General Acid–Base Catalysis of α-Glucan Phosphorylases: Stereospecific Glucosyl Transfer from D-Glucal Is a Pyridoxal 5′-Phosphate and Orthophosphate (Arsenate) Dependent Reaction†

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ABSTRACT: D-Glucal, containing a highly reactive double bond, can replace glucose 1-phosphate as the glucosyl donor in phosphorylase-catalyzed glucosyl transfer to a suitable oligo- or polysaccharide acceptor: D-glucal + P_i + $(glucose)_n$ → 2-deoxy-α-D-glucosyl$(glucose)_n$ ⇌ 2-deoxy-α-D-glucose-1-P + $(glucose)_n$. This reaction is catalyzed by α-glucan phosphorylases from rabbit skeletal muscle, potato tuber, and *Escherichia coli*. D-Glucal is only measurably consumed by α-glucan phosphorylases when orthophosphate or arsenate is present. With saturating concentrations of these anions and a glucosyl acceptor, the D-glucal reaction proceeds at rates comparable with the rates of glucosyl transfer from glucose 1-phosphate and of phosphorolysis or arsenolysis of poly- or oligosaccharides. Furthermore, for the reaction to proceed, the enzyme must be in the active conformation containing the cofactor pyridoxal 5′-phosphate in its dianionic form. On the basis of proton nuclear magnetic resonance spectra, it is proposed that protonation at C-2 of D-glucal gives rise to a hypothetical 2-deoxy-β-D-glucose intermediate, yielding as a final product (2-deoxy-α-D-[2(e)-^{2}H]glucose)$_n$ α(1→4) saccharides. These 2-deoxy-α-D-glucose oligo- or polysaccharides are degraded by α-glucan phosphorylases by phosphorolysis or arsenolysis like natural linear and branched α-glucans. The absolute requirement of the D-glucal reaction for phosphate (or arsenate) and its dependency on the dianionic form of the pyridoxal 5′-phosphate bound to phosphorylase are rationalized in terms of a proton transfer relay involving juxtaposed phosphates. Phosphate–phosphate interactions were postulated by Withers et al. [Withers, S. G., Madsen, N. B., Sykes, B. D., Takagi, M., Shimomura, S., & Fukui, T. (1981) *J. Biol. Chem. 256*, 10759–10762].

α-Glucan phosphorylases[1] catalyze in vitro the reversible reaction: $(glucose)_n$ + P_i ⇌ $(glucose)_{n-1}$ + α-D-glucose 1-phosphate. We reported some time ago a reaction of D-glucal with potato phosphorylase in the presence of α-cyclodextrin and made use of this reaction in an attempt to characterize a putative glucosyl intermediate (Klein et al., 1981). We had, however, at that time overlooked the fact that formation of (2-deoxy-α-D-glucose)$_n$ oligosaccharides from D-glucal obligatorily requires arsenate (or phosphate) because residual arsenate was present in the enzyme preparation. Arsenate had been carried over from the repeated arsenolysis used to free the large amounts of concentrated potato phosphorylase of primer. In addition, the α-cyclodextrin used quite likely also contained primer. We have since repeated these experiments using an enzyme and α-cyclodextrin both freed from primer by an improved method using Sepharose-bound α-glucosidase (Schiefer et al., 1978). When 1×10^{-4} M of this preparation was incubated with 100 mM D-[1-^{3}H]glucal, sp act. 500 cpm/nmol, and with 5 mM α-cyclodextrin, radioactivity was incorporated into potato phosphorylase only when $\geq 1 \times 10^{-4}$ M arsenate was present. When excess arsenate and D-glucal were removed and the α-cyclodextrin was replaced by 5 mM maltotetraose, the enzyme-bound radioactivity was transferred to the acceptor as we described (Klein et al., 1981).

In this paper, we report that α-glucan phosphorylases catalyze the D-glucal reaction at saturating concentrations of arsenate or phosphate at rates comparable with the rates of the reaction with glucose-1-P. This encouraged us to study this reaction in detail and to focus attention on mechanistic aspects including the stereochemical course of the reaction and the dependence on pyridoxal-5′-P. Our studies indicate that the general concept developed by Hehre et al. (1973), whereby glycosylic compounds such as glucal on protonation of the double bond give rise to a 2-deoxyglucosyl residue, also applies to α-glucan phosphorylases. Since this concept is not in any way restrictive with respect to the nature of the proton source, it has consequences for the mechanism of the phosphorylase reaction and for the role of pyridoxal-P in catalysis.

Experimental Procedures

Reagents. Glucose 1-phosphate, 5′-AMP, and NADP were purchased from Boehringer, Mannheim, West Germany. Oyster glycogen was obtained from Merck, Darmstadt, West Germany. Limit dextrin was prepared by exhaustive arsenolysis of glycogen with rabbit skeletal muscle phosphorylase *a*, and the product was passed over a Dowex 1 × 8 Cl^- exchange resin and precipitated from aqueous solution by addition of absolute ethanol. α-Cyclodextrin (Serva) was treated with Sepharose-bound α-glucoamylase and recrystallized several times from an aqueous solution in order to remove linear saccharides. Maltotetraose and homologous higher linear oligosaccharides were a generous gift of Boehringer, Mannheim, West Germany. Sodium phosphite and sodium monofluorophosphate (Riedel-de Haen, Hannover, West Germany) were recrystallized as described by Voigt & Gallais (1953) and Hill & Audrieth (1950), respectively. Only freshly

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[1] Abbreviations: NMR, nuclear magnetic resonance; ppm, parts per million; glucose-1-P, α-D-glucopyranose 1-phosphate; pyridoxal-P, pyridoxal 5′-phosphate; P_i, inorganic (ortho) phosphate; As, orthoarsenate; 5′-AMP, adenosine 5′-phosphate; Mops, 3-(*N*-morpholino)propanesulfonic acid; α-glucan phosphorylases, 1,4-α-D-glucan:orthophosphate α-glucosyltransferase (EC 2.4.1.1); D-glucal, 1,5-anhydro-2-deoxy-*arabino*-hex-1-enitol; NADP, nicotinamide adenine dinucleotide phosphate; EDTA, ethylenediaminetetraacetic acid.

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prepared solutions of fluorophosphate were used to avoid hydrolysis to orthophosphate. The ^{31}P NMR spectrum of the monofluorophosphate anion exhibited a 1:1 doublet, which is expected for a single fluorine atom covalently bonded to the phosphorus. All other biochemicals and chemicals were of the highest grade commercially available. α-D-Glucopyranose 1,2-phosphate was prepared by cyclization of glucose-1-P with carbodiimide in aqueous pyridine according to Zmudzka & Shugar (1964). [^{14}C]Glucal (2.79 mCi/mmol) was prepared from uniformly labeled α-D-[^{14}C]glucose (Amersham), according to Lehmann & Schröter (1972) and purified as described by Legler et al. (1979). 2-Deoxy-D-[U-^{14}C]glucose (2-deoxy-D-*arabino*-hexose) was prepared from D-[U-^{14}C]-glucal by acid hydrolysis according to Lehmann & Schröter (1972). Radioactivity was measured in a Triton X-100 liquid scintillation cocktail in a Packard Tri-Carb Model 3380.

Coenzymes. Pyridoxal 5′-phosphate and pyridoxal were products of Merck, Darmstadt, West Germany. Pyridoxal 5′-phosphate monomethyl ester, prepared according to Pfeuffer et al. (1972), was a generous gift of Dr. Klaus Schnackerz of this laboratory. Pyridoxal 5′-diphosphate was synthesized according to Shimomura & Fukui (1978). Pyridoxal 5′-(fluorophosphate) was prepared by treatment of pyridoxal 5′-phosphate with 2,4-dinitrofluorobenzene in the presence of triethylamine, analogous to the Wittmann (1962) procedure.

