INITIATION OF GALACTITOL METABOLISM IN AEROBACTER AEROGENES

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ABSTRACT

Initiation of Galactitol Metabolism
in Aerobacter aerogenes

by

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The metabolism of galactitol in Aerobacter aerogenes is shown to be initiated by a phosphoenolpyruvate-dependent sugar phosphotransferase system. The product of the reaction catalyzed by this system is conclusively established as L-galactitol 1-phosphate by showing that it exhibits the same physical, chemical, and enzymatic characteristics as does chemically synthesized L-galactitol 1-phosphate. Furthermore, this product can be enzymatically oxidized using L-galactitol 1-phosphate dehydrogenase to yield D-tagatose 6-phosphate, which was characterized with respect to gas-liquid chromatography, acid lability, periodate oxidation, and enantiomeric configuration.

INITIATION OF GALACTITOL METABOLISM IN *AEROBACTER AEROGENES*

By

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A THESIS

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Dedicated to my family

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I thank my major professor, R. L. Anderson, who more than fulfilled his responsibility as a teacher by increasing my scientific abilities. I also thank the other members of the laboratory who's suggestions have also guided in my scientific training. For her enduring patience I thank my wife, Maria.

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ABBREVIATIONS

ATP	adenosine triphosphate
EDTA	ethylenediamine tetraacetate
P_i	inorganic phosphate
P_{org}	organic phosphate
TMS	trimethylsilyl
BSA	bis-trimethylacetamide
TMCS	trimethylchlorosilane
NAD^+	nicotinamide adenine dinucleotide
NADH	reduced nicotinamide adenine dinucleotide
$NADP^+$	nicotinamide adenine dinucleotide phosphate
NADPH	reduced nicotinamide adenine dinucleotide phosphate
PEP	phosphoenolpyruvate
E_{II}^{mtl}	enzyme II mannitol
E_{II}^{gltl}	enzyme II galactitol
PTS	phosphoenolpyruvate-dependent sugar phosphotransferase system
E_I	enzyme I
HPr	histidine-containing phosphotransfer protein
Tris	tris(hydroxymethyl)aminomethane
TCA	trichloroacetic acid
DEAE	diethylaminoethyl
GLC	gas-liquid chromatography

INTRODUCTION

An interest in bacterial hexitol metabolism led to my investigating the route of galactitol dissimilation in Aerobacter aerogenes PRL-R3. Galactitol catabolism has not been elucidated for any organism except for a Pseudomonas species which was found to directly oxidize the polyol to D-tagatose by utilization of a nonspecific dehydrogenase and NAD^+ (see Literature Review). However, in A. aerogenes, the occurrence of dehydrogenases specific for the phosphate esters of the naturally occuring hexitols (mannitol, sorbitol, and galactitol) suggested that the initiation of hexitol metabolism for this organism could proceed by phosphorylation prior to oxidation. Since it was known that A. aerogenes can simultaneously transport and phosphorylate specific sugars including sorbitol by means of the phosphoenolpyruvate-dependent sugar phosphotransferase system (PTS), it was highly plausible that galactitol biodegradation could be initiated through this system.

The present work confirms that galactitol metabolism in A. aerogenes is initiated through phosphorylation of the hexitol by means of the PTS in lieu of initiation by direct oxidation of galactitol. The product of the PTS-catalyzed reaction is identified as L-galactitol 1-phosphate by chemical,

physical, and enzymatic analysis. Furthermore, a dehydrogenase for L-galactitol 1-phosphate is shown to oxidize the product of the PTS-catalyzed reaction to D-tagatose 6-phosphate.

