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THE SUBSTRATES OF D-GALACTOSE 6-PHOSPHATE ISOMERASE:
AN ANALYSIS OF D-GALACTOSE 6-PHOSPHATE AND
D-TAGATOSE 6-PHOSPHATE

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THE SUBSTRATES OF D-GALACTOSE 6-PHOSPHATE ISOMERASE: AN ANALYSIS OF D-GALACTOSE 6-PHOSPHATE AND D-TAGATOSE 6-PHOSPHATE

By

William Charles Wenger

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ABSTRACT

THE SUBSTRATES OF D-GALACTOSE 6-PHOSPHATE ISOMERASE:
AN ANALYSIS OF D-GALACTOSE 6-PHOSPHATE AND
D-TAGATOSE 6-PHOSPHATE

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William Charles Wenger

D-Galactose 6-phosphate at anomeric equilibrium was determined by gas-liquid chromatography and mass-spectrometry to exist 96 percent in the pyranose form. This is contrary to literature reports of the abundant presence (20-50 percent) of furanose forms. The substances misidentified by others as the furanose anomers were determined to be by-products of a common synthetic procedure for galactose 6-phosphate. These impurities, present in commercial preparations of D-galactose 6-phosphate labeled 98-100 percent pure, were identified as the 3- and 5-phosphates of D-galactose. Purification of D-galactose 6-phosphate was achieved by ion-exchange chromatography.

A modified synthesis of D-tagatose 6-phosphate was devised which resulted in increased purity of the final product. The unmodified standard synthesis was found to result in an impure product contaminated by significant amounts of the isopropylidene derivative(s) of tagatose 6-phosphate.

William Charles Wenger

The equilibrium composition of the D-galactose 6-phosphate isomerase reaction was determined to be 90 percent galactose 6-phosphate and 10 percent tagatose 6-phosphate.

This work is dedicated to the memory of Eric Earl.

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LIST OF ABBREVIATIONS

ATP	adenosine 5'-triphosphate
Bicine	N,N-bis(2-hydroxyethyl)glycine
BME	β-mercaptoethanol; 2-thioethanol
DEAE	Diethylaminoethyl
EDTA	ethylenediamine tetraacetic acid
g	acceleration of gravity
GLC	gas-liquid chromatography
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethane-sulfonic acid
mU	milli Unit
NAD^+	Nicotinamide adenine dinucleotide
NADH	reduced nicotinamide adenine dinucleotide
$NADP^+$	nicotinamide adenine dinucleotide phosphate
NADPH	reduced nicotinamide adenine dinucleotide phosphate
NMR	nuclear magnetic resonance
PEP	phospho(enol)pyruvate
TMS	trimethylsilyl
Tris	tris(hydroxymethyl)aminomethane

INTRODUCTION

Lactose is commonly metabolized through hydrolytic cleavage to glucose and galactose, with further metabolism of galactose through the well known Leloir pathway (1). However, in certain bacteria (2), including Staphylococcus aureus (3), lactose is phosphorylated during transport and the resulting lactose phosphate is cleaved to glucose and galactose 6-phosphate. The pathway through which galactose 6-phosphate is further metabolized was unknown for any organism until Bissett and Anderson (4) reported its catabolism through what was termed the tagatose 6-phosphate pathway (see Literature Review). During the course of further investigations by Bissett (5), data were accumulated which suggested that D-galactose 6-phosphate failed to mutarotate in aqueous solution, contrary to the behavior of all known reducing sugars.

One original goal of this thesis research was to establish the cause of this apparent lack of mutarotation of galactose 6-phosphate. During the initial investigations it became apparent that the application of such analytical techniques as gas-liquid chromatography, ^{13}C-nuclear magnetic resonance spectroscopy, and mass spectrometry could provide new information about established syntheses of galactose 6-phosphate and tagatose 6-phosphate, and about the anomeric distribution of galactose 6-phosphate. Through the use of an enzyme highly specific for the two sugar phosphates under investigation, and through the application of the analytical techniques previously mentioned, a

systematic analysis of preparations of D-galactose 6-phosphate and D-tagatose 6-phosphate was undertaken.

In the following presentation, product analyses of common synthetic procedures for galactose 6-phosphate and tagatose 6-phosphate are detailed, and modifications leading to increased product purity are described. New information on the anomeric distribution of D-galactose 6-phosphate in aqueous solution is provided which is contrary to that reported in the literature. Definitive evidence for the mutarotation of D-galactose 6-phosphate is presented, and finally, the elucidation of the equilibrium composition of the D-galactose 6-phosphate isomerase-catalyzed reaction is described.

LITERATURE REVIEW

Sugar Phosphates: Syntheses and Analyses

Literature dealing with studies on the chemistry and metabolic role of sugar phosphates is voluminous and well beyond the scope of this present review. However, several excellent general reviews do exist (6, 7, 8) as well as more restricted reviews on the chemical syntheses of sugar phosphates (9, 10), and on the history of work which led to the isolation of hexose phosphates (11). More recently, reviews on keto-sugars and their biologically important phosphate esters (12), and on enzyme specificities and anomerization rates of biologically important sugar phosphates (13) have appeared.

Hexose monophosphates have been synthesized by both chemical and enzymatic means (6). Enzymatic methods have involved phosphorylation with ATP using different kinases, aldose/ketose interconversions using isomerases, and specific epimerases which lead to inversions of configuration.

Carbohydrate monoesters were commonly synthesized nonenzymatically through the use of phosphochloridic acids. The earliest chemical syntheses used phosphoryl chloride to prepare the sugar phosphate from an aqueous solution of the sugar (14). This method was improved by Fischer (15) using anhydrous pyridine as the solvent. To avoid the possibility of side reactions, later methods employed substituted

compounds such as diphenyl phosphochloridate (16) and dibenzyl phosphochloridate (17, 18) as phosphorylating reagents. After phosphorylation of the sugar, the phenyl or benzyl groups were removed by hydrogenolysis, or by hydrolysis under mild conditions using dilute NaOH or liquid ammonia.

Hexose monophosphates have also been synthesized from the sugar oxide derivative (ethylene oxide ring) with Na_2HPO_4 in aqueous solution at room temperature (17, 19). Syntheses of acid-stable 6-phosphates have involved the reaction of the free sugar with tetraphosphoric (20) or metaphosphoric (21) acids. Dicyclohexylcarbiimide has been a widely used reagent in the preparation of monoesters. Benzyl phosphate or β-cyanoethyl phosphate is transformed into the diester of the compound to be phosphorylated by the action of dicyclohexylcarbiimide. The benzyl or β-cyanoethyl group may then be removed by hydrogenolysis or with alkali (22).

Migration of the phosphate group is known to occur after phosphorylation of the sugar as in the case of D-xylose 3-phosphate (23). Phosphorylation of 1,2-isopropylidene xylose with diphenyl phosphochloridate gave the 5-phenyl phosphate which formed a 3,5-cyclic phosphate when the phenyl group was removed by prolonged alkaline treatment. Acid hydrolysis gave a mixture of the 3- and 5-D-xylose phosphates. This intramolecular migration of phosphate groups from one hydroxyl group to another is known to be catalyzed by acid (24, 25, 26). The migration is believed to involve a cyclic intermediate. The steric requirements for the formation of cyclic phosphates have been studied by Khorana (27). When the hydroxyl group next to the phosphate is free, a five-membered ring is formed. For example, xylose 3-phosphate

gives the 3,4- or 2,3-cyclic phosphate. If the hydroxyl group next to the phosphate forms part of a furanose or pyranose ring, then it is the next hydroxyl which reacts. For example, treatment with dicyclohexylcarbiimide leads to the formation of the 4,6-cyclic phosphate from glucose 6-phosphate.

Classical methods for the isolation of sugar phosphates have relied on the different solubilities of their salts (28). The usual procedure is to make the solution slightly alkaline, form the barium salt, and precipitate the salt by the addition of several volumes of ethanol. Calcium, magnesium, mercury, and lead salts have also been used (29).

The applications of paper and ion exchange chromatography to the purification and identification of sugar phosphates have been excellently reviewed (30). Recent advances have seen the use of high pressure column chromatography with automated detection systems that enable the rapid separation of sugar phosphates at the 10 nmole level (31).

An especially effective and widely used technique in the separation of sugar phosphate esters has involved the formation of borate complexes. Studies on the interaction of boric acid with carbohydrates have been reviewed by Boeseken (32). Khym and Cohn introduced the use of borate complexing to separate the commonly encountered sugar monophosphates by anion exchange chromatography in an alkaline chloride system (33). Separation was effected by using a sequence of eluting solutions of varying borate concentration, chloride concentration, and pH. By a similar method they were able to separate effectively all five of the ribose phosphates from one another (34). Through ion exchange studies of various sugar phosphates in the presence of borate, and through the earlier work on borate-sugar interactions summarized

by Boeseken, three generalizations have been made about the reactions of borate with sugars (35): (i) cis-α-glycols are strongly complexed, (ii) pyranose systems are not complexed relative to furanose systems, (iii) the stronger the complex, the greater the ionization and thus the greater the affinity for anion exchangers. The generalization that pyranose systems are not complexed relative to furanose systems was initially based on empirical observations of the elution positions of a variety of sugar phosphates. For example, ribose 5-phosphate and fructose 6-phosphate, which can only form furanose structures, elute from the anion exchange resin much slower than does glucose 6-phosphate, which can form both the furanose and pyranose rings. From direct experimental evidence it was demonstrated that borate interacts preferentially with furanose structures. When glucose is substituted in the 4-position to prohibit the formation of a furanose ring (as in the case of 4-O-methyl-D-glucose) the zone electrophoresis migration of the sugar-borate complex is decreased to 25 percent of that of the unsubstituted sugar complexed to borate (36), suggesting the importance of the furanose form in the reaction with borate. Further, when glucose is complexed with borate a proton shift occurs that is identical to the shift seen when 5-O-methyl-D-glucose is complexed with borate, as measured by proton-NMR (37). This is strong evidence that when a sugar exists in a furanose-pyranose equilibrium, the furanose is favored for borate complexing. Whereas borate ion exchange chromatography has been an effective method for separating sugar phosphates, the recovery of the sugar phosphates after most of these separations has been troublesome and time consuming. Lefebvre *et al.* (38) introduced a method which achieved separation of sugar phosphates on an anion

exchange column and permitted their almost quantitative recovery. It was based on the use of linear gradient elution with ammonium or triethylammonium borate, and on the removal of these salts after freeze-drying by distillation with methanol.

The analysis of carbohydrates was greatly enhanced by the introduction by Sweeley et al. (39) of a method to form the trimethylsilyl derivatives of sugars and related substances which were suitable for subsequent GLC analysis. TMS derivation occurred rapidly at room temperature in pyridine containing hexamethyldisilazane and trimethylchlorosilane. Silylation of all free hydroxyl groups occurred and the yield of the TMS derivative was quantitative. Initial attempts to trimethylsilylate directly all the hydroxyl groups and the acidic groups as well, of sugar phosphates were not successful. However, a two-step procedure was developed (40) in which the phosphoric acid residue was esterified with diazomethane followed by TMS derivation using established conditions reported for free sugars (39). Hashizume and Sasaki (41) reported a direct, one-step trimethylsilylation of sugar phosphates in 1966. The sugar phosphate was treated with hexamethyldisilazane and trimethylchlorosilane in anhydrous pyridine, and complete derivation occurred after refluxing for one hour. In 1973, Harvey and Horning (42) published a comprehensive paper on the GLC of sugar phosphates, listing derivation and chromatographic conditions for a variety of aldose- and ketose- 1,-4,-5, and -6 phosphates. Also noted were the retention times for the oxime -TMS derivatives of the C_4-C_7 sugar phosphates. Sherman et al. (43) noted difficulties common to the GLC of sugar phosphates. Impurities frequently encountered in the trimethylsilylating reagents gave rise to spurious

GLC peaks which seemed to increase with time. These impurities were found in silylating reagents from different sources and were enhanced in sample-reagent mixtures. The instability of chromatographic columns in the presence of sugar phosphates was also noted. One difficulty was termed the "priming effect". This was seen as an increasing but ultimately stabilized response toward repeated injections of samples of the same size. This effect was believed to be due to the interaction of highly reactive TMS esters with adsorbed matter on the columns or with other reactive components of the system such as alcoholic or silanol hydroxyls. A second difficulty encountered with the chromatographic columns was the sudden failure of a column which had previously been satisfactory. It has been suggested that this is caused by a phosphate ester which becomes adsorbed to the surfaces of the instrument where it undergoes thermal degradation to produce phosphoric acid, which then catalyzes further degradation of phosphate esters. These columns which fail to chromatograph TMS sugar phosphates remain functional in the analysis of nonphosphorylated compounds.

Sweeley et al. (39) noted the applicability of the gas chromatographic technique to the determination of the anomeric distribution of carbohydrates in solution. When aqueous equilibrium solutions were evaporated to dryness and trimethylsilylated, peak areas due to the α and β anomers agreed well with the known anomeric compositions established by optical rotation and bromine oxidation studies. Their studies revealed a possible generalization for the effect of structure on retention time for the TMS derivatives of aldoses: when the sugar normally exists in the C1 or 1C chair conformation and there is no evidence of conformational instability, then that anomer in which the

anomeric hydroxyl group is equatorial has the longest retention time. For example, for the following D-sugars that exist in the C1 conformation, the β anomer has a longer retention time than the α anomer: glucose, galactose, mannose, talose, xylose, and lyxose. For D-arabinose, which exists in the 1C conformation, the α anomer possesses an equatorial hydroxyl group and has a longer retention time than the corresponding β anomer. In 1967, Bentley and Botlock (44) described a method by which the anomeric composition of an aqueous sugar solution could be measured directly by gas-liquid chromatography. This required the preparation of TMS derivatives in the presence of water in such a way that the anomeric composition of the sugar did not change. A small sample (about 5 μl) of the sugar solution to be analyzed was diluted with dimethylformamide, frozen in liquid nitrogen, and reacted with a mixture of pyridine, hexamethyldisilazane, and trimethylchlorosilane (4:1:1). Anomeric distribution was determined by gas-liquid chromatography. Values for the mutarotation coefficients of glucose, galactose, arabinose, and xylose, determined by this method were identical to those values determined by polarimetry.

Combined gas chromatography-mass spectrometry has also been used in the analysis of sugar phosphates. Zinbo and Sherman (45) studied the mass spectra of a variety of sugar phosphates, including aldohexose phosphates, ketose 1- and 6-phosphates, aldopentose phosphates, and phosphohexonates. They were shown to undergo fragmentations which in many cases could be related to their structures. Fragment ions were identified which contained specific carbon atoms of the carbohydrate. Phosphorus-containing ions, some highly rearranged and some bearing specific carbon atoms of the parent sugar, were noted. A summary of

ions useful for carbohydrate structural identification was presented. Harvey and Horning (42) presented a tabulation of the partial mass spectra of the TMS derivatives of selected sugar phosphates showing the variation in the relative abundance of the main rearrangement ions.

Britmaier et al. (46) have provided a review of ^{13}C-nuclear magnetic resonance spectroscopy and its applications to the analysis of carbohydrates. ^{13}C-NMR provides a direct means of determining the anomeric distribution of sugar mono- and diphosphates in aqueous solution. For example, the anomeric distribution of the furanose forms of D-fructose 6-phosphate and D-fructose 1,6-diphosphate were determined by ^{13}C-NMR to be 19 ± 2 percent α, and 81 ± 2 percent β (47).

D-Tagatose 6-Phosphate

The synthesis of the ketohexose monophosphate, D-tagatose-6-phosphate, was first described by Totton and Lardy (48) in 1949. D-Tagatose was converted to 1,2;3,4-diisopropylidene-D-tagatose by the procedure of Reichstein and Bosshard (49). The 6-position was phosphorylated with diphenylchlorophosphonate and the resulting diphenylphosphate was converted to 1,2;3,4-diisopropylidene-D-tagatose 6-phosphate by hydrogenolysis. The isopropylidene groups were removed by hydrolysis and the D-tagatose 6-phosphate was collected as the barium salt. In 1973, Bissett and Anderson (4) reported the synthesis of D-tagatose-6-phosphate from D-galacturonic acid by a procedure that involved the combination of three published syntheses (48, 50, 51). D-Galacturonate was isomerized in alkaline solution to D-tagaturonate. The 1,2;3,4-diisopropylidene derivative of tagaturonate was formed, esterified by diazomethane, and reduced with $LiAlH_4$ to 1,2;3,4-

diisopropylidene D-tagatose. The procedure of Totton and Lardy was then followed to yield D-tagatose 6-phosphate. In 1976, Koerner et al. (52) modified the method of Totton and Lardy to increase the yield of intermediates and final product. The intermediate 1,2;3,4-diisopropylidene-tagatose-6-diphenylphosphate was obtained in 93 percent yield by using an excess of diphenyl phosphochloridate (1.5 molar equivalents) and longer reaction time (60 hours at 5°C). D-Tagatose 6-phosphate was isolated as its barium salt. In aqueous solution, D-tagatose 6-phosphate was determined by ^{13}C-NMR analysis to have the following anomeric distribution; 20 ± 2 percent α furanose, and 80 ± 2 percent β furanose (53).

Totton and Lardy reported that a beef brain extract containing phosphofructokinase and aldolase converted D-tagatose 6-phosphate to the triose phosphates dihydroxyacetone phosphate and D-glyceraldehyde 3-phosphate, presumably through the intermediate D-tagatose 1,6-diphosphate (48). D-Tagatose 6-phosphate was phosphorylated at about half the rate of D-fructose 6-phosphate. Tagatose 6-phosphate was also reported to be phosphorylated by a highly purified rabbit muscle phosphofructokinase (54) which was found to be specific for the β furanose anomer (53). Shiota et al. (55) found that D-tagatose 6-phosphate was the product of an enzymatic deamination of D-galactosamine 6-phosphate. Markwell et al. (56) reported that D-tagatose 6-phosphate was an intermediate in the pathway of galactitol catabolism in Klebsiella pneumoniae. L-Galactitol 1-phosphate is oxidized with NAD^+ by a dehydrogenase to yield D-tagatose 6-phosphate, which is then phosphorylated with ATP by a kinase to form D-tagatose 1,6-diphosphate. D-Tagatose 6-phosphate

was also discovered to be an intermediate in lactose and galactose metabolism in Staphylococcus aureus (4) and Group N streptococci (57) by Bissett and Anderson. D-Galactose 6-phosphate is converted to D-tagatose 6-phosphate by an isomerase. It has been suggested that D-tagatose 6-phosphate may have physiological significance as part of a mammalian galactose metabolizing pathway in galactosemia (52) but this remains to be proven.

D-Galactose 6-Phosphate

The synthesis of D-galactose 6-phosphate was first described in 1931 by Levene and Raymond (58). It was prepared by treatment of 1,2;3,4-diisopropylidene D-galactose with phosphorus oxychloride, followed by hydrolysis of the protecting groups. D-galactose 6-phosphate has also been reported to be synthesized from D-galactose and polyphosphoric acid by a procedure analogous to that used for the synthesis of D-glucose 6-phosphate (20).

D-Galactose 6-phosphate has been reported to exist in aqueous solution as a mixture of four anomers: the α and β furanoses, and the α and β pyranoses (5, 45, 59, 60). The amount of furanose forms present has been reported anywhere from 26 percent (5) to about 50 percent (45).

D-Galactose 6-phosphate was a reported product of the enzymatic hydrolysis of lactose phosphate by a phospho-β-galactosidase of Staphylococcus aureus (61, 62, 63). Simoni and Roseman proposed that D-galactose-6-phosphate was further catabolized by conversion to 6-phosphogalactonate (64). But in 1973, Bissett and Anderson showed that in S. aureus, galactose 6-phosphate was catabolized through what was termed the tagatose 6-phosphate pathway (4):

D-galactose 6-phosphate is converted to D-tagatose 6-phosphate by an isomerase, and D-tagatose 1,6-diphosphate is produced by the action of a kinase with ATP as phosphoryl donor. The diphosphate is cleaved by an aldolase to yield dihydroxyacetone and D-glyceraldehyde 3-phosphate. The isomerase, kinase, and aldolase were inducible on lactose and galactose, and were distinct from the corresponding enzymes of glucose 6-phosphate metabolism. D-Galactose 6-phosphate was also found to be an intermediate of galactose catabolism in the group N streptococci (57). Galactose was phosphorylated at C-6 by PEP during entrance into the cell via the PEP-dependent phosphotransferase system (65) and then catabolized through the tagatose 6-phosphate pathway.

D-Galactose 6-phosphate has been reported in extracts of the algae Chlorella vulgaris and Scenedesmus obliquus (65). It has also been identified as a hydrolytic product from the cell wall of the fungus Fusicoccum amygdali (66). Davidson (67) reported D-galactose 6-phosphate to be the product of phosphorylation of D-galactose by a hexokinase from Aspergillus parasiticus. D-Galactose was phosphorylated at 88 percent the rate of D-glucose. D-Galactose 6-phosphate also has been found in mammalian systems. Inouye et al. (68) found galactose 6-phosphate in galactosemic erythrocytes, and Musick and Wells (59) reported that galactose 6-phosphate accumulated in mammalian tissues that were incubated with high concentrations of D-galactose to mimic the galactosemic state.

D-Galactose 6-phosphate has been reported to serve as a substrate for a variety of other enzymes. It is acted upon by phosphoglucoisomerase (69), phosphoglucomutase (70), glucose 6-phosphate

dehydrogenase (71), liver galactose 6-phosphate dehydrogenase (72), liver hexose 6-phosphate dehydrogenase (73), NADP L-hexonate dehydrogenase (74), and UDP-glucose: α-1,4-α-4-glucosyltransferase (75). In every case, the K_m values for galactose 6-phosphate were high and the activity low so it is doubted that it serves as a physiologically significant substrate for these enzymes.

D-Galactose 6-Phosphate Isomerase

The inducible D-galactose 6-phosphate isomerase catalyzes the initial step in the D-tagatose 6-phosphate pathway in Staphylococcus aureus, the isomerization of galactose 6-phosphate to tagatose-6-phosphate. The enzyme has been partially purified and characterized from extracts of D-galactose-grown cells (5). The isomerization is reversible and the isomerase catalyzing the reaction is specific for the sugar phosphates D-galactose 6-phosphate and D-tagatose 6-phosphate. The molecular weight of the enzyme is estimated to be about 100,000 both by gel filtration and by sedimentation in a sucrose density gradient. The pH optimum varies from 8.2 to 9.2 depending on the buffer but the enzyme is unstable at pH values below 6.5. The enzyme is induced only by growth of the organism in the presence of D-galactose (specific activity, 0.20 μmole/min/mg protein) and lactose (specific activity, 0.064). The apparent K_m values for D-galactose 6-phosphate and D-tagatose-6-phosphate are reported to be 12 mM and 2.9 mM, respectively.

It is believed that the aldose/ketose interconversion catalyzed by isomerases proceeds through a cis- (rather than a trans-) enediol intermediate (76, 77). Experimental evidence has accumulated favoring a cis-

enediol intermediate in all isomerases examined (78, 79). Therefore, on mechanistic grounds the anomeric configurations of the aldose and ketose products of the D-galactose 6-phosphate isomerase catalyzed reaction would be expected to be α D-tagatose 6-phosphate and α D-galactose 6-phosphate.

MATERIALS AND METHODS

Bacterial Strains and Cell Growth

Bacterial Strains

The organism used as a source of D-galactose-6-phosphate isomerase was Staphylococcus aureus NCTC 8511. Strain GD 29, a mutant of S. aureus NCTC 8511 lacking D-galactose 6-phosphate isomerase (80), was utilized as a source of biosynthetic D-galactose-6-phosphate.

Characterization of Bacteria

The bacterial strains were monitored for contamination by microscopic examination and by observation of colonies plated on nutrient agar. Identification was insured by confirming the carbohydrate utilization patterns for each strain (80) using bromocresol purple broth (81).

Media

Inoculum cultures were grown in media containing 2 percent (w/v) proteose peptone, 0.1 percent (w/v) yeast extract, and 0.2 percent (w/v) K_2HPO_4 (82). When S. aureus was grown in the presence of carbohydrate, the proteose peptone concentration was lowered to 1 percent (w/v), and 1 percent (w/v) D-galactose was added. For short-term storage (1 to 3 months), the cells were maintained at 4°C on an agar slant, which contained 1.5 percent (w/v) agar in addition to the components of the inoculum medium.

Sterilization Technique

The media were sterilized in a Castle autoclave, model Thermomatic 60, for 20 minutes at 121°C and 15 lbs/in^2. Carbohydrates were autoclaved separately from other components of the medium.

Growth Conditions

Cells were grown aerobically by agitation on a New Brunswick Scientific Co. gyrotory shaker, model G10, at 37°C, in the dark. Inoculum cultures were grown overnight in culture tubes (18 x 150 mm) containing 7 ml of the 2 percent proteose peptone medium. The cells were grown in Fernbach flasks containing 1.0 to 1.5 l of 1 percent proteose peptone medium supplemented with D-galactose.

Centrifugation

All centrifugation was carried out in a Sorvall automatic refrigerated centrifuge, model RC2-B, at 4°C, using either the SS-34 or GSA rotor. Exact conditions of time and centrifugal force are specified in each procedure.