Enzymes. Phosphorylase *b* was prepared from frozen rabbit skeletal muscle as described by Fischer & Krebs (1958). The enzyme was at least 3 times recrystallized and stored at 4 °C under toluene vapor. Phosphorylase *a* was prepared from phosphorylase *b* with phosphorylase kinase (EC 2.7.1.38), Mg^{2+}, and ATP according to Krebs et al. (1964). Phosphorylase kinase was a generous gift of Dr. H. Jenissen, Department of Biochemistry, University of Bochum, West Germany. Rabbit skeletal muscle apophosphorylase *b* was prepared by the procedure of Shaltiel et al. (1967). Reconstitution of apophosphorylase *b* with pyridoxal 5′-phosphate or analogues of pyridoxal 5′-phosphate was carried out as described by Hedrick et al. (1966) [see also Pfeuffer et al. (1972) and Shimomura & Fukui (1978)]. Maltodextrin phosphorylase from *Escherichia coli* K 12 Hfr H 8000 was purified to electrophoretic homogeneity according to Schächtele et al. (1978). Potato phosphorylase was prepared from potato tubers according to Staerk & Schlenk (1967). The purified enzyme was homogeneous in sodium dodecyl sulfate–polyacrylamide gel electrophoresis. In order to remove primer, we treated potato phosphorylase repeatedly with Sepharose-bound α-glucoamylase according to Schiefer et al. (1978).

Analytical Procedures. Thin-layer chromatography was performed on HP–TLC cellulose and silica gel 60 plates from Merck. Paper chromatography was carried out in descending mode with Whatman No. 1 paper. Solvent systems used were (A) 1-butanol/acetic acid/water, 5:3:2, and (B) 1-butanol/pyridine/water, 6:4:3. Sugars were visualized on paper and cellulose plates with anilin hydrogen phthalate (Block et al., 1958); silica gel plates were sprayed with H_2SO_4 and heated for 10 min at 100 °C. Radioactive samples on thin-layer plates were traced with a thin-layer scanner II of the Berthold Company, West Germany. Radioautograms were obtained by exposure to Kodak Definix medical film for 24–72 h. The protein concentration was determined spectrophotometrically with the following extinction coefficients ($A_{280}^{1\%}$): 13.2 for rabbit skeletal muscle phosphorylase (Kastenschmidt et al., 1968), 13.6 for *E. coli* maltodextrin phosphorylase (Schächtele et al., 1978), and 11.7 for potato phosphorylase (Kamogawa et al., 1968). Molar concentrations were expressed in terms of monomers of 97412 daltons for muscle phosphorylase *a* (Titani et al., 1977), 81 000 daltons for maltodextrin phosphorylase (Schächtele et al., 1978), and 108 000 daltons for potato phosphorylase (Iwata & Fukui, 1973). When protein concentrations were determined with the Lowry method (Lowry et al., 1951), bovine serum albumin was used as standard.

Activity Measurements. Activity assays were carried out routinely in the direction of glycogen synthesis. In the case of rabbit muscle phosphorylases *a* and *b*, 16 mM glucose-1-P and 1% glycogen in 25 mM potassium maleate buffer containing 25 mM 2-mercaptoethanol and 2 mM Na_2EDTA, pH 6.8, at 30 °C were used. With maltodextrin and potato phosphorylase, 10 mM glucose-1-P and 10 mM maltoheptaose were used. Orthophosphate was measured by the method of Fiske & Subbarow (1925). In some instances, phosphorolysis of polysaccharides was measured with the assay of Helmreich & Cori (1964a), and arsenolysis of polysaccharides was measured as described by Helmreich & Cori (1964b). D-Glucal utilization was measured at 30 °C with 1×10^{-6} M phosphorylases (or 1×10^{-5} M phosphorylase derivatives) in 100-μL samples containing 50, 75, 100, 120, 150, 200, or 250 mM D-[^{14}C]glucal in 50 mM Mops/25 mM 2-mercaptoethanol buffer, pH 6.8. In the case of rabbit muscle phosphorylases *a* and *b*, incubations were with 5 mM maltotetraose or 1% limit dextrin and 50 mM arsenate or 25 mM phosphate. With muscle phosphorylase *b*, 1 mM 5′-AMP was also added. Assay conditions are further detailed in the legends to the figures and tables. With phosphate and arsenate, aliquots of 5 μL were withdrawn every 2 or 5 min, respectively, and spotted on to Whatman No. 1 paper. The reaction was terminated by adding 10 μL of absolute methanol to the spots. Rates were determined from 4 to 6 time points in the linear part of the progress curves. In general, less than 10% of the substrate was utilized during the reaction. Chromatograms were developed in solvent system B, stained with anilin phthalate, and scanned for radioactivity. D-Glucal, 2-deoxy-D-glucose, 2-deoxy-α-D-glucose 1-phosphate, and (2-deoxy-D-glucose)$_n$ oligosaccharides were determined quantitatively in triplicates by integration of the peak areas. Alternatively, the incorporation of radioactive 2-deoxy-α-D-glucose residues into glycogen was measured with the filter paper assay of Thomas et al. (1968). K_m values were calculated from double-reciprocal plots.

NMR Measurements. Fourier-transformed ^{31}P NMR spectra were recorded at 72.8 MHz on a Bruker WH-180 wide-bore superconducting spectrometer. The sample (1.0 mL) was placed in a concentric 5-mm NMR tube containing 20% D_2O for field/frequency lock. When required, broad-band proton decoupling (0.4 W) was applied. In general, a 1200-Hz spectral width was acquired in 8192 data points with a 60° pulse and a 3.4-s repetition time. Continuous air flow through the spectrometer probe head kept the temperature constant at 28 ± 1 °C. Orthophosphoric acid (85%) enclosed in a capillary tube was used for chemical shift referencing. Values downfield from the standard are positive values. ^{13}C NMR spectra were recorded at 45.28 MHz with proton decoupling (2.0 W) in 10-mm tubes also with a Bruker WH-180 spectrometer. A spectral width of 8000 Hz and a data table of 16 384 points were used, giving a pulse interval of 4 s and digital resolution of 1.0 Hz per point. The flip angle was 60°; typically 2000–3000 transients were averaged before Fourier transformation; the free induction decay was multiplied by an exponential function, giving a line broadening of 1 Hz. Chemical shifts were measured with respect to a dioxane

standard but are given relative to tetramethylsilane. The chemical shift of dioxane with respect to Me_4Si is 67.4 ppm. ^1H NMR spectra were recorded at 400 MHz in a Bruker WM-400 spectrometer in 5-mm tubes. In general, a 8000-Hz spectra width was recorded in 32 768 data points at 30 ± 1 °C. For ^1H NMR spectroscopic studies of the D-glucal reaction with potato phosphorylase and rabbit skeletal muscle phosphorylase *a*, the reactants, maltotetraose, D-glucal, arsenate, phosphate, and the phosphorylase, were extensively lyophilized and/or dialyzed against D_2O to exchange labile hydrogens for deuterium atoms. At zero time, samples were prepared by adding 0.400 mL of (3–8) × 10^{-6} M phosphorylase solution in D_2O, pD 6.8 ± 0.2, to 0.4 mL of a solution containing 200 mM D-glucal, 6 mM maltotetraose, and 100 mM arsenate or 50 mM phosphate in 99.7% D_2O, pD 6.8 ± 0.2. The solution was transferred to a 5-mm NMR tube, and ^1H NMR spectra were recorded once every 256 s for the first 30 min and once every 15 min for the next 2 h. Each spectrum consisted of 128 free-induction decays with 2-s repetition times. The data were stored on a memory disc and subsequently Fourier transformed.

Results

Reaction of D-*Glucal with α-Glucan Phosphorylases.* In Figure 1 radioautograms of reaction mixtures of D-[U-^{14}C]-glucal with phosphorylases from rabbit muscle (a) and potato tuber and *E. coli* (b) are shown. D-Glucal was utilized only by active or activated forms of phosphorylase in the presence of a glycosyl acceptor, limit dextrin or maltooligosaccharides, and phosphate or arsenate. Potato phosphorylase has been shown to bind α- or β-cyclodextrin (Kokesh & Kakuda, 1977). It is therefore worth noting (see Figure 1b, lane R) that, in contrast to our preliminary findings (see the introduction), potato phosphorylase could not catalyze the D-glucal reaction when primer was removed completely and replaced by α-cyclodextrin freed of primer. Moreover, glucose 1,2-phosphate, a competitive inhibitor with respect to glucose-1-P with K_i values ~0.05 and ~0.5 mM for potato phosphorylase and rabbit muscle phosphorylases *a* and *b*, respectively (Kokesh et al., 1977; Hu & Gold, 1978), inhibited completely the D-glucal reaction with both muscle and potato phosphorylases (Figure 1a, lane E; Figure 1b, lane N).