LITERATURE REVIEW

The literature is plethoric with studies concerning polyol metabolism in both bacterial and mammalian systems. There are a number of review articles available which pertain to the metabolism of these carbohydrates (1-8) and therefore a detailed account will not be given here. Instead, bacterial polyol biodegradation will be circumscribed to the naturally existing hexitols galactitol, D-glucitol (sorbitol), and mannitol, with converging interest in galactitol. These hexahydric alcohols have been recognized since the nineteenth century commencing with the discovery of mannitol in 1806 and the identification of galactitol in 1850 and sorbitol in 1872. Since the last quarter of the nineteenth century, these hexitols have been instituted as an aid in differentiating various bacteria (8) such as the constituents of the Enterobacteriaceae family which have distinguishable fermentative properties in the presence of galactitol (9). Until 1951, the metabolism of galactitol and the other polyols has been examined utilizing acid and/or gas production (10-13), oxygen consumption (14-17), or formation of unidentified reducing sugars (18-20) as criteria. The first hexitol dehydrogenase was isolated by Blakely (21) who demonstrated its catalytic requirement in the oxidation of different polyols

to their corresponding ketohexoses. It was later shown that NAD^+ was required as a coenzyme for the reaction (22). In 1955 (23) and 1956 (24) a second route of hexitol dissimilation was reported which involved phosphorylation prior to the oxidation catalyzed by a hexitol phosphate dehydrogenase requiring NAD^+ as the coenzyme. The corresponding dehydrogenase for L-galactitol 1-phosphate was implicated in 1955 (25) followed by isolation of a sorbitol 6-phosphate dehydrogenase in 1962 (26). The function of these dehydrogenases in relation to carbohydrate metabolism remained obscure to the investigators (26) since the phosphorylative mechanism was not definitively established. The resolution to this problem began in 1964 when an attempt to ascertain the existence of a kinase for sialic acids in bacteria resulted in the serendipitous discovery of a novel phosphotransferase system in Escherichia coli (27). In 1971 this system was further characterized by Roseman and coworkers (28-30). The system catalyzed the phosphorylation of hexoses utilizing phosphoenolpyruvate (PEP) as the phosphoryl donor and was consequently named the phosphoenolpyruvate-dependent phosphotransferase system (PTS). Concurrent with Roseman's investigations have been the sugar transport studies of Anderson and associates (31-34), Kaback and associates (35-37), Kornberg and associates (38-42), Hengstengstenberg and associates (43-46), and many others. Since many reviews on sugar transport have recently appeared (47-52), only the salient aspects will be reviewed here so

that a relationship of transport with bacterial hexitol metabolism may become apparent.

The significance of the phosphoenolpyruvate-dependent sugar phosphotransferase system to both gram negative and gram positive bacteria is the fact that it simultaneously transports and phosphorylates sugars. There are many sundry aspects to the process by which this occurs, contingent upon the organism investigated, but the basic mechanism is constituted by a two-step reaction sequence (48):

$$\text{PEP} + \text{HPr} \xrightleftharpoons{E_I,\ Mg^{+2}} \text{Pyruvate} + \text{P}\sim\text{HPr}$$

$$\text{P}\sim\text{HPr} + \text{Sugar} \xrightarrow{E_{II},\ Mg^{+2}} \text{Sugar-P} + \text{Pyruvate}$$

$$\text{PEP} + \text{Sugar} \longrightarrow \text{Sugar-P} + \text{Pyruvate}$$

The above reaction delineates the assignment of three proteins, HPr, E_I, and E_{II}, which effect the transfer of the phosphoryl group from phosphoenolpyruvate (PEP) to the carbohydrate. The transport of the phosphorylated carbohydrate is consummated by the enzyme II (E_{II}) component. The participation of other proteins elicits variations of the reaction schematic, but only those catalysts exhibited shall be expounded upon.

HPr: In E. coli HPr has been purified to homogeneity and the molecular weight determined to be approximately 9,500 (29). It is committed to the transfer of the phosphoryl group from a phosphoenzyme I intermediate (53) to E_{II}. Consequently HPr is not a true enzyme or substrate. The amino acid

composition reveals that there are two moles of histidine per mole of HPr. Stoichiometric studies demonstrated that one mole of ^{32}P is transferred from ^{32}P-enolpyruvate to one mole of HPr and that the phosphate is bound to the N-1 position of one of the histidine imidazole rings (48). HPr is a constitutive protein and is located in the periplasmic space (54). In Aerobacter aerogenes HPr has been partially purified and appears to be similar to HPr in E. coli (55). Recently the purification of an inducible protein has been reported which supplants HPr during the phosphorylation of low concentrations of fructose (31, 56). It has been designated PTP and functions with the inducible E_{II} for fructose (55). Both PTP and HPr are soluble proteins.

