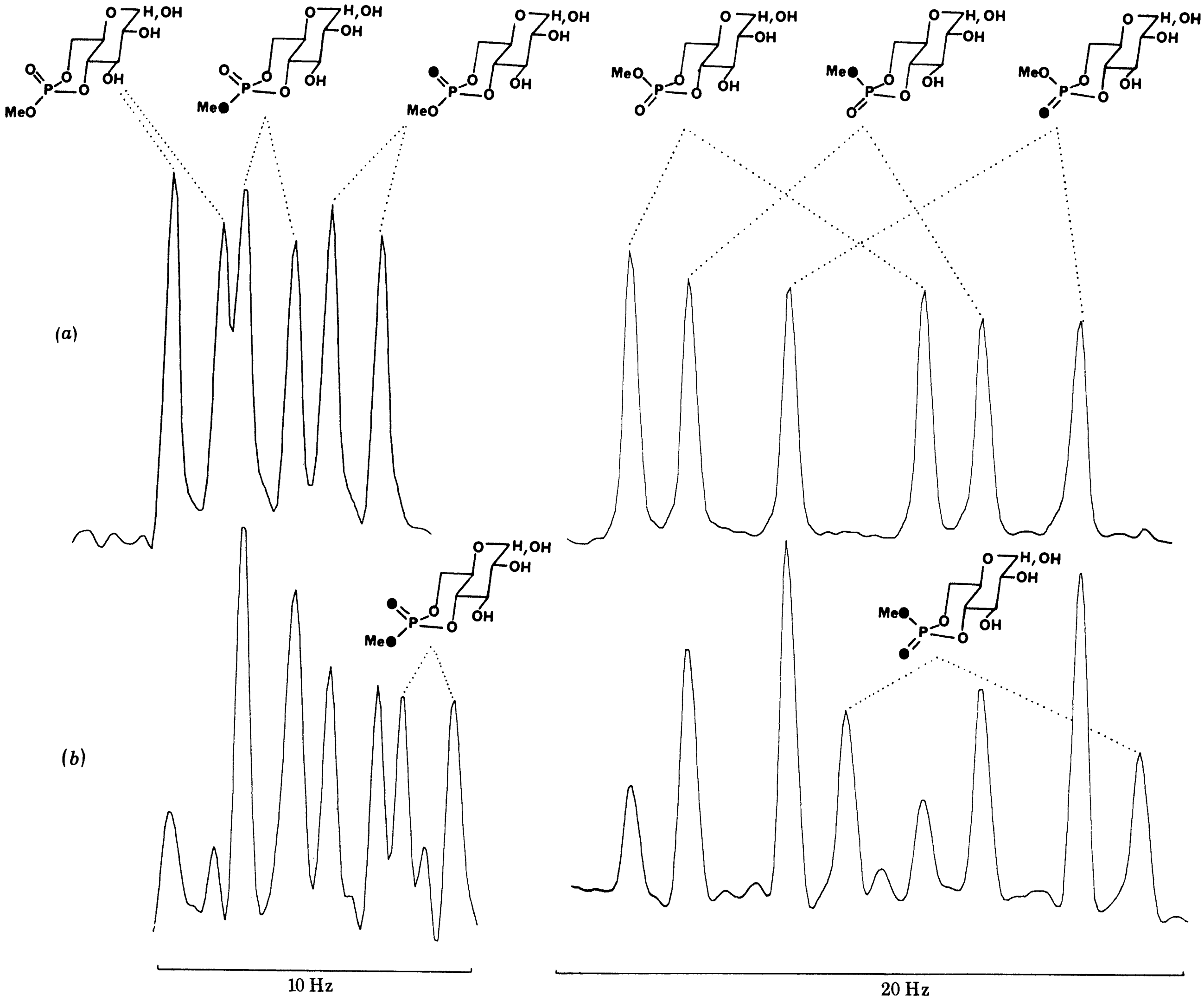


FIGURE 8. The ^{31}P nuclear magnetic resonance spectra (121.5 MHz) of the equatorial and axial triesters derived by cyclization and subsequent methylation of (*a*) D-glucose-6-[^{18}O]phosphate and (*b*) D-glucose-6-[(*S*)-^{16}O,^{17}O,^{18}O]phosphate.

whose ^{31}P chemical shift difference are comparable to the expected isotope shifts. However, by a suitable choice of solvent a convenient separation can be achieved.

D-Glucose-6-[^{18}O]phosphate (estimated by ^{31}P nuclear magnetic resonance spectroscopy to contain 95 at.% ^{18}O) was cyclized and methylated as in figure 7; the ^{31}P nuclear magnetic resonance spectrum of the product is shown in figure 8*a*. As expected, the axial and equatorial esters each appear as two sets of three resonances due to the α- and β-anomers; the assignments are shown on the spectrum. The integration shows that about 4% loss of isotope occurs in the course of this chemical transformation.

