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D-GALACTOSE 6-PHOSPHATE: ANOMERIC DISTRIBUTION AND ANALYSIS OF ITS SYNTHESIS FROM D-GALACTOSE AND POLYPHOSPHORIC ACID

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ABSTRACT

D-Galactose 6-phosphate as synthesized by direct phosphorylation of D-galactose with polyphosphoric acid is contaminated with two of its positional isomers. These were separated from D-galactose 6-phosphate and from each other, and identified as D-galactose 3- and 5-phosphate by enzymic, chromatographic, and mass-spectral analysis. The previous misidentification of these isomers as furanose forms of D-galactose 6-phosphate has led to erroneous reports concerning the anomeric distribution of D-galactose 6-phosphate. The anomeric distribution of D-galactose 6-phosphate in a purified preparation was determined by gas–liquid chromatography and ^{13}C-n.m.r. spectroscopy to be 32% α-pyranose, 64% β-pyranose, and no more than 4% furanose anomers.

INTRODUCTION

D-Galactose 6-phosphate is now recognized as a metabolite or component of a wide spectrum of organisms. In *Staphylococcus aureus*[1–3] and Group N streptococci[4], it is an intermediate of lactose and D-galactose metabolism. It has been detected in extracts of the algae *Chlorella vulgaris* and *Scenedesmus obliquus*[5], was identified as a hydrolytic product from the cell wall of the fungus *Fusicoccum amygdali*[6], and was a reported product of a hexokinase purified from *Aspergillus parasiticus*[7]. In animal systems, D-galactose 6-phosphate has been found in galactosemic erythrocytes[8], in the brains of D-galactose-intoxicated chicks[9], and in rat hearts perfused with D-galactose[9].

Although the biochemical importance of galactose 6-phosphate is now established, information on its anomeric distribution is contradictory. Based on gas–liquid-chromatographic (g.l.c.) analyses, Zinbo and Sherman[10] reported that two pyranose and two furanose forms of galactose 6-phosphate exhibited peaks of nearly equal size, whereas Musick and Wells[9] reported that the four forms gave peaks of differing size, and Harvey and Horning[11] reported two pyranose forms and two to three peaks for furanoses, some of which were designated "probably an impurity".

Determination of anomeric distribution requires samples of defined purity.

D-Galactose 6-phosphate offered by commercial suppliers, including those used in the cited investigations[9-11], is generally obtained by chemical synthesis from galactose and polyphosphoric acid (Sigma Chemical Co., personal communication). The synthesis of a hexose monophosphate by direct phosphorylation of a sugar with polyphosphoric acid[12] is attractive because of the utilization of readily available, inexpensive precursors and the avoidance of time-consuming, site-blocking processes. However, the purity of D-galactose 6-phosphate from such a synthesis has not been unequivocally established.

We report here that the synthesis of D-galactose 6-phosphate from D-galactose and polyphosphoric acid gives positional isomers of D-galactose 6-phosphate as by-products. The isomers were separated from D-galactose 6-phosphate and from each other, and identified as galactose 3-phosphate and D-galactose 5-phosphate. Their misidentification by others as furanose anomers of D-galactose 6-phosphate has contributed to the contradictory reports of the anomeric distribution of D-galactose 6-phosphate. We have determined the anomeric distribution of D-galactose 6-phosphate in a purified preparation by g.l.c. analysis and by ^{13}C-n.m.r. spectroscopy.

