Stereochemical Course of the Reactions Catalyzed by the Bacterial Phosphoenolpyruvate:Glucose Phosphotransferase System†

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ABSTRACT: The overall stereochemical course of the reactions leading to the phosphorylation of methyl α-D-glucopyranoside by the glucose-specific enzyme II (enzyme II^{Glc}) of the *Escherichia coli* phosphotransferase system has been investigated. With [(*R*)-^{16}O,^{17}O,^{18}O]phosphoenolpyruvate as the phosphoryl donor and in the presence of enzyme I, HPr, and enzyme III^{Glc} of the phosphotransferase system, membranes from *E. coli* containing enzyme II^{Glc} catalyzed the formation of methyl α-D-glucopyranoside 6-phosphate with *overall inversion* of the configuration at phosphorus (with respect to phosphoenolpyruvate). It has previously been shown that sequential covalent transfer of the phosphoryl group of phosphoenolpyruvate to enzyme I, to HPr, and to enzyme III^{Glc} occurs before the final transfer from phospho-enzyme III^{Glc} to the sugar, catalyzed by enzyme II^{Glc}. Because overall inversion of the configuration of the chiral phospho group of phosphoenolpyruvate implies an odd number of transfer steps, the phospho group has been transferred *at least five times*, and transfer from phospho-enzyme III^{Glc} to the sugar must occur in two steps (or a multiple thereof). On the basis that no membrane protein other than enzyme II^{Glc} is directly involved in the final phospho transfer steps, our results imply that a covalent phospho-enzyme II^{Glc} is an intermediate during transport and phosphorylation of glucose by the *E. coli* phosphotransferase system.

The phosphoenolpyruvate-dependent sugar phosphotransferase system (PTS)[1] concomitantly transports and phosphorylates a number of mono- and disaccharides as well as certain sugar alcohols in many prokaryotes (Postma & Roseman, 1976; Saier, 1977; Dills et al., 1980). This system, first described by Roseman and co-workers (Kundig et al., 1964), is comprised of a series of phosphotransferases that culminate in the phosphorylation of the sugar as it is translocated across the cytoplasmic membrane:

$$\text{phosphoenolpyruvate} + \text{HPr} \xrightleftharpoons{\text{enzyme I}} \text{pyruvate} + \text{phospho-HPr}$$

$$\text{phospho-HPr} + \text{sugar (out)} \xrightleftharpoons[\text{enzyme III}]{\text{enzyme II}} \text{HPr} + \text{phosphosugar (in)}$$

Transfer of the phospho group from phosphoenolpyruvate to a low molecular weight, heat-stable protein (HPr) occurs in the cytoplasm and is catalyzed by enzyme I via a covalent phospho-enzyme I intermediate. Both HPr and enzyme I are proteins involved in the phosphorylation and transport of all the sugars recognized by the PTS. The subsequent enzymatic steps are sugar specific and result in the transfer of the phospho group from phospho-HPr to the sugar by way of one of several membrane-bound enzyme II proteins that act as specific permeases for phospho group transfer. This transfer may or may not also require a sugar-specific enzyme III, depending on the sugar being transported (Postma & Roseman, 1976; Saier, 1977; Dills et al., 1980). For example, the transport of glucose in *Escherichia coli* and *Salmonella typhimurium* via the glucose enzyme II (enzyme II^{Glc}) involves a specific enzyme III^{Glc} that intervenes between phospho-HPr and enzyme II^{Glc}, while for the transport of mannitol via enzyme II^{Mtl}, phospho-HPr apparently interacts directly with enzyme II^{Mtl} (Jacobson et al., 1979).