Harvesting of Cells

Cells were harvested when the pH of the medium dropped just below 6 (about 5 hours of incubation). Harvesting at this point was critical, because it was noted that D-galactose 6-phosphate isomerase activity decreased significantly if the medium was allowed to become more acidic. Cells were harvested by centrifugation (14,000 X g for 10 minutes in the GSA rotor). The cells were washed once by suspension in cold 0.85 percent (w/v) NaCl and recollected by centrifugation (12,000 X g for 10 minutes in the SS-34 rotor). The cells were either used immediately or stored at -20°C. Some loss (about 20

percent) of D-galactose 6-phosphate isomerase activity was incurred upon storage at -20°C.

Enzyme Purification

Preparation of Buffer Solutions

All buffer solutions were prepared at room temperature (about 25°C). Measurements of pH were carried out on a Beckman Zeromatic pH meter, model 9600, using a Fischer Scientific Co. combination electrode, model 13-639-90. With buffers containing BME, pH readings were taken prior to the addition of this component, since it has been reported that the presence of BME causes erroneous pH readings with many electrodes (83).

Preparation of Glass Beads

Glass beads (88 to 125 μm diameter), were washed by suspension in 3 N HCl and rinsed to neutrality with distilled water. The beads could be reused by washing them free of cellular debris by repeated decantation with hot water, followed by acid-treatment as previously described.

Preparation of Crude Extract

Harvested cells were suspended in 40 mM Tris-HCl buffer (pH 8.5) containing 15 percent (v/v) glycerol, 0.2 percent (v/v) BME, and 0.2 mM EDTA (about 5 ml buffer per gram of wet cells). Cells were broken by exposing the buffered suspension to sonic vibration (ten 1-minute bursts) at 0°C in the presence of twice the packed cell volume of glass beads. (Care must be taken to avoid excessive heating of the suspension. Typically a 1-minute burst of sonic vibration will raise the temperature of the solution from 0°C to 10°C.) The instrument

used was a Heat Systems-Ultrasonics, Inc. model W-185D sonifer cell disrupter, equipped with a micro-probe and set at an output of 100 watts. Glass beads were added to the suspension because they are known to be effective in the disruption of S. aureus (84). Cellular debris and glass beads were removed by centrifugation (10 minutes at 40,000 X g in the SS-34 rotor) and the resulting supernatant solution was used as the crude extract.

Bentonite Treatment

The crude extract was stirred at 4°C, and bentonite (40 mg per ml of crude extract) was added. After 10 minutes of vigorous stirring, the suspension was clarified by centrifugation (14,000 X g for ten minutes in the SS-34 rotor). The precipitate was discarded. (Typically, a two-fold increase in specific activity was obtained by this step with about 90 percent recovery of total activity.)

Hydroxyapatite Chromatography

Hydroxyapatite resin (Bio-Gel HTP) was suspended in 40 mM Tris-HCl buffer (pH 7.5) containing 15 percent (v/v) glycerol, 0.2 percent (v/v) BME, and 0.2 mM EDTA. Columns were poured at 4°C and washed with the above buffer until the effluent had the same pH as the buffer. The supernatant solution from the bentonite treatment was loaded onto the column which was then washed with the above buffer until no more protein eluted. (Typically, about a two-fold increase in specific activity was achieved by this step, but with only about a 50 percent recovery of total activity. Also, if the resin were not exhaustively washed with buffer, total inactivation of the enzyme occurred. For these reasons, in most purifications DEAE-cellulose chromatography replaced this step.)

DEAE-Cellulose Chromatography

DEAE-cellulose was pretreated with sequential washes of NaOH, HCl, and NaOH (85). Columns were poured at 4°C and washed with buffer until the effluent had the same pH as the buffer. The supernatant from the bentonite treatment was layered onto the column which was then washed with buffer until no more protein eluted. The enzyme then eluted with a 0.1 M to 0.8 M KCl gradient (10 times the column bed volume). (Typically, a five-fold increase in specific activity was achieved by this step, with a 60 percent recovery of total activity.) To prepare the DEAE-cellulose for further use, the column was washed with 2 M NaCl to remove any tightly bound material. When not in use, the DEAE-cellulose was stored at 4°C in 1 M NaCl.

Sephadex G-100 Chromatography

Sephadex G-100 gel was pretreated by soaking for 24-48 hours at room temperature in the desired buffer. Columns were poured at 4°C and washed with about five bed volumes of the appropriate buffer. The sample from a previous purification step was applied to the column and chromatographed with the same buffer used to equilibrate the gel. After use, the gel was washed with 0.02 percent (w/v) sodium azide and stored at 4°C.

Ultrafiltration

Purified enzyme solutions were concentrated by ultrafiltration using the Amicon Diaflo, model 10, filtration cell with an Amicon Diaflo UM-10 membrane (25 mm) under 30 lbs/in^2 N_2 at 4°C.

Preparation of Substrates

Removal of Barium from Sugar Phosphates

An aqueous solution of the barium salt of the sugar phosphate

was treated at room temperature with Dowex 50W-X8 resin (H^+ form) until the pH was 1.5. The solution was separated from the resin and carefully titrated to pH 7.0 with aqueous NaOH. The resultant disodium salt of the sugar phosphate was stored at -20°C either in aqueous solution or as the lyophilized powder.

Biosynthesis of D-Galactose 6-Phosphate

Staphylococcus aureus, strain GD 29, was reconstituted from a frozen glycerol suspension. An overnight routine broth culture was inoculated into one liter of induction broth consisting of 1 percent (w/v) proteose peptone, 0.1 percent (w/v) yeast extract, and 0.2 percent (w/v) K_2HPO_4, in a Fernbach flask. After 5 hours on a rotary shaker at 37°C, D-galactose was added to a concentration of 1 percent (w/v). Incubation was continued for an additional 5 hours.

The cells were harvested by centrifugation (12,000 X g for 10 minutes in the GSA rotor), and washed with 0.85 percent (w/v) NaCl. The pellet was extracted by suspension for 5 minutes in 80 percent (v/v) ethanol (10 ml per gram of wet cells); ethanol has been shown to lyse effectively S. aureus (86). Cell debris was collected by centrifugation (14,000 X g for 10 minutes in the SS-34 rotor) and the bright-yellow supernatant was saved. The pellet was washed twice with 30 ml portions of H_2O, and the three supernatant fractions were combined.

The ethanol was removed in vacuo with a Buchi rotary evaporator, model R, and the remaining solution was lyophilized using a Virtis Automatic Freeze-Dryer, model 10-010. The lyophilized powder was dissolved in 1.5 ml H_2O and clarified by centrifugation (6,000 X g for 10 minutes in the SS-34 rotor). This supernatant contained the

accumulated metabolite, D-galactose 6-phosphate, as determined by enzymatic assay.

Chemical Synthesis of D-Galactose 6-Phosphate

The procedure, detailed below, was that of Seegmiller and Horecker (20), with modifications.

Polyphosphoric acid (23 g) was weighed into a 100 ml beaker. Water (2.3 ml) was carefully added (exothermic reaction), and the solution was cooled to 5-10°C in the cold room. D-Galactose (10 g) was added to the viscous solution while being vigorously stirred with a glass rod to disperse the sugar uniformly. The mixture was allowed to stand at room temperature for 16 hours, during which time it turned light brown. The reaction was terminated by the addition of 57.5 ml of distilled H_2O.

Anhydrous sodium carbonate (30 g) was added to the reaction mixture with stirring. To remove CO_2, the mixture was warmed to 60°C and evacuated using a water aspirator. The solution (the pH is now 7.3-7.6) was diluted with 150 ml of H_2O and cooled overnight at 4°C with stirring. (To reduce the rate of cooling, the solution was placed in a large water bath which was initially at 40°C.) The resulting dense slurry of sodium polyphosphate crystals which resulted was cooled in an ice bath with stirring for 4 hours and thoroughly suction-filtered. The filtrate contained the polyphosphorylated galactose.

To the filtrate was added concentrated (8.9 N) HBr (0.2 ml acid per ml of filtrate). The mixture was refluxed for 24 hours at 100°C in a round-bottomed flask equipped with stirring bar and water-cooled condenser.

The brown hydrolysate was treated at room temperature with barium carbonate (0.2 g per ml of hydrolysate) and stirred for 4 hours. The addition of several drops of n-octanol was helpful in reducing foaming. After 4 hours, the pH of the mixture was 6.4. The mixture was suction-filtered and the residue was washed twice by suspending it in 10 ml of distilled water. The filtrate and washings were combined and treated with 4 volumes of 95 percent ethanol. The flocculant precipitate was allowed to settle and was collected by centrifugation (14,000 X g for 10 minutes in the GSA rotor). The precipitate was extracted four times with 10 ml portions of distilled H_2O and the final brown residue was discarded. The extracts were pooled, and the amber solution decolorized by treatment with 50 mg of activated charcoal for 2 hours with stirring. The solution was suction-filtered and the filtrate treated with four volumes of 95 percent ethanol. The resulting white precipitate of the barium salt of D-galactose 6-phosphate was collected by gentle suction-filtration. Yield: 1.9 g.

Synthesis of D-Tagatose 6-Phosphate

The procedure followed for the synthesis of D-tagatose 6-phosphate from D-galacturonic acid involved the combination of three published syntheses (48, 50, 51) and was essentially as outlined by Bissett (5), with some modifications. Because of the complexity of the synthesis, and because certain modifications led to increased yields of some steps and higher purity of final product, a detailed description of the procedure will be presented.

$Ca(OH)_2$ (3.8 g) was dissolved in 2500 ml of distilled water at room temperature. The cloudy solution was clarified by a single pass through Whatman No. 2 filter paper using gentle suction-filtration. The alkaline solution (pH 13) was equilibrated to 4°C in the cold room. D-Galacturonic acid (10 g; 51 mmoles) was added to the cold solution with vigorous stirring. The D-galacturonate had previously been purified by recrystallization according to the procedure of McCready (87). The solids dissolved easily, and stirring was continued at 4°C for one hour. The solution was removed to room temperature and allowed to stand for 10 days, during which time crystals of the calcium salt of D-tagaturonic acid precipitated. The pH of the supernatant solution was 10.5. The crystals were collected by suction-filtration, washed once with cold water and dried over $CaSO_4$. Yield: 9.1 g (39 mmoles).

The calcium salt of D-tagaturonic acid (9.1 g; 39 mmoles) was added to a solution of dry acetone (distilled over Na_2SO_4) and H_2SO_4, and stirred for 4 hours at room temperature in a vessel equipped with a water-cooled condenser (for each gram of D-tagaturonate, 19.5 ml of dry acetone and 0.8 ml of concentrated H_2SO_4 were present). The solution was rapidly neutralized with an excess of $Ca(OH)_2$ suspended in H_2O (about 1.5 g $Ca(OH)_2$ for each ml of concentrated H_2SO_4 initially present). Rapid neutralization was critical because in an acidic medium in the presence of H_2O, hydrolysis of isopropylidene groups will occur, resulting in reduced yields. Once the pH reached neutrality, the mixture was rapidly suction-filtered and the supernatant, which turned slightly basic, was

adjusted to pH 7.0 with H_2SO_4. The solution was lyophilized to a fine white powder, the calcium salt of 1,2;3,4-di-O-isopropylidene-D-tagaturonic acid. Yield: 6.0 g (19 mmoles).

The calcium salt of 1,2;3,4-di-O-isopropylidene-D-tagaturonate (6.0 g; 19 mmoles) was converted to the free acid by addition to a solution of 100 ml H_2O, 100 ml of ethyl ether, and 32.8 ml of 10 percent (w/v) H_2SO_4 with vigorous stirring for 5 minutes at 4°C. The ether phase was washed 4 times with 100 ml portions of cold water in a separatory funnel, dried over Na_2SO_4, and evaporated to dryness on the Buchi rotary evaporator. It was found that the yield could be increased from that previously reported by backwashing the water phases (the initial water phase and the 4 water washes) with ether, and evaporating the ether washes to dryness as above. It was noted that the white crystals of product were discolored yellow if dried in the presence of even trace amounts of H_2O. It was important to avoid this discoloration because it would interfer with the detection of excess diazomethane, crucial in the next step of the synthesis. Yield: 4.1 g of 1,2;3,4-di-O-isopropylidene-D-tagaturonic acid (15 mmoles).

Ethereal diazomethane, used as an esterifying agent in the next step of the synthesis, was generated from Diazald (N-methyl-N-nitroso-p-toluenesulfonamide) as detailed by Bissett (5). Because of the toxic and potentially explosive nature of diazomethane, special precautions (88) were taken in its generation and handling.

1,2;3,4-di-O-isopropylidene-D-tagaturonic acid (4.1 g; 15 mmoles) was dissolved in a minimal amount of ether (about 30 ml of ether per gram), and ethereal diazomethane was added to excess, which

was detected when the solution turned a bright yellow. The solution was allowed to stand for 45 minutes to insure completion of the esterification reaction, and concentrated to a golden-yellow syrup, the methyl ester of 1,2;3,4-di-O-isopropylidene tagaturonic acid, by removal of ether on the rotary evaporator at 30°C. The syrup was dissolved in 150 ml of anhydrous ether in a round-bottom flask fitted with a drying tube and water-cooled condenser. Finely crushed $LiAlH_4$ (3.7 g) was gradually added with rapid stirring and the reaction was allowed to proceed for 5 hours. Because of the potentially explosive nature of the reaction, all work was done behind protective shields in a hood. The reaction was terminated by the addition of ethyl acetate to destroy excess $LiAlH_4$; the end-point was detected as the absence of bubbling upon the careful addition of several drops of H_2O. Water (150 ml) was added, and the organic solvents were removed by rotary evaporation at room temperature. The solution (pH 11.5) was adjusted to near neutrality with 10 percent (v/v) acetic acid, filtered, and neutralized (from pH 7.5). The solution was extracted with an equal volume of chloroform. The chloroform phase was washed with a smaller volume of water, dried over Na_2SO_4, and evaporated to a thin, transparent syrup on the rotary evaporator at room temperature. Three recrystallizations from petroleum ether (glass-distilled) at 4°C gave white needles of 1,2;3,4-di-O-isopropylidene-D-tagatose. Recrystallization was difficult, and the critical volume (about 50 ml) of petroleum ether had to be determined empirically. Yield: 1.6 g (6 mmoles).

In the following step of the synthesis, the modifications of Koerner et al. (52) were adopted. 1,2;3,4-di-O-isopropylidene-D-tagatose (1.5 g; 5.8 mmoles) was dissolved in 14 ml of cold, dry pyridine (distilled over KOH). With constant stirring at 0°C, 1.9 ml (1.5 molar equivalents) of diphenyl chlorophosphate were added dropwise. A precipitate formed and the mixture was stirred for an additional 30 minutes at 0°C before being placed at 4°C for 70 hours. The reaction was terminated by pouring the mixture onto 200 ml of finely cracked distilled water ice with vigorous stirring. The slurry was stirred for 30 minutes at 4°C, poured into a separatory funnel, and extracted with 125 ml of $CHCl_3$. The chloroform phase was washed once with 25 ml of 10 percent (w/v) HCl, 4 times with 25 ml portions of H_2O, dried over Na_2SO_4, and concentrated to a syrup on the rotary evaporator. The syrup was taken up in 200 ml of ethanol, and distilled H_2O (about 250 ml) was added until the solution became turbid. Flocculant, white silky crystals of 1,2;3,4-di-O-isopropylidene-D-tagatose 6-diphenylphosphate readily formed upon storage at 4°C for 48 hours. The crystals were collected and dried by suction-filtration using a large (2000 ml) Buchner funnel to maximize surface area. The product was stored over $CaSO_4$. Yield: 2.3 g (4.7 mmoles).

1,2;3,4-di-O-isopropylidene-D-tagatose 6-diphenylphosphate (2.3 g; 4.7 mmoles) was dissolved in 50 ml of absolute ethanol. PtO_2 (0.21 g) was added and the mixture was shaken in a Parr pressure reaction apparatus for 5 hours under 20 lbs/in^2 H_2. (The PtO_2 catalyst had previously been activated by wetting with a small amount of absolute ethanol and shaking in the Parr pressure

apparatus for 10 minutes under 20 lbs/in^2 H_2). When the reaction was complete (no more H_2 being consumed), the supernatant was carefully pipetted away from the catalyst and clarified by centrifugation (12,000 X g for 10 minutes in the SS-34 rotor). The solution was concentrated to a clear syrup on the rotary evaporator. The syrup was taken up in 100 ml of pentane, a few drops of water were added, and the solution was placed at 4°C for 48 hours. White needles of 1,2;3,4-di-O-isopropylidene-D-tagatose 6-phosphate were collected by suction-filtration and dried over $CaSO_4$. Yield: 1.3 g (3.8 mmoles).

1,2;3,4-di-O-isopropylidine-D-tagatose 6-phosphate (1.3 g; 3.8 mmoles) was dissolved in 15 ml of 0.05 N H_2SO_4 and heated on a steam bath for 30 minutes. Aqueous $Ba(OH)_2$ (CO_2-free) was then added until the pH was 10.2. The $BaSO_4$ which resulted was removed by repeated filtration through Whatman No. 5 paper. The solution was treated with 4 volumes of 95 percent (v/v) ethanol to precipitate the barium salt of D-tagatose 6-phosphate, and stored at 4°C for 24 hours. The product was collected by centrifugation (14,000 X g for 10 minutes in the GSA rotor), and washed with absolute ethanol, absolute ethanol:ethyl ether (4:1, 1:1, 1:4), and ethyl ether (twice). Yield: 1 g (2.5 mmoles).

Colorimetric Determinations

Protein Determination

Protein was determined by the method of Bradford (89). This method was selected because the presence of BME in samples to be assayed does not interfere with color development. Bovine serum albumin was used as the standard.

Total Carbohydrate Determination

Total carbohydrate was determined by the phenol-sulfuric acid method of Smith et al. (90).

Aldohexose Determination

Aldohexose was determined by the o-aminodiphenyl method of Timell et al. (91). D-Glucose and D-glucose 6-phosphate were used as standards. (o-Aminodiphenyl is a suspected carcinogen and this assay was used only when it was necessary to distinguish an aldohexose in the presence of a ketohexose.)

Ketohexose Determination

Ketohexose was determined by the resorcinol method of Roe (92) as described by Ashwell (93). D-Fructose, D-fructose-6-phosphate, and D-fructose 1,6-diphosphate were used as standards.

Phosphate Determinations

Inorganic phosphate was determined by the Fiske-SubbaRow method (94) as modified by Clark (95). Total phosphate was determined by the method of Umbreit et al. (96). KH_2PO_4 was used as the standard.

Enzymatic Assays

Assays that involved the oxidation of NADH were carried out in a total volume of 0.15 ml in quartz microcurvettes with a 1.0 cm light path. Absorbance at 340 nm was monitored using a Beckman recording spectrophotometer, model DUR, equipped with a Gilford absorbance indicator, model 220, and interfaced to a Sargent recorder, model SRL. The temperature of the assays was maintained at 25°C with a Precision Scientific Co. constant temperature

circulating water bath, model 10-R-1. One unit of enzyme activity was defined as that amount of enzyme which catalyzed the transformation of 1.0 μmole of substrate per minute at 25°C.

D-Galactose 6-Phosphate Isomerase

D-Galactose 6-phosphate isomerase activity was determined by the method of Bissett (5), using an assay coupled to NADH, with D-galactose 6-phosphate as substrate.

D-Galactose 6-Phosphatase

D-Galactose 6-phosphatase activity was assayed as described by Bissett (5), using Bicine buffer (pH 8.2).

D-Galactose 6-Phosphate Isomerase Coupled Reaction

In experiments where it was desired to drive the D-galactose 6-phosphate isomerase-catalyzed reaction to completion, the reaction was coupled to rabbit muscle fructose 6-phosphate kinase. Rabbit muscle fructose 6-phosphate kinase phosphorylates D-tagatose 6-phosphate (97), the product of the isomerase reaction. The reaction mixture (0.2 ml) consisted of 16 μmoles of Bicine buffer (pH 8.2), 3.0 μmoles of $MgCl_2$, 1.5 μmoles of ATP, 1.0 μmoles of D-galactose 6-phosphate, 20 mU of D-galactose 6-phosphate isomerase, and non-limiting amounts of rabbit muscle fructose 6-phosphate kinase.

Enzymatic Determinations of Substrate Concentration

Because of the ability of sugar phosphates to readily form hydrates and because of the possible presence of contaminants, the determination of substrate concentration on a by-weight basis was not considered accurate enough in many cases. In such cases, substrate concentrations were determined by enzymatic methods of analysis.

Knowing the stoichiometry of the enzymatic reaction, the molar extinction of NADH (or NADPH), and the volume of the reaction mixture, the total number of μmoles of substrate converted in the reaction was calculated. With this value, and knowing the volume of the substrate solution added to the assay, the concentration of the substrate solution could be calculated. Absorbances at 340 nm were monitored with the Beckman spectrophotometer described earlier. All assays were carried out at 25°C in quartz microcurvettes. The reactions were allowed to proceed to completion, and the total changes in absorbance were determined.

D-Galactose 6-Phosphate

D-Galactose 6-phosphate concentration was determined as described by Musick and Wells (59). The reaction mixture (0.15 ml) consisted of 15 μmoles of Tris-HCl buffer (pH 8.1), 0.075 μmole of NAD^+, 75 mU of β-galactose dehydrogenase, 400 mU of E. coli alkaline phosphatase, and D-galactose 6-phosphate. The control to correct for possible NAD reductase activity was minus D-galactose 6-phosphate. Typically, the reaction reached completion in about 60 minutes.

D-Tagatose 6-Phosphate

The concentration of D-tagatose 6-phosphate was determined by phosphorylating it to an end-point with rabbit muscle fructose 6-phosphate kinase. The reaction mixture (0.15 ml) contained 10 μmoles of glycylglycine buffer (pH 7.5), 1.0 μmole of $MgCl_2$, 0.5 μmole of ATP, 0.5 μmole of PEP, 0.05 μmole of NADH, non-limiting amounts of pyruvate kinase, lactate dehydrogenase, and fructose 6-phosphate

kinase, and D-tagatose 6-phosphate. The control to correct for ATPase and NADH oxidase was minus D-tagatose 6-phosphate.

D-Fructose 6-Phosphate

The assay was identical to that just described for D-tagatose 6-phosphate except that it contained D-fructose 6-phosphate instead of D-tagatose 6-phosphate as the substrate for the kinase.

D-Glucose 6-Phosphate

The concentration of D-glucose 6-phosphate was determined by reacting it to completion with glucose 6-phosphate dehydrogenase. The reaction mixture (0.15 ml) contained 10 μmoles of glycylglycine buffer (pH 7.5), 1.0 μmole of $MgCl_2$, 0.1 μmole of $NADP^+$, a non-limiting amount of glucose 6-phosphate dehydrogenase, and D-glucose 6-phosphate. The control to correct for $NADP^+$ reductase activity was minus D-glucose 6-phosphate.

D-Tagatose 1,6-Diphosphate

The concentration of D-tagatose 1,6-diphosphate was determined using D-tagatose 1,6-diphosphate aldolase. The reaction mixture (0.15 ml) consisted of 10 μmoles of HEPES buffer (pH 7.0), 1.0 μmole of $MgCl_2$, 0.05 μmole of NADH, non-limiting amounts of triose phosphate isomerase, α-glycerophosphate dehydrogenase, and tagatose 1,6-diphosphate aldolase, and D-tagatose 1,6-diphosphate.

D-Fructose 1,6-Diphosphate

The concentration of D-fructose 1,6-diphosphate was determined by using rabbit muscle aldolase. The reaction mixture (0.15 ml) contained 10 μmoles of glycylglycine buffer (pH 7.5), 1.0 μmole of $MgCl_2$, 0.05 μmole of NADH, non-limiting amounts of triose phosphate isomerase, α-glycerophosphate dehydrogenase, and rabbit

muscle aldolase, and D-fructose 1,6-diphosphate. Controls to correct for NADH oxidase activity were minus fructose 1,6-diphosphate.

Instrumental Methods of Analysis

Gas-Liquid Chromatography

All samples were lyopholized and either silylated immediately or stored in a desiccator. Trimethylsilyl derivatives of the carbohydrates and carbohydrate-phosphates used in this study were prepared with the silylating reagent of Bentley and Botlock (44). The lyophilized sample (0.3-1.0 mg) was treated with 0.2-0.4 ml of silylating reagent, vigorously stirred, and allowed to stand at room temperature for 5 minutes, at which time the reaction was complete. The samples, stored in tightly closed vials, were stable for 12-14 hours. Trimethylsilyl O-methyloxime derivatives of D-galactose 6-phosphate and D-tagatose 6-phosphate were prepared by the method of Laine and Sweeley (98).

Samples containing 1-10 μg were injected into a Hewlett-Packard Gas Chromatograph, model 5830A, with an injector port temperature of 250°C, and a flame-ionization detector temperature of 320°C. The recorder chart speed was 0.60 cm/min; attenuation settings were varied to accommodate the particular sample being analyzed. The GLC column had dimensions of 1.8 meters X 2 mm. A variety of column packings were used during the course of the study. Column temperature and carrier gas (N_2) flow-rate for each packing material are listed in parentheses: 3% XE-60 (175°C, 23 ml/min), 3% OV-17 (190°C, 25 ml/min), 3% OV-7 (220°C, 23 ml/min),

and 3% OV-1 (200°C, 30 ml/min for sugar phosphates: 160°C, 19 ml/min for free sugars).

Combined Gas-Liquid Chromatography-Mass Spectrometry

Samples were silylated as described previously. Analyses were carried out on an LKB 9000 combined gas-liquid chromatograph-mass spectrometer. Instrument conditions were as follows: accelerating voltage of 3555 V, ionizing electron energy of 70 eV, ion source temperature of 290°C, molecular separator temperature of 250°C, and a repetitive scanning range of 50-650 M/e. The GLC column dimensions were 1.8 m X 3 mm. The packing materials used and the column temperatures for each were as follows: 3% XE-60 (175°C), 3% OV-1 (200°C), and 3% OV-17 (190°C). Mass spectral data were compiled in tabular form and plotted as bar graphs using a computerized data-acquisition system interfaced to the mass spectrometer.