RESULTS AND DISCUSSION

Synthesis and analysis of D-*galactose 6-phosphate*. — D-Galactose 6-phosphate

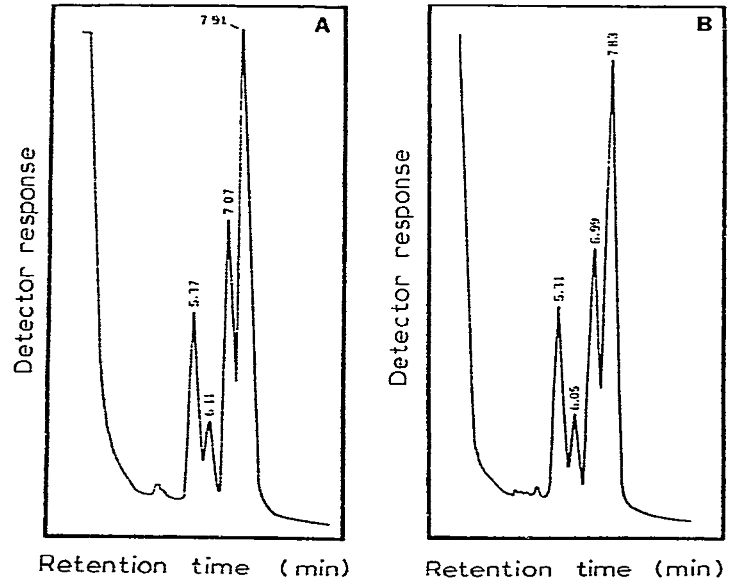
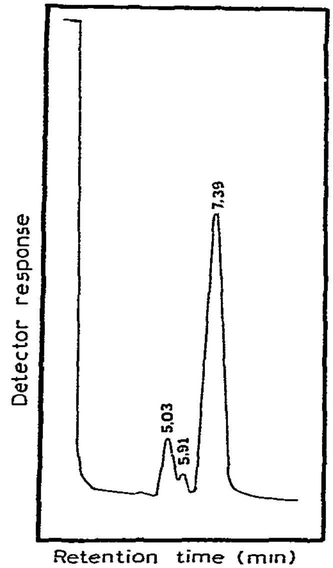


Fig. 1. Chromatogram (g.l.c.) of the product synthesized from D-galactose and polyphosphoric acid. Trimethylsilyl derivatives of commercial D-galactose 6-phosphate labeled 98–100% pure (A) and the product synthesized in this laboratory (see text) (B) were analyzed on a Hewlett–Packard gas chromatograph, model 5830A, equipped with an integrator. The column (1.8 m × 2 mm) was packed with 3% OV-7 on Gas-chrom Q (80–100 mesh). Temperatures were as follows: injector port, 250°; column, 220°; flame-ionization detector, 320°. Carrier gas: nitrogen at a flow rate of 23 mL/min.

was prepared from D-galactose and polyphosphoric acid by the procedure used by Seegmiller and Horecker[12] for the synthesis of D-glucose 6-phosphate, and was obtained in similar yield (9 mol%). Based on g.l.c. analysis, the product was identical to D-galactose 6-phosphate obtained from a commercial source and labeled 98–100% pure (Fig. 1). However, only 77–80% of the total carbohydrate in the product, as



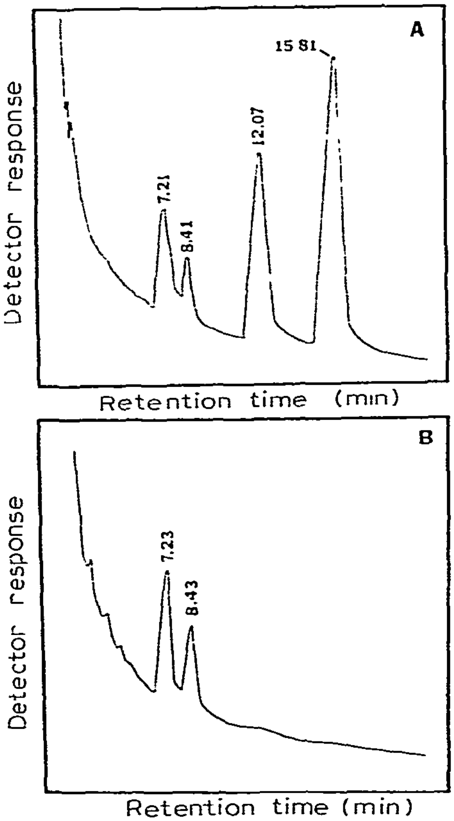


Fig. 2. Chromatogram (g.l.c.) of the Me_3Si-*O*-methyloxime derivatives formed from the chemically synthesized D-galactose 6-phosphate preparation. Instrument and conditions were identical to those described in the legend for Fig. 1, except that the column packing was 3% OV-17 on Gas-chrom Q (80–100 mesh), the column temperature was 190°, and the flow rate of the carrier gas was 25 mL/min. The peak at 7.39 min was identified as the derivative of D-galactose 6-phosphate, whereas peaks at 5.03 min and 5.91 min were subsequently identified as positional isomers of D-galactose 6-phosphate (refer to text).