Both of the cytoplasmic components, enzyme I and HPr, have been purified from enteric bacteria, and the phosphorylated intermediates of both proteins have been shown to involve histidine residues (Postma & Roseman, 1976; Anderson et al., 1971; Robillard et al., 1979; Waygood & Steeves, 1980). Enzyme III^{Glc} has been purified from *E. coli* and is phosphorylated on a protein carboxyl group by phospho-HPr (Kundig, 1974). The phospho-enzyme III^{Glc} is active in the phosphorylation of glucose in the presence of enzyme II^{Glc} (Kundig, 1974). It is not known, however, whether phospho group transfer proceeds directly between phospho-enzyme III^{Glc} and glucose (catalyzed by enzyme II^{Glc}) or whether a phospho-enzyme II^{Glc} is an obligatory intermediate. Kinetic studies have suggested that such an intermediate may occur in the PTS's of *Bacillus subtilis* that are responsible for the transport of methyl α-D-glucoside (Marquet et al., 1978) and fructose (Perret & Gay, 1979), the glucose PTS of *Streptococcus faecalis* (Hüdig & Hengstenberg, 1980), and the β-glucoside PTS (Rose & Fox, 1971) and the mannitol PTS (C. Lee, personal communication) of *E. coli*. Definitive evidence for a phospho-enzyme II intermediate, however, has yet to be obtained, although *E. coli* enzyme II^{Mtl} has been purified (Jacobson et al., 1979), characterized (G. R. Jacobson, C. A. Lee, J. E. Leonard, and M. H. Saier, Jr., unpublished results), and functionally reconstituted into proteoliposomes (J. E. Leonard and M. H. Saier, unpublished results). Determination of whether such an intermediate exists will obviously be important in the development of a model to explain the mechanism of transport and phosphorylation by the PTS enzymes II.

As a first step in this direction, we are studying the reaction catalyzed by enzyme II^{Glc} of *E. coli*, which is specific for the transport and phosphorylation of glucose and of methyl α-D-glucoside. The pathway of phospho transfer in this system is as shown in Scheme I. It is the transfer catalyzed by enzyme II^{Glc} (4 and 5 in Scheme I) in this sequence that is the least understood mechanistically and which could occur

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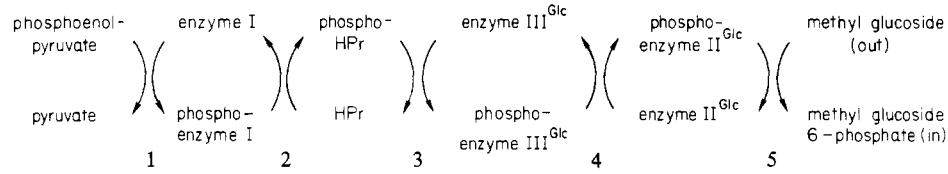
[1] Abbreviations: PTS, phosphoenolpyruvate-dependent sugar phosphotransferase system; HPr and enzyme I, soluble, general phospho transfer proteins of the PTS; enzyme III^{Glc}, phospho transfer protein of the PTS specific for glucose; enzyme II^{Glc}, enzyme II^{Mtl}, and enzyme II^{Man}, membrane-bound enzymes/permeases of the PTS specific for glucose, mannitol, and mannose, respectively; Tris, tris(hydroxymethyl)aminomethane; DEAE, diethylaminoethyl.

0006-2960/82/0421-5552$01.25/0

Scheme I



either directly or via a phospho-enzyme II^{Glc} intermediate. If enzyme II^{Glc} is phosphorylated, then the phospho group of phosphoenolpyruvate would be transferred *five* times in going to the product methyl glucoside 6-phosphate. But if enzyme II^{Glc} catalyzes the direct transfer of the phospho group from phospho-enzyme III^{Glc} to methyl α-D-glucoside, only *four* phospho transfers would be involved. To distinguish between these possibilities, we have determined the overall stereochemical course of the glucose PTS system, using as substrate, [(*R*)-^{16}O,^{17}O,^{18}O]phosphoenolpyruvate, chiral at phosphorus. Since there is clear evidence that each act of enzyme-catalyzed phospho transfer proceeds with inversion of the configuration at phosphorus (Knowles, 1980, 1982), we predict that if *four* phospho transfers occur, the configuration at phosphorus in the methyl glucoside 6-phosphate product will be the same as the starting phosphoenolpyruvate (i.e., overall retention), whereas if *five* phospho transfers are required, the product will have the opposite configuration at phosphorus (i.e., overall inversion).