Carbon-13 Nuclear Magnetic Resonance Spectroscopy

Non-^{13}C-enriched (natural abundance) samples of sugar-phosphates were prepared at a concentration of 0.5 M in a total volume of 0.3 ml. The instrument used was a Bruker WP-60, 15.08 MHz Fourier-transform spectrometer equipped with quadrature detection. Spectra were obtained at 23°C, with 4 K 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 were given relative to external tetramethylsilane (Me_4Si) and were accurate to within ± 0.1 ppm.

Ion Exchange Chromatography

Commercial D-galactose 6-phosphate was purified by anion-exchange chromatography using a triethylammonium tetraborate

gradient (38). Dowex 1-X4 (200-400 mesh) resin was washed according to the procedure of Brewer, Pesce, and Ashworth (99). A column (1.0 X 30 cm) was poured, and washed with 0.8 M potassium tetraborate until all the chloride had been displaced. The column was then washed with about 10 bed volumes of H_2O. A commercial sample of the disodium salt of D-galactose 6-phosphate (10-100 mg) was converted to the free ester by treatment to pH 1.5 with Dowex 50W-X8 (H^+ form) resin. The supernatant solution was adjusted to pH 8.0 with ammonium hydroxide, and loaded onto the column. The column was washed with about 3 bed volumes of H_2O, and eluted with a linear gradient of 0.1-0.4 M triethylammonium tetraborate (400 ml). Following the gradient elution, the column was washed with 0.4 M triethylammonium tetraborate (100 ml). Fractions (1.5 ml) were collected during both the gradient and 0.4 M tetraborate elutions. Fractions were assayed for total carbohydrate as described previously. Peak fractions were pooled and desalted by repeated evaporations to dryness with methanol. The samples were dissolved in H_2O and converted to the disodium salts by titration to pH 7.0 with NaOH.

Sources of Materials

S. aureus NCTC 8511 was supplied by the National Collection of Type Cultures, London, England. S. aureus, mutant GD 29, D-tagatose 1,6-diphosphate aldolase, and the D-tagatose 6-phosphate used in the initial phase of the project, were graciously supplied by D.L. Bissett. Nutrient agar, proteose peptone, and yeast extract were purchased from Difco Laboratories. D-Galactose, D-galacturonic-acid, D-glucose, D-fructose, L-arabinose, D-glucose 6-phosphate, D-galactose 6-phosphate, D-fructose 6-phosphate, D-fructose 1,6-

diphosphate, BME, Tris, Bicine, glycylglycine, DEAE-cellulose, polyphosphoric acid, bovine serum albumin, HEPES, ATP, PEP, NADH, NAD^+, NADPH, $NADP^+$, (Type III) rabbit muscle fructose 6-phosphate kinase (E.C.2.7.1.11), β-galactose dehydrogenase (E.C.1.1.1.48), (Type III) E. coli alkaline phosphatase (E.C.3.1.3.1), (Type II) pyruvate kinase (E.C.2.7.1.40), (Type III) lactate dehydrogenase (E.C.1.1.1.27), (Type XI) glucose 6-phosphate dehydrogenase (E.C.1.1.1.49), (Type III, mixed crystalline suspension) α-glycerophosphate dehydrogenase (E.C.1.1.1.8)/triose phosphate isomerase (E.C.5.3.1.1.), and (Grade I) rabbit muscle aldolase (E.C.4.1.2.13) were purchased from Sigma Chemical Co. Diazald was supplied by Aldrich Chemical Co. Sephadex G-100 was from Pharmacia Fine Chemicals, Inc. Trimethylchlorosilane and hexamethyldisilazane were purchased from Regis Chemical Co., EDTA from Mallinckrodt Chemical Co., bentonite from Fisher Scientific Co., and hydroxyapatite from Biorad Laboratories. D-Galactitol 6-phosphate, prepared from commercial D-galactose 6-phosphate by sodium borohydride reduction, was kindly supplied by G.T. Shimamoto. Glass beads (88 to 125 μm diameter) were from LaPine Scientific Co.

RESULTS AND DISCUSSION

Evidence for Mutarotation of D-Galactose 6-Phosphate

One goal of this thesis research was to confirm, and establish the cause of, the apparent lack of mutarotation of D-galactose 6-phosphate reported in a previous study (5; also see the General Discussion Section of this thesis). An initial effort was undertaken to confirm this apparent lack of mutarotation by different methods than those used in the previous study.

The reverse D-galactose 6-phosphate isomerase-catalyzed reaction (i.e., with D-tagatose 6-phosphate as initial substrate) was investigated by natural abundance ^{13}C-NMR spectroscopy. If D-galactose 6-phosphate did, in fact, fail to mutarotate, one would predict that the resonance of only one anomeric carbon of D-galactose 6-phosphate (that of the active anomer produced by the isomerase) would be observed in the ^{13}C-NMR spectrum of the reverse reaction. The technique of ^{13}C-NMR spectroscopy is particularly well-suited to such an investigation because the resonances of the anomeric carbons are at substantially lower fields than those of the remaining carbons, and the identification of these resonances in each spectrum is therefore facilitated (46). Also, the anomeric carbon atoms of the α and β pyranose tautomers of galactose 6-phosphate (100; also see the following text) and the α and β

furanose tautomers of tagatose 6-phosphate (53) appear as four separate and distinct resonances, and are readily identified by their characteristic chemical shifts. It should be noted that no ^{13}C-NMR data for galactose-6-phosphate exists in the literature. Therefore, assignments of resonances due to the α and β anomeric carbons of galactopyranose 6-phosphate must be made by comparison to the characteristic chemical shifts reported for D-galactopyranose (100). Such a comparison is justified since phosphorylation at C-6 of a hexose has little effect (a few ppm) on the chemical shift of the anomeric carbon (47, 101, 102, 103).

Reaction conditions were set up to closely parallel those under which it was reported that D-galactose 6-phosphate had failed to mutarotate (5). D-Tagatose 6-phosphate (0.15 mmole) was incubated with 450 mU of D-galactose 6-phosphate isomerase in 0.3 ml of 0.14 M Bicine buffer (pH 8.2) at 23°C. The progress of the reaction was followed by measuring the disappearance of ketohexose using the Roe method (93). When the reaction neared apparent equilibrium (40 percent ketohexose), a ^{13}C-NMR spectrum was obtained. Surprisingly, two anomeric peaks for D-galactose 6-phosphate were observed (Figure 1). Furthermore, the ratio of the peak areas for the α and β anomers were similar to those obtained from a ^{13}C-NMR spectrum of commercial D-galactose 6-phosphate (Figure 2) known to be at anomeric equilibrium. These data indicate that D-galactose-6-phosphate did mutarotate under the conditions of the reverse isomerase reaction, and that mutarotation occurred to the extent that anomeric equilibrium was achieved within the time frame of the experiment (about 5 hours).

Figure 1. Natural abundance ^{13}C-NMR spectroscopy of the reverse galactose 6-phosphate isomerase-catalyzed reaction mixture at equilibrium. σ values are relative to TMS = 0. Resonances due to the α and β anomeric carbons of D-galactopyranose 6-phosphate occur at -93.6 ppm and -97.7 ppm, respectively (100).

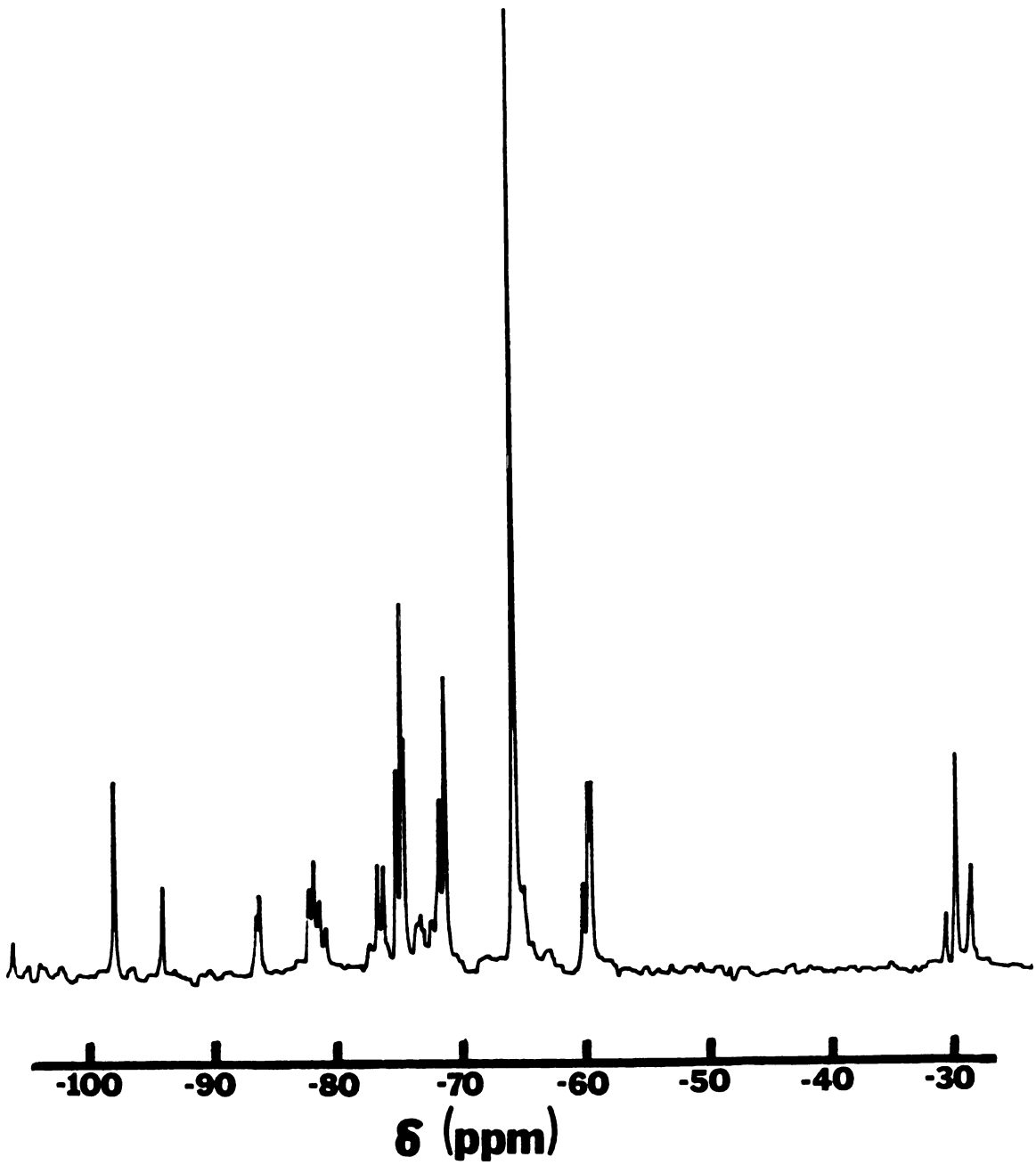


Figure 1.

Figure 2. Natural abundance ^{13}C-NMR spectroscopy of commercial D-galactose 6-phosphate. The 0.5 M sample was run in 0.3 ml of 0.14 M Bicine, pH 8.2, at 23°C. σ values are relative to TMS = 0. Resonances due to the α and β anomeric carbons of D-galactopyranose 6-phosphate occur at -93.2 ppm and -97.2 ppm, respectively (100).

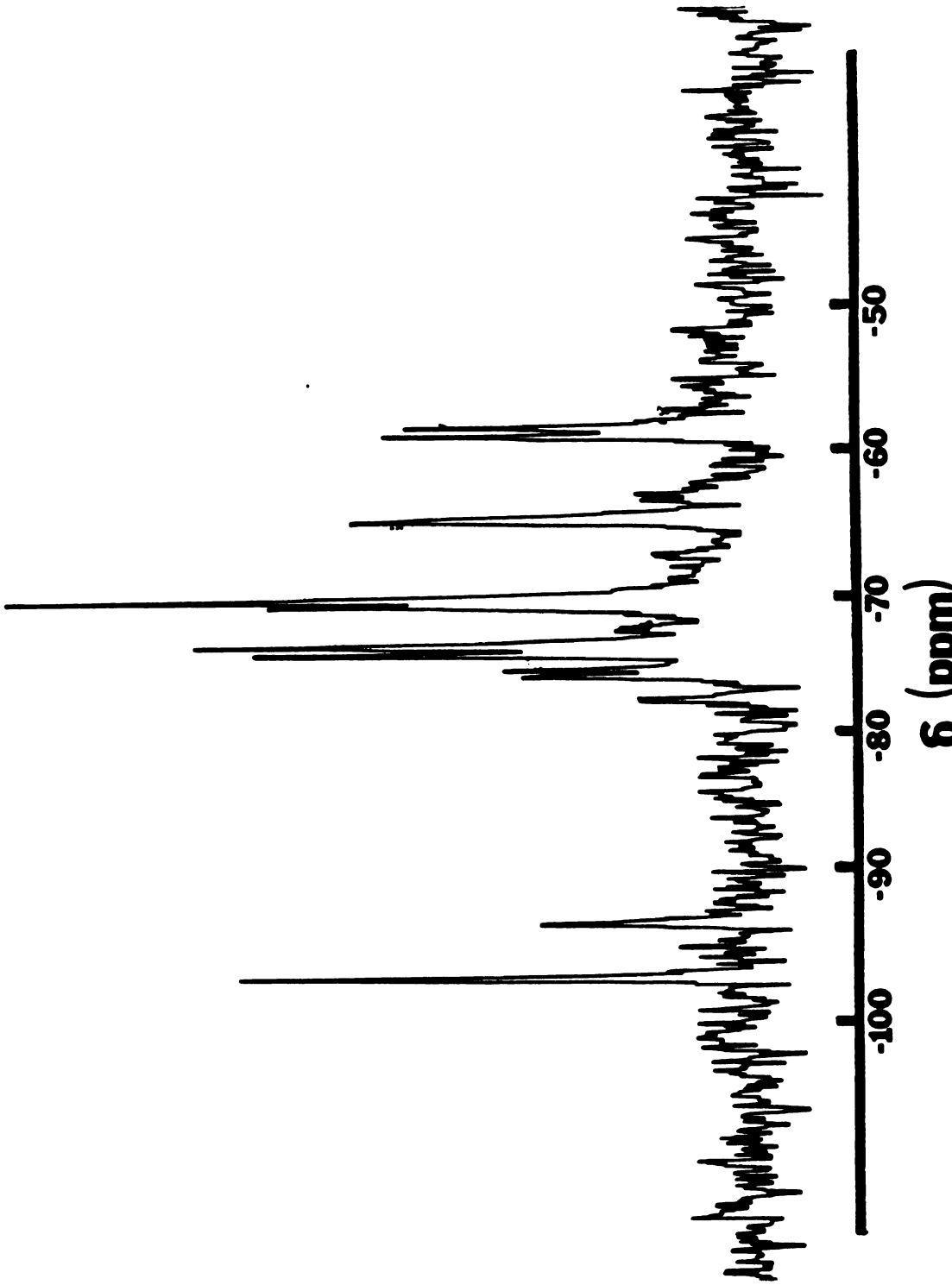


Figure 2.

Since the above results conflict with the findings of Bissett (5) obtained by another method (GLC), an attempt was made to determine the reason for the discrepancy. Effects of temperature and pH were ruled out since these parameters in the present investigation were identical to those used by Bissett. Possible differences in the purity of the isomerase preparations were considered inconsequential since Bissett reported a lack of mutarotation whether a crude extract or a purified isomerase preparation was used.

A difference in reaction conditions of the two studies was the molar ratio of Bicine to D-galactose 6-phosphate: it was 1:2 in the present investigation and 15:1 in Bissett's experiments. Through the use of molecular models I theorized that Bicine could bind to D-galactose 6-phosphate in such a way as to prevent mutarotation (see General Discussion). Since Bicine was present in excess in Bissett's experiments but not even present in stoichiometric amount in the ^{13}C-NMR study, the lack of mutarotation of D-galactose 6-phosphate observed by Bissett and the presence of mutarotation in the ^{13}C-NMR experiment could be explained by the 'Bicine-binding' theory.

Molecular models suggested that Bicine could similarly bind to D-glucose 6-phosphate in such a way as to inhibit its mutarotation. The glucose 6-phosphate dehydrogenase reaction was used to test this hypothesis. The dehydrogenase is specific for the β anomer of D-glucopyranose 6-phosphate (104). The anomeric composition of D-glucose 6-phosphate in aqueous solution is 39 percent α pyranose and 61 percent β pyranose (104), so one would predict the dehydrogenase reaction to go to 61 percent completion and stop if mutarotation

were inhibited in the presence of Bicine. It was found, however, that the dehydrogenase reaction went rapidly to 100 percent completion even when the molar ratio of Bicine to sugar phosphate was over 1000:1. This was strong evidence that Bicine did not bind to D-glucose 6-phosphate in such a way as to prevent mutarotation.

Next, data were obtained to determine the effects of Bicine on the mutarotation of D-galactose 6-phosphate. It is known that cyanide adds only to the open-chain form of an aldo-sugar (105) in the well known Kiliani cyanohydrin reaction (106). Since there appears to be a relationship between mutarotation rate and the proportion of the open form of the sugar (107), investigators have measured the rate of the cyanohydrin reaction to gain an approximation of mutarotation rate (108). D-galactose 6-phosphate was reacted with NaCN in the presence of Bicine (pH 8.2), at 25°C; the molar ratio of Bicine to sugar phosphate was 20:1. The progress of the reaction was followed by measuring the disappearance of reducing sugar using the phenol/H_2SO_4 assay (90). Cyanide added readily to D-galactose 6-phosphate (Figure 3) indicating that mutarotation occurred in the presence of Bicine. Furthermore, the data indicated that mutarotation of D-galactose 6-phosphate proceeded even faster than that of D-glucose 6-phosphate (Figure 3).

Further evidence that D-galactose 6-phosphate freely mutarotated was obtained by coupling the forward D-galactose 6-phosphate isomerase reaction (i.e., with D-galactose 6-phosphate as initial substrate) to rabbit muscle fructose 6-phosphate kinase. Fructose 6-phosphate kinase phosphorylates D-tagatose 6-phosphate (97), the product of the

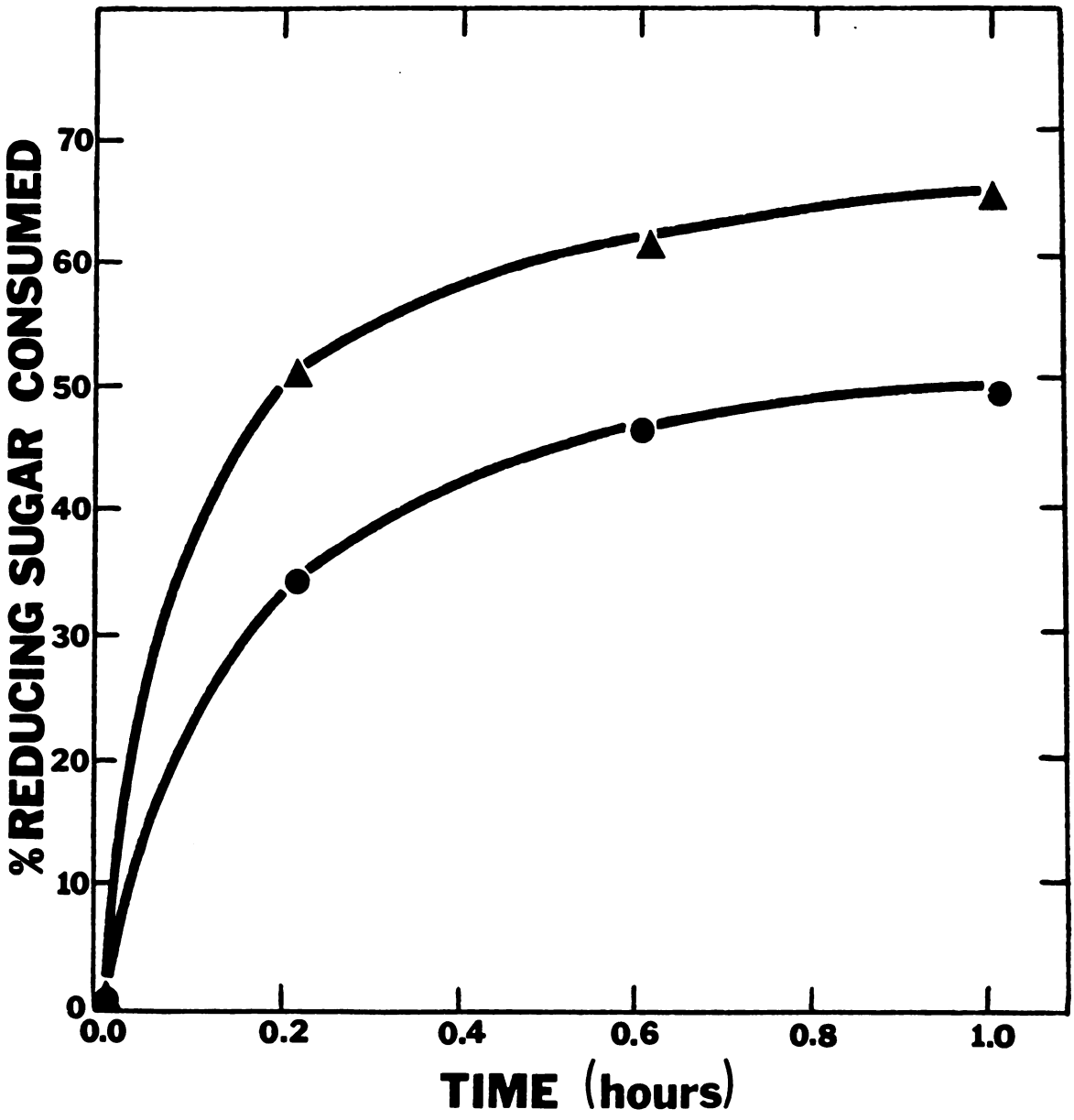


Figure 3. Cyanohydrin reaction rate with D-galactose 6-phosphate: evidence for mutarotation. The reaction mixture (2.0 ml, 25°C) contained 1.0 M Bicine buffer (pH 8.2), 0.1 N NaCN, and 0.05 M of either D-galactose 6-phosphate (▲), or D-glucose 6-phosphate (●) in a small flask sealed with a rubber septum. Samples were removed at timed intervals and assayed for reducing sugar. D-glucose 6-phosphate, which is known to mutarotate in the presence of Bicine, was included as a control.

isomerase reaction, thereby driving the forward isomerase reaction to completion. However, if D-galactose 6-phosphate did not mutarotate, only the active anomer, believed to be α D-galactopyranose 6-phosphate (5), would serve as a substrate for the isomerase and the percent completion of the coupled reaction would be determined by the percent of the active anomer initially present. GLC analysis of a commercial sample of D-galactose 6-phosphate at anomeric equilibrium indicated that the anomer tentatively identified as the α pyranose (39, 42) was present in the amount of 28 percent (Figure 4). It was found that when the forward D-galactose 6-phosphate isomerase reaction was coupled to fructose 6-phosphate kinase, the reaction proceeded to 78 percent completion (Figure 5). The progress of the reaction was followed by determining the amount of D-tagatose-1,6-diphosphate produced, using the Roe procedure (93) with D-fructose 1,6-diphosphate as the standard. The molar absorptivities of D-tagatose 1,6-diphosphate and D-fructose 1,6-diphosphate are known to be very nearly the same in this assay (109). The data obtained with the colorimetric assay were verified by measuring the amount of D-tagatose 1,6-diphosphate produced in the coupled isomerase reaction via a D-tagatose 1,6-diphosphate aldolase end-point assay. The coupled isomerase reaction was also found to go to 77 percent completion as measured by a D-galactose 6-phosphate isomerase end-point assay (data not shown).