Identification of Reaction Products. Structures were assigned to the products of the reaction of D-glucal and arsenate with phosphorylases by comparison with reference compounds. Assignments are based on R_f values on thin-layer chromatograms (see Figure 1a, lanes A and G, and Figure 1b, lanes J and O) and on ^{13}C NMR spectra of a reaction mixture of potato phosphorylase and D-glucal and maltotetraose in the presence of arsenate (Figure 2C). ^{13}C NMR spectra of 2-deoxy-D-glucose and D-glucal are shown in parts B and A of Figure 2, respectively. Spectra of the reaction mixture (Figure 2C) indicate the formation of 2-deoxy-D-glucose in an α,β-anomer equilibrium mixture.

The time course of the utilization of D-glucal and the formation of 2-deoxy-D-glucose by 5′-AMP-activated rabbit muscle phosphorylase *b* in the presence of limit dextrin and arsenate conclusively demonstrated the absolute requirement for arsenate. Contrary to our expectations from previous experiments (Klein et al., 1981; see the introduction), no glycosyl transfer took place in the absence of arsenate (Figure 3A). Moreover, if the arsenate had participated only in the arsenolysis of glycosidic bonds formed from 2-deoxy-D-glucose and limit dextrin, one would have expected radioactive saccharides to accumulate in the preincubation period without arsenate. However, no radioactive dextrin was formed without

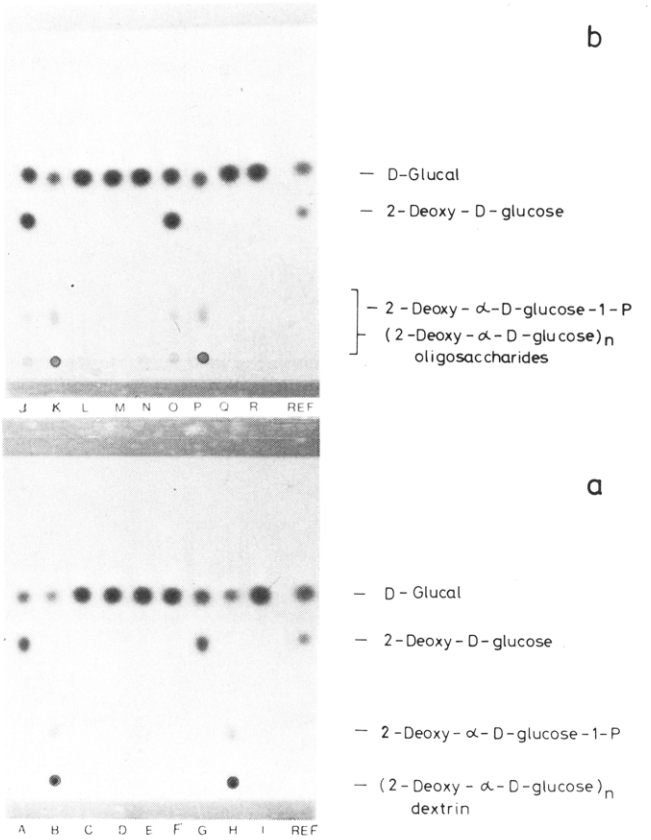


FIGURE 1: Radioautograms of D-glucal phosphorylase reaction mixtures. All reaction mixtures contained in 25 μL of 100 mM D-[U-^{14}C]glucal (50 cpm/nmol), 1 × 10^{-5} M enzyme, 25 mM Mops, 25 mM 2-mercaptoethanol, 2 mM Na_2EDTA buffer, pH 6.8, and the additions listed below. The substrate concentrations were 1% limit dextrin, 3 mM maltotetraose, and 25 mM arsenate or phosphate, respectively. All incubations were at 30 °C and for the times specified. Samples of 2 μL were spotted on silica gel 60 plates and chromatographed in solvent system B (see Experimental Procedures). The reference compounds were D-glucal and 2-deoxy-D-glucose. (a) Rabbit skeletal muscle phosphorylases *b* and *a*; (b) Potato and *E. coli* maltodextrin phosphorylases. (Lanes A–E) Rabbit skeletal muscle phosphorylase *a* and limit dextrin. Further additions and omissions as stated: (A) 40 min, arsenate; (B) 10 min, phosphate; (C) 60 min, phosphate, no limit dextrin; (D) 40 min, no arsenate or phosphate; (E) 60 min, phosphate and 5 mM glucose 1,2-phosphate; (F–I) rabbit skeletal muscle phosphorylase *b* with 1 mM 5′-AMP and limit dextrin; (F) 30 min, phosphate, no 5′-AMP; (G) 40 min, arsenate; (H) 10 min, phosphate; (I) 20 min, no arsenate or phosphate; (J–N and R) potato phosphorylase; (O–Q) *E. coli* maltodextrin phosphorylase with maltotetraose; (J) 20 min, arsenate; (K) 20 min, phosphate; (L) 60 min, phosphate, no maltotetraose; (M) 60 min, no arsenate or phosphate; (N) 60 min, arsenate and 2 mM glucose 1,2-phosphate; (O) 20 min, arsenate; (P) 20 min, phosphate; (Q) 40 min, no arsenate or phosphate; (R) 240 min, 10 mM cyclodextrin, arsenate, no maltotetraose.

arsenate (see Figure 1a, lane I). This is also reflected in the lag period preceding the formation of 2-deoxy-D-glucose (Figure 3A), which is probably the time needed to build up sufficient amounts of radioactive dextrin from D-[U-^{14}C]glucal for subsequent arsenolysis. Incidentally, release of radioactive glucose, presumably via α-D-glucose 1-arsenate in phosphorylase-catalyzed arsenolysis of glycogen and starch, proceeds without a lag [cf. Katz & Hassid (1951) and Helmreich & Cori (1964b)]. About 30 min after addition of arsenate (2-deoxy-D-glucose)$_n$ dextrin concentrations approached a steady state with equal rates of formation and degradation by arsenolysis. A satisfactory correspondence between substrate (D-glucal) depletion and product (2-deoxy-D-glucose) formation is apparent from the data in Figure 3B. The amount of

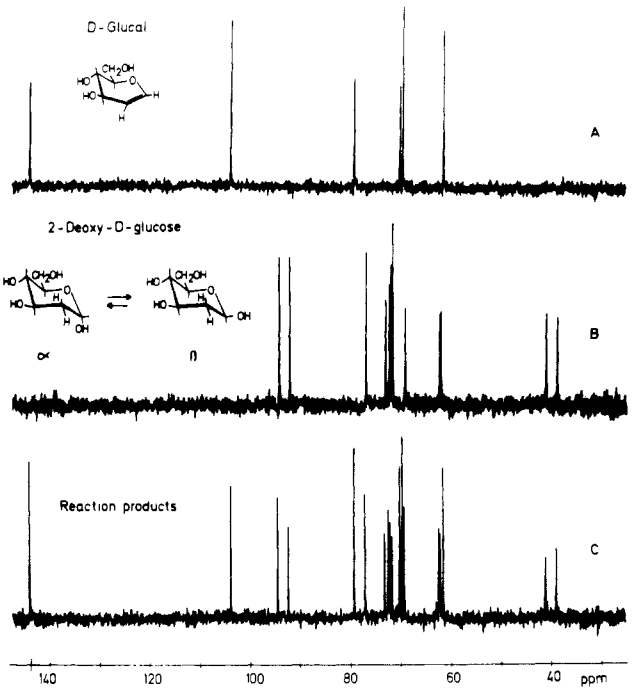


FIGURE 2: ^{13}C NMR spectra of a D-glucal phosphorylase reaction mixture: (A) 2341 transients, 100 mM D-glucal, 25 mM arsenate and 3 mM maltotetraose in 20% D_2O, pH 6.8 at 30 °C, without enzyme; (B) 2500 transients, 50 mM 2-deoxy-D-glucose, 25 mM arsenate in 20% D_2O, pH 6.8 at 30 °C, without enzyme; (C) 2134 transients, 100 mM D-glucal, 3 mM maltotetraose and 1.2×10^{-5} M potato phosphorylase in 25 mM arsenate buffer containing 20% D_2O, pH 6.8. The spectra were recorded after 2 h at 30 °C.