Materials and Methods

Materials. Methyl α-D-[^{14}C]glucopyranoside (275 mCi/mmol) was a product of New England Nuclear Corp. (Lexington, MA). The following were purchased from Sigma Chemical Co. (St. Louis, MO): methyl α-D-glucopyranoside, 3-phospho-D-glycerate, 2,3-bis(phospho)-D-glycerate, phosphoenolpyruvate (tricyclohexylammonium salt), phenylmethanesulfonyl fluoride, phosphoglycerate mutase (rabbit muscle), and enolase (yeast). 3-[(*R*)-^{16}O,^{17}O,^{18}O]Phospho-D-glycerate was synthesized by the method described earlier (Abbott et al., 1979; Blättler & Knowles, 1980). All other chemicals were obtained from Aldrich Chemical Co. (Milwaukee, WI).

Bacterial Strains. *E. coli* strain ML308 (*manA58*) was generously supplied by Dr. Milton Saier and was used as a source of membranes containing enzyme II^{Glc}. This strain was used because it is defective in enzyme II^{Man} (often referred to as the enzyme II-A/II-B system), which also recognizes glucose as a substrate and is independent of enzyme III^{Glc}. *S. typhimurium* strain LJ144 (*cpd-401, cysA1150/F'198*), also supplied by Dr. Saier, contains the PTS operon on an *E. coli* episome and overproduces enzyme I, HPr, and enzyme III^{Glc} about 5-fold relative to wild-type cells (Saier & Feucht, 1975). This strain was used as a source of these three soluble PTS components.

Spectroscopy and Chromatography. ^{1}H and ^{31}P NMR spectra were recorded on a Bruker WM-300 instrument. ^{31}P NMR chemical shifts are relative to external 85% H_3PO_4 (downfield positive). Mass spectra were recorded on an AEI MS9 double-focusing instrument. Ion-exchange chromatography was performed on Dowex-1 (AG1-X8, 200–400 mesh, 8% cross-linked) from Bio-Rad Laboratories (Richmond, CA) and Dowex-50 (100–200 mesh, 8% cross-linked) from Sigma Chemical Co. (St. Louis, MO).

Cell Growth and Preparation of Cell-Free Fractions. *E. coli* strain ML308 (*manA58*) was grown at 37 °C to midexponential phase (A_{550nm}^{10mm} = 1.1) in 5 L of medium 63 (Saier et al., 1976), with 0.5% (w/v) glucose as a carbon source. Phenylmethanesulfonyl fluoride in absolute ethanol was then added to a final concentration of 1 mM, and the cells were harvested by centrifugation at 10000*g* and then washed with medium 63 at 4 °C. The cell paste was resuspended in 100 mL of 20 mM Tris-HCl buffer, pH 7.5, containing dithiothreitol (1 mM), and broken in a French pressure cell at 10000 psi. After removal of unbroken cells (by centrifugation at 10000*g* for 5 min), membranes were collected by centrifugation at 100000*g* for 90 min at 4 °C. The membrane fraction was resuspended in 50 mL of the same buffer and stored at −70 °C.

S. typhimurium strain LJ144 was grown in a 2-L culture as described above for *E. coli*. The cells were harvested, washed, and then resuspended in 20 mL of 20 mM Tris-HCl buffer, pH 8.4, containing dithiothreitol (1 mM). After cell lysis in the French press as described above, unbroken cells were removed, and the membranes were pelleted by centrifugation at 100000*g* for 90 min at 4 °C. The supernatant was then recentrifuged to remove residual membranes. The supernatant from this second centrifugation was then dialyzed overnight at 4 °C against 1 L of 20 mM Tris-HCl buffer, pH 8.4, containing dithiothreitol (1 mM), and stored at −20 °C. This solution was used as a source of the soluble PTS proteins: enzyme I, HPr, and enzyme III^{Glc}.