The initial concentration of D-galactose 6-phosphate in the above studies was accurately determined both colorimetrically with D-glucose 6-phosphate as a standard, and enzymatically with alkaline

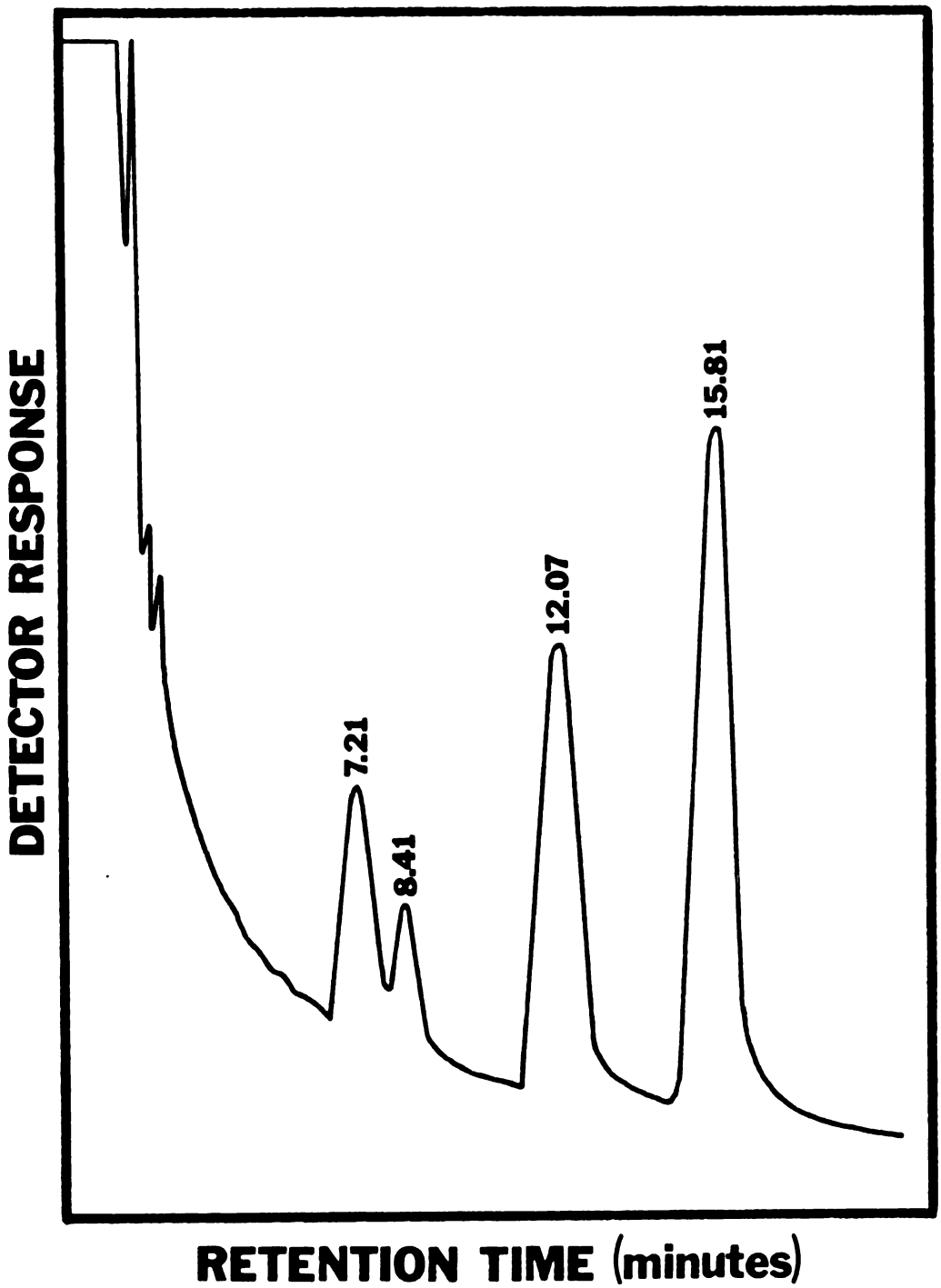


Figure 4. Gas-liquid chromatography of the trimethylsilyl derivatives of commercial D-galactose 6-phosphate. 3% XE-60 column packing was used. Retention times were printed out on the chromatogram and peak integrations were performed by the programmed instrument. The peak appearing at 12.07 minutes is tentatively identified as the α pyranose (39, 42) and has a peak area of 28 percent (in terms of percent of the total area for the four peaks).

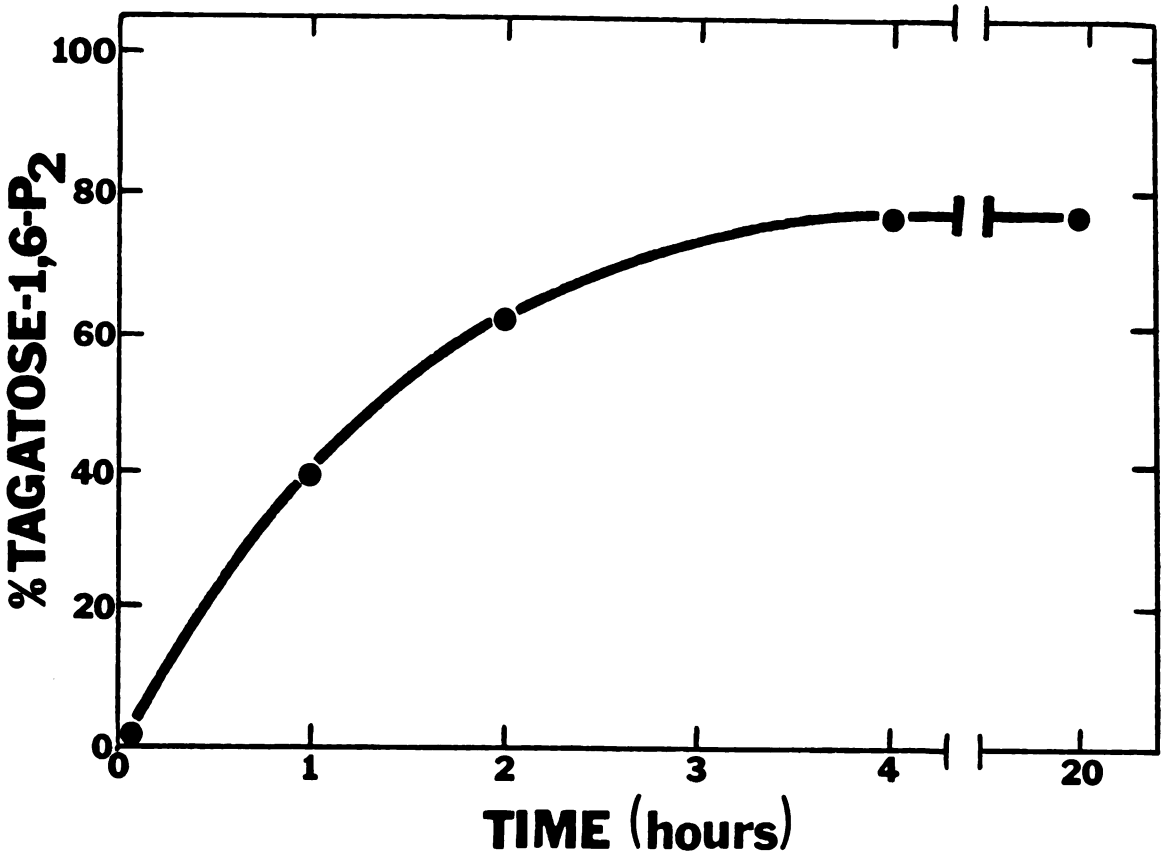


Figure 5. Forward D-galactose 6-phosphate isomerase reaction coupled to D-fructose 6-phosphate kinase. The reaction components and conditions were as described in the Materials and Methods section. At specified intervals, samples were withdrawn and assayed for D-tagatose 1,6-diphosphate. One-hundred percent D-tagatose 1,6-diphosphate was defined as the amount equimolar to the initial D-galactose 6-phosphate present.

phosphatase coupled to β galactose dehydrogenase in an end-point assay. The D-glucose 6-phosphate used in the colorimetric assay was validated as a standard via a glucose 6-phosphate dehydrogenase end-point assay.

The fact that the coupled isomerase reaction consumed a greater percentage of D-galactose 6-phosphate than was initially present as the active α pyranose anomer, indicated that D-galactose 6-phosphate was free to mutarotate. However, it was curious that the coupled reaction terminated after only about 80 percent of the D-galactose 6-phosphate initially present was consumed. It was determined that this premature termination was not due to limiting reagents or inactivation of the enzymes involved. The presence of D-galactose 6-phosphatase activity in the partially purified isomerase preparation was also ruled out (Table 1). The presence of impurities in the commercial preparation of D-galactose 6-phosphate could explain the data; however, such impurities would have to serve as substrates for both alkaline phosphatase and β galactose dehydrogenase (after dephosphorylation) since both these enzymes were used to determine the initial concentration of the commercial D-galactose 6-phosphate. The resolution of this problem will be described in the subsection of the Results and Discussion dealing with the investigation of D-galactose 6-phosphate.

Investigation of D-Tagatose 6-Phosphate

One piece of evidence that led a previous investigator to believe that D-galactose 6-phosphate was not mutarotating was the unusual equilibrium composition he determined for the D-galactose

Table 1

Absence of galactose 6-phosphatase activity in the D-galactose 6-phosphate isomerase preparation.

Reaction mixture	Inorganic phosphate (μmole)
Complete[a] :35 minute reaction time	0.05
Complete :90 minute reaction time	0.05
Complete minus isomerase (90 minutes)	0.05

[a] The reaction mixture (0.20 ml) contained 14 μmoles of Bicine buffer (pH 8.2), 1.0 μmoles of $MgCl_2$, 1.9 μmoles of D-galactose 6-phosphate, and 15 mU of partially purified D-galactose 6-phosphate isomerase. At the specified times the reactions were terminated and the mixtures analyzed for inorganic phosphate.

6-phosphate-isomerase catalyzed reaction (5). When D-galactose 6-phosphate was the initial substrate for the isomerase, the equilibrium composition was 92 percent aldohexose, and 8 percent ketohexose. When D-tagatose 6-phosphate was the initial substrate, the equilibrium composition was 60 percent aldohexose and 40 percent ketohexose. This discrepancy in the equilibrium compositions of the forward and reverse reactions was explained by reasoning that there was only one active anomer of D-galactose 6-phosphate and that mutarotation did not occur under the conditions of the reaction (5; see also the General Discussion). But the present investigation has clearly demonstrated that D-galactose 6-phosphate freely mutarotated under the conditions of the isomerase reaction. So the unusual equilibrium composition of the isomerase reaction determined in the previous study remained to be explained.

An attempt was made to confirm the findings of Bissett (5). When D-galactose 6-phosphate was used as the initial substrate for the isomerase, the equilibrium composition was found to be 92 percent aldohexose, and 8 percent ketohexose, which is identical to Bissett's findings. The initial concentration of D-galactose 6-phosphate was determined by colorimetric assay (90) using D-glucose 6-phosphate as a standard. Ketohexose present at equilibrium was determined by the Roe method (93) using D-fructose 6-phosphate as a standard, and by a fructose 6-phosphate-kinase-linked end-point assay. When D-tagatose 6-phosphate was used as the initial substrate for the isomerase, the equilibrium composition was determined to be 60 percent aldohexose and 40 percent ketohexose,

again in agreement with Bissett. Initial and equilibrium concentrations of D-tagatose 6-phosphate were determined by the Roe method using D-fructose 6-phosphate as a standard. However, when initial and equilibrium concentrations of D-tagatose 6-phosphate were determined by enzymatic end-point assay using fructose 6-phosphate kinase, it was found that the equilibrium composition of the reverse isomerase reaction was 90 percent aldohexose and 10 percent ketohexose, very close to the composition one would predict if D-galactose 6-phosphate were free to mutarotate.

An explanation for these observed discrepancies in equilibrium compositions would be the presence of impurities in the tagatose 6-phosphate preparation used both by Bissett and in the present investigation. This hypothesis was tested. The concentration of total ketohexose in an aqueous solution of the tagatose 6-phosphate preparation was determined by the colorimetric method of Roe (93) using D-fructose 6-phosphate as the standard. D-Fructose 6-phosphate was validated as a standard for the colorimetric assay by enzymatic end-point analysis using fructose 6-phosphate kinase. The same solution of the tagatose 6-phosphate preparation was then assayed for tagatose 6-phosphate using both fructose 6-phosphate kinase and tagatose 6-phosphate kinase end-point assays. The results of these analyses revealed that only 65 mole-percent of the total ketohexose present in the preparation was D-tagatose 6-phosphate. Since the tagatose 6-phosphate preparation was synthesized by Bissett using established literature procedures (48, 50, 51), it was reasoned that an investigation into the

identity and source of the ketohexose contaminant(s) might lead to an improved method for the synthesis of D-tagatose 6-phosphate.

The impurity in the tagatose 6-phosphate preparation was identified in the following manner. Natural abundance ^{13}C-NMR spectroscopy of the D-tagatose 6-phosphate preparation (Figure 6) revealed the presence of peaks at resonance frequencies characteristic of methyl carbons (46). Since 1,2;3,4-di-O-isopropylidene-D-tagatose-6-phosphate (Figure 7) is an intermediate in the synthesis of D-tagatose 6-phosphate, it was reasoned that the methyl carbon resonances observed in the ^{13}C-NMR spectrum were due to the presence of isopropylidene groups. 1,2;3,4-di-O-isopropylidene-D-tagatose 6-phosphate was then synthesized from D-galacturonic acid (48, 50, 51). It was shown upon GLC analysis that the impurity in Bissett's preparation of D-tagatose 6-phosphate had an identical retention time to authentic 1,2;3,4-di-O-isopropylidene-D-tagatose 6-phosphate (Figure 8. The other two peaks in Figure 8B were identified as the α and β anomers of tagatose 6-phosphate; refer to the following text).

The presence of the isopropylidene derivative suggested that the conditions of hydrolysis specified in the literature (48) for the synthesis of D-tagatose 6-phosphate from 1,2;3,4-di-O-isopropylidene-D-tagatose 6-phosphate were inadequate to remove the isopropylidene groups. This hypothesis was verified by hydrolyzing a sample of 1,2;3,4-di-O-isopropylidene-D-tagatose 6-phosphate according to the literature procedure (48). GLC analysis of the hydrolysis products indicated that a substantial

Figure 6. Natural abundance ^{13}C-NMR spectroscopy of the impure tagatose 6-phosphate preparation. Analysis of the 0.5 M aqueous sample was carried out at 23°C. σ values are relative to TMS = 0. Resonances due to the methyl carbons of the impurity occur between -26.5 ppm and -24.4 ppm (46).

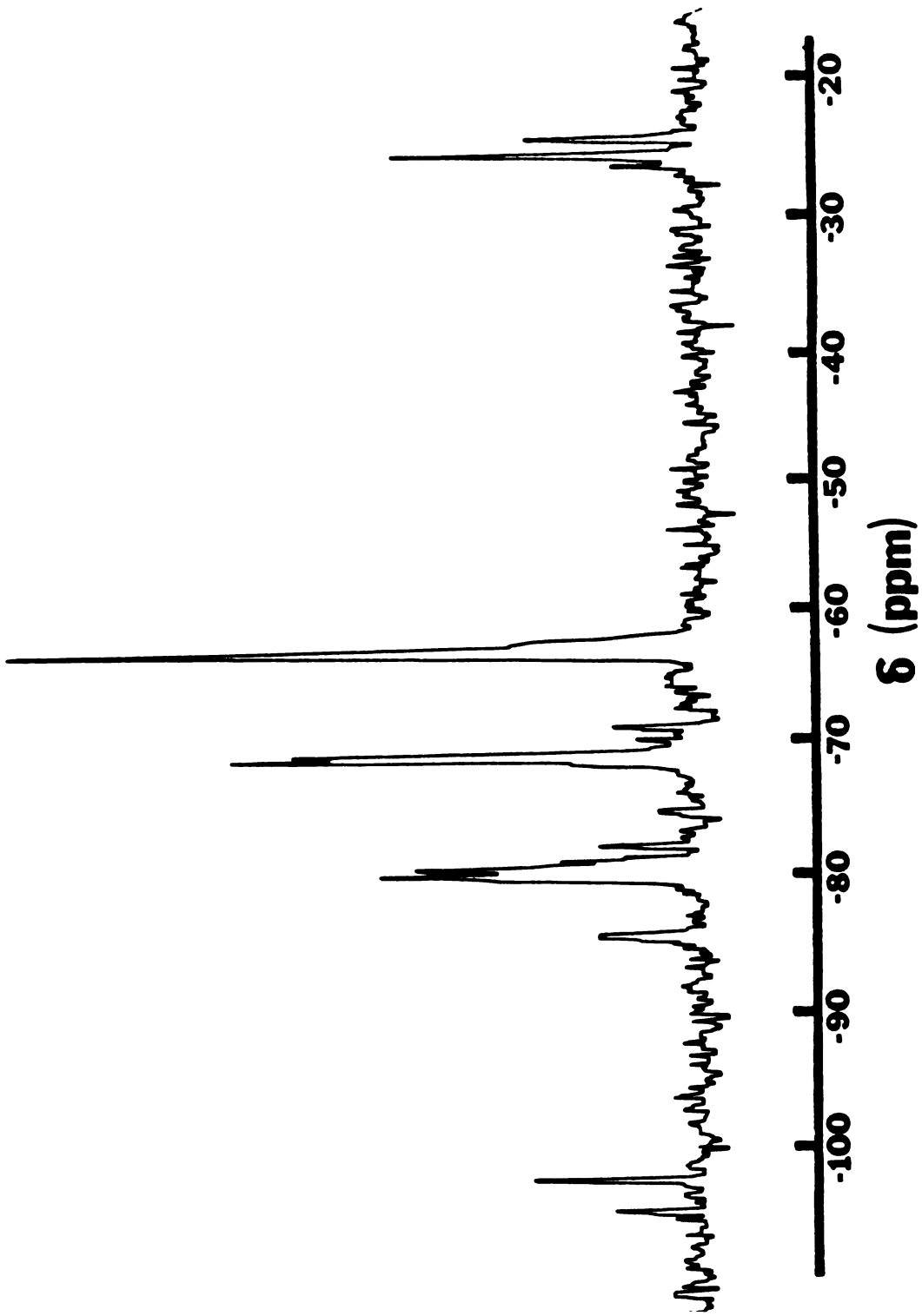


Figure 6.

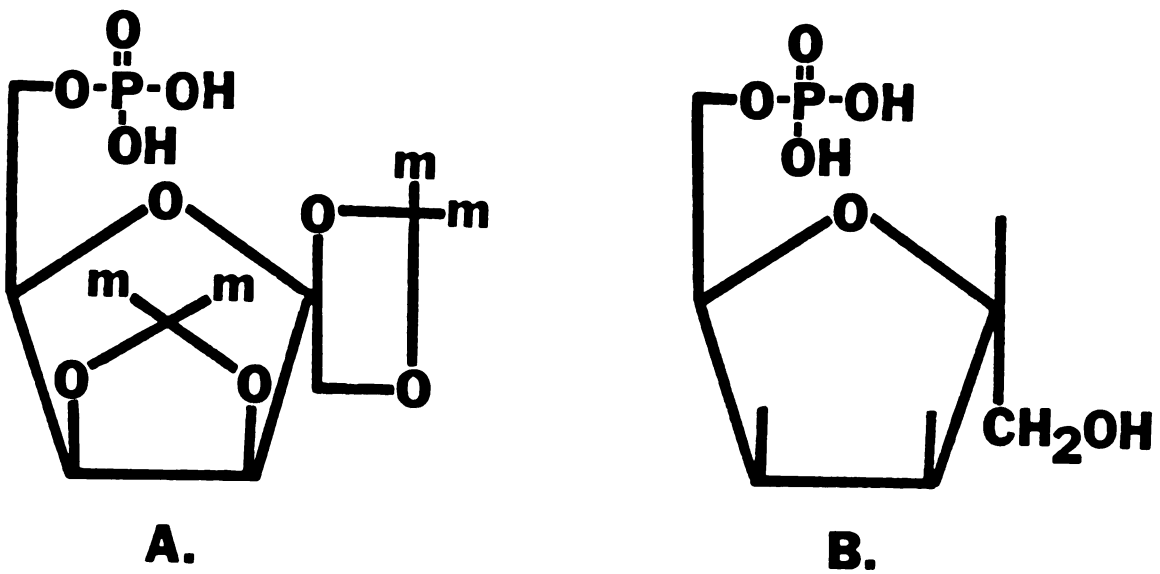


Figure 7. Structures of 1,2;3,4-di-O-isopropylidene-D-tagatose 6-phosphate (A) and D-tagatose 6-phosphate (B). Symbols: m, methyl group.

Figure 8. GLC analysis of the impurity in the D-tagatose 6-phosphate preparation. TMS derivation conditions were as described in Materials and Methods. 3% OV-17 column packing was used. Retention times were printed out on the chromatogram by the programmed instrument.

A. TMS derivative of chemically synthesized 1,2;3,4-di-O-isopropylidene-D-tagatose 6-phosphate.
B. TMS derivatives of the D-tagatose 6-phosphate preparation of Bissett (5).

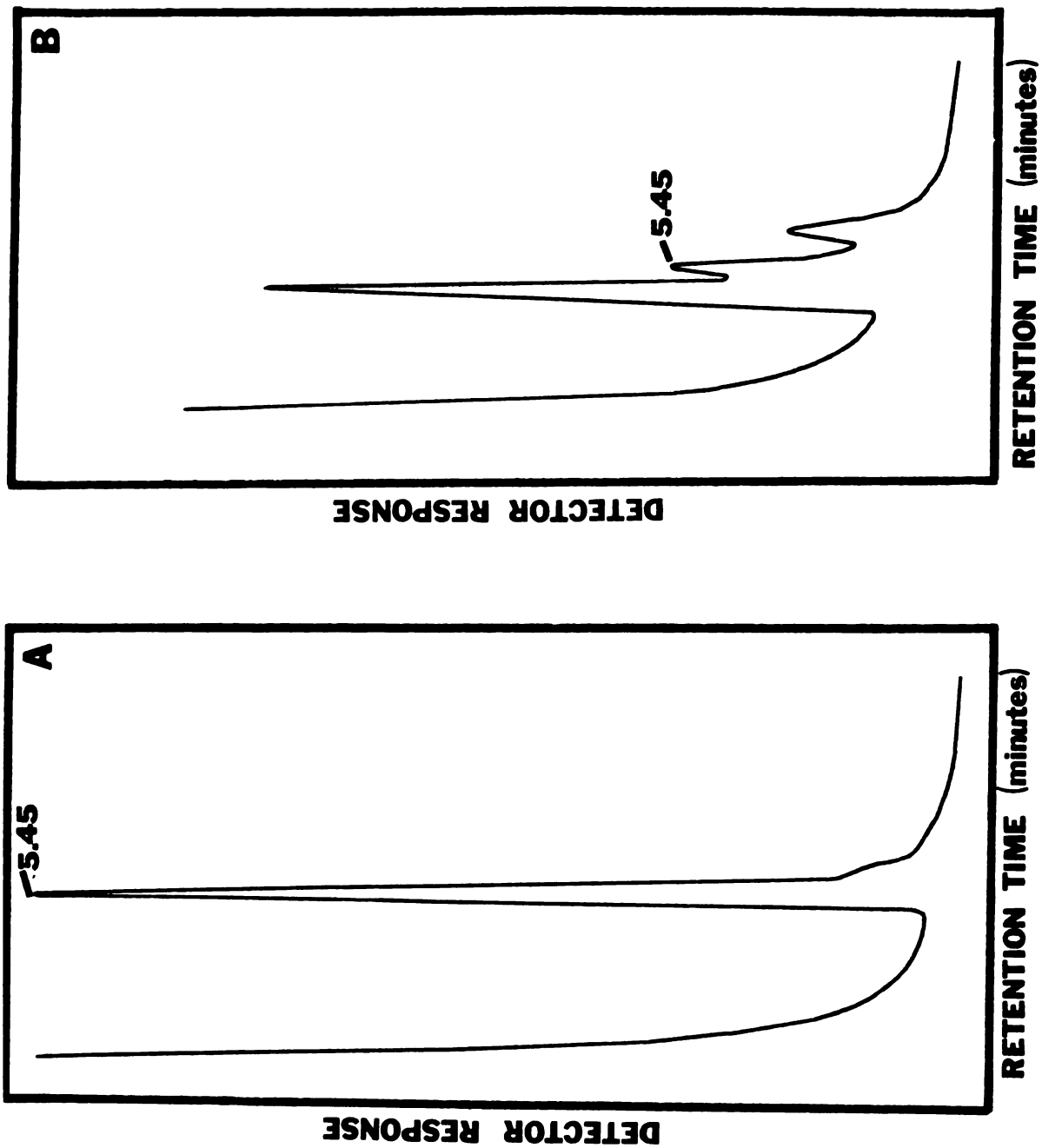

B
5.45
RETENTION TIME (minutes)
DETECTOR RESPONSE
A
5.45
RETENTION TIME (minutes)
DETECTOR RESPONSE

Figure 8.

amount (about 30 percent) of the isopropylidene derivative remained intact (Figure 9A). Conditions of hydrolysis were then modified to insure removal of the isopropylidene groups. Hydrolysis of 1,2;3,4-di-O-isopropylidene-D-tagatose 6-phosphate was carried out in 0.05 N H_2SO_4 for 30 minutes at 100°C. GLC examination of the hydrolysis mixture indicated that the isopropylidene derivative had been completely removed under the modified conditions (Figure 9B). Analysis of the D-tagatose 6-phosphate produced under the modified hydrolysis conditions showed that colorimetric and enzymatic assays were in agreement (Table 2), giving additional evidence that the isopropylidene contaminant had been completely removed.

The substances giving rise to the two peaks in Figure 9B were identified as anomers of tagatose 6-phosphate on the following basis. The substances were stable to prolonged acid treatment (Figure 10) which is consistent with their identification as ketohexose 6-phosphates (28), and indicates that they are not isopropylidene derivatives which are known to be acid-labile (110). The two substances served as substrates for rabbit muscle fructose 6-phosphate kinase (Figure 11) and therefore are identified as either D-tagatose 6-phosphate or D-fructose 6-phosphate since the substrate specificity of fructose 6-phosphate kinase is limited to these two sugar phosphates (111). D-Fructose 6-phosphate was ruled out as a possibility on the following basis. The D-tagatose 6-phosphate preparation was phosphorylated with rabbit muscle fructose 6-phosphate kinase and then treated with

Figure 9. GLC analysis of the hydrolysis products of 1,2;3,4-di-O-isopropylidene-D-tagatose 6-phosphate. TMS derivation conditions were as described in the Materials and Methods section. 3% OV-17 column packing was used. Retention times were printed out on the chromatogram by the programmed instrument.