Fig. 3. Enzymic removal of D-galactose 6-phosphate from a standard preparation. The preparation of D-galactose 6-phosphate was analyzed by g.l.c. before (A) and after (B) incubation with D-galactose 6-phosphate isomerase. The assay mixture (total volume, 0.4 mL) contained 64 μmol of Bicine buffer (pH 8.2), 12 μmol of magnesium chloride, 6 μmol of ATP, 4 μmol of the D-galactose 6-phosphate preparation, and 60 milliunits of the isomerase. In addition, D-fructose 6-phosphate kinase was included to catalyze the phosphorylation of D-tagatose 6-phosphate[17], the product of the isomerase, thereby allowing the isomerization reaction to proceed to completion. Lyophilized samples were analyzed as their Me_3Si derivatives. Instrument and conditions were as described in the legend of Fig. 1, except that the column packing was 3% XE-60 on Gas-chrom Q (80–100 mesh) and the column temperature was 175°.

well as in the commercial sample, was D-galactose 6-phosphate, as determined by a specific enzymic assay (see Experimental section).

The D-galactose 6-phosphate preparation formed multiple *O*-methyloxime derivatives (Fig. 2), indicating that it contained sugar phosphate impurities. On some g.l.c. packings, the *syn*, *anti*, or cyclic forms of the Me_3Si-*O*-methyl-oxime derivative might be expected to be partially resolved[11,13-15], resulting in a peak with a following shoulder or small peak, but such was not the case in our investigation. The major peak in Fig. 2, which comprised 80% of the total peak area, gave a mass spectrum identical to that reported for the Me_3Si-*O*-methyloxime derivative of galactose 6-phosphate[16]. The estimation of purity (80%) was the same as that obtained by the enzymic analysis.

In reports by other investigators[9,10], the initial two large peaks on gas–liquid chromatograms of D-galactose 6-phosphate preparations (for instance, see Fig. 1 or 3A) have been ascribed to furanose anomers. However, when we selectively removed D-galactose 6-phosphate from standard preparations by a coupled enzymic reaction initiated by D-galactose 6-phosphate isomerase, the initial two peaks remained (Fig. 3). Although isomerases may exhibit anomeric specificity, the rapid mutarotation of sugar phosphates[18] would preclude the selective removal of only certain anomers by the isomerase. Therefore, the initial two peaks could not have been anomers of D-galactose 6-phosphate.

The probability that the initial two peaks on gas–liquid chromatograms constituted by-products of the chemical synthesis of D-galactose 6-phosphate was suggested by analyses of D-galactose 6-phosphate derived from biological sources. The bacterium *S. aureus* GD29, a mutant of *S. aureus* NCTC 8511 deficient in D-galactose 6-phosphate isomerase[2], was grown in the presence of D-galactose. Under these conditions, this strain accumulates D-galactose 6-phosphate, the further metabolism of which is blocked by the absence of the isomerase[2]. D-Galactose 6-phosphate extracted from these cells, as well as galactose 6-phosphate synthesized from tagatose 6-phosphate by purified D-galactose 6-phosphate isomerase[3], was subjected to g.l.c. analysis. In neither case were substances observed having retention times corresponding to those of the initial two peaks of chemically synthesized D-galactose 6-phosphate.