Glucose PTS Reaction Conditions. Phosphoenolpyruvate was formed from 3-[(*R*)-^{16}O,^{17}O,^{18}O]phosphoglycerate by including phosphoglycerate mutase and enolase in the PTS reaction mixture. The reaction was conducted in 25 mM Tris-HCl buffer, pH 8.0, containing $MgCl_2$ (5 mM), KF (10 mM), dithiothreitol (1 mM), 3-[(*R*)-^{16}O,^{17}O,^{18}O]phosphoglycerate (1 mM), 2,3-bis(phospho)glycerate (5 μM), phosphoglycerate mutase (1000 units), enolase (1000 units), methyl α-D-glucopyranoside (1 mM), *S. typhimurium* cytoplasm (10 mL), and *E. coli* membrane suspension (50 mL). The final volume was 100 mL. A reaction mixture (1 mL) of identical composition was prepared and supplemented with methyl α-D-[^{14}C]glucopyranoside (to 0.5 μCi/μmol) and used to determine the time course of the reaction. Both reaction mixtures were incubated at 37 °C with shaking for 5 h, after which time the conversion of methyl α-D-[^{14}C]glucopyranoside to the 6-phospho compound had ceased, as judged by the DEAE filter disc technique (Jacobson et al., 1979). Under these conditions, 51% of the 3-phosphoglycerate and methyl glucoside substrates were converted into the products, hexose 6-phosphate and pyruvate. Membranes were then removed by centrifugation at 100000*g* for 90 min at 4 °C. More than 90% of the methyl α-D-glucoside 6-phosphate product remained in the supernatant, as judged by a similar centrifugation of the radioactive reaction mixture. The supernatant from the 100-mL incubation mixture was stored at −20 °C.

Purification and Stereochemical Analysis of Methyl α-D-Glucopyranoside 6-[^{16}O,^{17}O,^{18}O]Phosphate. The frozen supernatant obtained above was thawed at room temperature and passed through Amicon CF-25 Centriflo ultrafiltration cones (at 1200*g*) to remove most soluble enzymes. The filtrate was then chromatographed on a column (30 mL) of Dowex-1 (HCO_3^- form) equilibrated with 25 mM triethylammonium bicarbonate buffer, pH 7.5, and eluted with a linear gradient

(10–250 mM) of the same buffer. The methyl glucoside 6-phosphate product was converted into its bis monocyclohexylammonium salt, which was recrystallized from acetone–water (20:1 v/v). The isolated yield was 35% (based on 3-[(*R*)-^{16}O,^{17}O,^{18}O]phospho-D-glycerate). The methyl α-D-glucopyranoside 6-[^{16}O,^{17}O,^{18}O]phosphate was identical (by ^{1}H NMR and mass spectrometry) with an authentic unlabeled sample synthesized by the method of Rose et al. (1974).

The bis(cyclohexylammonium) salt of the product sample of methyl α-D-glucopyranoside 6-[^{16}O,^{17}O,^{18}O]phosphate was converted to the mono(tri-*n*-butylammonium) mono(tri-*n*-octylammonium) salt by passage through a column (2 mL) of Dowex-50 (H^+ form), followed by neutralization with 35 μmol of tri-*n*-butylamine and 35 μmol of tri-*n*-octylamine in dioxane (5 mL). The resulting solution was evaporated to dryness 4 times and freeze-dried once, from dry dioxane. Dry dioxane (750 μL) was added to the resulting white solid, followed by 10 molecular sieves (4 Å). The solution was stirred for 2 h, and freshly distilled diphenyl phosphorochloridate (31.5 μmol) was then added. After the solution was stirred for a further 30 min, it was frozen and the dioxane removed by lyophilization. To the resulting syrup was added a solution of potassium *tert*-butoxide (0.3 g) in dry dimethylformamide (10 mL), and the mixture was stirred for 10 min. The reaction was then quenched by pouring it onto an aqueous slurry of Dowex-50 (pyridinium form) (20 mL). The mixture was filtered and the filtrate evaporated to dryness. The cyclic diester product (in ~50% yield as estimated by ^{31}P NMR [δ_P (D_2O) −1.89 when H decoupled; d, J_{PH} 22 Hz when H undecoupled]) was purified by ion-exchange chromatography on a column (5 mL) of Dowex-1 (HCO_3^- form) as described above. The purified diester was then converted into the potassium salt by passage down a column (10 mL) of Dowex-50 (K^+ form). The product, methyl α-D-glucopyranoside cyclic 4,6-phosphate, was evaporated to dryness.