Enzyme I (E_I): E_I, like HPr, is a soluble and constitutive protein. It catalyzes the reversible phosphorylation of HPr with PEP. In E. coli partially purified E_I has been shown to be irreversibly inactivated with N-ethylmalaimide (28) or with vinylglycolate (57). Inhibition by sulfhydryl reagents such as mecuribenzoate or dithionitrobenzoic acid can be reversed by 2-mercaptoethanol, glutathion, or cystein (28). This implies that sulfur-containing amino acids are present and may function in the active center of E_I. In A. aerogenes E_I has been partially purified to demonstrate its participation in the PTS system, but has not been characterized (56). As with HPr, mutants that lack E_I exhibit pleiotropism since they are incompetent in the utilization of various carbohydrates. Pleiotropic mutants have been isolated

from A. aerogenes (58), E. coli (59), Salmonella typhimurium (60), and Staphylococcus aureus (61). The Salmonella typhimurium mutants cannot ferment D-glucose, D-mannose, D-fructose, maltose, melibiose, N-acetylglucosamine, and the polyols D-glucitol, mannitol, and glycerol. The Staphylococcus aureus mutants are incapable of transporting sucrose, maltose, lactose, trehalose, D-galactose, D-fructose, D-ribose, and the hexitol mannitol. The mutants of E. coli are defective in the utilization of D-glucose, D-fructose, D-mannose, D-galactose, maltose, lactose, melibiose, succinate, and the polyols mannitol and glycerol. The A. aerogenes mutants cannot transport D-glucose, D-fructose, D-mannose, and the hexitols D-glucitol, and mannitol. As evidenced by the listed compounds, not all of the pleiotropic mutants are capable of dissimilating glycerol or succinate which are not phosphorylated by the PTS. However, exogenously furnished cyclic AMP restores the capacity to flourish on these substances, thus implying the existence of a regulatory responsibility for the PTS (62). Credible control mechanisms may be catabolite repression (63, 64), transient repression (65), and inducer exclusion (66).

Enzyme II (E_{II}): In A. aerogenes the constitutive E_{II}'s catalyze irreversible phosphorylation of D-fructose (31), and apparently D-mannose (55), D-glucose (55), and L-sorbose (34). However, the preponderant E_{II}'s are inducible sugar-specific proteins which are integrally associated with the membrane (50). A schematic model for the mechanism of action

of E_{II} is delineated as a contiguous membrane constituent which complexes with both HPr and E_I such that the external sugar becomes phosphorylated causing a vectorial passage of the product through a diffusion barrier (50). It has been substantiated that membrane vesicles of E. coli are contingent upon PEP in order to take up sugars as their phosphorylated derivatives and that treatment of the vesicles with phospholipase D inhibits vectorial phosphorylation of α-methylglucoside by phosphatidylglycerol hydrolysis (35, 36, 50). Furthermore, accumulation of sugar phosphate esters in whole cells has been attributed to the PTS (61, 67, 68, 69, 70, 71, 72, 32, 73, 74, 59, 75, 76). The constitutive E_{II} of E. coli has been solubilized into three basic components: two proteins and phosphatidyl glycerol (30). One of the proteins, designated II-A, has been further resolved into three proteins which ascribe phosphorylation specificity to D-glucose, D-fructose, and D-mannose respectively. The remaining protein, II-B, has not been fractionated further but the molecular weight was determined to be 36,000 (30). The II-B protein functions as a ligand between one of the II-A proteins and phosphatidylglycerol thereby constructing the active complex. The specificity of phosphorylation usually occurs at C-6 (35). However, D-fructose (31) and L-sorbose (34) are phosphorylated at C-1 in A. aerogenes. In E. coli D-fructose is phosphorylated at C-1 at low concentrations and at C-1 and C-6 at high concentrations of the sugar (40).