The initial two peaks in gas–liquid chromatograms of chemically synthesized D-galactose 6-phosphate were nevertheless identified as galactose monophosphates as follows: (*i*) they were present in the monophosphate fraction[19] obtained after application to a Dowex 1-X8 hydrogencarbonate column and elution in a stepwise gradient of 0.15, 0.30, and 0.45M potassium hydrogencarbonate; (*ii*) they reacted quantitatively with alkaline phosphatase and the resulting free carbohydrate moieties reacted >99% with β-D-galactose dehydrogenase. β-D-Galactose dehydrogenase is specific for D-galactose and L-arabinose[9]. Galactose and arabinose are effectively resolved in g.l.c. by using 3% OV-1 column packing (160°), and may be identified by their characteristic retention-times (peaks at 4.07 and 4.63 min for arabinose and 11.13, 13.05, and 16.04 min for galactose). Subsequent g.l.c. analysis confirmed that galactose was the sole carbohydrate product of the phosphatase treatment and that, in particular, arabinose was absent. It must, therefore, be concluded that the initial two peaks on the chromatograms were positional isomers of galactose monophosphate.

Identification of the positional isomers of D-*galactose 6-phosphate.* — As the initial two peaks did not serve as substrates for D-galactose 6-phosphate isomerase (see foregoing), they could not have been D-galactose 6-phosphate. The 4-phosphate is also ruled out because it would presumably be structurally unstable because of the sterically unfavorable, axial position of the bulky, polar phosphate group; studies of esterification reactions have confirmed that the 4-hydroxyl group of galactose exhibits low reactivity[20]. The 2-phosphate is ruled out because of the presence of ions *m/z* 160 and 561 in the mass spectra of the trimethylsilyl-*O*-methyloxime derivatives of the positional isomers (Fig. 4). The presence of these ions indicates that C-2–C-3 cleavage had occurred. In particular, ion *m/z* 160 incorporates C-1 and C-2 of the carbon chain[13], and would not be present had the sugar contained a phosphate group at the 2-position. With regard to the 1-phosphate, it forms stable trimethylsilyl (Me_3Si) derivatives only under specially defined conditions[11] not used in this study. We have shown that the positional isomers present in the product mixture consistently formed stable Me_3Si derivatives (Figs. 1, 3, 5). Furthermore, the 1-phosphate is acid-labile[21] and would not have survived the harsh acidic conditions inherent in the synthetic procedure of Seegmiller and Horecker[12]. The positional isomers in question have been shown to be acid-stable: they were isolated as their barium salts after being subjected to hydrolysis in 1.6M hydrobromic acid for 24 h at 100° (Fig. 1). By hydrolyzing the mixture of positional isomers under the foregoing conditions for prolonged periods (up to 48 h) and analyzing the hydrolyzates by g.l.c., we determined that the two isomers exhibited a similar high degree of acid-