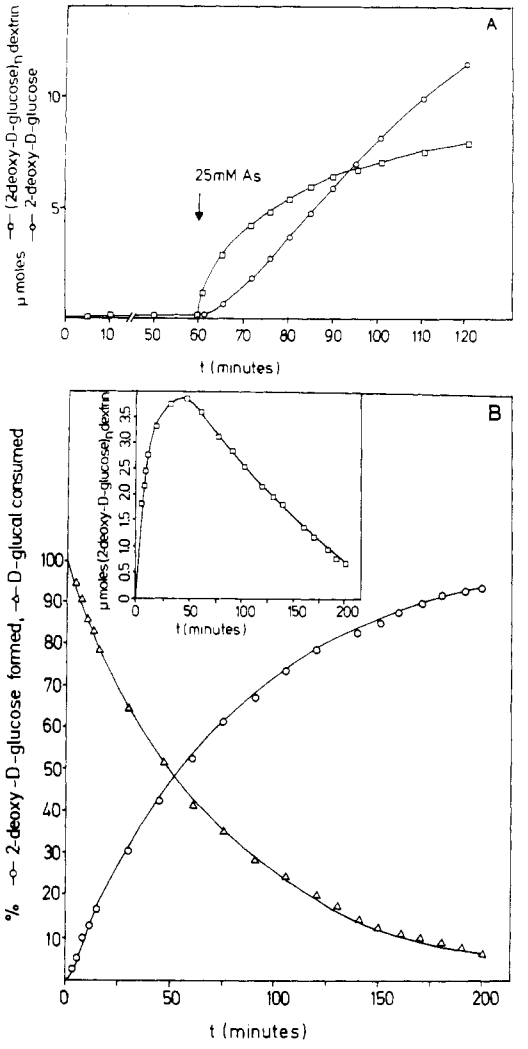


FIGURE 3: Formation of 2-deoxy-D-glucose from D-glucal by muscle phosphorylase *b*. (A) The reaction was carried out at 30 °C with 100 mM D-[U-^{14}C]glucal (50 cpm/nmol), 1.5×10^{-5} M muscle phosphorylase *b*, 1 mM 5'-AMP, 1% limit dextrin in 25 mM Mops, 25 mM 2-mercaptoethanol, and 2 mM Na_2EDTA buffer, pH 6.8. The final volume was 1.0 mL. At given times, 5-μL aliquots were withdrawn; the reaction was quenched by adding 10 μL of MeOH, and the products were analyzed in triplicates after separation by paper chromatography in solvent B, as described under Experimental Procedures. As indicated by the arrow, the reaction was started after 60 min by the addition of arsenate to a final concentration of 25 mM. (B) Conditions are as in (A) but with 3.5×10^{-5} M muscle phosphorylase *b*; the reaction was initiated by the addition of 100 mM arsenate at zero time. 100% D-glucal corresponds to 100 μmol.

radioactive polysaccharide accumulated (insert to Figure 3B) was likewise less. This is explained by assuming faster turnover and more rapid arsenolysis. The polysaccharide synthesized peaked around 4 μmol after 50 min and declined steadily thereafter. Simultaneously, the formation of 2-deoxy-D-glucose slowed down and finally ceased when D-glucal was exhausted. The experimental conditions necessary for the formation of radioactive oligosaccharides and dextrins from D-glucal are further detailed in Figure 1 (see Figure 1a, lanes B and H, Figure 1b, lanes K and P). The relatively low sensitivity of ^{13}C NMR spectroscopy did not permit the detection of oligosaccharides under the experimental conditions employed (see Figure 2C). Oligosaccharides were, however, readily detected in ^1H NMR spectra. This is shown later (see Figure 6). A sugar phosphate, a product of the reaction of D-glucal with phosphorylase *a* in the presence of orthophosphate and limit dextrin, was identified by ^{31}P NMR spectroscopy (Figure 4). The proton-decoupled ^{31}P NMR spectrum (Figure 4B) shows two resonance lines at 1.85 and 1.35 ppm. The signal at lower field was assigned to orthophosphate, while the singlet at 1.35 ppm is that of a phosphate monoester as indicated by its splitting into a doublet in the undecoupled spectrum (Figure 4A). When incubation was prolonged for 2.5 h, a high molecular weight polysaccharide actually began to settle. The precipitate was removed by centrifugation, and a ^{31}P NMR spectrum was recorded with the supernatant solution. This spectrum (not shown) was like that of Figure 4. The phosphate ester was further characterized by HP–TLC chromatography (not shown). Moreover, the isolated radioactive phosphate monoester was incubated with potato phosphorylase in the presence of maltotetraose. Radioactivity was found in a series of homologous oligosaccharides, suggesting that the phosphate ester is 2-deoxy-α-D-glucose 1-phosphate, which can serve as glycosyl donor and which is enzymatically transferred to oligosaccharide acceptors. This assumption was proved to be correct, and the structure of 2-deoxy-α-D-glucose 1-phosphate was subsequently established by ^1H NMR spectroscopy (see Figures 6 and 7A). The radioactive high molecular weight material was likewise characterized and subjected to arsenolysis with muscle phosphorylase *a*. In autoradiograms (not shown) we found predominantly one spot that comigrated with authentic 2-deoxy-α-D-glucose. Thus, the 2-deoxyglycosyl residues transferred to poly- or oligosaccharides were linked by α(1→4) glycosidic bonds, as was expected from the known specificity of phosphorylase catalyzed phosphorolysis or arsenolysis of α(1→4) glycosidic bonds (Brown & Cori, 1961).

Rates of D-*Glucal Utilization.* The data in Table I indicate that the rates of the D-glucal reaction are significant when compared with the rates of the natural phosphorylase reaction. Thus, the rate of the reaction with 100 mM D-glucal was between 11.6% and 24.6% of the rate of phosphorolysis of

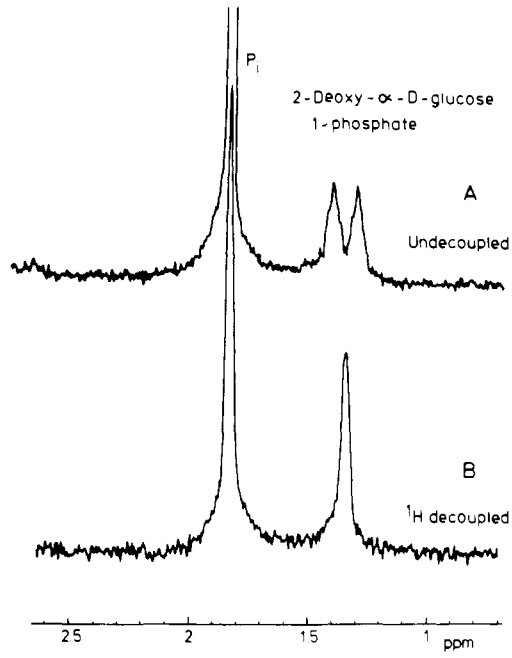


FIGURE 4: ^{31}P NMR spectra of a D-glucal phosphorylase reaction mixture. The reaction was carried out with 100 mM D-glucal, 25 mM orthophosphate, 1% limit dextrin, and 1×10^{-5} M rabbit skeletal muscle phosphorylase *a* in 25 mM Mops, 25 mM 2-mercaptoethanol, and 2 mM Na_2EDTA buffer and 20% D_2O, pH 6.6. The total volume was 1 mL. (A) Proton-coupled spectrum recorded after 30 min at 30 °C (929 transients); $J_{H_1,^{31}P} = 7.4 \pm 1$ Hz. The spectra in (B) are like those in (A) but proton decoupled (300 transients) and recorded after 90 min of incubation at 30 °C.