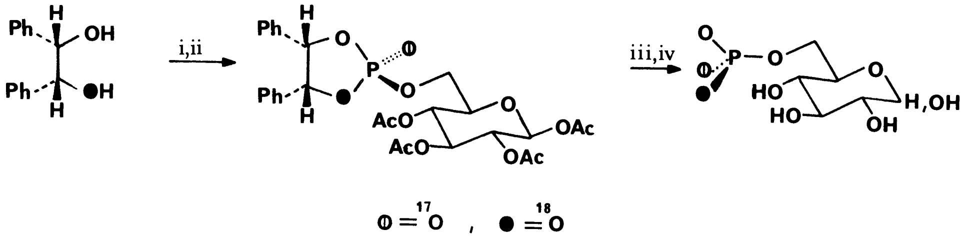


FIGURE 9. Synthesis of D-glucose-6-[(*S*)-^{16}O,^{17}O,^{18}O]phosphate. Reagents: i, P⦶Cl_3 in C_5H_5N; ii, 1,2,3,4-tetraacetyl-β-D-glucose in C_5H_5N; iii, H_2–Pd/C; iv, KOMe in MeOH.

D-Glucose-6-[(*S*)-^{16}O,^{17}O,^{18}O]phosphate was prepared by the general method of synthesis as outlined in figure 9. After cyclization and methylation steps as before, the ^{31}P nuclear magnetic resonance spectrum shown in figure 8*b* was obtained. All the resonances observed in figure 8*a* are present, together with the anomers of the [$^{18}O_2$] axial and equatorial triesters. It is easy to see that the most intense resonances correspond to **7** and **10** (figure 6). To account quantitatively for the spectrum and hence determine the stereoselectivity of the cyclization, a number of factors need to be considered. First, the isotope content of phosphorus [^{17}O]oxychloride (determined by mass spectrometry after conversion to trimethyl phosphate) was 3.3 at.% ^{16}O, 43.5 at.% ^{17}O and 53.2 at.% ^{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 at.% ^{18}O, and contained 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, 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 label during the cyclization step was allowed for. Taking all these factors into consideration the relative intensities of the ^{31}P resonances expected were calculated for inversion and retention of configuration at phosphorus and are compared in table 2 with the observed intensities from figure 8*b*. Clearly, the cyclization occurred with inversion of configuration at phosphorus, and with a stereoselectivity in excess of 94%.

HEXOKINASE

Adenosine-5′-[γ-(*S*)-^{16}O,^{17}O,^{18}O]triphosphate was prepared by the general method of synthesis as outlined in figure 10. It was then incubated with D-glucose and yeast hexokinase, and after phosphoryl transfer was complete, the D-glucose-6-[^{16}O,^{17}O,^{18}O]phosphate was

TABLE 2. THE OBSERVED RELATIVE PEAK INTENSITIES OF THE ^{31}P RESONANCES (FROM FIGURE 8*b*, AVERAGE OF BOTH ANOMERS) OF THE ^{18}O-LABELLED DIASTEREOISOMERIC TRIESTERS DERIVED BY CYCLIZATION FOLLOWED BY METHYLATION OF D-GLUCOSE-6-[(*S*)-$^{16}O,^{17}O,^{18}O$]PHOSPHATE, COMPARED WITH THE EXPECTED VALUES FOR CYCLIZATION WITH RETENTION AND INVERSION OF CONFIGURATION AT PHOSPHORUS

(The stereoselectivity is determined by the ratio of the intensities of the two mono-^{18}O-triesters, compared with the calculated value.)

	equatorial triester				axial triester	
	observed	calculated		observed	calculated	
		retention	inversion		retention	inversion
MeO—P=O	†	0.28	0.28	0.41	0.28	0.28
Me●—P=O	1.00	0.74	1.00	0.76	1.00	0.74
MeO—P=●	0.72	1.00	0.74	1.00	0.74	1.00
Me●—P=●	0.65	0.49	0.49	0.62	0.49	0.49

† Too inaccurate.

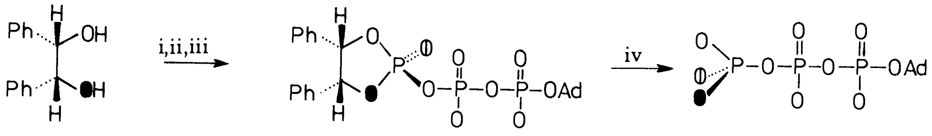


FIGURE 10. Synthesis of adenosine-5′-[γ(*S*)-$^{16}O,^{17}O,^{18}O$]triphosphate. Reagents: i, PⓄCl_3 in C_5H_5N; ii, 3 equiv. $(C_8H_{17})_3N$; iii, ADP$[(C_8H_{17})_3NH^+]_2$; iv, H_2–Pd/C in DMF, $(C_8H_{17})_3N$.

isolated. After cyclization and methylation steps as before, the ^{31}P nuclear magnetic resonance spectrum shown in figure 11 was obtained. Since the relative intensities of the [^{18}O]triesters are reversed from those found in the analysis of D-glucose-6-[(*S*)-$^{16}O,^{17}O,^{18}O$]phosphate, the D-glucose-6-[$^{16}O,^{17}O,^{18}O$]phosphate obtained by the hexokinase catalysed reaction must have the (*R*)-configuration at phosphorus. Hence phosphoryl transfer has occurred with inversion of configuration. From the data in table 3, giving the actual composition of the isotopically labelled ATP (determined by ^{31}P nuclear magnetic resonance spectroscopy), the expected ratios of the isotopically labelled D-glucose-4,6-phosphate triesters can be calculated. The calculated values for phosphoryl transfer occurring with inversion and retention of configuration at phosphorus are compared in table 4 with the experimental values derived from the ^{31}P nuclear magnetic resonance spectrum. From these data it follows that yeast hexokinase catalyses phosphoryl transfer from ATP to D-glucose with inversion of configuration and with a stereoselectivity in excess of 94% (Lowe & Potter 1981).