The cyclic diester was dissolved in H_2O (3 mL), and the cyclic polyether 18-crown-6 (20 mg) was added. The solution was then evaporated to dryness and then freeze-dried several times from dry dioxane. To the anhydrous product was then added dry dimethyl sulfoxide (750 μL), followed by methyl iodide (100 μL), and the mixture was stirred for 20 h. Dry dimethyl-d_6 sulfoxide (2 mL) was then added and the solution transferred to a dry 10-mm NMR tube for ^{31}P NMR analysis.

Results and Discussion

A number of control experiments established that phospho group transfer from 3-[^{16}O,^{17}O,^{18}O]phospho-D-glycerate to methyl α-D-glucopyranoside took place by the expected pathway via enzyme II^{Glc} of the PTS. Formation of methyl α-D-glucopyranoside 6-phosphate was not detected under the reaction conditions if 3-phosphoglycerate, *S. typhimurium* cytoplasm, or *E. coli* membranes were omitted from the mixture. Moreover, inclusion of a 10-fold molar excess of 2-deoxyglucose over methyl α-D-glucoside in the reaction mixture did not affect the phosphorylation rate of the methyl glucoside significantly. This confirms that sugar phosphorylation occurred via enzyme II^{Glc} and not via enzyme II^{Man}. The former enzyme has been shown to prefer methyl α-D-glucoside as a substrate while the latter preferentially phosphorylates 2-deoxyglucose (Curtis & Epstein, 1975). This result was expected, since the membranes used in these experiments were isolated from an *E. coli* strain defective in enzyme II^{Man}.

The chiral sample of [(*R*)-^{16}O,^{17}O,^{18}O]phosphoenolpyruvate was generated *in situ* from 3-[(*R*)-^{16}O,^{17}O,^{18}O]phospho-D-glycerate in the presence of phosphoglycerate mutase and enolase. [We have previously shown (Blättler & Knowles, 1980) that rabbit muscle phosphoglycerate mutase proceeds with overall retention of the configuration at phosphorus.] The phosphoglycerate was synthesized by the method described earlier (Abbott et al., 1979; Blättler & Knowles, 1980), and the configuration at phosphorus was confirmed by independent stereochemical analysis (Blättler & Knowles, 1980). The chiral phospho group was transferred to methyl α-D-glucoside catalyzed by the PTS^{Glc} system, and the configuration at phosphorus in the product, methyl α-D-glucoside 6-[^{16}O,^{17}O,^{18}O]phosphate, was determined by using the approach of Jarvest et al. (1981).

Table I: Peak Integrations for the ^{31}P NMR Spectra of Figure 2

	peak no.[a]							
	1	2	3	4	5	6	7	8
predicted for *S* phospho[b]	25.6	42.2	23.8	8.4	25.6	23.8	42.2	8.4
predicted for *R* phospho[b]	25.6	23.8	42.2	8.4	25.6	42.2	23.8	8.4
obsd	22.4	43.4	25.1	9.1	25.2	23.0	43.4	8.4

[a] Reading from low field up. [b] On the basis of the known isotopic composition of the methyl α-D-glucopyranoside 6-phosphate used.