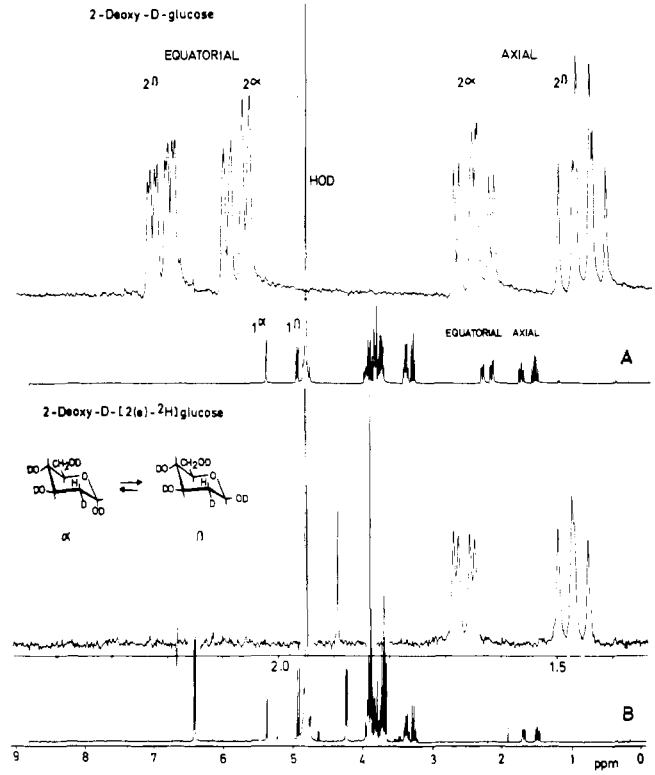


FIGURE 5: ^1H NMR spectra of a D-glucal phosphorylase reaction mixture in the presence of arsenate. Formation of α- and β-2-deoxy-D-glucose: (A) 50 mM 2-deoxy-D-glucose in 1 mL of 99.7% D_2O containing 50 mM arsenate, pD 6.8 at 30 °C; (B) reaction mixture (0.8 mL) of 3×10^{-6} M potato phosphorylase, 100 mM D-glucal, and 3 mM maltotetraose in 50 mM arsenate and 99.7% D_2O, pD 6.8. The spectrum was recorded after 120 min at 30 °C. The upper lines in (A) and (B) show the C-2 proton resonances on an 8 times expanded scale. Note the abscissa.

glycogen or oligosaccharides and about 7.1–11.3% of the rate of glycogen synthesis with glucose-1-P. K_m and V_{max} values for D-glucal and potato phosphorylase, *E. coli* maltodextrin phosphorylase, and rabbit muscle glycogen phosphorylase *a*, respectively, were estimated as described under Experimental Procedures in the presence of 25 mM orthophosphate, 5 mM maltotetraose, or 1% limit dextrin at D-glucal concentrations ranging from 50 to 250 mM. The corresponding K_m and V_{max} values extrapolated from double-reciprocal plots for the three phosphorylases were 160 mM, 145 mM, and 200 mM and 6, 5, and 20 μmol min^{-1} mg^{-1}, respectively. Thus, the V_{max} values for D-glucal range between one-fifth and one-third of the corresponding V_{max} values for the utilization of glucose-1-P. Moreover, the activity of nonactivated muscle phosphorylase *b* without 5′-AMP is as low in the D-glucal reaction as it is in the natural reaction (≤1%). The small residual activity can be accounted for by assuming the presence of traces of nucleotide in the enzyme and the substrates. Also given in Table I are data on the activities of muscle phosphorylase *b* derivatives containing pyridoxal, pyridoxal 5′-diphosphate, pyridoxal 5′-phosphate monomethyl ester, and pyridoxal 5′-(fluorophosphate). All these cofactor analogues lack the dianionic 5′-monophosphate group of the natural cofactor. The phosphorylase derivatives containing these analogues are inactive except for the pyridoxal-containing phosphorylase in the presence of high concentrations of phosphate or arsenate (see Table I). The residual activities of the apoenzymes and the other phosphorylase derivatives can be accounted for by postulating the presence of traces of unresolved active holophosphorylase. The pyridoxal containing phosphorylase *b* derivative had about 7% of the activity of a muscle phosphorylase *b* preparation reconstituted with the natural cofactor pyridoxal-5′-P and assayed in the presence of 50 mM orthophosphate or arsenate. This activity was significantly higher than the 1.9% for apophosphorylase *b*. We explain this finding in the light of the experiments of Parrish et al. (1977), who have noted significant activity of pyridoxal phosphorylase *b* in glycogen synthesis from glucose-1-P at high orthophosphate and phosphite concentrations. Additional experiments were carried out under the conditions described (see legend to Table I and Materials and Methods) both with the pyridoxal phosphorylase derivative and pyridoxal-5′-P-containing holoenzyme, with D-glucal as substrate and 50 mM concentrations of fluorophosphate, phosphite, carbonate, acetate, and sulfate, respectively. Apart from phosphate and arsenate, none of these anions participated in the D-glucal reaction. Moreover, none of these anions could replace phosphate or arsenate in glycogenolysis catalyzed by the pyridoxal-5′-P-containing holoenzyme (Parrish et al., 1977). Thus, the transfer of 2-deoxy-α-D-glucose residues from D-glucal to poly- or oligosaccharides seems, like glucosyl transfer from glucose-1-P, to require a dianionic phosphate of pyridoxal-5′-P (or a dianionic phosphate bound adjacent to pyridoxal) in addition to orthophosphate or arsenate. Moreover, the muscle enzyme must be allosterically (or covalently) activated (see Added in Proof).

Mechanism of D-*Glucal Reaction.* Use was made of ^1H NMR spectroscopy to clarify the stereochemical course of the D-glucal reaction with phosphorylase. In 1977, Hehre et al. (1977) had already employed ^1H NMR spectroscopy in their studies of the mechanism of α- or β-glucosidases. The reaction of D-glucal with potato phosphorylase was monitored in the presence of arsenate and maltotetraose in D_2O (Figure 5B). The spectrum was recorded after half of the D-glucal was converted to 2-deoxy-D-glucose. The ^1H NMR spectrum of a sample of authentic 2-deoxy-α-D-glucose is shown in Figure 5A. Hehre et al. (1977) have assigned the resonance signals

Table I: Rates of Glucosyl Transfer by α-Glucan Phosphorylases and Phosphorylase Derivatives[a]

enzymes	anions	rates with glycosyl donors (μmol min^{-1} mg^{-1}) glucal[b]	saccharides[c]	G-1-P[d]	glucal[b] reactions: comparative ratios × 100 (%) glucal/ saccharides[c]	glucal (+P_i)/ G-1-P[d]	phosphorylase derivatives/ phosphorylase *b*
potato phosphorylase	50 mM As	0.93	1.50		62.00		
	50 mM P_i	2.14	12.00		17.80		
	none			25.00		8.60	
E. coli maltodextrin phosphorylase	50 mM As	0.46	0.91		47.20		
	50 mM P_i	1.85	16.00		11.60		
	none			26.00		7.10	
muscle phosphorylase *a*	50 mM As	0.28	1.04		27.00		
	25 mM P_i	7.89	32.00		24.60		
	none			70.00		11.30	
muscle phosphorylase *b* reconstituted from apophosphorylase *b* and pyridoxal-5′-P	50 mM As	0.27	1.00		27.00		100.00
	25 mM P_i	6.64	30.00		22.00		100.00
	none			65.00		10.20	
muscle phosphorylase *b* reconstituted from apophosphorylase *b* and pyridoxal 5′-(fluorophosphate)	50 mM As 25 mM P_i none	0.10		0.60			
muscle apophosphorylase *b*	50 mM P_i	0.13					1.90
muscle phosphorylase *b* reconstituted from apophosphorylase *b* and pyridoxal	50 mM As	0.02					7.00
	50 mM P_i	0.45					6.80
muscle phosphorylase *b* reconstituted from apophosphorylase *b* and pyridoxal-5′-di-P	50 mM As	0.01					3.70
	50 mM P_i	0.14					2.10
muscle phosphorylase *b* reconstituted from apophosphorylase *b* and pyridoxal 5′-phosphate monomethyl ester	50 mM As	0.01					3.70
	50 mM P_i	0.14					2.10