PHOSPHOFRUCTOKINASE

Sn-Glycerol-3-[(*S*)-$^{16}O,^{17}O,^{18}O$]phosphate was prepared by the general method of synthesis, as outlined in figure 12. This was incubated with the enzymes and cosubstrates shown in figure 13. 3-Chloroacetol phosphate was added to the reaction mixture to inhibit possible traces of triose phosphate isomerase (Hartman 1977), which would catalyse the interconversion of dihydroxyacetone phosphate and D-glyceraldehyde-3-phosphate. In the absence of triose phosphate isomerase, the [$^{16}O,^{17}O,^{18}O$]phosphoryl group should be exclusively transferred to

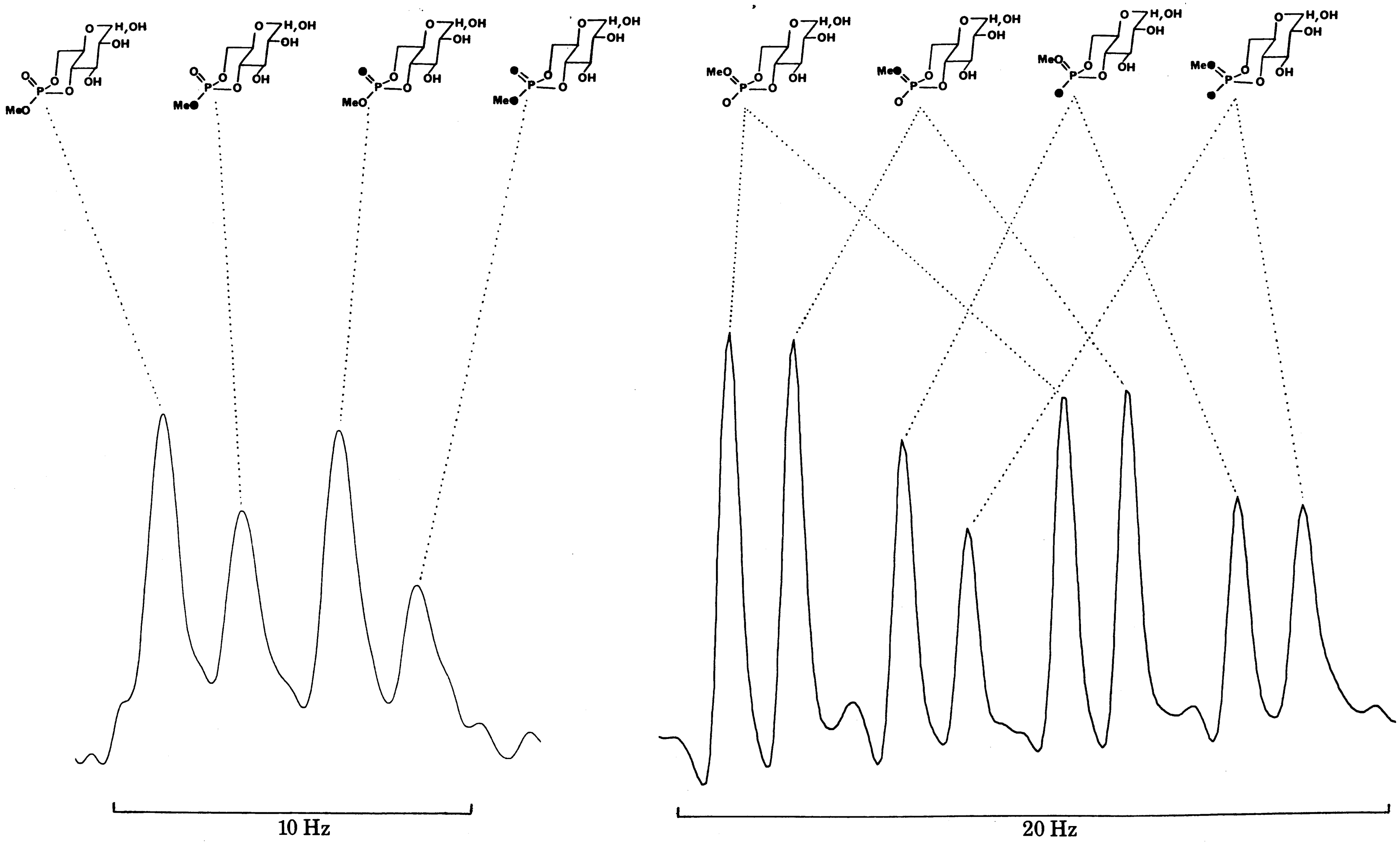


FIGURE 11. The ^{31}P nuclear magnetic resonance spectrum (121.5 MHz) of the equatorial and axial triesters derived by cyclization and subsequent methylation of D-glucose-6-[$^{16}O^{17}O,^{18}O$]phosphate obtained by yeast hexokinase-catalysed transfer of the γ-phosphoryl group from adenosine-5-′-[γ(*S*)-$^{16}O,^{17}O,^{18}O$]triphosphate to D-glucose.