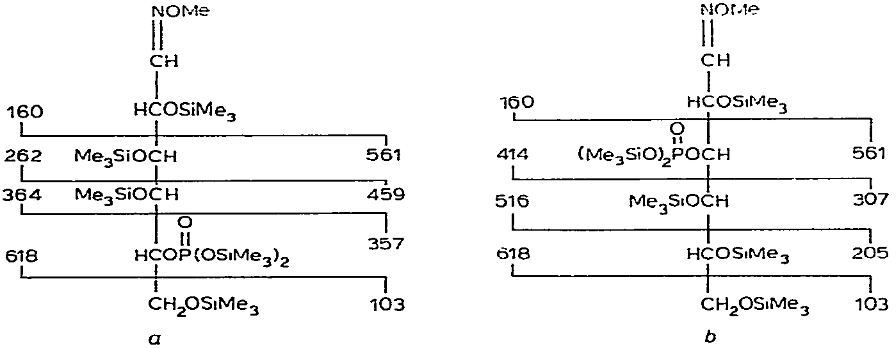


Fig. 4. Mass-spectral fragmentation patterns of derivatized, positional isomers found in preparations of galactose 6-phosphate. Mass-spectral data were obtained with an LKB model 9000 combined g.l.c.–mass spectrometer equipped with a column (1.8 m × 3 mm) of 3% OV-17 on Gas-chrom Q (80–100 mesh). Instrument conditions were as follows: ionizing energy, 70 eV; repetitive scanning-range, 50–650 *m/z*; molecular separator, 250°; ion source, 290°; injector port, 250°; and column, 190°. Data from the mass spectra are noted in parentheses and are expressed as percent intensity, relative to *m/z* 73 = 100%. The structures depict the Me_3Si-*O*-methyloxime derivatives of (*a*) D-galactose 5-phosphate: *m/z* 618 (0.5), 561 (7.9), 459 (3.9), 364 (0.3), 357 (13.8), 262 (1.1), 160 (10.1), and 103 (6.1); and (*b*) D-galactose 3-phosphate: *m/z* 618 (0.2), 561 (1.9), 516 (1.5), 414 (2.6), 307 (2.0), 205 (7.9), 160 (4.7), and 103 (11.9).

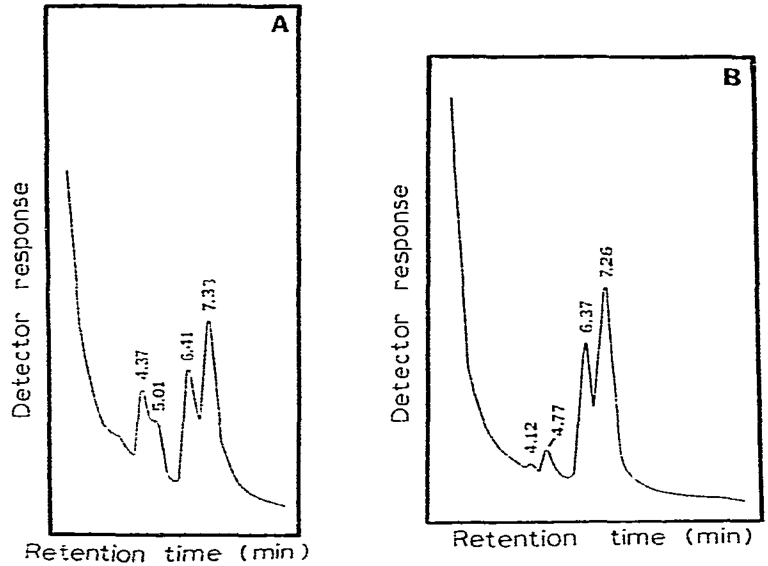


Fig. 5. Chromatogram (g.l.c.) of the galactose 6-phosphate preparation before (A) and after (B) removal of the positional isomers by anion-exchange chromatography. Galactose 6-phosphate was separated from its positional isomers by anion-exchange (Dowex 1-X4) chromatography using a triethylammonium tetraborate elution-gradient[29]. Fractions (1.5 mL) were assayed for total carbohydrate; the contents of peak fractions were identified by g.l.c. after removal of borate. The isomers eluted in non-overlapping peaks as follows: D-galactose 3-phosphate (peak at fraction 150); D-galactose 5-phosphate (peak at fraction 185); D-galactose 6-phosphate (peak at fraction 220). Me_3Si derivatives were analyzed by g.l.c. with the instrument and conditions identical to those described in the legend for Fig. 1, except that the column packing was 3% OV-17 on Gas-chrom Q (80–100 mesh), the column temperature was 190°, and the carrier-gas flow-rate was 25 mL/min.

stability (about 60% as stable as D-galactose 6-phosphate). Besides D-galactose 6-phosphate, only D-galactose 3-phosphate[22] and possibly D-galactose 5-phosphate[23] have been shown to be acid-stable phosphates of galactose. From the foregoing considerations, it seems likely that the positional isomers present in preparations of D-galactose 6-phosphate are the 3- and the 5-phosphates of D-galactose, a conclusion substantiated by the analyses described next.