[a] The reactions were carried out at 30 °C. Rates were calculated from the linear part of the progress curves. The phosphorylase concentrations were 1×10^{-6} M except in the case of the inactive phosphorylase derivatives where the concentrations were 1×10^{-5} M. The assay was performed in 25 mM Mops, 25 mM 2-mercaptoethanol, and 2 mM Na_2EDTA buffer, pH 6.8, and contained 1 mM AMP in reactions with phosphorylase *b* (derivatives). [b] The reaction mixture contained in each case 100 mM D-[U-^{14}C]glucal (50 cpm/nmol) and arsenate or orthophosphate as indicated. The glucosyl acceptor was 1% limit dextrin with muscle phosphorylase *b* (derivatives) and phosphorylase *a* or 5 mM maltotetraose with potato and maltodextrin phosphorylase. [c] The reaction mixture contained 10 mM maltoheptaose in the case of potato and maltodextrin phosphorylase and 1% glycogen in the case of phosphorylase *a* and *b* and arsenate or phosphate as indicated. [d] The concentration of G-1-P (glucose-1-P) was 10 mM except for muscle phosphorylase *a* and *b* where it was 16 mM; glucosyl acceptors as in *b*. For further details see Experimental Procedures.

at 5.4, 2.1, and 1.7 ppm to the C-1 proton and to the equatorial and axial protons of C-2 of 2-deoxy-α-D-glucose, respectively. For the β anomer of 2-deoxy-D-glucose, the corresponding resonance lines are at 4.9, 2.25, and 1.5 ppm, respectively. On comparing the spectrum of the product of the D-glucal reaction in Figure 5B with that of the reference in Figure 5A, it becomes apparent that one hydrogen atom at C-2 is replaced by a deuterium atom. This deuterium must have originated from the equatorial position at C-2 since the resonance line is missing in that region. Anomerization is apparent from the two resonance signals at 5.4 (J_{H_1,H_2} = 3.5 Hz) and 4.9 (J_{H_1,H_2} = 10 Hz) ppm of the C-1 proton of the α and β anomer and at 1.7 and 1.5 ppm for the C-2 axial protons of 2-deoxy-α- and 2-deoxy-β-D-[2(e)-^{2}H]glucose. No epimerization at C-2 was observed even during prolonged incubation. Hence, the configurational stability at C-2 provides additional assurance that phosphorylase-catalyzed protonation (deuteration) of D-glucal is equatorial at the C-2 position.

We repeated the experiment with a 2.5 times higher phosphorylase concentration and recorded the ^{1}H NMR spectrum after 15 min of incubation at 30 °C. But even under these conditions, the β conformer had already appeared. After 30 and 45 min, an equilibrium between α and β anomer was established (not shown). However, a corresponding ^{1}H NMR study of the D-glucal reaction in D_2O in the presence of orthophosphate allowed unequivocal assignments of anomeric conformations because formation of the sugar phosphate prevented further anomerization (see Figure 6). After 20 min, the ^{1}H NMR spectrum in the region of anomeric protons

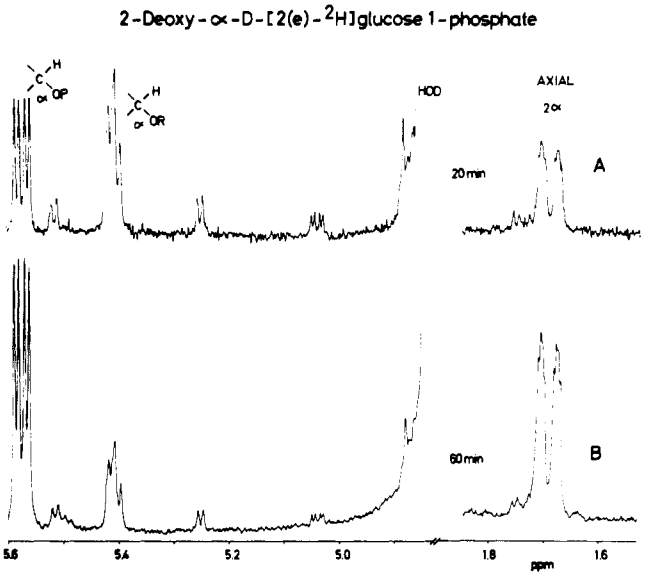


FIGURE 6: ^{1}H NMR spectra of a D-glucal phosphorylase reaction mixture in the presence of orthophosphate. Conditions were described in the legend to Figure 5B; 50 mM phosphate, pD 6.6, was used and potato phosphorylase was replaced by 6×10^{-6} M rabbit skeletal muscle phosphorylase *a*. Only the C-1 and C-2 proton resonance regions are shown: (A) after 20 min at 30 °C; (B) after 60 min at 30 °C.

shows two nearly equally large resonances at 5.6 and 5.4 ppm (Figure 6A). We have assigned the resonance quartet at lower

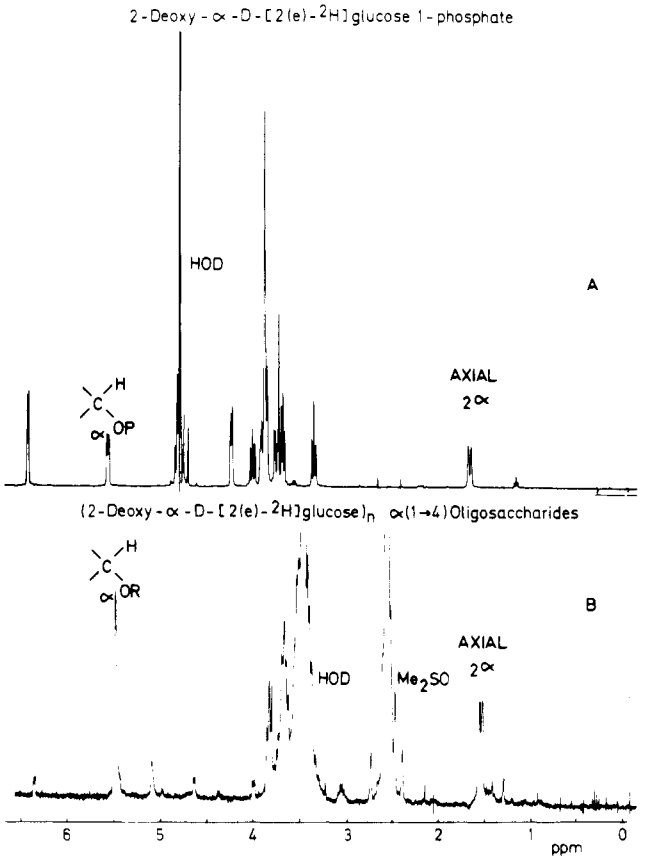


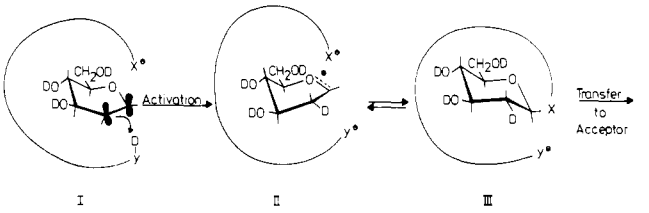
FIGURE 7: ^1H NMR spectra of the products of the D-glucal phosphorylase reaction in the presence of orthophosphate. After 90-min incubation at 30 °C with 6×10^{-6} M rabbit skeletal muscle phosphorylase *a*, 100 mM D-glucal, 3 mM maltotetraose, 50 mM orthophosphate in 99.7% D_2O, pD 6.6, the reaction mixture was filtered, the removed polysaccharides were collected and resuspended without further purification in [$^2H_6$]dimethyl sulfoxide: (A) reaction mixture freed from polysaccharides; (B) (2-deoxy-α-D-[2(e)-^2H]glucose)$_n$ polysaccharides resuspended in dimethyl sulfoxide. The resonances at 2.58 and 3.5 ppm are due to dimethyl sulfoxide and DOH.