The stereoanalytical method depends upon the different perturbations of the NMR signal of a ^{31}P atom by the oxygen isotopes that are attached to it. Illustrated in Figure 1 is the hypothetical situation where the atom percent excess at each of the three peripheral oxygens of the *S* phospho group is 100%. (In fact, the ^{17}O position only contains 41% atom excess of ^{17}O; this is discussed later.) On cyclization, a 1:1:1 mixture of the three cyclic diesters is produced, which on methylation gives six cyclic triesters, three with the methyl group axial and three with the methyl group equatorial. Now, the electrical quadrupole moment of ^{17}O so broadens the ^{31}P NMR resonance of the ^{31}P nucleus to which it is attached that no species containing a bond between ^{17}O and ^{31}P is observed in the ^{31}P NMR spectrum (Lowe et al., 1979; Tsai, 1979). Only the two boxed triesters are seen, and the ^{31}P NMR signals for these species are shifted upfield (relative to the materials containing only ^{16}O) by the attached ^{18}O (Cohn & Hu, 1978; Lowe & Sproat, 1978). The axial isomer (which contains a double bond between ^{18}O and ^{31}P) is shifted upfield by some 0.04 ppm, and the equatorial isomer (which has only a single ^{18}O–^{31}P bond) is shifted upfield by about 0.02 ppm (Lowe et al., 1979; Cohn & Hu, 1980). If the original phospho group had been of the *R* configuration (opposite to that illustrated in Figure 1), the ^{18}O would have been in the axial position, and the equatorial isomer would have suffered the larger upfield shift. The fact that, in practice, the ^{17}O position of the original phospho group was contaminated with 27% ^{16}O and 32% ^{18}O (this derives from the imperfect isotopic enrichment of the "$H_2{}^{17}O$" used synthetically) leads to the appearance of resonances in the observed ^{31}P NMR spectrum due to species containing ^{16}O in both the axial and equatorial positions, axial ^{18}O with equatorial ^{16}O, and ^{18}O in both the axial and equatorial positions. These additional signals do not affect the stereochemical analysis since they derive from prochiral phospho groups, and the dominant peaks in the ^{31}P NMR spectrum of the cyclic triesters from an *S* phospho group are those due to the boxed species in Figure 1. The observed spectra of the product glucoside 6-phosphate after cyclization and methylation are shown in Figure 2, from which it is clear that the configuration at phosphorus is *S* (see also Table I). According to the quality index for such experiments (Buchwald et al., 1982), Q_{max} (which is a measure of the maximum relative size