The distribution of the PTS appears to be restricted to the anaerobic and facultatively anaerobic bacteria with the apparent exception of Arthrobacter pyridinolis (77). Most investigations have been concerned with the facultative anaerobes. Among the other organisms studied besides those mentioned, have been Bacillus megaterium (78), Bacillus cereus (78), Achromobacter parvulus (78), Lactobacillus arabinosus (27), Streptococus lactis (83), Bacillus subtilis (78, 79, 35, 54, 80), Clostridium perfringes (69), Clostridium thermocellum (81, 82), and Corynebacterium ulcerans (78). The majority of these organisms have also been examined in regard to the metabolism of polyols. However, the first resolute endeavor to associate hexitol catabolism with the PTS was conducted by Tanaka and coworkers in 1967 (58, 83). It was shown that an E. coli mutant which failed to grow on mannitol was deficient in an apparently inducible E_{II} for mannitol. Further work showed that a mutant in either E_I or HPr also failed to grow on mannitol but revertants of these components regained the wild type growth characteristics (58). In 1971, Kelker and Anderson (33) established the pathway of D-glucitol metabolism in A. aerogenes. The results were consonant with growth on D-glucitol as being dependent upon the PTS and militated against the initiation of glucitol metabolism by a hexitol dehydrogenase. A partially purified glucitol 6-phosphate dehydrogenase was used in the identification of the PTS-catalyzed reaction product, a type of

experiment omitted by Tanaka in his studies with A. aerogenes and E. coli. Corroborative genetic evidence provided substantiation of the glucitol pathway where E_I and glucitol 6-phosphate dehydrogenase mutants were unable to grow on glucitol whereas the corresponding revertants could be expressed on the hexitol.

The investigation of hexitol metabolism in A. aerogenes is extended in this thesis to the initiation of galactitol metabolism. As with D-glucitol catabolism, it might be expected that galactitol is also transported by the PTS in lieu of dissimilation by a galactitol dehydrogenase (85).

MATERIALS AND METHODS

Bacterial Strains

A uracil auxotroph of Aerobacter aerogenes PRL-R3 was selected for the investigation of galactitol catabolism. The uracil genetic marker of the organism was utilized in order to detect contaminations and to be consistent with any future use of this strain as the prototroph for mutant analysis of the pathway.

Media

All media used for growth of uracil auxotrophs was supplemented with 0.005% (w/v) of uracil. Uracil and sugars were autoclaved separately from the rest of the media constituents. The final pH of all the media was adjusted to 7.0 before sterilization in a Castle autoclave for 15 minutes at 121°C.

Mineral Medium

The medium was prepared by dilution, with distilled water, of concentrated stock solutions. The constituents and their final concentrations (w/v) are listed below: 0.71% Na_2HPO_4, 0.15% KH_2PO_4, 0.3% $(NH_4)_2SO_4$, 0.01% $MgSO_4$, 0.0005% $FeSO_4 \cdot 7H_2O$, and 0.5% of the specified sugar.

Nutrient Broth Medium

Two procedures were used for preparing this medium. The first method involved dissolving 5.0 g of Bactopeptone (Difco) and 3.0 g of Bacto beef extract (Difco) in 1.0 L of distilled water. The second procedure consisted of dissolving 8.0 g Bacto nutrient broth (Difco) in 1.0 L of distilled water.

Nutrient Agar Medium

All strains were kept viable by transferring them to a new nutrient agar slant every three months and stored under refrigeration. The medium was prepared by dissolving 23 g of Bacto nutrient agar (Difco) in 1.0 L of distilled water.

Growth of Cultures

Cells utilized for enzyme purification were routinely grown in Fernbach flasks containing 1.0 L quantities of mineral medium. The cultures were incubated at 30°C on a gyrorotary shaker set at speed 3. Cells were also grown in 16 liter and 40 liter quantities of mineral media in Pyrex carboys under the same conditions except that aeration was achieved by sparging with air filtered through a cotton-containing flask. Media contained in Fernbach flasks were usually inoculated with 2.0 ml of an overnight nutrient broth culture whereas media in glass carboys were inoculated with 1.0 liter of an overnight culture grown in mineral medium.