Table 3. The composition of the isotopically labelled ATP sample determined from the ^{31}P nuclear magnetic resonance spectrum

(From the known chiral purity of the [^{18}O]-diol used in the synthesis of the [^{16}O,^{17}O,^{18}O]ATP, 87 % will have the (*S*) and 13 % the (*R*) configuration.)

ATP species	ATP	[^{18}O]ATP	[$^{18}O_2$]ATP	[^{16}O,^{17}O,^{18}O]ATP
percentage of total	20	15	37	28

Table 4. The observed relative peak intensities of the ^{31}P resonances (from figure 11, average of both anomers of the axial triesters except for the axial [$^{18}O_2$]-triester, in which only the low field anomer peak was used) of the ^{18}O-labelled diastereoisomeric triesters derived by cyclization and subsequent methylation of the D-glucose-6-[^{16}O,^{17}O,^{18}O]phosphate formed by yeast hexokinase from adenosine-5′-[γ(*S*)-^{16}O,^{17}O,^{18}O] triphosphate, and the calculated values expected for phosphoryl transfer with retention and inversion of configuration from the known isotopic composition of the isotopically labelled ATP and the known chiral purity of the [^{18}O]-diol used in its synthesis (table 3)

	equatorial triester			axial triester		
	observed	calculated		observed	calculated	
		retention	inversion		retention	inversion
MeO—P=O	1.04	0.99	0.99	0.99	0.99	0.99
Me●—P=O	0.75	1.00	0.73	1.00	0.73	1.00
MeO—P=●	1.00	0.73	1.00	0.73	1.00	0.73
Me●—P=●	0.56	0.49	0.49	0.54	0.49	0.49

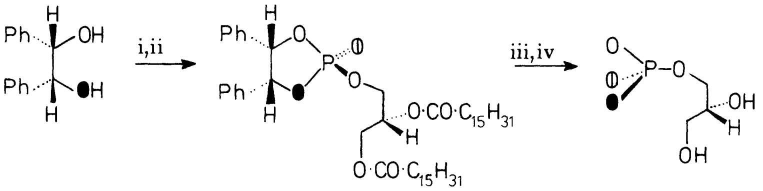


Figure 12. Synthesis of *sn*-glycerol-3-[(*S*)-^{16}O,^{17}O,^{18}O]phosphate. Reagents: i, P⦶Cl_3 in C_5H_5N; ii, 1,2-dipalmitoyl-*sn*-glycerol in C_5H_5N; iii, H_2–Pd/C; iv, NaOH in MeOH–$CHCl_3$.

ADP, in a reaction sequence involving only one phosphoryl transferase, namely phosphofructokinase. The reaction was driven by converting the D-fructose-6-phosphate to D-glucose-6-phosphate and then to D-gluconolactone-6-phosphate, which hydrolyses spontaneously. The adenosine-5′-[^{16}O,^{17}O,^{18}O]triphosphate was isolated and then incubated with D-glucose and yeast hexokinase, and the D-glucose-6-[^{16}O,^{17}O,^{18}O]phosphate isolated and analysed as before. The ^{31}P nuclear magnetic resonance spectrum of the cyclized and methylated material is shown in figure 14. Since the relative intensities of the [^{18}O]triesters are now similar to those found in the analysis of D-glucose-6-[(*S*)-^{16}O,^{17}O,^{18}O]phosphate, and since yeast hexokinase catalyses phosphoryl transfer with inversion of configuration, the phosphoryl transfer catalysed by phosphofructokinase must also occur with inversion of configuration at phosphorus. From the known chiral purity of the [^{18}O] diol, the isotopic composition of the phosphorus [^{17}O]oxychloride and the expected loss of isotope during chemical manipulation (as before) it was possible to calculate the expected ratios of the isotopically labelled D-glucose-4,6-phosphate triesters. The unexpectedly large resonances for the unlabelled esters suggest that in spite of the addition of 3-chloroacetol phosphate to the enzymes before incubating with substrates, some D-glyceraldehyde-3-phosphate has been converted to dihydroxyacetone phosphate. This could be estimated from the isotopic composition of the labelled ATP (see legend to table 5). The