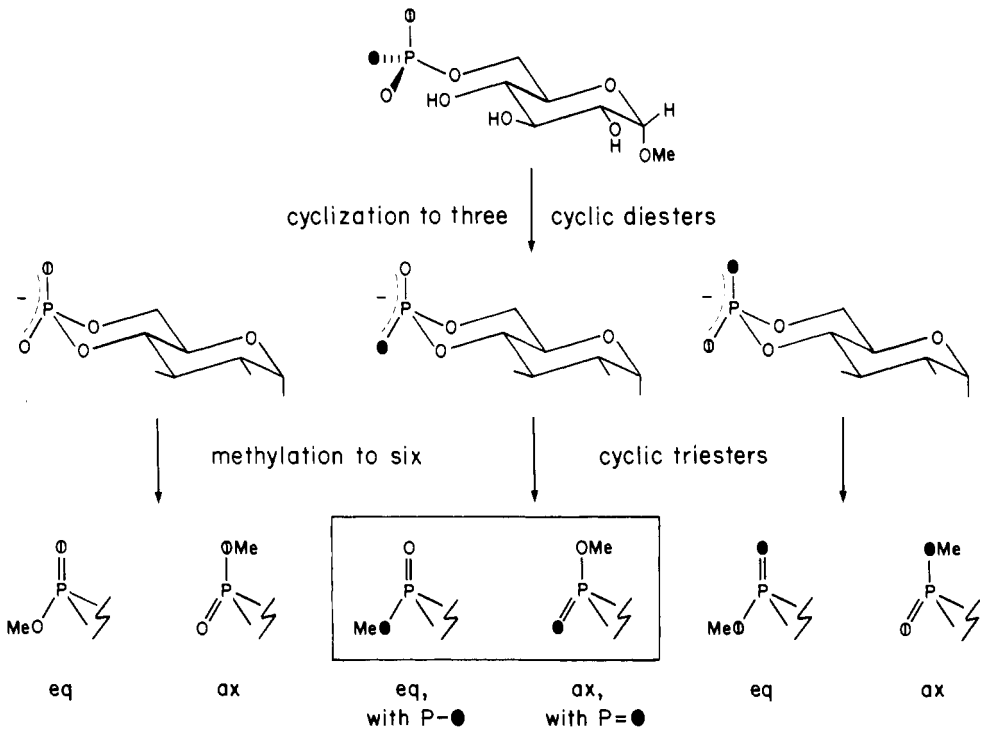


FIGURE 1: Illustration of the stereochemical analysis for methyl α-D-glucopyranoside 6-[(*S*)-^{16}O,^{17}O,^{18}O]phosphate: ^{16}O (O), ^{17}O (Φ), ^{18}O (●); eq, equatorial methoxy group; ax, axial methoxy group. In the starting phosphoric monoester, double bonds and charges on the phospho group are omitted.