A. TMS derivatives of hydrolysis products of 1,2;3,4-di-O-isopropylidene-D-tagatose 6-phosphate hydrolyzed at 100°C for 3 minutes in aqueous solution.
B. TMS derivatives of hydrolysis products of 1,2;3,4-di-O-isopropylidene-D-tagatose 6-phosphate hydrolyzed at 100°C for 30 minutes in 0.05 N H_2SO_4.

Refer to Figure 8A for the retention time of authentic 1,2;3,4-di-O-isopropylidene-D-tagatose 6-phosphate.

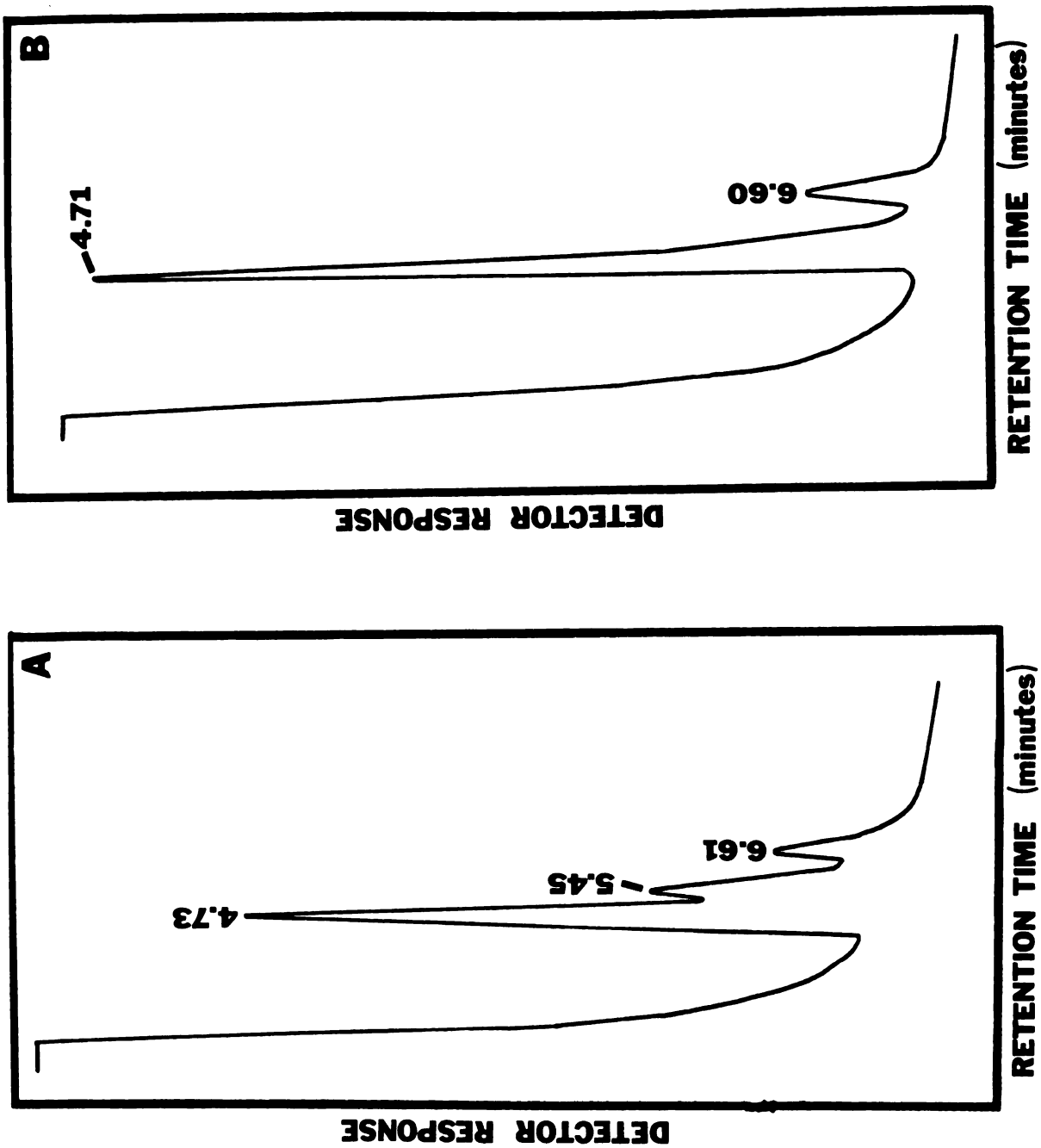

B
4.71
6.60
DETECTOR RESPONSE
RETENTION TIME (minutes)
A
4.73
5.45
6.61
DETECTOR RESPONSE
RETENTION TIME (minutes)

Figure 9.

Table 2

Comparison of colorimetric and enzymatic analyses of D-tagatose 6-phosphate preparations.

Sample	Colorimetric assay[a] (µg ketose/50 µl)	Enzymatic assay[b] (µg ketose/50 µl)
Tagatose 6-phosphate preparation: Literature hydrolysis conditions[c].	404 ± 22	264 ± 11
Tagatose 6-phosphate preparation: Modified hydrolysis conditions[d].	420 ± 20	436 ± 5

[a]The colorimetric assay used was the method of Roe (93). D-Fructose 6-phosphate was used as the standard. Assays were run in triplicate.

[b]A fructose 6-phosphate kinase end-point assay was used. Rabbit muscle fructose 6-phosphate kinase is known to phosphorylate D-tagatose 6-phosphate (97). Assays were run in triplicate.

[c]1,2;3,4-di-O-isopropylidene-D-tagatose 6-phosphate was hydrolyzed under conditions outlined in the literature (48).

[d]1,2;3,4-di-O-isopropylidene-D-tagatose 6-phosphate was hydrolyzed at 100°C for 30 minutes in 0.05 N H_2SO_4.

Figure 10. GLC analysis of the acid stability of the substances in the D-tagatose 6-phosphate preparation. TMS derivation conditions were as described in the Materials and Methods section. 3% OV-17 column packing was used.

A. TMS derivatives of the D-tagatose 6-phosphate preparation hydrolyzed at 100°C for 30 minutes in 0.05 N H_2SO_4.

B. TMS derivatives of the D-tagatose 6-phosphate preparation hydrolyzed at 100°C for 4 hours in 0.05 N H_2SO_4.

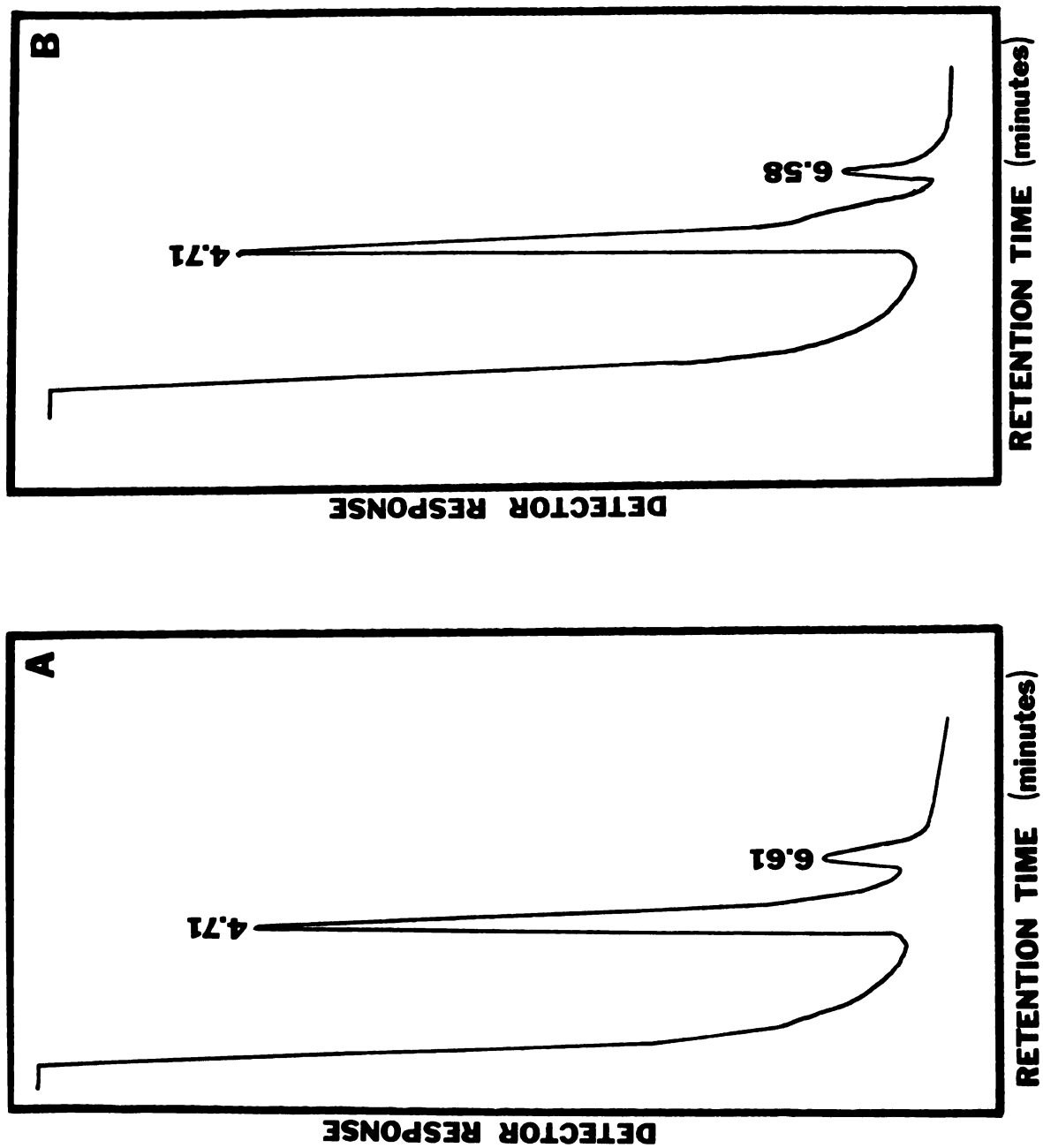

B
6.58
4.71
DETECTOR RESPONSE
RETENTION TIME (minutes)
A
6.61
4.71
DETECTOR RESPONSE
RETENTION TIME (minutes)

Figure 10.

Figure 11. GLC analysis of the D-tagatose 6-phosphate preparation treated with rabbit muscle fructose 6-phosphate kinase. TMS derivation conditions were as outlined in Materials and Methods. 3% OV-7 column packing was used. The reaction mixture (0.7 ml) contained 65 μmoles of glycylglycine buffer (pH 7.5), 10 μmoles of ATP, 4.5 μmoles of D-tagatose 6-phosphate, and 4 Units of rabbit muscle fructose 6-phosphate kinase. The progress of the reaction was followed by determining the amount of D-tagatose 6-phosphate remaining at specified time points by a fructose 6-phosphate kinase end-point assay.

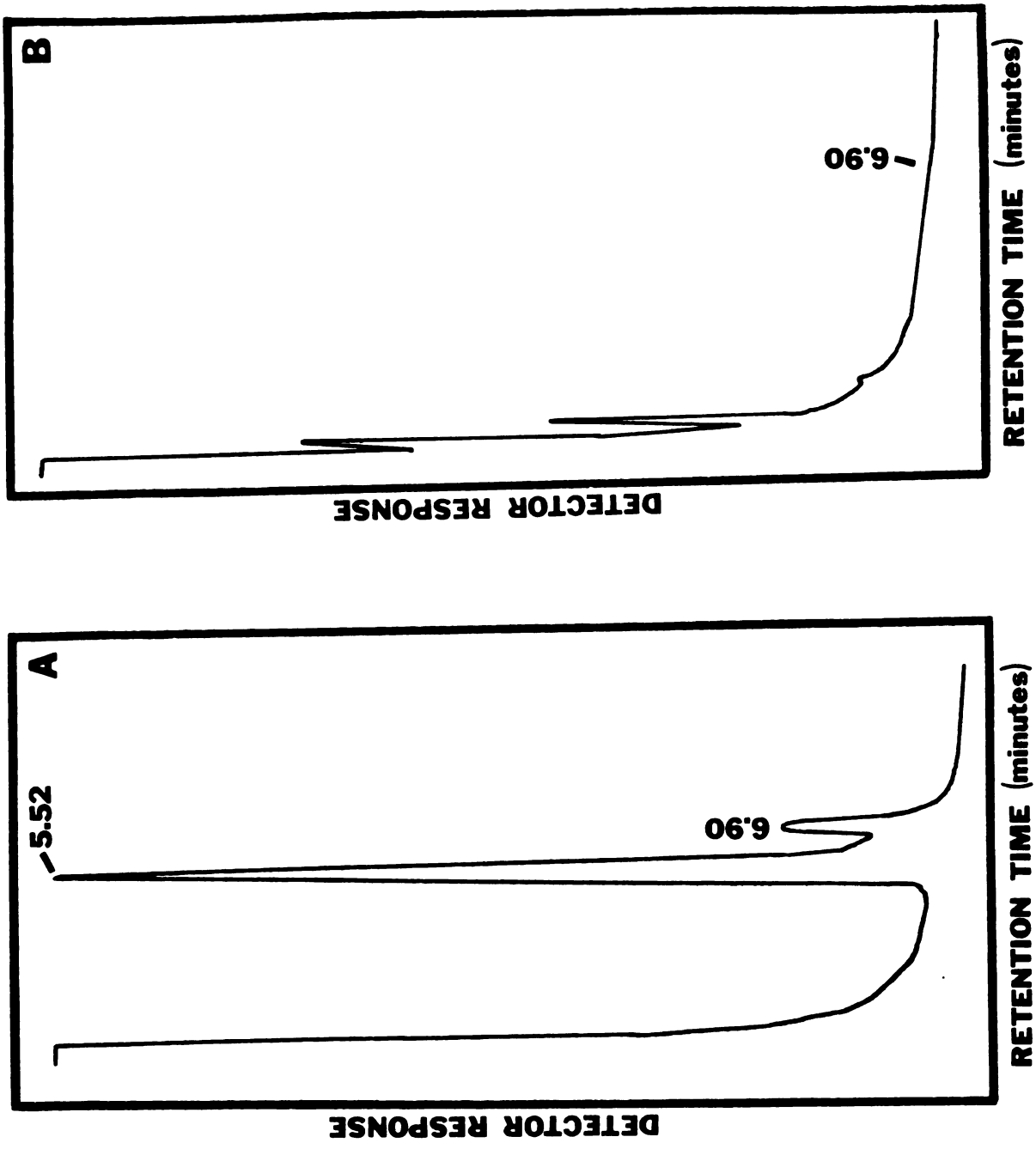

B
6.90
RETENTION TIME (minutes)
DETECTOR RESPONSE
A
5.52
6.90
RETENTION TIME (minutes)
DETECTOR RESPONSE

Figure 11.

rabbit muscle aldolase. Rabbit muscle aldolase cleaves D-fructose 1,6-diphosphate but not D-tagatose 1,6-diphosphate (112). There was no detectable aldolase activity, indicating that there was no D-fructose 6-phosphate present in the D-tagatose 6-phosphate preparation.

GLC analysis of an aqueous solution of the pure tagatose 6-phosphate preparation showed that the area distribution of the two peaks was 78 percent, 22 percent (Figure 12). It is known from the literature that the anomeric distribution of D-tagatose 6-phosphate is 80 percent β and 20 percent α, as determined by ^{13}C-NMR analysis (53). This distribution was confirmed by the ^{13}C-NMR analysis of D-tagatose 6-phosphate in the present investigation (Figure 6) which showed 78 percent β and 22 percent α anomer, based on the peak areas of the resonances attributed to the anomeric carbons.

On the basis of these data, the substance with a retention time of 4.61 minutes (Figure 12) is identified as β-D-tagatose 6-phosphate, and the substance with a retention time of 6.47 minutes (Figure 12) is identified as α-D-tagatose 6-phosphate.

GLC (Figure 9), colorimetric and enzymatic (Table 2) analyses reveal that D-tagatose 6-phosphate is the sole product resulting from the modified conditions for the hydrolysis of 1,2;3,4-di-O-isopropylidene-D-tagatose 6-phosphate. The modified synthesis of tagatose 6-phosphate described in this thesis results in a pure product.

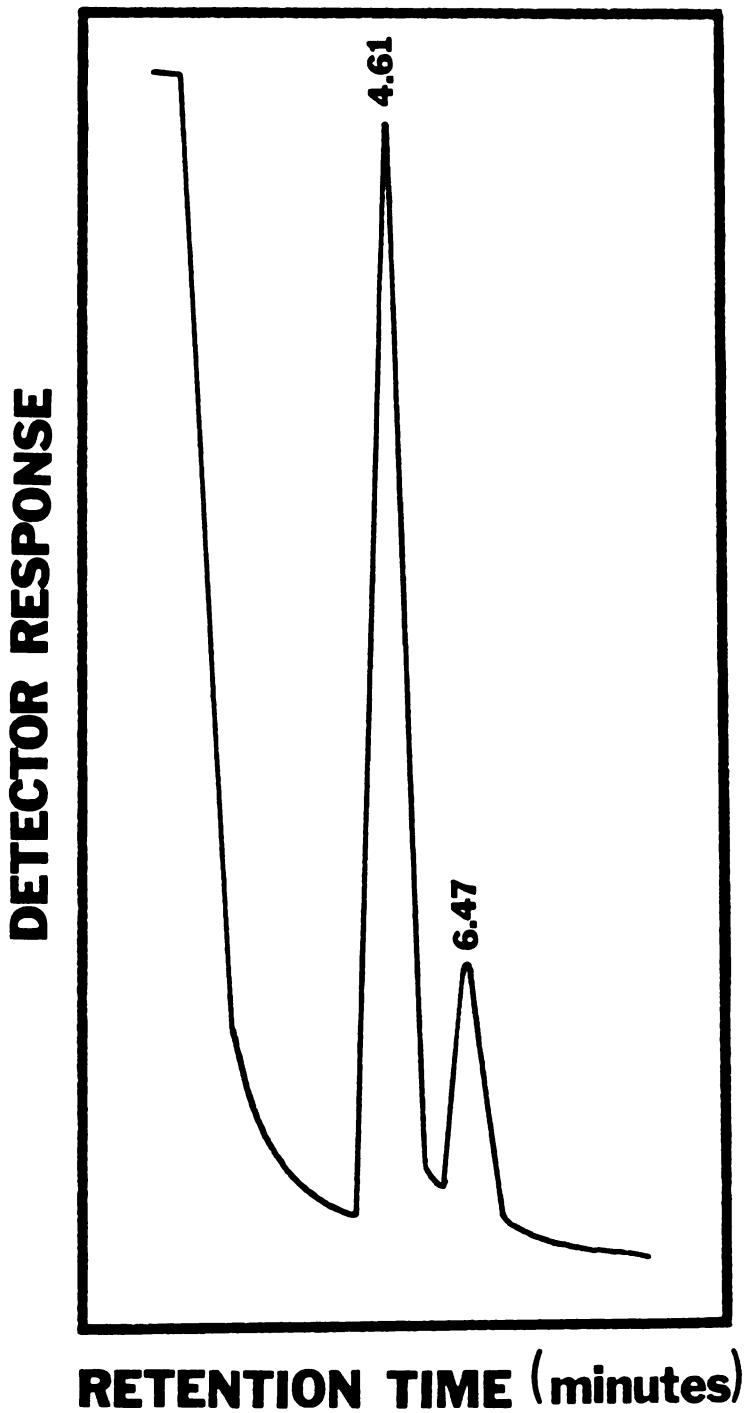


Figure 12. GLC analysis of the D-tagatose 6-phosphate preparation. TMS derivation of the sample lyophilized from an aqueous solution was carried out as described in Materials and Methods. Retention times and peak areas were determined by the programmed instrument. The peak appearing at 4.61 minutes contained 78 percent of the total peak area. The peak with a retention time of 6.47 minutes contained 22 percent of the total peak area. 3% OV-17 column packing was used.

Investigation of D-Galactose 6-Phosphate

It was noted earlier that when the D-galactose 6-phosphate-isomerase-catalyzed reaction (with D-galactose 6-phosphate as the initial substrate) was coupled to rabbit muscle fructose 6-phosphate kinase, the reaction only went to about 80 percent completion (Figure 5). A possible explanation for this behavior was that the commercial preparation of D-galactose 6-phosphate was impure. This possibility was investigated.

TMS-O-methyloxime derivatives were prepared (98) from a commercial sample of D-galactose 6-phosphate and subjected to GLC analysis. The O-methyloxime derivative forms a straight chain structure so the presence of anomers is prohibited. Therefore, if the preparation of D-galactose 6-phosphate was pure, only one TMS-O-methyloxime derivative would be formed and only one peak would be observed in the GLC analysis of the sample. (It is possible that the syn and anti isomers of the TMS-O-methyloxime derivative would be partially resolved; 42, 98, 113. In such a case, one would predict observing one peak and an accompanying shoulder if the preparation of D-galactose 6-phosphate was pure.) However, at least four distinct TMS-O-methyloxime derivatives could be prepared from the commercial D-galactose 6-phosphate sample (Figure 13). The large peak appearing at 9.43 minutes contained 82 percent of the total peak area and was identified by mass-spectrometry as the TMS-O-methyloxime derivative of D-galactose 6-phosphate (data not shown). The shoulder at 10.00 minutes is probably due to the separation of the syn and anti isomers of the methyloxime. Peaks with retention times of 4.87 minutes, 7.57 minutes, and 8.40 minutes were assumed

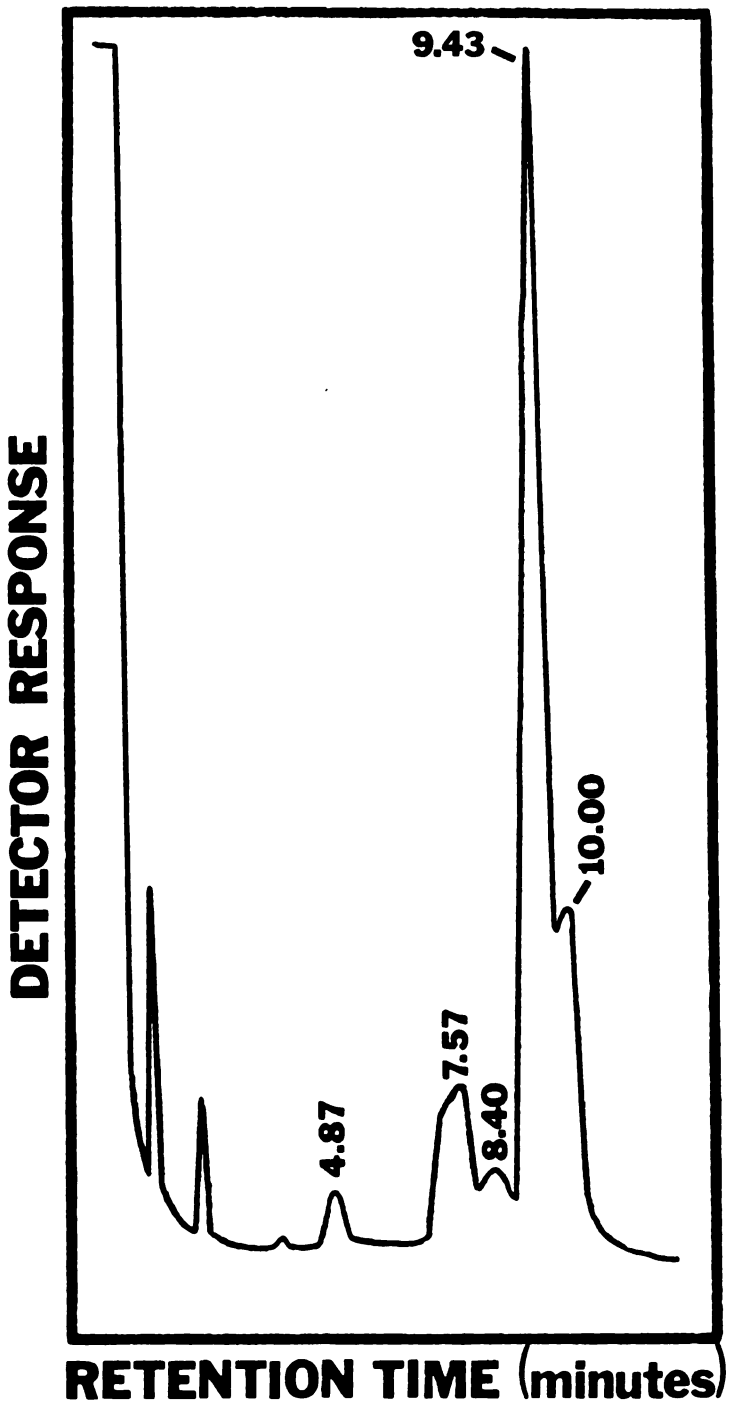


Figure 13. GLC analysis of TMS-O-methyloxime derivatives of a commercial D-galactose 6-phosphate preparation. Derivation conditions were as described in Materials and Methods. 3% OV-1 column packing was used. Retention times and peak areas were determined by the programmed instrument.

to be due to impurities present in the commercial preparation of D-galactose 6-phosphate and comprised 18 percent of the total peak area. Peaks with retention times less than 4 minutes are believed to be due to degradation products inherent in the derivation process because their retention times and peak areas varied from sample to sample.

When the commercial preparation of D-galactose 6-phosphate was reduced with sodium borohydride (60) and the reaction products analyzed by GLC, two distinct peaks were evident, offering further proof of the presence of impurities in the commerical preparation (Figure 14).