The major positional isomer of D-galactose 6-phosphate (peak at 5.37 min in Fig. 1A or 5.31 min in Fig. 1B) is considered to be the 5-phosphate, because its borate complex was retained longer on an anion-exchange resin than was the borate complex of the minor isomer (peak at 6.05 or 6.11 min, Fig. 1) whereas, on all g.l.c. packings used (Figs. 1, 3, 5), the major isomer migrated ahead of the minor one. The longer retention-time of the major isomer in the presence of borate may be explained as follows: because of the position of the phosphate group, galactose 5-phosphate can only exist as a furanose. It is known that furanoses form stronger borate complexes than do pyranoses[24–26], and therefore would be retained longer on an anion-exchange resin.

Consistent with the identification of the positional isomers as the 3- and the

5-phosphates were results of combined g.l.c.–m.s. of Me_3Si-*O*-methyloxime derivatives (Fig. 4). Such an analysis has been shown effective for locating the sites of substituents on non-phosphorylated sugars[13]. Although phosphate-group migrations and rearrangements[10,27,28] in the mass spectrometer complicate such an analysis when applied to sugar phosphates, we were still able to obtain useful information by examining the relative intensities of certain key ions. For each isomer, all eight predicted ions arising from cleavage along the carbon chain were present. Those ions (*m/z* 205, 307, 414, and 516) predicted for the 3-phosphate, but not the 5-phosphate, were of greater intensity for the isomer identified as D-galactose 3-phosphate. That they were present at all in the spectrum of the 5-phosphate may be due to alternative fragmentation-pathways or to the lack of complete resolution of the isomers by g.l.c. (see Fig. 2).

The lack of vicinal, alkoxyl groups impedes cleavage along the carbon chain[13]. By this criterion, one would predict less of *m/z* 103 and 618, and more of *m/z* 160 and 561 in the spectrum of the 5-phosphate as compared to the 3-phosphate (see Fig. 4). This prediction held true except for *m/z* 618, an ion whose intensity was so low that its use for comparison purposes is questionable.

In light of these findings, we conclude that the substance identified by Zinbo and Sherman[10] as α-galactofuranose 6-phosphate was primarily D-galactose 5-phosphate. They contrasted its mass spectrum to that of galactopyranose 6-phosphate, drawing special reference to the mass-spectral differences between furanose and pyranose forms of phosphorylated carbohydrates. Fortuitously, since galactose 5-phosphate cannot exist as a pyranose, their assignments of fragmentation ions characteristic of furanoses remain valid. The reported presence of a relatively large g.l.c. peak thought to be α-D-galactofuranose 6-phosphate in brain extracts of D-galactose-intoxicated chicks[9] is difficult to evaluate, because a chromatogram of brain extracts of normal chicks was not presented. However, it may be that chick brain extracts contain some other sugar phosphate having a retention time similar to that of D-galactose 5-phosphate.

Determination of the anomeric distribution of D-*galactose 6-phosphate.* — Chromatographic profiles of D-galactose 6-phosphate before and after removal of the positional isomers by anion-exchange chromatography are shown in Figs. 5A and 5B, respectively. D-Galactose 6-phosphate thus purified was used for the determination of anomeric distribution.

The anomeric distribution was determined by g.l.c. At anomeric equilibrium, 96% of the galactose 6-phosphate existed in the pyranose forms (peaks at 6.37 and 7.26 min, Fig. 5B). Mass-spectral analysis of these peaks yielded major ions having *m/z* values of 204, 357, and 387, which are characteristically abundant for hexopyranose 6-phosphates[10,11]. The components giving small peaks (4% of the total) at 4.12 and 4.77 min do serve as substrates for D-galactose 6-phosphate isomerase and, therefore, are probably the furanose forms of D-galactose 6-phosphate. Of the D-galactose 6-phosphate, 32% exists as the α pyranose, and 64% as the β pyranose