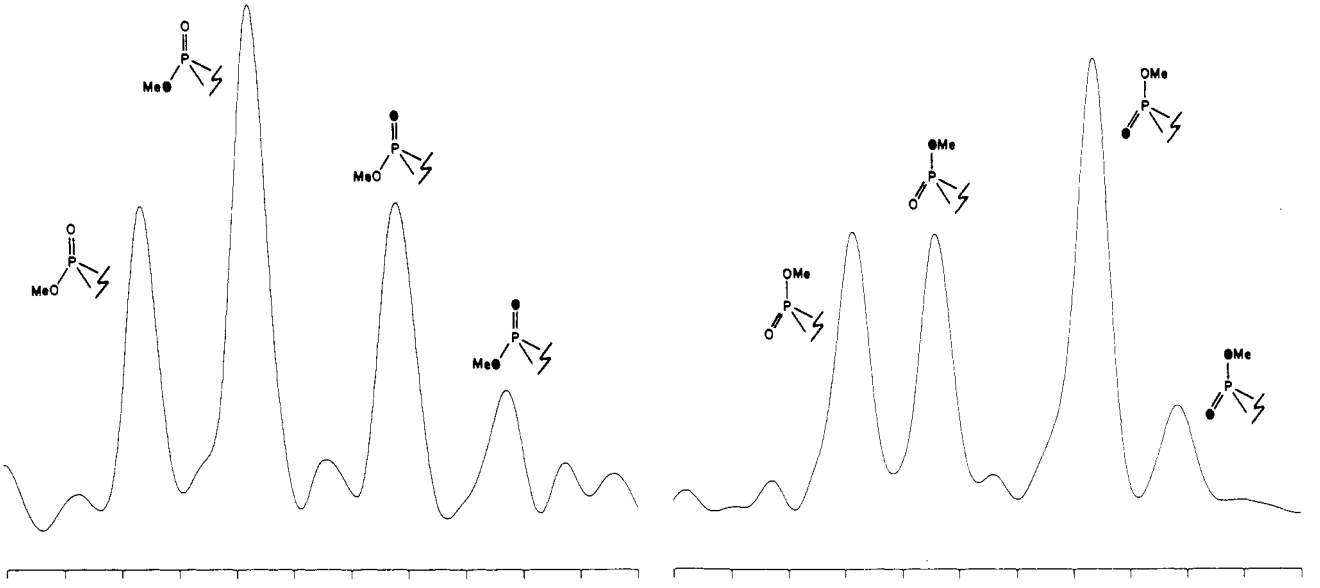


FIGURE 2: ^{31}P NMR spectra of axial and equatorial methyl esters of methyl α-D-glucopyranoside cyclic 4,6-phosphate derived from stereochemical analysis of the methyl α-D-glucopyranoside 6-[(*S*)-^{16}O,^{17}O,^{18}O]phosphate obtained in the overall PTS^{Glc} reaction. The spectra were run on a Bruker WM-300 instrument at 121.50 MHz with a deuterium field lock and broad-band decoupling: spectral width 500 Hz, acquisition time 4.1 s, pulse width 25.0 μs, number of transients 6255, Gaussian multiplication in 4K (Gaussian broadening 0.1 Hz; line broadening −0.5 Hz), and Fourier transform in 16K. The gain control for the equatorial triesters is twice that for the axial triesters. The chemical shifts for the equatorial resonances are −3.1800, −3.2041, −3.2302, and −3.2483 ppm and for the axial resonances are −5.1532, −5.1673, −5.1954, and −5.2095 ppm, upfield from 85% phosphoric acid. The scale used was 0.01 ppm per division.

of the stereochemically informative resonances) is 0.44, Q_{pred} (which is the predicted value of Q on the basis of the isotopic content of the product) is 0.44, and the values of Q_{obsd} (observed, from Figure 2) are 0.42 and 0.47. Within experimental error, therefore, the PTS^{Glc} system catalyzes the transfer of the phospho group from phosphoenolpyruvate to methyl α-D-glucopyranoside with *complete, overall, stereochemical inversion.*

Overall inversion implies an odd number of phospho transfers between phosphoenolpyruvate and methyl α-D-glucopyranoside 6-phosphate. Three such transfers are already known and have been confirmed biochemically: phosphoenolpyruvate to enzyme I, phospho-enzyme I to HPr, and phospho-HPr to enzyme III^{Glc}. If *direct* phospho group transfer between phospho-enzyme III^{Glc} and methyl glucoside catalyzed by enzyme II^{Glc} occurred, overall retention of the configuration of the chiral phospho group would have been expected (four transfers). Since, however, overall inversion is observed, we must conclude that two phospho transfers (or a multiple thereof) must have occurred between phospho-en-

zyme III^{Glc} and methyl glucoside. The simplest interpretation of this result is that a phospho-enzyme II^{Glc} is an obligatory intermediate in the reaction sequence and that transfers occur from phospho-enzyme III^{Glc} to enzyme II^{Glc} and from phospho-enzyme II^{Glc} to methyl glucoside. If this is indeed the case, any mechanistic model that is formulated to explain transport and phosphorylation of glucose by enzyme II^{Glc} must take such an intermediate into account. The obligatory coupling of transport and sugar phosphorylation in the bacterial PTS's therefore may also reflect the need for covalent modification of the permease in order for transport to occur. Such coupling is also evident in the covalent phosphorylations of the Na^+,K^+- and Ca^{2+}-translocating ATPases of animal cells, which are believed to provide a driving force for active transport by these enzymes (Kyte, 1981).

Finally, it should be noted that although the existence of a phospho-enzyme II^{Glc} intermediate most easily explains our results, other interpretations are possible. For example, phosphorylation of another unidentified membrane protein by phospho-enzyme III^{Glc} followed by direct transfer catalyzed by enzyme II^{Glc} cannot be ruled out. However, extensive genetic and biochemical studies have failed to reveal any additional membrane or cytoplasmic proteins necessary for sugar phosphorylation by enzyme II^{Glc} (Postma & Roseman, 1976; Saier, 1977; Dills et al., 1980). We anticipate that analysis of the stereochemical course of the sugar:phosphosugar transphosphorylation reactions, which are known to be catalyzed by the PTS enzymes II alone (Saier, 1977), will provide additional evidence concerning the existence of phospho-enzyme II intermediates during transport. Because optimal transphosphorylation rates occur at phosphosugar/sugar ratios of about 10^2–10^3 (Saier et al., 1977), these analyses are technically more difficult. However, since transphosphorylation can occur in a vectorial manner in whole cells and membrane vesicles (Saier et al., 1977), its mechanism may parallel that with phosphoenolpyruvate as the phospho group donor. Furthermore, the recent purification of the mannitol enzyme II from *E. coli* (Jacobson et al., 1979) should allow similar analyses to be carried out on a more homogeneous system.

Acknowledgments

We are most grateful to Professor Milton Saier for having initiated this collaboration and for helpful comments and advice.

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