field, a doublet of doublets (J_{H_1,H_2} = 3.5 Hz; $J_{H_1,^{31}P}$ = 7.4 Hz), to the C-1 proton of an α-phosphate ester of 2-deoxy-D-glucose. The triplet-like peak at higher field, which probably represents overlapping doublets (J_{H_1,H_2} = 3.5 Hz), is assigned to the C-1 protons of the (2-deoxy-α-D-glucose)$_n$ oligosaccharides, which were also seen when the reaction was carried out with arsenate instead of phosphate. The resonances of the C-2 axial protons are at 1.7 ppm. After 1 h of incubation, the resonances of the 2-deoxy-α-D-[2(e)-^2H]glucose 1-phosphate became the dominant signals (Figure 6B). After 1.5 h of incubation, polysaccharides precipitated and were removed by filtration. The ^1H NMR spectrum of the filtered solution is shown in Figure 7A and clearly indicates the presence of 2-deoxy-α-D-[2(e)-^2H]glucose 1-phosphate (and D-glucal). (2-Deoxy-α-D-glucose)$_n$ polysaccharides are missing. The removed polysaccharides were redissolved in [$^2H_6$]dimethyl sulfoxide and their structure was determined (Figure 7B). The resonances at 2.6 and 3.5 ppm are solvent peaks of dimethyl sulfoxide and DHO. The signals at 5.5 and 1.55 ppm were assigned to the protons at C-1 of α-anomeric 2-deoxyoligosaccharides and to the axial protons at C-2, respectively.

Discussion

Mechanism of D-*Glucal Reaction.* The data in Table I demonstrate conclusively that α-glucan phosphorylases catalyze effectively glucosyl transfer reactions with D-glucal instead of glucose-1-P as substrate. From a comparison of molecular models of D-glucal and glucose-1-P, one would expect D-glucal to attach itself to the glucose subsite of the glucose-1-P site via hydrogen bonds through the C-3, C-4 and C-6 hydroxyl groups [cf. Johnson et al. (1980) and Jenkins et al. (1981)]. This is supported by complete inhibition of the D-glucal reaction in the presence of saturating concentrations of glucose 1,2-phosphate (Figure 1a, lane E; Figure 1b, lane N). The much weaker binding of D-glucal compared to that of glucose-1-P might be due to a lack of ionic contacts and hydrogen bonding.

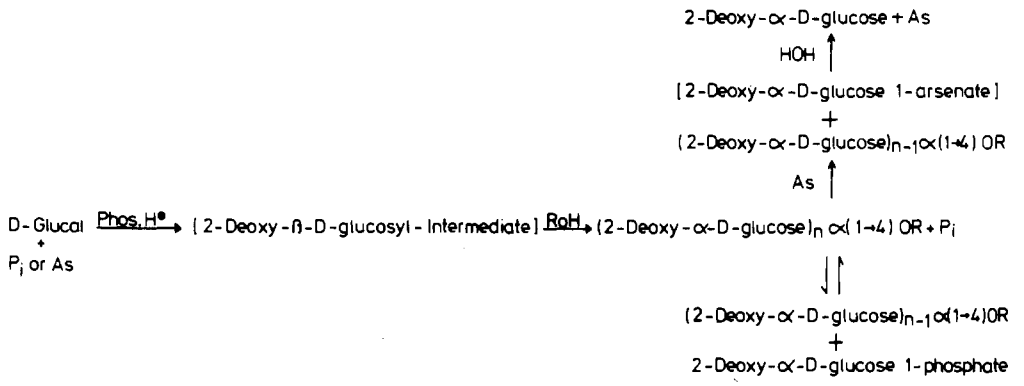
Scheme I: Protonation of D-Glucal[a]



[a] I is D-glucal; II and III are hypothetical cationic and covalent 2-deoxy-β-D-glucose–enzyme intermediates, respectively. X^- is a nucleophilic group of the enzyme. y-D is a deuterated proton-donor group of the enzyme.

Lehmann & Schröter (1972) reported that glycals carrying a reactive enol ether bond that is readily protonated by general acid catalysis (Salomaa et al., 1966) are utilized by glycosidases, yielding an incipient glucosyl residue. Subsequently, Hehre et al. (1977) used D-glucal in investigations of the catalytic mechanism of α- and β-glucosidases and demonstrated by ^1H NMR spectroscopy in deuterated water the formation of the corresponding 2-deoxy-D-glucose anomers. The product formed by α-glucan phosphorylases from D-glucal in D_2O is 2-deoxy-α-D-[2(e)-^2H]glucose oligo- or polysaccharide (Figures 6 and 7) with the deuterium atom and the saccharide acceptor in cis configuration. In the case of the glycosylases studied by Hehre et al. (1977), the D-glucal reaction involves a trans addition of water to the double bond. In the case of the α-glucan phosphorylase reaction, a plausible sequence of steps would be first the attack of an enzyme derived proton on the double bond from below C-2 of the pyranose ring assisted by a general base from above C-1 (β position) forming a hypothetical β-glucosyl intermediate that may be stabilized by a counter ion or may form a covalent bond on attack of a nucleophile or base. Noncovalently and covalently bound intermediates may equilibrate (Scheme I). However, the β configuration of the postulated enzyme-bound intermediate has not been verified. We have tried to isolate and characterize an enzyme-bound intermediate formed by potato phosphorylase from D-glucal in the presence of cyclodextrin and have published a preliminary report of these experiments (Klein et al., 1981), but further efforts to stabilize the putative covalent 2-deoxy-β-glucosyl residue and then to determine the amino acid side chain to which the intermediate becomes attached have been unsuccessful so far (H. W. Klein, E. Schiltz, and E. J. M. Helmreich, unpublished experiments). Therefore, other possibilities must be considered, but nevertheless, we would like to suggest that α configuration in the final product (see Figure 7B) is a consequence of nucleophilic displacement with inversion of configuration at C-1 (S_N2 mechanism). Thus, the first step in the D-glucal reaction catalyzed by α-glucan phosphorylases would correspond to a glycosylase mechanism but with notable exceptions: The D-glucal reaction by α-glucan phosphorylases is strictly dependent on phosphate (arsenate) anions and requires the dianionic form of pyridoxal 5′-phosphate. This common re-

Scheme II: Sequence of Phosphorylase-Catalyzed D-Glucal Reaction[a]



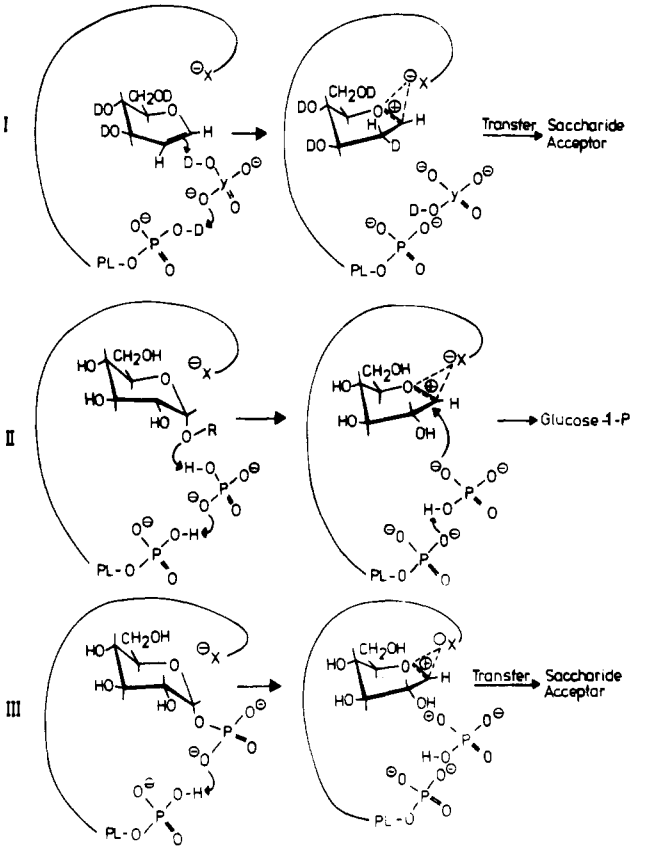
[a] ROH are linear or branched α(1⟶4) poly- or oligosaccharides. Reaction intermediates in brackets have not been isolated.

quirement of the D-glucal reaction and the natural reaction for phosphate implies a close relationship. In addition, the fact that α-glucan phosphorylases (and glycosylases) also react with 2,6-anhydro-1-deoxy-D-*gluco*-hept-1-enitol (Hehre et al., 1980), whereby the phosphorylases again obligatorily require phosphate or arsenate, indicates that the dependency on anions is not a pecularity of the D-glucal reaction (H. W. Klein, M.-J. Im, and J. Lehmann, unpublished experiments).