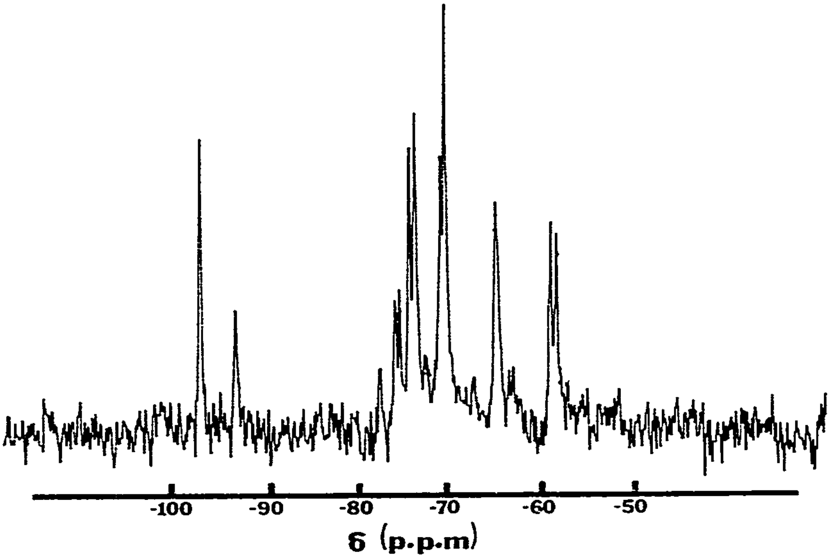


Fig. 6. Natural-abundance ^{13}C-n.m.r. spectrum of D-galactose 6-phosphate. The 0.5M sample (total volume, 0.3 mL) contained 140 mM Bicine buffer (pH 8.2). The instrument used was a Bruker WP-60, 15.08 MHz Fourier-transform spectrometer equipped with quadrature detection. The spectrum was obtained at 23°, with 4000 spectral points, a spectral width of 3000 Hz, and a filter setting of 2400 Hz. The spectrometer was locked to the resonance of D_2O in a capillary. Chemical shifts are given relative to external tetramethylsilane and are accurate to within ±0.1 p.p.m. Resonances of the α and β anomeric carbon atoms of D-galactopyranose 6-phosphate occur at −93.2 and −97.2 p.p.m., respectively[34].

(Fig. 5B). Confirmation of this distribution was achieved by ^{13}C-n.m.r. spectroscopy: the ratio of β pyranose to α pyranose was 2 to 1 (Fig. 6).

EXPERIMENTAL

Commercial D-galactose 6-phosphate (disodium and barium salts), D-galactose, polyphosphoric acid, L-arabinose, rabbit muscle D-fructose 6-phosphate kinase, β-D-galactose dehydrogenase and *Escherichia coli* alkaline phosphatase were purchased from Sigma Chemical Co., St. Louis, MO. D-Tagatose 6-phosphate was synthesized from D-galacturonate, and D-galactose 6-phosphate isomerase was purified from D-galactose-grown *S. aureus* NCTC 8511 as described by Bissett *et al.*[3].

The enzymic end-point assay for D-galactose 6-phosphate utilized D-galactose 6-phosphate isomerase, which is specific for D-galactose 6-phosphate and D-tagatose 6-phosphate[3]. The absence of the ketohexose phosphate in all preparations analyzed was confirmed by the resorcinol method of Roe *et al.*[30] as described by Ashwell[31], and by g.l.c. The enzymic end-point assay was as described by Bissett *et al.*[3], except that limiting amounts of D-galactose 6-phosphate were added to the incubation mixtures.

O-Trimethylsilyl derivatives of the sugars and sugar phosphates were prepared according to the procedure of Bentley and Botlock[32]. Me_3Si-*O*-methyloxime derivatives were prepared as described by Laine and Sweeley[14]. Prior to derivatizing samples in fractions from columns of Dowex-1 (borate), the borate was removed by repeated evaporation of methanol[29]; we found that rigorous removal of borate was essential for prevention of interference with the silylation reaction. The silylating reagents (chlorotrimethylsilane and hexamethyldisilazane) were purchased from Regis Chemical Co., Morton Grove, IL.

Total carbohydrate was determined by the phenol–sulfuric acid method of Dubois *et al.*[33].

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