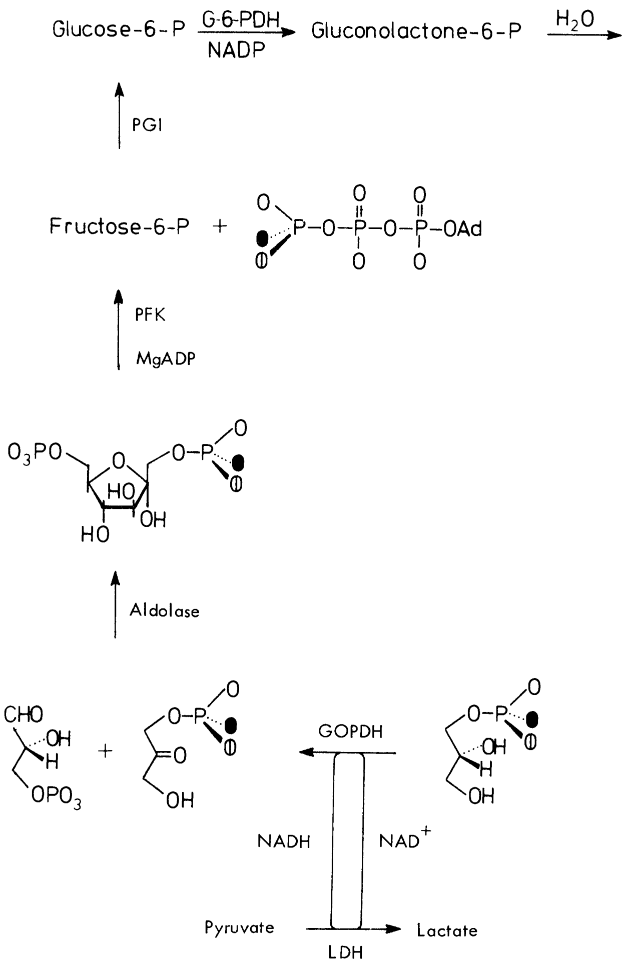


FIGURE 13. The enzymic route used for converting the phosphoryl group of *sn*-glycerol-3-[(*S*)-$^{16}O,^{17}O,^{18}O$]phosphate into adenosine-5′-[γ-$^{16}O,^{17}O,^{18}O$]triphosphate. The only phosphoryl transfer reaction is that catalysed by phosphofructokinase. ATP synthesis is made favourable by converting fructose-6-phosphate to 6-phosphogluconate. G6PDH is glucose-6-phosphate dehydrogenase, PGI is phosphoglucose isomerase, PFK is phosphofructokinase, GOPDH is glycerophosphate dehydrogenase and LDH is lactate dehydrogenase.

calculated values for phosphoryl transfer occurring with inversion and retention of configuration at phosphorus are compared in table 5 with the experimental values derived from the ^{31}P nuclear magnetic resonance spectrum. From these data it follows that *Bacillus stearothermophilus* phosphofructokinase catalyses phosphoryl transfer between D-fructose-1,6-bisphosphate and ADP with inversion of configuration at phosphorus with a stereoselectivity in excess of 94 %. Rabbit muscle phosphofructokinase similarly catalyses phosphoryl transfer with inversion of configuration at phosphorus (R. L. Jarvest, G. Lowe & B. V. L. Potter, unpublished).