Proof of impurities in the commercial preparations was intriguing for the following reasons. These impurities were found in preparations of galactose 6-phosphate labeled by the manufacturer to be 98-100 percent pure. Also, as I have reported earlier, all the reducing sugar present in the preparations reacted quantitatively with β-galactose-dehydrogenase after being dephosphorylated with alkaline phosphatase, a finding which is consistent with the manufacturer's claim of a pure product. Further investigation was warranted.

The D-galactose 6-phosphate isomerase-catalyzed reaction, with commercial D-galactose 6-phosphate as the substrate, was coupled to rabbit muscle fructose 6-phosphate kinase. As before (Figure 5), the reaction only went to about 80 percent completion. The reaction mixture was further analyzed via GLC. It was shown, conclusively, that the substances giving rise to the first two peaks in the

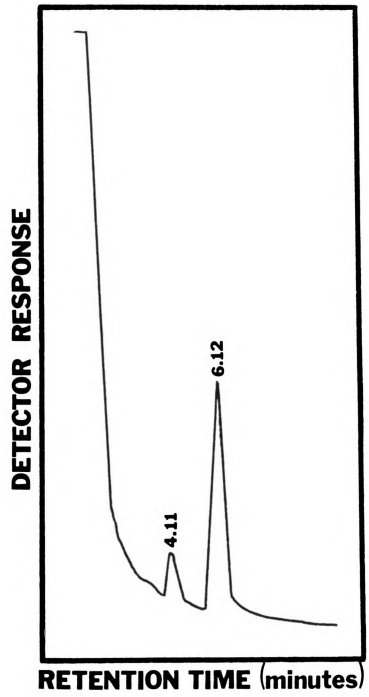


Figure 14. GLC analysis of TMS-derivatives of the reaction products formed by sodium borohydride reduction of a commercial preparation of D-galactose 6-phosphate. The reduced products were kindly supplied by G.T. Shimamoto. TMS derivation conditions were as described in Materials and Methods. 3% OV-17 column packing was used.

chromatogram did not serve as substrates for the isomerase (Figure 15) and therefore could not be authentic galactose 6-phosphate. This finding was most surprising since these two substances have been reported by others to be the α and β furanose anomers of D-galactose 6-phosphate (5, 45, 59, 60). These two substances comprise 21 percent of the total peak area in the chromatogram (Figure 15A), which is nearly identical to the amount of impurities determined to be present in the commercial samples.

From these data, it was concluded that the substances thought by others to be α- and β-galactofuranose 6-phosphate, were in fact impurities present in commercial preparations of galactose 6-phosphate.

Verification of this conclusion was obtained by analyzing preparations of D-galactose 6-phosphate derived from biological sources.

When D-galactose 6-phosphate was enzymatically synthesized from D-tagatose 6-phosphate by D-galactose 6-phosphate isomerase, GLC analysis of the reaction mixture indicated that the two presumed furanose peaks were absent (Figure 16). Peaks with retention times of 9.45 minutes and 10.88 minutes were due to D-tagatose 6-phosphate and isopropylidene-D-tagatose 6-phosphate, respectively. Peaks at 12.25 minutes and 15.88 minutes were due to α and β galactopyranose-6-phosphate. These identifications were based on the comparison of retention times to those of known standards. The peak with retention time of 7.65 minutes was believed to be an authentic furanose anomer of D-galactose 6-phosphate (42).

Another biosynthetic source of D-galactose 6-phosphate was

Figure 15. GLC analysis of the forward D-galactose 6-phosphate isomerase reaction coupled to fructose 6-phosphate kinase. The reaction mixture (0.4 ml) contained 64 μmoles of Bicine buffer (pH 8.2), 12.0 μmoles of $MgCl_2$, 6.0 μmoles of ATP, 4.0 μmoles of D-galactose 6-phosphate, 60 mU of D-galactose 6-phosphate isomerase, and 8 Units of fructose 6-phosphate kinase. The progress of the reaction was monitored by determining the amount of D-tagatose 1,6-diphosphate produced using the Roe method (20) with D-fructose-1,6-diphosphate as the standard. At specified times, samples were withdrawn and lypophilized. TMS derivation conditions were as described in Materials and Methods. 3% XE-60 column packing was used. Retention times and peak areas were determined by the programmed instrument.

A. Reaction mixture at initial time (before addition of the isomerase). The peak areas in terms of percent of the total area for the four peaks were (from left to right) 14.4, 6.3, 28.4, 50.9.

B. Reaction mixture at completion.

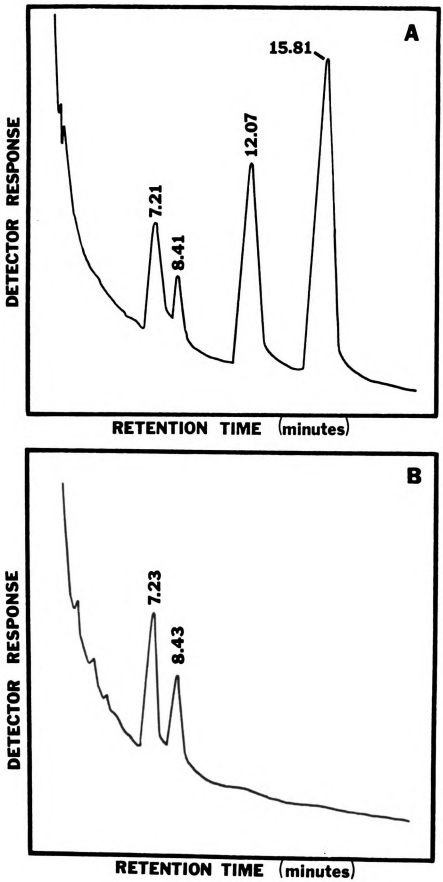

A
15.81
12.07
7.21
8.41
DETECTOR RESPONSE
RETENTION TIME (minutes)
B
7.23
8.43
DETECTOR RESPONSE
RETENTION TIME (minutes)

Figure 15.

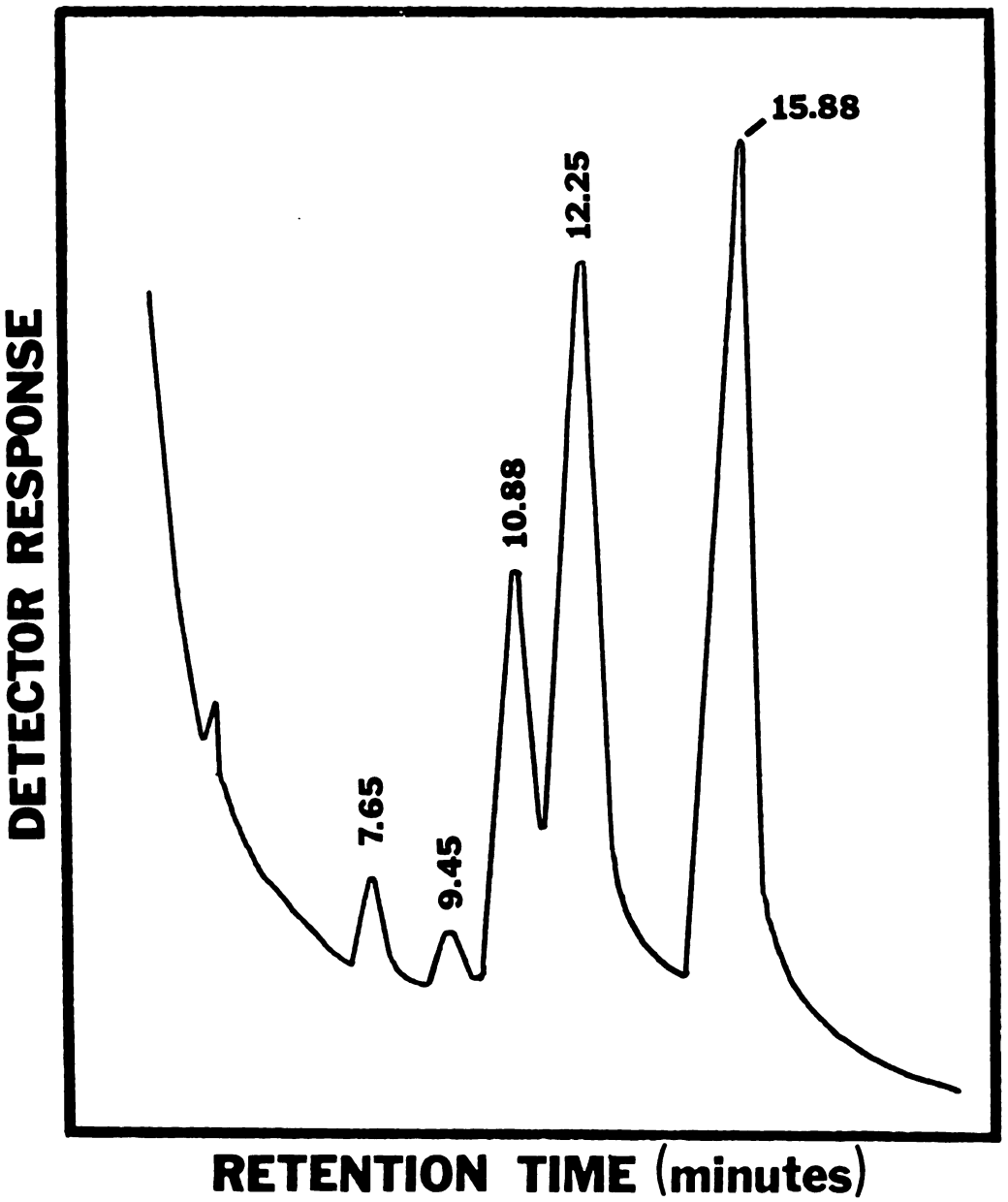


Figure 16. GLC analysis of D-galactose 6-phosphate enzymatically synthesized from D-tagatose 6-phosphate. The reaction mixture (0.4 ml) contained 64 μmoles of Bicine buffer (pH 8.2), 4.0 μmoles of D-tagatose 6-phosphate, and 60 mU of D-galactose 6-phosphate isomerase. The progress of the reaction was followed by measuring the disappearance of D-tagatose 6-phosphate using fructose 6-phosphate kinase in an end-point assay. When equilibrium was achieved, the reaction mixture was lyophilized, and TMS derivatives were formed as described in Materials and Methods. 3% XE-60 column packing was used. Retention times were determined by the programmed instrument. For identification of peaks, refer to the text.

also investigated. Staphylococcus aureus strain GD29, a mutant of S. aureus NCTC 8511 deficient in D-galactose 6-phosphate isomerase (80), was grown in the presence of D-galactose, and the accumulated metabolite, D-galactose 6-phosphate, was analyzed by GLC. The presumed furanose peaks present in the commercial preparation of D-galactose 6-phosphate (Figure 17A) were absent in the D-galactose 6-phosphate accumulated by S. aureus GD29 (Figure 17B). Peak identities in the S. aureus extract chromatogram were as follows: 3.4 minutes, unknown, but possibly 2-deoxy-D-ribose 5-phosphate; 3.7 minutes, D-ribose 5-phosphate; 5.0 minutes, tentatively identified as D-galactofuranose 6-phosphate; 6.3 minutes, D-tagatose 6-phosphate; 7.0 minutes and 8.0 minutes, α and β D-galactopyranose 6-phosphate. These identities were established by comparing retention times to those of known standards.

Because the impurities found in commercial preparations of galactose 6-phosphate were not detected in preparations of galactose 6-phosphate derived from biological sources, it was suspected that these impurities were by-products of the chemical synthesis used by the commercial suppliers. Through conversation with a representative of Sigma Chemical Co., a major commercial supplier of D-galactose 6-phosphate, it was established that D-galactose 6-phosphate was synthesized from D-galactose and polyphosphoric acid using the procedure of Seegmiller and Horecker (20).

The next step in my investigation was to determine conclusively that the impurities were by-products of this chemical synthesis. The synthesis was carried out and the barium salt of

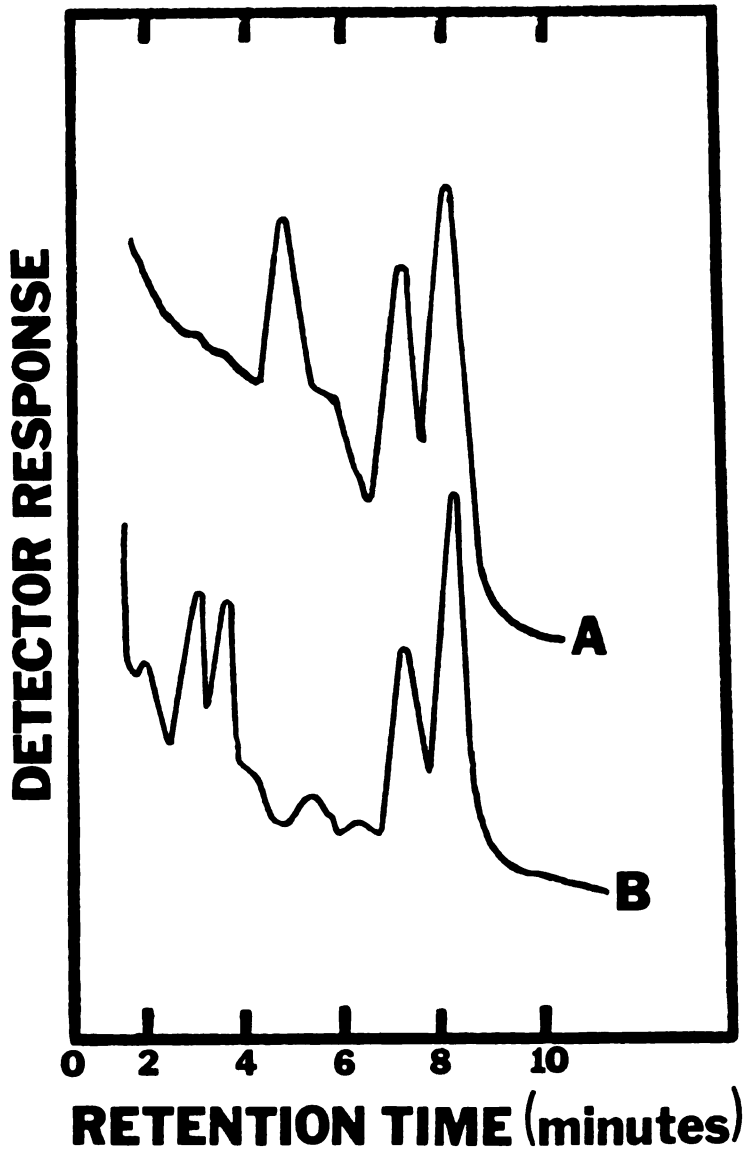


Figure 17. GLC analysis of D-galactose 6-phosphate synthesized by S. aureus GD29. Conditions for bacterial growth, extraction of metabolites, and TMS derivation were as described in Materials and Methods. 3% OV-17 column packing was used. For this particular experiment, a Hewlett-Packard model 402 gas chromatograph was used, with a column temperature of 230°C.

A. Commercial preparation of D-galactose 6-phosphate.

B. Metabolite extract from S. aureus GD29.

Refer to text for identities of the components of the metabolite extract.

D-galactose 6-phosphate was produced in similar yield (9 mole-percent) to that reported in the literature. GLC analysis of the product mixture revealed the presence of the impurities previously identified in commercial preparations (Figure 18). The synthesis was further investigated to determine if conditions could be modified to decrease or eliminate the presence of the impurities.

Seegmiller and Horecker reported that in the direct phosphorylation of an aldohexose with polyphosphoric acid, esterification occurs not only at the 6 position but at other positions as well (20). They further stated that since aldohexose 6-phosphates are resistant to acid hydrolysis, other esters formed could be removed by this means with little loss of the 6-phosphate. With these statements in mind, the effect of acid hydrolysis in the synthesis of D-galactose 6-phosphate was examined.

Phosphate-to-sugar ratios of product obtained after varying lengths of acid hydrolysis were determined (Table 3). The phosphate-to-sugar ratio of 2.3 prior to hydrolysis suggests that either D-galactose is phosphorylated in multiple positions or that polyphosphoric ester linkages are present. Phosphate-to-sugar ratios of less than 1 obtained upon extended hydrolysis reflect the acid lability of the hexose monophosphates.

The effects of acid hydrolysis were further investigated by GLC analysis of the product mixture. It was found that the impurities were quite stable to extended periods of acid hydrolysis, though to a slightly lesser degree than authentic

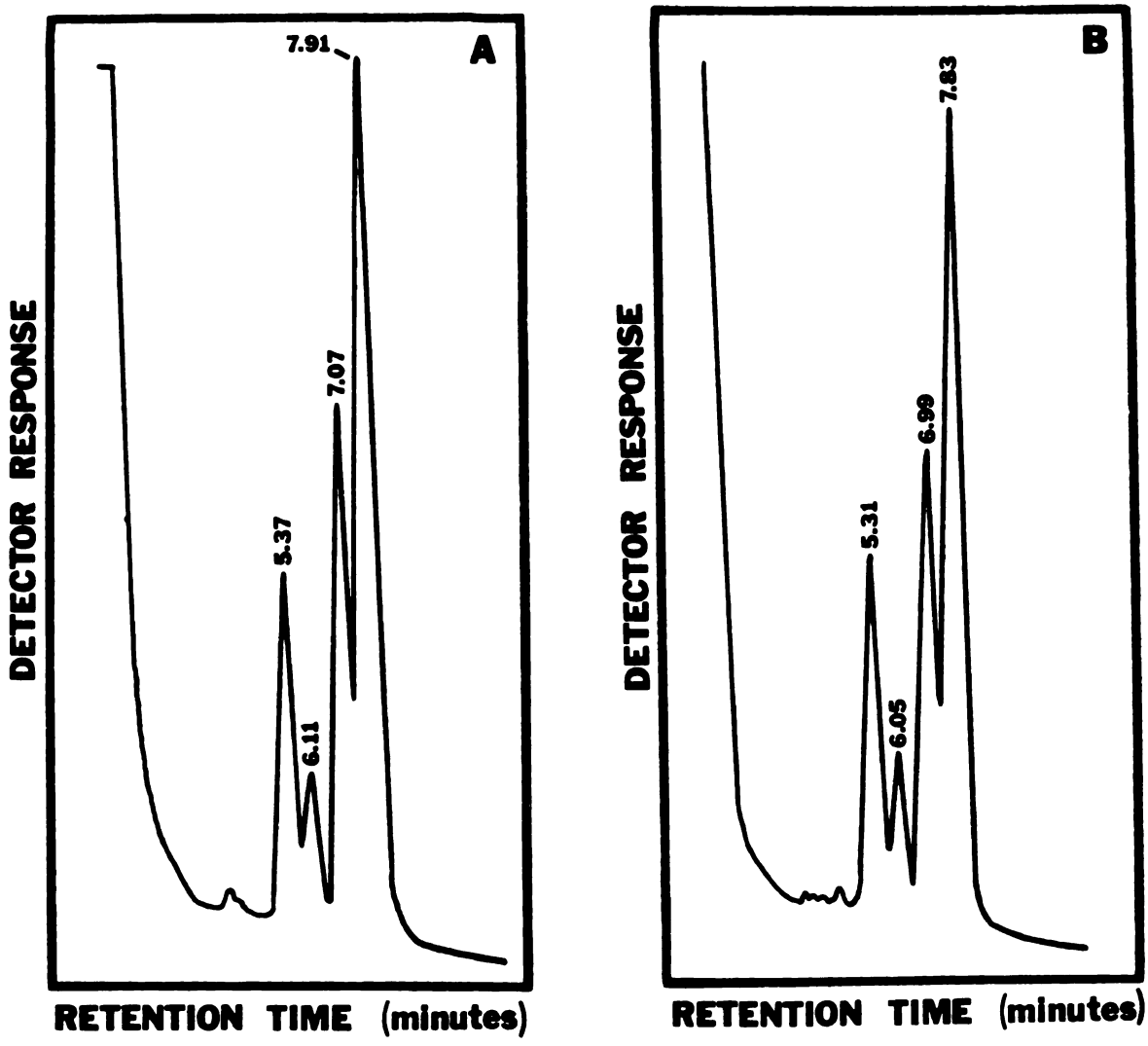


Figure 18. GLC analysis of chemically synthesized D-galactose 6-phosphate. D-Galactose 6-phosphate was synthesized from D-galactose and polyphosphoric acid as described in Materials and Methods. TMS derivation conditions were as described in Materials and Methods. 3% OV-7 column packing was used.

A. Commercial preparation of D-galactose 6-phosphate.
B. D-Galactose 6-phosphate chemically synthesized in this laboratory.

Table 3

Effect of acid hydrolysis on phosphate-to-sugar ratios in the synthesis of D-galactose 6-phophate.

Time of hydrolysis[a] (hours)	Organic Phosphate ($\frac{mmoles}{ml}$)	Total Carbohydrate ($\frac{mmoles}{ml}$)	Phosphate/sugar ratio[b]
No hydrolysis	0.39	0.17	2.3
0.1	0.15	0.17	0.9
8.0	0.13	0.16	0.8
32.0	0.12	0.17	0.7

[a]Hydrolysis was carried out in 1.6 N HBr for the specified period of time at 100°C.

[b]Total carbohydrate, total phosphate, and inorganic phosphate were determined as described in the Materials and Methods section.

galactose 6-phosphate (Figure 19). This fact explains the observation that the amount of impurities in the commercial preparations differed from lot to lot, since variations will occur depending upon the time of acid hydrolysis to which the samples were subjected.

The stability of the impurities to acid hydrolysis precluded any simple modifications in procedure that would lead to a pure preparation of D-galactose 6-phosphate. Therefore, a means of separating D-galactose 6-phosphate from its impurities was pursued.

Authentic D-galactose 6-phosphate was separated from the impurities by forming the borate complexes of the commercial D-galactose 6-phosphate preparation and passing them over a strongly basic Dowex ion-exchange resin using the procedure of Lefebvre et al. (38). All of the authentic D-galactose 6-phosphate layered on the column was recovered. This was consistent with the literature which reported 91-100 percent recoveries by this method (38). For an elution profile of the Dowex (borate) column, refer to Figure 20.

Not only was the borate column effective in separating authentic D-galactose 6-phosphate from its impurities, it was also found to be an effective method for separating the impurities from each other. GLC analysis of the borate column elution peaks revealed the presence of two major impurities in the commercial preparation of D-galactose 6-phosphate whose GLC retention times correspond to the two peaks which were once thought to be the authentic furanose forms of D-galactose 6-phosphate (Figure 21).

Figure 19. GLC analysis of the effect of acid hydrolysis on the purity of chemically synthesized D-galactose 6-phosphate. D-Galactose 6-phosphate was synthesized from D-galactose and polyphosphoric acid as described in Materials and Methods. TMS derivation conditions were as described in Materials and Methods. 3% OV-17 column packing was used.

A. D-Galactose 6-phosphate preparation hydrolyzed for 8 hours in 1.6 N HBr at 100°C.

B. D-Galactose 6-phosphate preparation hydrolyzed for 48 hours in 1.6 N HBr at 100°C.

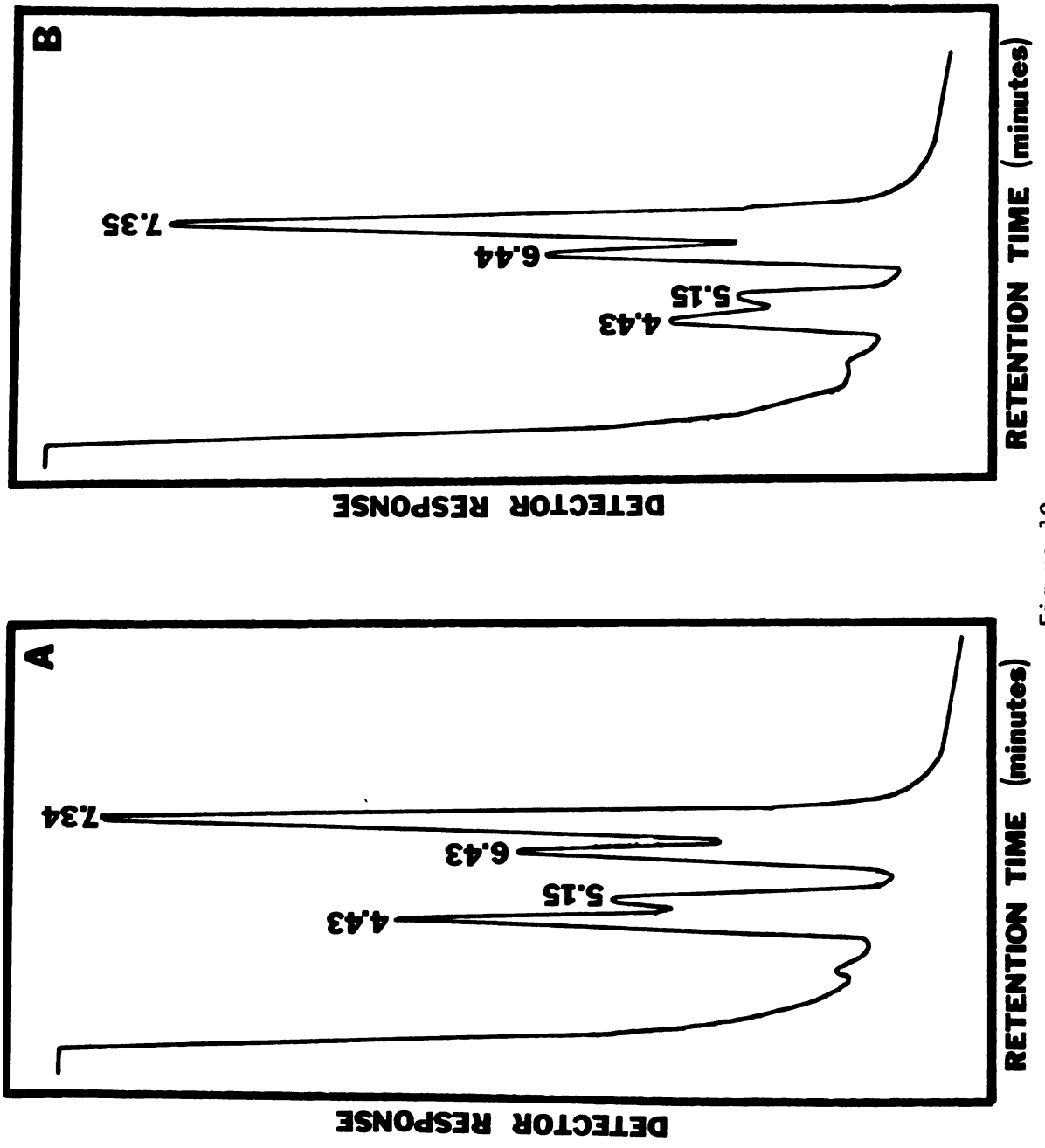

B
7.35
6.44
5.15
4.43
DETECTOR RESPONSE
RETENTION TIME (minutes)
A
7.34
6.43
5.15
4.43
DETECTOR RESPONSE
RETENTION TIME (minutes)

Figure 19.