Scheme II summarizes D-glucal utilization by α-glucan phosphorylases. In the presence of orthophosphate, an equilibrium between (2-deoxy-α-D-glucose)$_n$ α(1→4) oligosaccharides and 2-deoxy-α-D-glucose 1-phosphate is attained.

Role of Phosphate (Arsenate) and of the 5′-Phosphate Group of Pyridoxal-P. ^{31}P NMR studies of the 5′-phosphate of pyridoxal-P bound to activated forms of rabbit skeletal muscle phosphorylases *a* and *b* (Feldmann & Hull, 1977; Hörl et al., 1979) and to the active phosphorylases of *E. coli* and potato tuber (Palm et al., 1979; Klein & Helmreich, 1979) led us to suggest a role for the reversibly protonatable dianionic 5′-phosphate of the cofactor as proton-donor acceptor in general acid–base catalysis (Helmreich & Klein, 1980). Withers et al. (1981a,b) have questioned that mechanism and have proposed an alternative interpretation, suggesting that an immobilized and constrained dianionic 5′-phosphate group of the cofactor acts as an electrophile that facilitates nucleophilic attack by the substrate phosphate and labilizes the ester bond without protonation. Hence, the present controversy regarding the function of the 5′-phosphate group of the cofactor in phosphorylase catalysis centers around the question of whether the mechanism of α-glucan phosphorylases requires a Brønsted acid (proton donor) or a Lewis acid (electrophile). However, the activation of D-glucal (see Scheme I) is unlikely to proceed by an electrophilic mechanism without concomitant proton transfer. Moreover, the observations that (1) fluorophosphate, although it binds and competes with phosphate, does not substitute for phosphate in the D-glucal reaction and that (2) pyridoxal 5′-(fluorophosphate) reconstituted muscle phosphorylase is inactive (Table I) would seem to argue against an electrophilic mechanism of the type proposed by Withers et al. (1981a,b).[2] On the other hand, the findings reported here that the D-glucal reaction, like the reaction with natural substrates, is dependent on the dianionic 5′-phosphate group of the cofactor bound to phosphorylase (Table I) and on phosphate or arsenate (Figure 3) can be reconciled with the evidence recently obtained by Withers et al. (1981b) and Takagi et al. (1982). Both findings emphasize the importance of interactions between the 5′-phosphate group of pyridoxal-P

Scheme III: A Hypothetical Proton Transfer Relay[a]



[a] Orthophosphate (or arsenate) as intermediary in a proton transfer relay between pyridoxal phosphate and D-glucal, oligosaccharides, and glucose-1-P. This proposal is based on a mechanism suggested by Jenkins et al. (1981) [see Figure 2b, p 26, in Jenkins et al. (1981)]. Pyridoxal-5′-P is depicted in its monoanionic (protonated) form. This is the form that is seen in ^{31}P NMR spectroscopy on binding of glycosylic substances (Klein & Helmreich, 1979; Helmreich & Klein, 1980). (I) Reaction with D-glucal: formation of a 2-deoxyglucosyl intermediate and subsequent transfer to a saccharide acceptor. (II) Phosphorolysis of a saccharide: formation of glucose-1-P from a glucosyl intermediate. The phosphorolysis of a 2-deoxyglucosyl-containing saccharide would proceed accordingly. (III) Reaction with glucose-1-P: formation of a glucosyl intermediate and subsequent transfer to a saccharide acceptor.

[2] If it were true that the phosphate of glucose-1-P interacts directly with that of pyridoxal-P and the phosphate of the coenzyme acts as an electrophile like the metal ion in phosphoryl transfer mechanisms, as was postulated by Withers et al. (1981b), an inhibition by orthovanadate might be anticipated. However, 0.3–1.2 mM orthovanadate concentrations had no effect on *E. coli* maltodextrin phosphorylase activity measured in the direction of oligosaccharide breakdown with a coupled assay.

and the substrate phosphates. The plausible and attractive model of a phosphate–phosphate cluster in glycogen phosphorylases introduced by Withers et al. (1981b) can be readily accommodated within a general acid–base-catalyzed mechanism for α-glucan phosphorylases (see Scheme III). It must be acknowledged that Scheme III is as hypothetical as is the model of Withers et al. (1981b). The problem of how to accomodate simultaneously the bulky phosphate and the terminal glucosyl residues of the saccharides (see II in Scheme III) was discussed in detail by Withers et al. (1982a). No evidence is as yet available to support or disprove a concerted action of orthophosphate and the 5′-phosphate group of the cofactor in a hypothetical proton shuttle. Moreover, the structural information available is not of much help in the search for possible proton donors. A number of charged residues at the active site of muscle phosphorylase with appropriate p*K* values, for example, Asp-284, His-570, Glu-671, and the 5′-phosphate group of pyridoxal-P [cf. Fletterick & Madsen (1980) and Johnson et al. (1981)], could conceivably interact with D-glucal, but none of these is in such a clearly prefered position that one could select it over the others. Thus, at present, the possibility has not been ruled out that the intervention of phosphate is indirect and is due to an appropriate structural change bringing the actual proton donor into a position favorable for protonation of the substrate. In fact, the increased reactivity of Arg-568 toward site-specific reagents, when rabbit muscle phosphorylase is activated allosterically or covalently, attests to the possibility of such conformational changes on activation (Dreyfus et al., 1980; Miller et al., 1981). Furthermore, Jenkins et al. (1981) have speculated that a conformational change would allow the guanidino group of Arg-568 to interact with the substrate phosphates (or with the 5′-phosphate group of the cofactor). Quite clearly, the precise location of the phosphates and other possible functional groups in the catalytic site of the active (activated) enzymes needs to be clarified before a more definitive statement can be made about the functional groups participating in proton transfer.

In conclusion, the D-glucal reaction can be interpreted in the framework of a general acid–base-catalyzed reaction mechanism as previously formulated for α-glucan phosphorylases (Feldmann et al., 1978). In view of the findings of Withers et al. (1981b) and Takagi et al. (1982), we would like to speculate that the two negatively charged phosphates are juxtaposed. This would require charge neutralization either by protonation (Scheme III) or by interaction with positively charged amino acid side chains at the active site (Withers et al., 1981b). The former possibility, which we prefer, is attractive because the proximity of the phosphate groups should facilitate their function as proton donor and as glycosyl acceptor as well (see Scheme III).

Added in Proof

In the light of the recent paper of Withers et al. (1982), we have compared activities of the pyridoxal phosphorylase *b* derivative in the normal reaction with glucose-1-P and glycogen in the presence of a fluorophosphate sample purified as described in this paper and of a sample purified according to Withers et al. (1982). Withers' et al. (1982) sample activated pyridoxal phosphorylase *b* under their assay conditions to about 9% of the activity of the native holoenzyme, whereas our sample did not activate above the residual activity of the apoenzyme. This confirms Withers' et al. (1982) recent findings and the previous results reported by Parrish et al. (1977) as well. We have assumed that a 3–5% contamination of our fluorophosphate sample with polyphosphate was responsible for the suppression of activity. However, in the D-glucal reaction fluorophosphate, even when freed from polyphosphate and orthophosphate according to Withers et al. (1982), was inactive and could not replace phosphate in the hypothetical proton relay. This may be rationalized because enzyme-bound fluorophosphate (and phosphite) can either serve as proton donor or acceptor but not as both. To the contrary, phosphate and arsenate can function as proton shuttle.

Acknowledgments

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