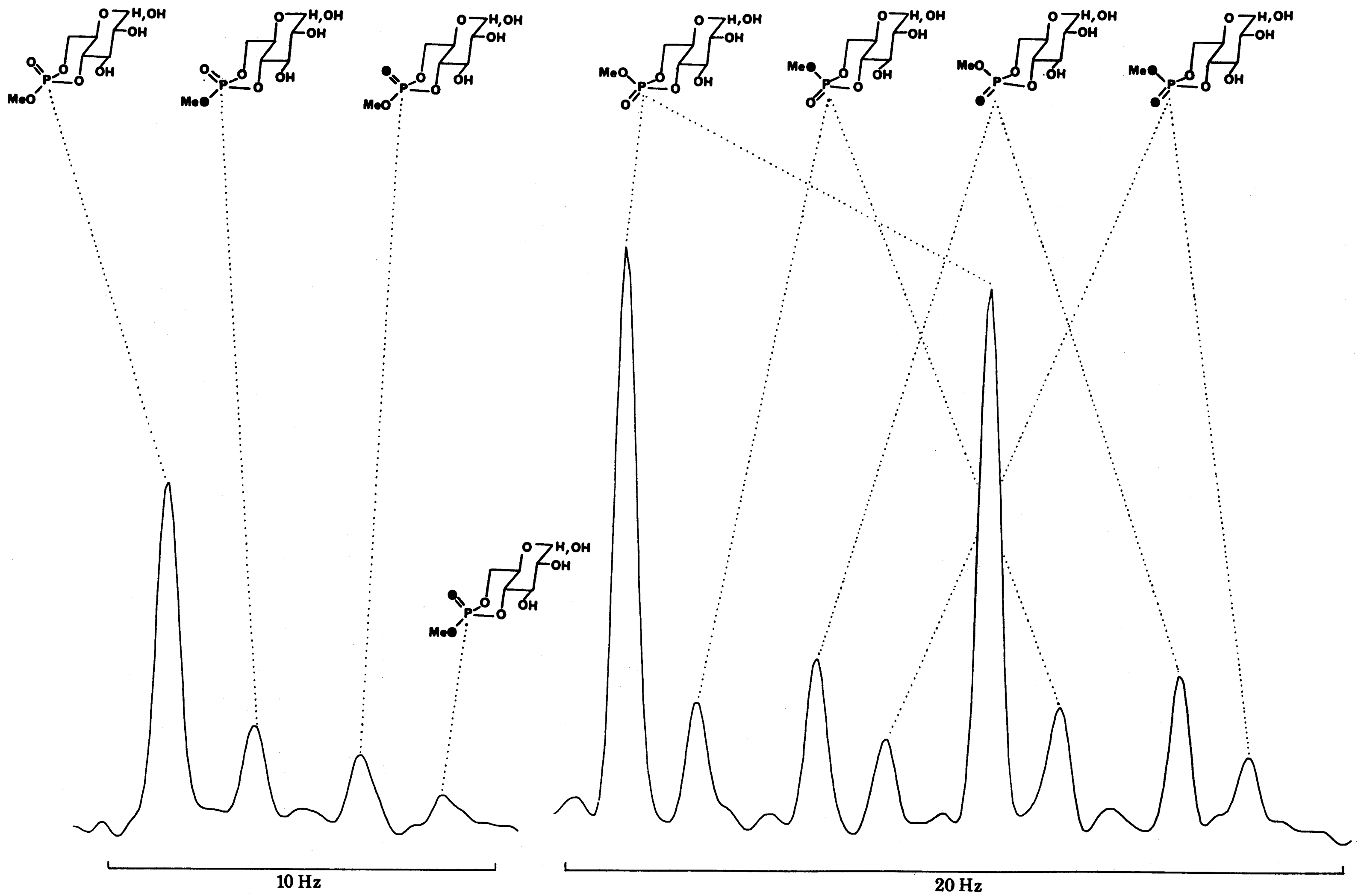


FIGURE 14. The ^{31}P nuclear magnetic resonance spectrum (121.5 MHz) of the equatorial and axial triesters derived by cyclization and subsequent methylation of D-glucose-6-[^{16}O,^{17}O,^{18}O]phosphate obtained by yeast hexokinase-catalysed transfer to D-glucose of the γ-phosphoryl group from adenosine-5′[γ-^{16}O,^{17}O,^{18}O]triphosphate formed by *B. stearothermophilus* phosphofructokinase, as outlined in figure 13.

TABLE 5. THE OBSERVED RELATIVE PEAK INTENSITIES OF THE ^{31}P RESONANCES (FROM FIGURE 14, AVERAGE OF BOTH ANOMERS OF THE AXIAL TRIESTERS) OF THE ^{18}O-LABELLED DIASTEREOISOMERIC TRIESTERS DERIVED BY CYCLIZATION AND SUBSEQUENT METHYLATION OF THE D-GLUCOSE-6-[^{16}O,^{17}O,^{18}O]PHOSPHATE FORMED BY YEAST HEXOKINASE FROM ADENOSINE-5′-[γ-^{16}O,^{17}O,^{18}O]TRIPHOSPHATE DERIVED AS OUTLINED IN FIGURE 13, AND THE CALCULATED VALUES EXPECTED FOR PHOSPHORYL TRANSFER BY *B. STEAROTHERMOPHILUS* PHOSPHOFRUCTOKINASE WITH RETENTION AND INVERSION OF CONFIGURATION

(The composition of the isotopically labelled ATP (determined from its ^{31}P nuclear magnetic resonance spectrum) was ATP (50 %), [^{18}O]ATP (8 %), [$^{18}O_2$]ATP (23 %) and [^{16}O,^{17}O,^{18}O]ATP (19 %).)

	equatorial triester			axial triester		
	observed	calculated		observed	calculated	
		retention	inversion		retention	inversion
MeO—P=O	3.32	3.52	3.52	3.45	3.52	3.52
Me●—P=O	1.00	0.77	1.00	0.77	1.00	0.77
MeO—P=●	0.71	1.00	0.77	1.00	0.77	1.00
Me●—P=●	0.30	0.47	0.47	0.51	0.47	0.47

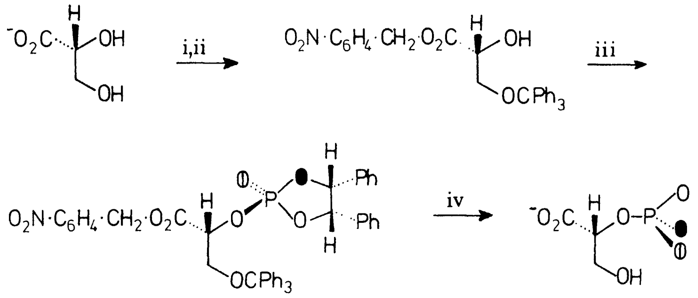


FIGURE 15. Synthesis of 2-[(*S*)-^{16}O,^{17}O,^{18}O]phospho-D-glycerate. Reagents: i, *p*-$O_2N.C_6H_4.CH_2Br$; ii, Ph_3CCl in C_5H_5N; iii, (1*R*,2*S*)-1,2-[1-^{18}O]dihydroxy-1,2-diphenylethane and PⓄCl_3 in C_5H_5N; iv, Na in liquid NH_3.