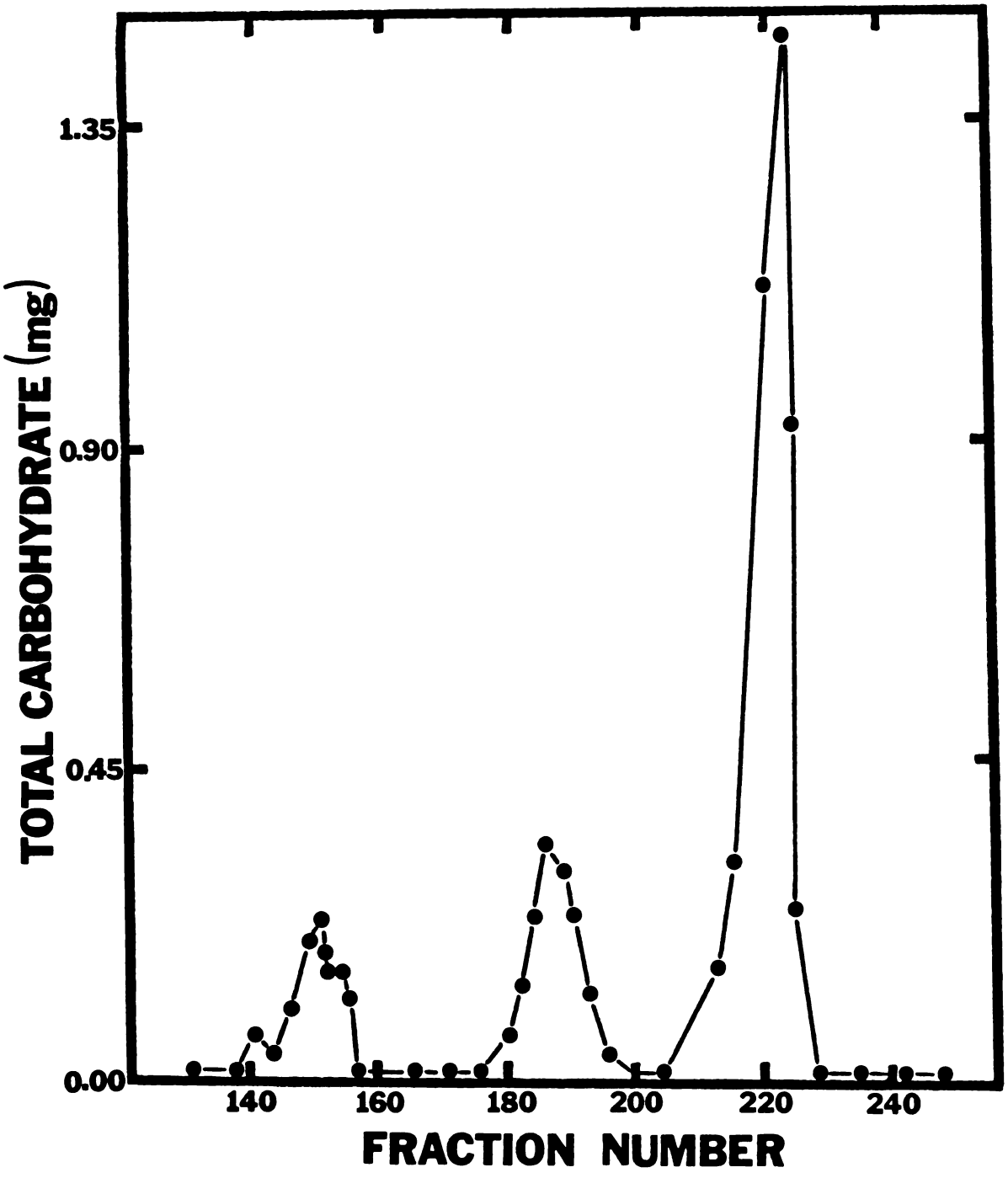


Figure 20. Ion-exchange chromatography of the commercial preparation of D-galactose 6-phosphate. The procedure followed was described in detail in the Materials and Methods section. Total carbohydrate was determined by the phenol/H_2SO_4 assay (90).

Figure 21. GLC analysis of the Dowex (borate) ion exchange chromatography elution peaks (refer also to Figure 20). Samples were treated with methanol as described in the Materials and Methods section, and lypophilized. TMS derivation conditions were as described in Materials and Methods. 3% OV-17 column packing was used. Retention times were determined by the programmed instrument.

A. Commercial preparation of D-galactose 6-phosphate.
B. Initial borate column elution peak (pooled fractions 139-157; refer to Figure 20).
C. Second borate column elution peak (pooled fractions 178-196; refer to Figure 20).

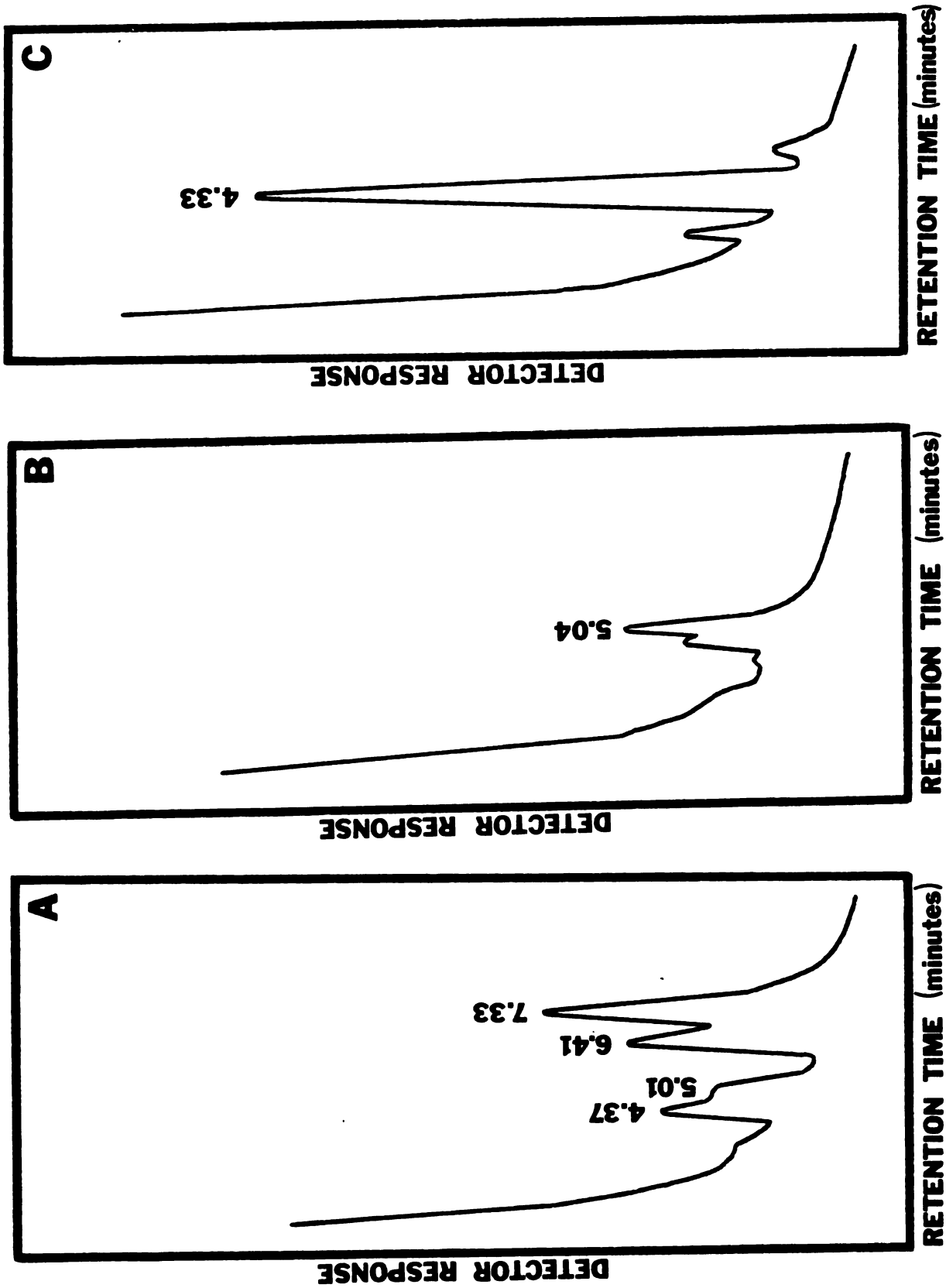

A
4.37
5.01
6.41
7.33
DETECTOR RESPONSE
RETENTION TIME (minutes)
B
5.04
DETECTOR RESPONSE
RETENTION TIME (minutes)
C
4.33
DETECTOR RESPONSE
RETENTION TIME (minutes)

Figure 21.

It was noted that the presence of borate interferred with both the phenol/H_2SO_4 assay for carbohydrate and the silylation reaction. Consistent with the literature for the corresponding free sugar (114), it was found that for D-galactose 6-phosphate there was a 20 percent decrease in color development in the phenol/H_2SO_4 assay in the presence of borate. The silylation reaction was almost completely inhibited in the presence of borate. However, it was found that desalting the sample with repeated methanol evaporations prior to silylating alleviated the problem.

Authentic D-galactose 6-phosphate eluted from the borate column in fractions 210-225 (refer to Figure 20). Its identity was confirmed by GLC analysis (Figure 22). GLC analysis also revealed that at anomeric equilibrium, 96 percent of the D-galactose 6-phosphate existed in the pyranose forms (peaks at 6.37 and 7.26 minutes, Figure 22). Mass-spectral analysis of these peaks yielded major ions with m/e values of 204, 357, and 387, which are characteristically abundant in hexopyranose 6-phosphates (42, 45). The small peaks (4 percent of the total, at 4.12 and 4.77 minutes, Figure 22) serve as substrates for galactose 6-phosphate isomerase and are probably the furanose forms of galactose 6-phosphate. This is the first report concerning the anomeric distribution of D-galactose 6-phosphate that was uncomplicated by the presence of impurities.

Identification of the impurities in commercial preparations of D-galactose 6-phosphate was the next goal of the investigation.

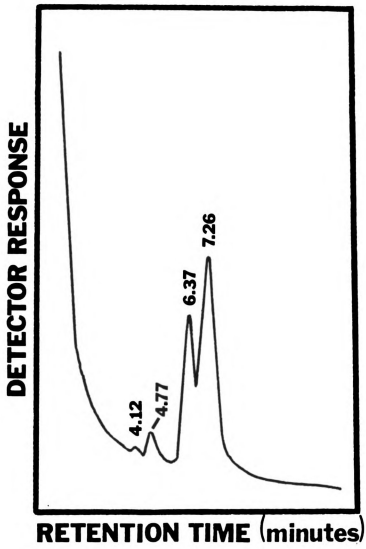


Figure 22. GLC analysis of authentic D-galactose 6-phosphate. Fractions 210-225 from the Dowex (borate) column (Figure 20) were pooled and analyzed. Sample preparation and TMS derivation conditions were as described in Materials and Methods. 3% OV-17 column packing was used. Retention times and peak areas were determined by the programmed instrument. (Refer to Figure 21A for a comparison of retention times to those of the commercial preparation.)

The impurities were identified as hexose monophosphates on the following basis. The commerical galactose 6-phosphate preparation was applied to a Dowex 1-X8 bicarbonate column and its constituents were eluted with a stepwise gradient of 0.15 M, 0.30 M, and 0.45 M $KHCO_3$. The impurities eluted with the monophosphate fraction (115). The impurities were determined to have molecular weights corresponding to hexose monophosphates by mass-spectral analysis of their TMS-O-methyloxime derivatives. Ions m/e 160 and 561, which result from cleavage between C-2 and C-3 of the carbon chain (113, also refer ahead to Figure 24), are indicative of a hexose monophosphate and are present in the mass spectra of the impurities (refer ahead to Table 4).

The impurities were identified as D-galactose-phosphates in the following manner. The impurities reacted quantitatively with alkaline phosphatase, and the carbohydrate moieties liberated by the phosphatase treatment reacted >99 percent with β galactose-dehydrogenase. β-Galactose dehydrogenase is specific for D-galactose and L-arabinose (59). Through the use of appropriate standards, it was determined that D-galactose and L-arabinose are effectively resolved by GLC using a 3 percent OV-1 column, and may be identified by their characteristic retention times (Figure 23). GLC analysis confirmed that D-galactose was the sole product resulting from phosphatase treatment of the impurities and that, in particular, L-arabinose was absent (Figure 23).

The position of the phosphate groups on the contaminating substances was determined in the following manner. The 1-phosphate

Figure 23. GLC analysis of the products of phosphatase-treated commercial D-galactose 6-phosphate. The reaction mixture (0.6 ml) contained 135 μmoles of Tris-HCl buffer (pH 8.1), 12.0 μmoles of the commercial D-galactose 6-phosphate preparation, and 5.5 Units of E. coli alkaline phosphatase. Reaction completion was confirmed by the absence of peaks in the sugar-phosphate region of the GLC chromatogram (not shown). TMS derivation conditions for the lypophilized samples were as described in the Materials and Methods section. 3% OV-1 column packing was used. Retention times were determined by the programmed instrument.

A. Product of phosphatase-treated commercial D-galactose 6-phosphate.
B. D-Galactose standard.
C. L-Arabinose standard.

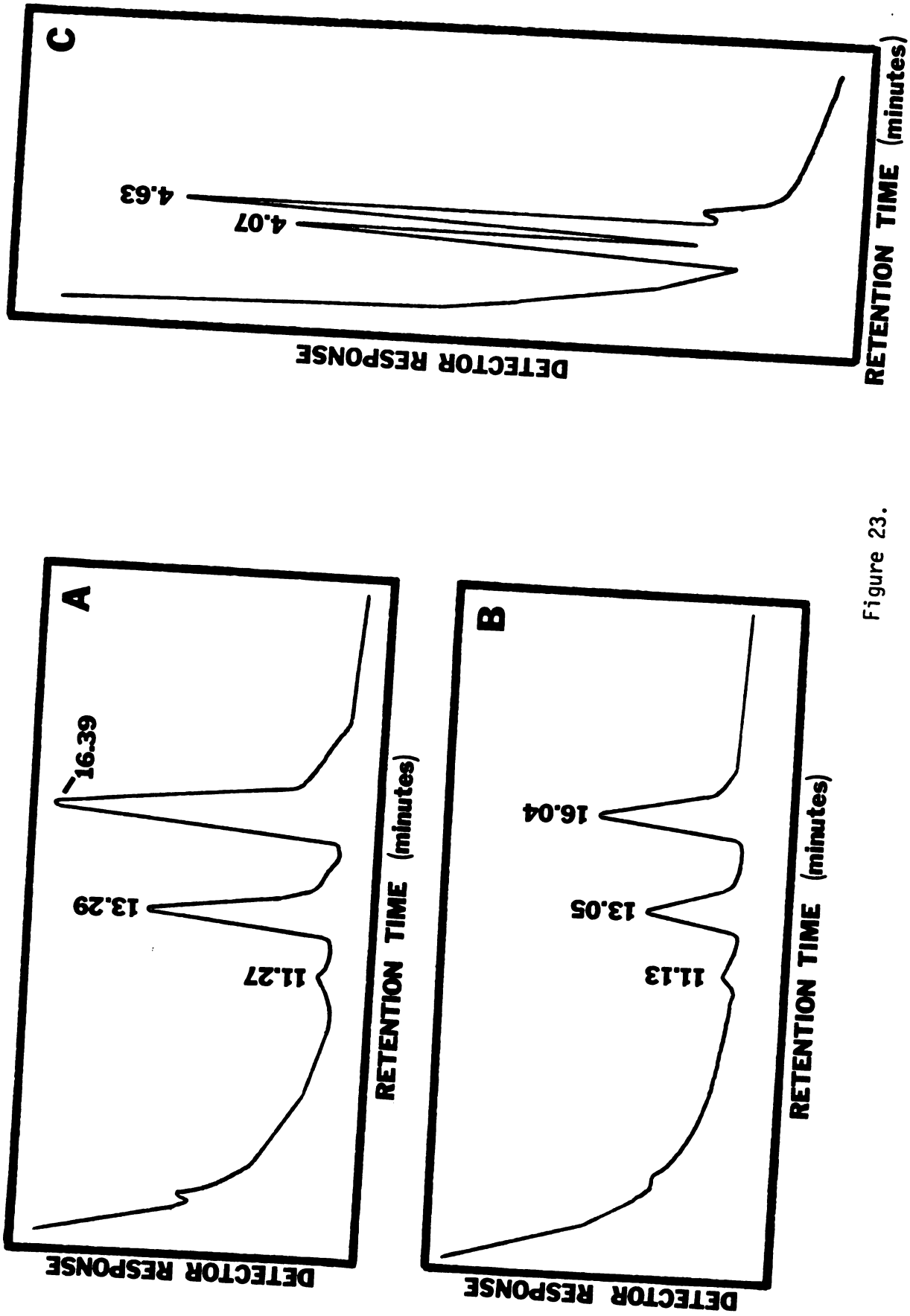

C
4.63
4.07
DETECTOR RESPONSE
RETENTION TIME (minutes)
A
16.39
13.29
11.27
DETECTOR RESPONSE
RETENTION TIME (minutes)
B
16.04
13.05
11.13
DETECTOR RESPONSE
RETENTION TIME (minutes)

Figure 23.

was ruled out as an impurity for the following reasons. The contaminants consistently formed stable TMS derivatives (Figures 4, 15, 18, 19). However, repeated attempts to form TMS derivatives of authentic galactose 1-phosphate under the conditions of trimethylsilylation used in this study were not successful. It is known that TMS derivatives of the aldose 1-phosphates are less stable than the hexose 6-phosphates (116). Specially defined conditions are required to form suitably stable TMS derivatives of aldose 1-phosphates (42). In addition, aldohexose 1-phosphates are known to readily hydrolyze in 1 N acid solution at 100°C (28). Galactose 1-phosphate could not have survived the harsh acid conditions involved in the galactose 6-phosphate synthesis (see Materials and Methods). The impurities are known to be acid-stable (Figure 19). Finally, O-methyloxime derivatives can be synthesized from the impurities in the commerical galactose 6-phosphate preparations (Figure 13). An O-methyloxime derivative of galactose 1-phosphate cannot be formed because of the lack of a free carbonyl function.

The impurities could not be the 6-phosphate of D-galactose because they did not serve as substrates for D-galactose 6-phosphate isomerase (Figure 15), and because of the obvious reason that the TMS-O-methyloxime derivative of authentic galactose 6-phosphate had a different retention time than the TMS-O-methyloxime derivatives of the impurities upon GLC analysis (Figure 13).

It is unlikely that the 2-phosphate could be an impurity because of the presence of ion m/e 160 in the mass spectra of the TMS-O-methyloxime derivatives of the contaminating substances (Table 4). Ion m/e 160 incorporates C-1 and C-2 of the carbon chain (113) and would not be present if the sugar contained a phosphate group at the 2-position. The concurrent presence of m/e 561 (Table 4 and Figure 24) in the mass spectra of the impurities supports the contention that cleavage of the carbon chain between C-2 and C-3 has occurred.

It is improbable that the 4-phosphate is an impurity. The existence of the 4-phosphate of D-galactose is unlikely due to structural instability. The bulky, polar, phosphate group would be positioned axially on the D-galactose moiety, a sterically unfavorable situation (117). Indeed, studies involving esterification reactions of D-galactose have shown that the 4-hydroxyl group exhibits low reactivity (118).

Therefore, by a process of elimination, the two impurities in the commercial preparations of galactose 6-phosphate were identified as the 5-phosphate and the 3-phosphate of D-galactose. Furthermore, the assignment of galactose 5-phosphate as the major impurity (retention time of 5.37 minutes, Figure 18A), and galactose 3-phosphate as the minor impurity (retention time of 6.11 minutes, Figure 18A) is supported by the chromatographic behavior of the two substances. On all the GLC packing used in this study, the major impurity migrated ahead of the minor impurity (Figures 4, 15, 18, 19). However, in the presence of

Table 4

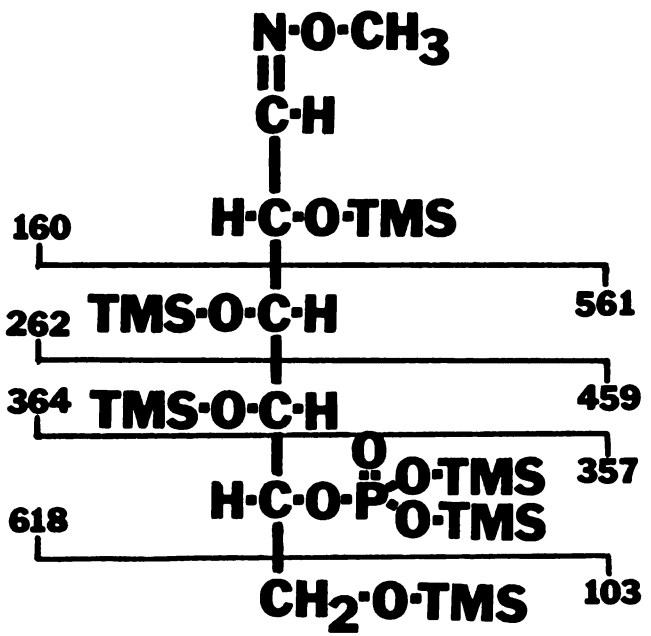
Mass-spectral analysis of the TMS-O-methyloxime derivatives of authentic galactose 6-phosphate and of the impurities found in commercial preparations of D-galactose 6-phosphate.

M/e	Major Impurity[a]	Minor Impurity[b]	Authentic Galactose 6P
103	6.1	11.9	4.9
160	10.1	4.7	10.3
205	6.7	7.9	2.3
255	0.4	0.5	0.2
262	1.1	0.5	0.7
307	0.8	2.0	0.9
357	13.8	9.0	21.5
364	0.3	0.2	1.2
414	0.3	2.6	0.0
459	3.9	3.5	0.8
466	0.1	0.0	0.1
516	0.4	1.5	0.0
561	7.9	1.9	1.3
618	0.5	0.2	0.0

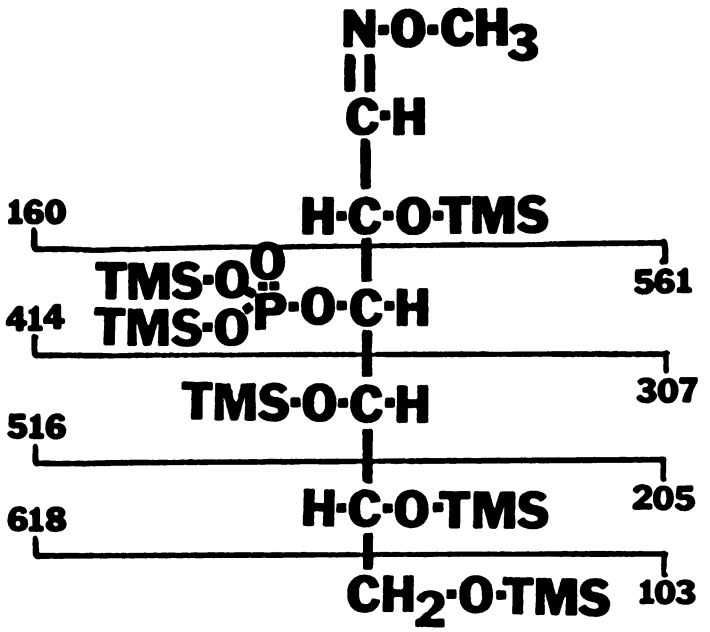
Mass spectra were obtained as described in Materials and Methods. The presence of each ion is recorded in terms of percent intensity, relative to m/e 73=100%.

[a]The designation 'major impurity' refers to the substance which gives rise to the peak with a retention time of 7.57 minutes in Figure 13. This substance has been identified as D-galactose 5-phosphate (see text).

[b]The designation 'minor impurity' refers to the substance which gives rise to the peak with a retention time of 8.40 minutes in Figure 13. This substance has been identified as D-galactose 3-phosphate (see text).



a.



b.

Figure 24. Predicted major fragmentation pattern for the TMS-O-methyloxime derivatives of the impurities in the commercial preparation of D-galactose 6-phosphate. Symbol: TMS= $(CH_3)_3Si$-.

a. TMS-O-methyloxime derivative of D-galactose 5-phosphate.

b. TMS-O-methyloxime derivative of D-galactose 3-phosphate.

borate, the minor impurity migrated ahead of the major impurity (Figure 20). This behavior may be explained by the fact that the 5-phosphate of galactose is necessarily a furanose; substitution at the 5-position prevents formation of a six-membered ring. Furanoses form much stronger borate complexes than do six-membered rings (32, 33, 37); they tend to interact more readily with the ion-exchange resin and, in general, have longer retention times than corresponding pyranoses (35). This observed elution behavior of the galactose phosphates was analogous to that reported for the ribose phosphates (35). In the absence of borate, the 5-phosphate of ribose eluted before the 3-phosphate. In the presence of borate, the 5-phosphate eluted after the 3-phosphate.

Unequivocal identification of the galactose monophosphate impurities as the 3- and the 5-phosphates was attempted through mass-spectral analysis of TMS-O-methyloxime derivatives of the impurities. Mass-spectral analysis of the trimethylsilyl O-methyloxime derivatives of partially methylated sugars has been successfully used to determine the location of substituents on a carbohydrate (113). However, this technique is not as successful when applied to sugar phosphates. The occurrence of phosphate group migrations and rearrangements in the mass spectrometer (45, 119, 120) precluded unambiguous fragmentation patterns like those reported for the non-phosphorylated sugars (113). While the mass-spectral data therefore cannot be used to provide conclusive identifications to the impurities, the data are nevertheless consistent with the assignment of the 5-phosphate and the 3-phosphate to the impurities.

Examination of the relative intensities of certain key ions support the identifications assigned to the impurities. For each impurity, all eight predicted ions arising from cleavage along the carbon chain are present (Table 4 and Figure 24). Ions with m/e values of 205, 307, 414, and 516 are predicted to arise from cleavage along the carbon chain of galactose 3-phosphate, but not the 5- or the 6-phosphate. These ions were found to be of the greatest intensity in the spectrum of the impurity identified as the 3-phosphate (Table 4). The presence of these ions in the spectra of the 5- and the 6-phosphates may be due to alternate fragmentation pathways (121), or to lack of complete resolution of the substances by GLC (Figure 13).

It is predicted that cleavage of the carbon chain at a point adjacent to the carbon containing a phosphate group is less likely to occur because of the lack of vicinal alkoxyl functions (113). Using this criterion, one would expect to observe less of m/e 103 and 618, and more of m/e 160 and 561 in the spectrum of the impurity identified as the 5-phosphate, when compared to the intensities of those ions in the spectrum of the impurity identified as the 3-phosphate (Figure 24). This was found to be true in all cases except m/e 618 (Table 4). The insubstantial presence of this ion in all three mass spectra, probably due to further rapid fragmentation of the large ion, rendered it unsatisfactory for comparison purposes. By the criterion under consideration, m/e 357 would not be expected to be abundant in the impurity identified as the 5-phosphate. However, m/e 357 is a rearrangement ion containing

a resonance stabilized phosphonium cation (42, 120) and has been reported as a significant ion in at least 15 sugar phosphates (42). It was found to be a major ion in the 3-, 5-, and 6-phosphate derivatives (Table 4).

Finally with regard to the mass-spectral analyses, it is of interest to note evidence suggesting the minor presence of a galactose cyclic-phosphate in the commercial preparations of galactose 6-phosphate. The substance tentatively identified as the TMS-O-methyloxime derivative of a galactose cyclic-phosphate appears with a retention time of 4.87 minutes in Figure 13. Pertinent data from a mass-spectral analysis of this substance is presented in Table 5. The largest ion present in the spectrum, m/e 559, corresponds to the molecular weight of a TMS-O-methyloxime galactose cyclic-phosphate. That this is the molecular ion is substantiated by the presence of m/e 544, formed by the loss of a methyl radical from a TMS group in the molecular ion. This (M-15) ion is a fragment of high mass commonly observed in the analysis of TMS derivatives (42). Ions m/e 160, 195, 262, 297, 364, and 399 are predicted for cleavage along the carbon chain of the TMS-O-methyloxime derivative of galactose 5,6-cyclic phosphate (Figure 25). Their presence in the mass spectrum of the substance in question (Table 5) has led to its identification as galactose 5,6-cyclic phosphate. Other ions listed in Table 5 arise from the common loss of TMS-OH groups (45) from the fragments derived from carbon chain cleavage. Their presence supports the identification of the substance as galactose 5,6-cyclic phosphate. While

Table 5

Mass-spectral analysis of the TMS-O-methyloxime derivative of a substance in commercial preparations of D-galactose 6-phosphate identified as galactose 5,6-cyclic phosphate. Refer also to Figure 25.

M/e	Percent intensity[a]
559 (M)	1.0
544 (M-15)	2.9
399	25.6
364	0.2
297	1.1
262	1.0
195	1.5
160	7.4
309 (399-90)	6.1
274 (364-90)	0.2
207 (297-90)	3.0
172 (262-90)	0.9
105 (195-90)	2.1
129 (399-270)	9.9

[a]Percent intensity is relative to m/e 73=100 percent. M = the molecular ion. (M-15) represents the loss of a methyl group from the molecular ion. Minus m/e = 90 (or a factor thereof) represents the common loss of TMS-OH. Refer to the text for further discussion.

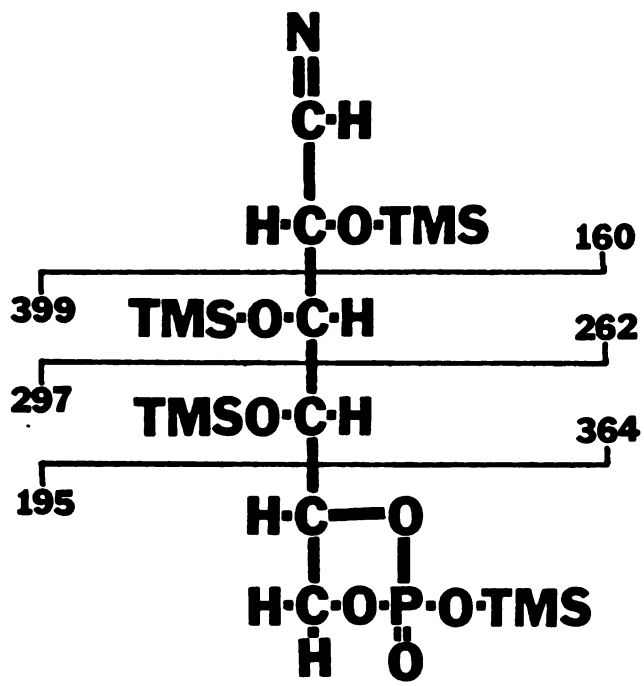


Figure 25. Predicted major fragmentation pattern for the TMS-O-methyloxime derivative of D-galactose-5,6-cyclic-phosphate. Symbol: TMS= $(CH_3)_3$-Si-.

the mass-spectral data could arise from any hexose 5,6-cyclic-phosphate, it is believed that the cyclic-phosphate is a derivative of D-galactose because the commercial preparation had been found to react quantitatively with β-galactose dehydrogenase after treatment with alkaline phosphatase. The presence of D-galactose 5,6-cyclic phosphate suggests that a phosphate group migration occurred during the preparation of D-galactose 6-phosphate, since a cyclic intermediate is proposed for such group migrations (24, 25, 26).

As a final note to the investigation of galactose 6-phosphate, it was observed that high concentrations of glycerol (42 μmoles/μmole galactose 6-phosphate) decreased the relative size of the β pyranose peak in the GLC analyses of galactose 6-phosphate. Glycerol was a major component (15 percent by volume) of the galactose 6-phosphate isomerase preparation used throughout the course of this project. Reasons for this effect are not known. Depression of the β pyranose peak was not observed when the glycerol concentration was lowered (10 μmoles glycerol/μmole galactose 6-phosphate).

Equilibrium Composition of the D-Galactose 6-Phosphate Isomerase-Catalyzed Reaction

With the investigation of the two substrates complete, the true equilibrium composition of the D-galactose 6-phosphate isomerase catalyzed reaction could be established. It was determined, using purified substrates, that at equilibrium the reaction mixture consisted of 90 percent D-galactose 6-phosphate and 10 percent D-tagatose 6-phosphate (Figure 26). Initial and

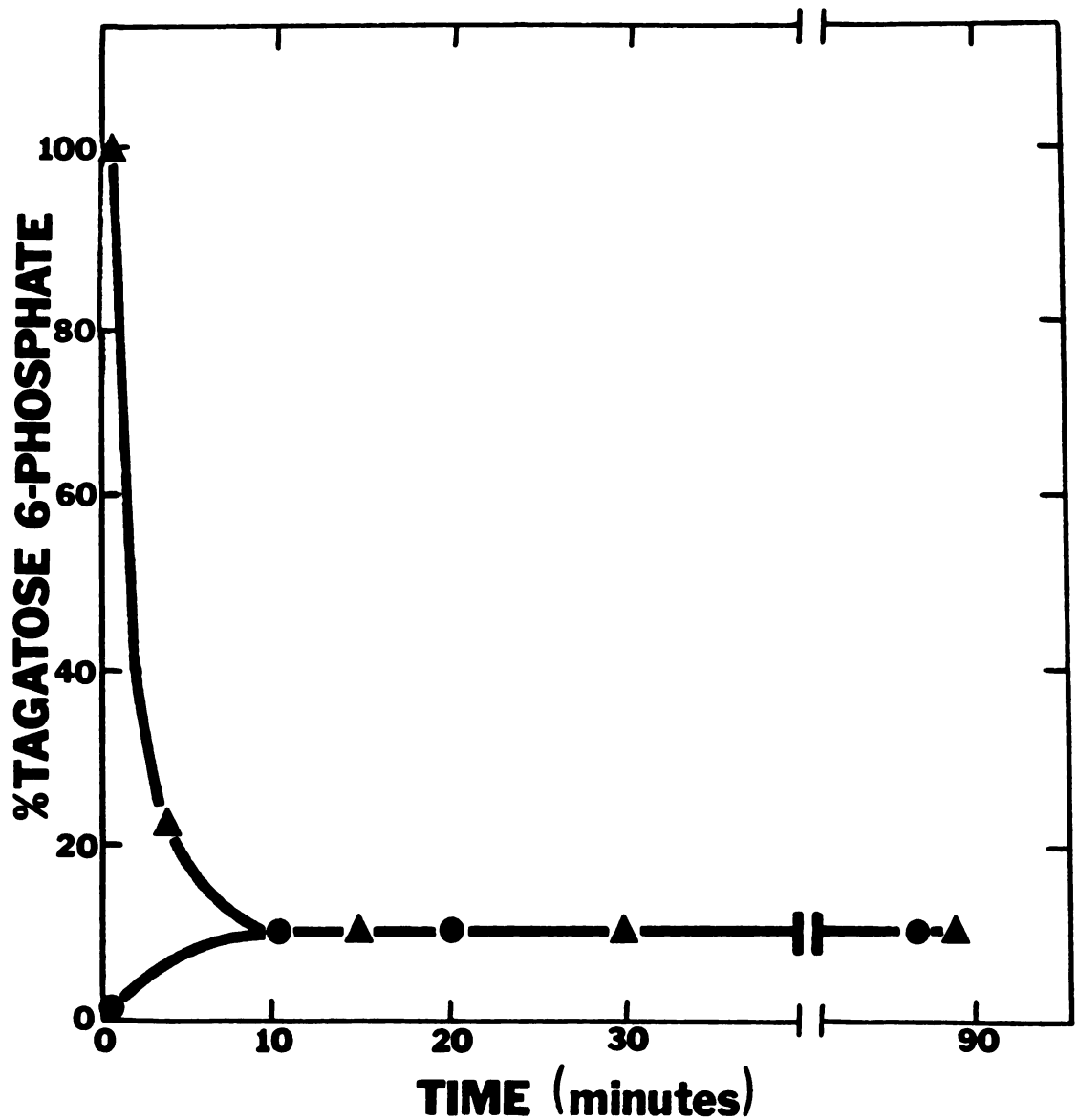


Figure 26. Equilibrium composition of the D-galactose 6-phosphate isomerase-catalyzed reaction. The reaction mixture contained 14 μmoles of Bicine buffer (pH 8.2), 1 μmole of tagatose 6-phosphate and 15 mU galactose 6-phosphate isomerase (▲), or 5 μmoles galactose 6-phosphate and 300 mU galactose 6-phosphate isomerase (●). At the specified times, samples were removed and assayed for tagatose 6-phosphate by the fructose 6-phosphate kinase-linked end-point assay.

equilibrium concentrations of D-tagatose 6-phosphate were determined by the fructose 6-phosphate kinase-linked end-point assay. Initial and equilibrium concentrations of D-galactose 6-phosphate were determined by the alkaline phosphatase coupled to β galactose-dehydrogenase end-point assay.

GENERAL DISCUSSION

The hypothesis, proposed by D.L. Bissett, that galactose 6-phosphate failed to mutarotate in aqueous solution was based on the following evidence (5). When D-tagatose 6-phosphate was the substrate in the D-galactose 6-phosphate isomerase-catalyzed reaction, the equilibrium mixture contained 40 percent ketohexose and 60 percent aldohexose. When D-galactose 6-phosphate was the substrate, the equilibrium mixture contained 8 percent ketohexose and 92 percent aldohexose. The same equilibrium values were obtained regardless of the amount or purity of the isomerase added, or the time of incubation. When the equilibrium mixture of the isomerase-catalyzed reaction with D-tagatose 6-phosphate as initial substrate was examined by gas-liquid chromatography, only two peaks were present: one corresponding to D-tagatose 6-phosphate, and the other to one of the anomers of D-galactose 6-phosphate. Treatment of a duplicate sample with HCl (to pH 1) prior to silylation resulted in the appearance of five peaks: one for D-tagatose 6-phosphate, and one each for the four reported anomers of D-galactose 6-phosphate. Furthermore, the ratio of D-tagatose 6-phosphate to the active anomer of D-galactose 6-phosphate was 0.67 at equilibrium for both the

forward and reverse isomerase reactions. These data led Bissett to propose that only one anomer of D-galactose 6-phosphate participated in the isomerization reaction, and that there was no mutarotation of D-galactose 6-phosphate under the conditions employed (25°C, pH 8.2).

My early attempts to explain the apparent lack of mutarotation of galactose 6-phosphate centered on possible interactions between the sugar-phosphate and other molecules in solution that could be inhibitory to the mutarotation process. Initial speculation was that Bicine [N,N-bis(2-hydroxyethyl) glycine] could be acting as a specific inhibitor of mutarotation. It was proposed that an ionic interaction could occur at pH 8.2 between the positively charged nitrogen atom of the Bicine molecule and the negatively charged phosphate group of D-galactose 6-phosphate. If such an interaction occurred the molecules could be oriented such that the two hydroxyl groups of Bicine could form hydrogen bonds to the ring oxygen of the carbohydrate. Mutarotation would therefore be inhibited by blocking the necessary protonation of the ring oxygen (122). Furthermore, in such a binding scheme the negatively charged carboxyl group of the Bicine molecule would be oriented away from the negatively charged phosphate group of the sugar molecule. While such a hypothesis was attractive both for its theoretical feasibility and as an explanation for the apparent lack of mutarotation of galactose 6-phosphate observed by Bissett (5), it was shown not to hold true. The results of the present investigation clearly indicated that galactose 6-phosphate

readily mutarotated in the presence of Bicine in aqueous solution. Although a mutarotation coefficient was not determined, it was noted (data not shown) that galactose 6-phosphate reached anomeric equilibrium in an aqueous solution at pH 8.2 in less than two minutes, indicating a rapid mutarotation consistent with previously studied sugar phosphates (13, 104).

In light of the findings of the present study, it may now be speculated how Bissett arrived at the erroneous conclusion that galactose 6-phosphate failed to mutarotate. The discovery that the preparation of tagatose 6-phosphate used by Bissett was substantially impure provides an explanation for the unusual equilibrium compositions that were reported for the forward and reverse galactose 6-phosphate isomerase-catalyzed reactions (5). Bissett had determined the equilibrium composition by measuring colorimetrically the amount of ketohexose present initially and at equilibrium: a determination which was based on the assumption that all of the ketohexose present was enzymatically active. However, it was found in the present study that the impurity in the tagatose 6-phosphate preparation, isopropylidene-D-tagatose 6-phosphate, gave a positive test for ketohexose in the colorimetric assay but failed to serve as a substrate for the isomerase. Therefore, a colorimetric determination of the equilibrium composition of the reverse isomerase reaction gave an artificially high value for D-tagatose 6-phosphate, when based on the assumption that all of the ketohexose present was enzymatically active.

Bissett could have based his assumption that the tagatose 6-phosphate preparation was pure on the observation of a single peak in the GLC analysis of the preparation (5). It should be noted that using the same instrument, column packing, and parameters as Bissett, I was unable to resolve the isopropylidene contaminant(s) in the tagatose 6-phosphate preparation.

It is troubling that Bissett (5) observed only one peak for galactose 6-phosphate in the GLC analysis of the reverse galactose 6-phosphate isomerase equilibrium mixture. The furanoses of authentic galactose 6-phosphate appear in such small quantities (Figure 22) that it is understandable how they might not be detected. It is more difficult to explain the absence of the β pyranose peak, the major anomer of galactose 6-phosphate. It is speculated that the absence of the β pyranose peak in Bissett's GLC analysis may have been due to the presence of adversely high concentrations of glycerol in his samples, since it was found in the present study that high concentrations of glycerol decreased the relative size of the β pyranose peak in the GLC analysis of galactose 6-phosphate (see Results and Discussion). It is also possible that the α and β pyranose anomers were inadequately resolved on the particular chromatographic column in use. In relation to this, W.D.L. Musick's thesis (123) also contains a GLC chromatogram of galactose 6-phosphate in which the β pyranose peak was not clearly resolved.

Despite repeated attempts, I was unable to define GLC conditions that led to results similar to those of Bissett concerning

the peak distribution in the reverse galactose 6-phosphate isomerase equilibrium mixture. Neither am I able to explain in light of the findings of this thesis research how Bissett was able to induce the appearance of the galactose-phosphate contaminants by treatment of the reverse galactose 6-phosphate isomerase equilibrium mixture with acid (5).

It has been conclusively shown that impure preparations of tagatose 6-phosphate resulted from incomplete acid hydrolysis of 1,2;3,4-di-O-isopropylidene tagatose 6-phosphate (see Results and Discussion). However, it has not been clearly resolved whether a single impurity is involved, or whether a mixture of isopropylidene-tagatose 6-phosphate derivatives is present. Possible candidates are 1,2;3,4-di-O-isopropylidene-tagatose 6-phosphate, 1,2-O-isopropylidene-tagatose 6-phosphate, and 3,4-O-isopropylidene-tagatose 6-phosphate. Both α and β forms of these substances are possible. Upon GLC analysis, the contaminating substance(s) appears as a single peak with a retention time identical to the di-isopropylidene derivative (Figure 8). It is possible, however, that a mixture of isopropylidene derivatives is present but is not resolved under the chromatographic conditions employed. ^{13}C-NMR analysis of the impure preparation of tagatose 6-phosphate suggests that a mixture of isopropylidene derivatives, rather than a single species, is involved. At least three peaks were present in the methyl carbon region of the spectrum (Figure 6). These peaks are present in the ratio of 4 to 2 to 1 (peak height ratios).

It is believed that the di-isopropylidene derivative is not present above trace amounts in the impure tagatose 6-phosphate preparation. The di-isopropylidene derivative was found to be insoluble in water, while the contaminating substances were readily soluble.

It is known that for cyclic acetals from ketoses in general, rings attached to the anomeric carbon atom are less readily hydrolyzed than those attached at other positions (124, 125). The relative stability of 1,2-isopropylidene derivatives to acid hydrolysis is well documented in the literature. The 1,2-cyclic acetal of di-isopropylidene derivatives is produced in high yield under well defined conditions of acid hydrolysis for the following: 1,2;3,4-di-O-isopropylidene-D-galactose (126), 1,2;3,4-di-O-isopropylidene-L-xylose (127), 1,2;5,6-di-O-isopropylidene-D-glucose (128), 1,2;4,5-di-O-isopropylidene-D-fructose (129), and 1,2;4,5-di-O-isopropylidene-D-psicose (130). Therefore, it is reasonable to assume that 1,2-O-isopropylidene-tagatose 6-phosphate is present in the impure preparation in a greater amount than 3,4-O-isopropylidene tagatose 6-phosphate.

It is known that the anomeric distribution of tagatose 6-phosphate is 80 percent β and 20 percent α (53). It is also known that the ^{13}C-NMR peak height ratios for methyl carbons in the impure preparations is 4 to 2 to 1. If an assumption is made that the methyl carbons of the β anomer of the 1,2-cyclic acetal resonate at a different frequency than the methyl carbons of its α anomer, and that the methyl carbons of the 3,4-cyclic acetal resonate at

identical frequencies for both its α and β anomers, then one may speculate that the impurities consisted of a mixture of the following: 1,2-O-isopropylidene-β-tagatose 6-phosphate (4 parts), 1,2-O-isopropylidene-α-tagatose 6-phosphate (1 part), and 3,4-O-isopropylidene-tagatose 6-phosphate (2 parts). That is, the mixture of isopropylidene-tagatose 6-phosphate impurities contained 70 percent 1,2-cyclic acetal, and 30 percent 3,4-cyclic acetal, by the above reasoning.

The misconception existing in the literature that the contaminants of commercial galactose 6-phosphate are the authentic furanose anomers holds particular importance in a paper by Zinbo and Sherman (45). They compared the mass spectra of trimethylsilyl-α-D-galactopyranose 6-phosphate and what they thought was α-D-galactofuranose 6-phosphate with special references being drawn to the mass spectral differences between furanose and pyranose forms of phosphorylated carbohydrates. Fortuitiously, what they thought was a furanose of galactose 6-phosphate has now been identified as galactose 5-phosphate, itself locked into the furanose ring, so that their assignments of fragmentation ions characteristic of furanose forms remain valid.

Harvey and Horning (42) suggested the possibility of impurities in commercial galactose 6-phosphate preparations in a footnote to their data concerning galactofuranose 6-phosphate. However, their claim was not substantiated and subsequent investigators assumed the major presence of furanose anomers of authentic galactose 6-phosphate (5, 59, 60).

Musick and Wells (59) reported observing in the gas chromatograms of extracts from chick brain, peaks with the retention times of what had been thought to be α and β-galactofuranose 6-phosphate. These peaks were probably due to pentose phosphates which have similar retention times to the impurities of commercial galactose 6-phosphate. Possible candidates are D-ribose 5-phosphate and 2-deoxy-D-ribose-5-phosphate.

D-Galactose 1-phosphate and D-galactose 6-phosphate are widely known in the literature. The only other galactose phosphates reported are D-galactose 3-phosphate and D-galactose 5-phosphate. D-Galactose-3-phosphate was prepared from 1,2-isopropylidene-4,6-ethylidene-D-galactopyranose and diphenyl phosphochloridate by Foster et al. (131). They studied the acid labilities of galactose 3-phosphate and galactose 6-phosphate by heating in 0.1 N H_2SO_4 at 100°C, and at given time intervals estimated the liberated phosphate colorimetrically. It was known that 6-phosphates were stable to acid hydrolysis, but it was found that the 3-phosphate was also quite stable in the presence of acid. After 5 hours of the above acid treatment, 87 percent of the D-galactose 3-phosphate remained (compared to 93 percent D-galactose 6-phosphate remaining after an identical acid treatment). This is in accord with the results of the present study which indicates that the contaminant identified as D-galactose 3-phosphate is quite stable to harsh acid conditions, but to a somewhat lesser degree than D-galactose 6-phosphate (refer to Figure 19). D-Galactose 5-phosphate was reported to be synthesized in 43 percent yield from methyl-α-D-galactopyranoside with $POCl_3$ in excess trimethylphosphate (132).

Two major impurities have been identified in commercial preparations of D-galactose 6-phosphate during the course of this study. Some evidence has accumulated suggestive of the possible presence of additional minor impurities. The existence of shoulders on several peaks in the chromatogram of TMS-O-methyloxime derivatives of commercial galactose 6-phosphate (Figure 13) might be interpreted as evidence for additional impurities. However, the appearance of shoulders on the peaks of TMS-O-methyloxime derivatives is common, and is presumed to be due to the existence of syn- and anti- isomers of the O-methyloxime (42, 98, 113). Prominent shoulders on the initial elution peak of Figure 20 are suggestive of additional minor impurities, as is the presence of multiple peaks in the GLC analyses of the Dowex (borate) ion-exchange chromatography elution peaks (Figure 21). However, it is known that borate-polyol systems frequently involve complicated equilibria between several types of sugar-borate complexes (32, 37, 133). The shoulders on the elution peak in Figure 20 might be due to the presence of alternate forms of the sugar-borate complex. The multiple peaks in the GLC analyses (Figure 21) could be due to incomplete derivation of the substance during the silylation process. It was discovered (see Results and Discussion) that the silylation reaction is completely inhibited in the presence of borate. Partial inhibition of the silylation reaction, leading to multiple GLC peaks, would be possible if traces of borate were present in the samples to be analyzed. Therefore, the presence of additional impurities in commercial preparations of galactose-6-phosphate remains questionable.

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