PYRUVATE KINASE

2[(*S*)-^{16}O,^{17}O,^{18}O]Phospho-D-glycerate was prepared by the general method of synthesis, as outlined in figure 15. This was incubated with the enzymes and cosubstrates shown in figure 16 so that the phosphoryl group was transferred to ADP by pyruvate kinase. The adenosine-5′-[^{16}O,^{17}O,^{18}O]triphosphate was isolated and then incubated with D-glucose and yeast hexokinase, and the D-glucose-6-[^{16}O,^{17}O,^{18}O]phosphate isolated and analysed as before. The ^{31}P nuclear magnetic resonance spectrum of the cyclized and methylated material is shown in figure 17. From the relative intensities of the [^{18}O]triesters it is again clear that pyruvate kinase catalyses phosphoryl transfer with inversion of configuration. The relative intensities of the [^{18}O]equatorial triesters and the [^{18}O]axial triesters compared with the calculated values (table 6) shows that pyruvate kinase catalyses phosphoryl transfer with inversion of configuration with a stereoselectivity in excess of 86 % (P. M. Cullis, G. Lowe & B. V. L. Potter, unpublished).

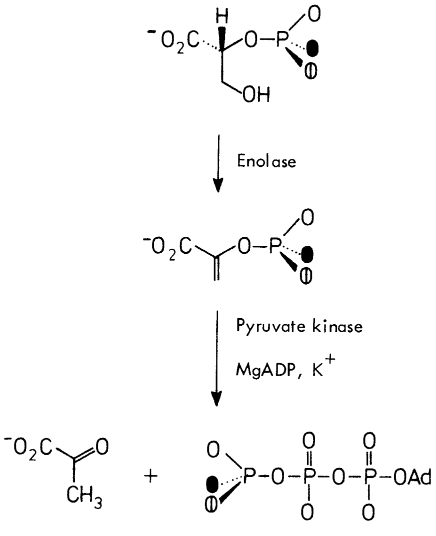


FIGURE 16. The enzymic route used for converting the phosphoryl group from 2-[(*S*)-^{16}O,^{17}O,^{18}O]phospho-D-glycerate into adenosine-5′-[γ-^{16}O,^{17}O,^{18}O]triphosphate. The only phosphoryl transfer step is that catalysed by pyruvate kinase.

DISCUSSION

Yeast hexokinase, *B. stearothermophilus* and rabbit muscle phosphofructokinase, and rabbit muscle pyruvate kinase all catalyse phosphoryl transfer with inversion of configuration, which is most simply interpreted in terms of an 'in line' transfer of the phosphoryl group between substrates in the ternary complex. To determine whether the transfer occurs by an associative or dissociative mechanism requires independent evidence, which can be provided by a positional isotope exchange experiment (Lowe & Sproat 1978*b*, 1980; Rose 1980).

Yeast hexokinase and pyruvate kinase have previously been shown to catalyse thiophosphoryl transfer with inversion of configuration at phosphorus (Orr *et al.* 1978), so that the stereochemical courses of three kinases have now been shown to occur with inversion of configuration both for thiophosphoryl and phosphoryl transfer. It therefore appears likely that in general when the natural substrate adopts an 'in-line' mechanism, the thiophosphate analogue will follow the same stereochemical course.

Phosphoglycerate mutase (both from wheat germ and rabbit muscle) is the only other glycolytic phosphoryl transferase that has been investigated by using isotopically labelled natural substrate and here retention of configuration was observed (Blättler & Knowles 1980). This is in accord with an adjacent mechanism with pseudorotation or a double displacement mechanism. Since this enzyme is known to form a kinetically competent phosphoryl-enzyme intermediate, the double displacement mechanism provides the simplest interpretation of all the experimental evidence. Since phosphoglucomutase also possesses a kinetically competent phosphoryl-enzyme intermediate, it is likely that this also follows a double displacement mechanism.

The question naturally arises as to why the phosphokinases have evolved a catalytic mechanism with 'in-line' phosphoryl transfer between substrates in the ternary complex, whereas the phosphomutases have evolved a double displacement mechanism involving a phosphoryl-enzyme intermediate. A possible explanation is provided by the fact that the two substrates for

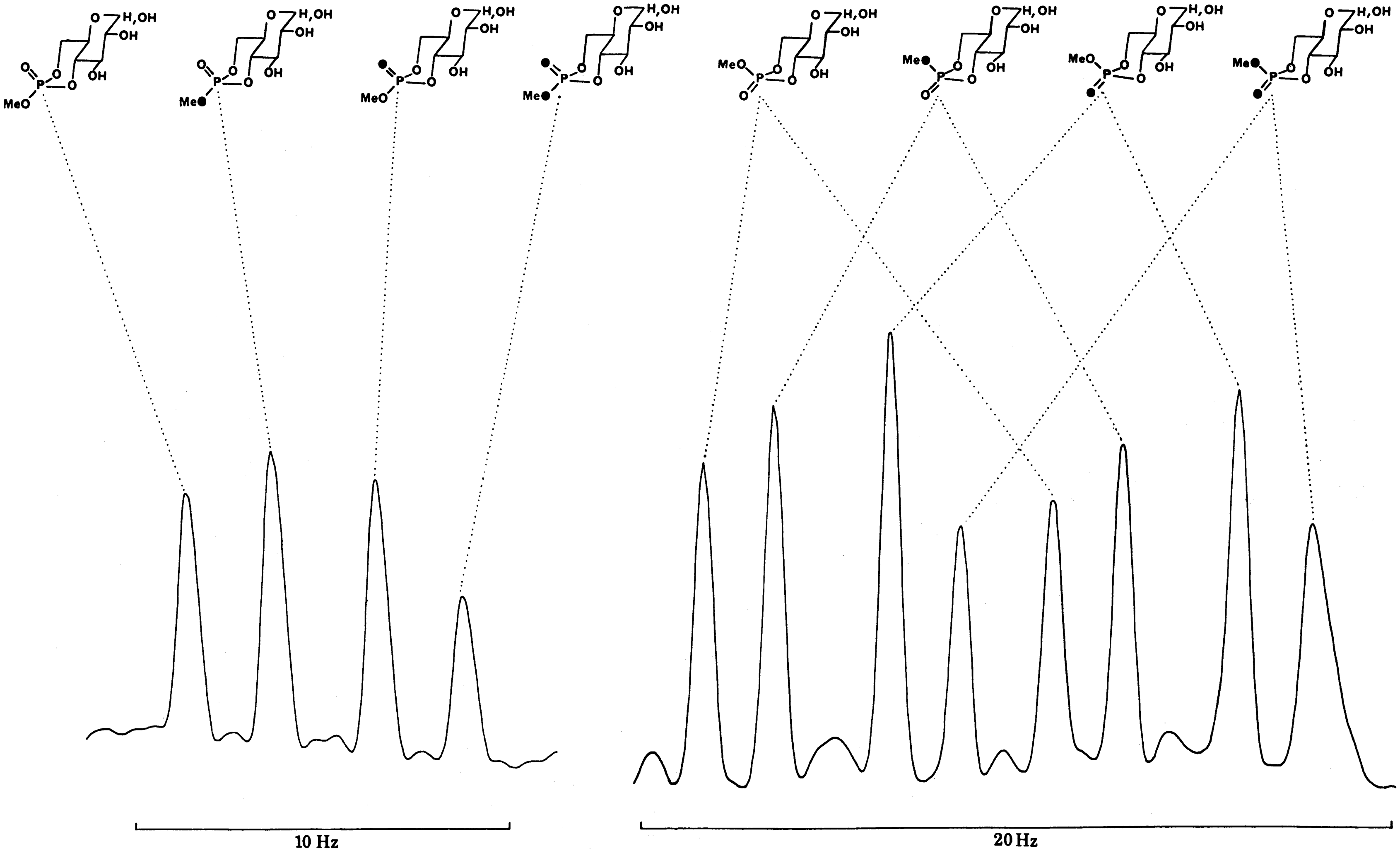


Figure 17. The ^{31}P nuclear magnetic resonance spectrum (121.5 MHz) of the equatorial and axial triesters derived by cyclization and subsequent methylation of D-glucose-6-[^{16}O,^{17}O,^{18}O]phosphate obtained by yeast hexokinase-catalysed transfer to D-glucose of the γ-phosphoryl group from adenosine-5′-[γ-^{16}O,^{17}O,^{18}O]triphosphate formed by pyruvate kinase, as outlined in figure 16.

Table 6. The observed relative peak intensities of the ^{31}P resonances (from figure 16, average of both anomers of the axial triesters except for the axial [$^{18}O_2$]-triester, in which only the low field anomer peak was used) of the ^{18}O-labelled diastereoisomeric triesters derived by cyclization and subsequent methylation of the D-glucose-6-[^{16}O,^{17}O,^{18}O]phosphate formed by yeast hexokinase from adenosine-5′-[γ-^{16}O,^{17}O,^{18}O]triphosphate derived as outlined in figure 16, and the calculated values expected for phosphoryl transfer by pyruvate kinase with retention and inversion of configuration

	equatorial triester			axial triester		
	observed	calculated		observed	calculated	
		retention	inversion		retention	inversion
MeO—P=O	0.86	0.72	0.72	0.71	0.72	0.72
Me●—P=O	1.00	0.85	1.00	0.85	1.00	0.85
MeO—P=●	0.90	1.00	0.85	1.00	0.85	1.00
Me●—P=●	0.52	0.65	0.65	0.62	0.65	0.65

a phosphokinase are grossly different, so that the enzyme must provide a distinctive binding site for each substrate. By requiring both substrates to be present simultaneously at the active site, the 'in-line' mechanism can be utilized. By contrast the two substrates (and cosubstrate) for the mutases possess the same carbon–oxygen skeleton, so that a single binding site could suffice to bind both substrates, but not simultaneously. The phosphoryl group to be transferred must therefore become temporarily bound to the enzyme while substrate exchange takes place. Since the phosphoryl transfer potential of the substrates that the mutases transform is low, the intervention of a covalent phosphoenzyme intermediate could also have a catalytic advantage. So by providing a refuge for the phosphoryl group on the enzyme, the overall activation energy for phosphoryl transfer should be lowered, and the need to evolve more than one substrate binding site unnecessary.

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