Preparation of Cell Extracts

All procedures were performed at 0°C to 4°C. Bacterial cultures grown in Fernbach flasks were harvested by centrifugation at 16,000 x g in a Sorvall refrigerated centrifuge for 15 minutes. Cells grown in glass carboys were harvested using a Sharples centrifuge at room temperature at a rate of 2.0 liters per minute. The cells were washed twice by resuspension in 200 ml of 0.85 percent NaCl using a large gauge needle and syringe. The suspended cells were harvested and resuspended in 0.02 M Tris-HCl buffer (pH 7.5) containing 0.028 M 2-mercaptoethanol at a concentration of 10 ml of buffer per liter of cells. A maximum of 35 ml of suspension was then disrupted by ultrasonic vibration for 30 minutes in a 10-kHz, 250-watt Raytheon sonicator equipped with an ice-water cooling jacket. Cellular debris was removed from the broken-cell preparation by centrifugation for 15 minutes at 45,000 x g in a refrigerated Sorvall centrifuge. The resulting supernatant was carefully removed with a pipette so as not to recover any of the sedimented debris. This supernatant was then used as the crude extract.

Qualitative Analyses

Dephosphorylation of Sugar Phosphates

Chemically and enzymatically synthesized sugar phosphates were dephosphorylated using commercially prepared Escherichia coli alkaline phosphatase which had been desalted

on a Biogel P-6 column (0.5 cm x 6 cm). Controls missing either enzyme or substrate were also used in order to validate the reaction and account for any background in the GLC analysis. The hydrolysis was carried out in a 12-ml ground-glass, stoppered conical centrifuge tube containing the following constituents in a final volume of 0.90 ml: 1 to 3 mg of sample, 0.85 mmole of Na_2CO_3-acetic acid buffer at pH 8.0, and 12 units of alkaline phosphatase. The reaction mixture was warmed to 30°C and then the hydrolysis was initiated by the addition of enzyme. The reaction was monitored for 6.5 hours by assaying for P_i liberation using the modified method of Fiske and SubbaRow (86). When the production of P_i ceased, the reaction vessels were heated at 100°C for 7 minutes and then cooled on ice. The precipitate was then removed by centrifugation at 12,000 x g for 10 minutes at 4°C. The supernatant was removed and treated with 20 ml of Dowex 50W-X8 H^+ to eliminate bicarbonate as CO_2. The resin was removed by suction filtration and then washed with 10 ml of triply distilled water. The filtrates were combined in a tared glass-stoppered 50-ml centrifuge tube and evaporated to dryness under reduced pressure at 30°C using a rotary evaporator. The centrifuge tube was then reweighed and the sample dissolved in triply distilled water to yield a final sugar concentration of 80 µg/ul.

Gas-Liquid Chromatography of the Polyacetate Derivatives of Various Polyols

The polyacetate derivatives of sugar alcohols were

prepared and analyzed by a slight modification of the procedure used by Jones and Albersheim (87). A 50-µl sample of the dephosphorylated sugar was transferred to a 300-µl Kontes microflex tube and evaporated to dryness at 60°C. A few crystals of sodium acetate were then added (88) followed by 50 µl of fresh anhydrous acetic anhydride. The reaction vessels were flushed with nitrogen, capped, and placed in a metal heating block at 121°C for one hour. The samples were then allowed to cool to room temperature and then were analyzed with a Perkin Elmer 900 Gas Chromatograph equipped with an Autolab System 4 computer. The standard mixture of polyols and all other sugars tested were prepared in the same way except the concentrations were 40 µg/50 µl of acetic anhydride. The instrument parameters and settings of the gas chromatograph were as follows: helium carrier flow rate = 40.0 ml/minute, manifold temperature = 250°C, injector temperature = 265°C, program rate = 1°C/minute, initial time = 4 minutes, final time = 12 minutes, program range = 130 to 180°C, attenuation = 3600 to 60. The glass columns employed were 6 feet x 2mm internal diameter and contained a liquid phase of 0.2% polyethyleneglycol adipate, 0.2% polyethylene glycol succinate, and 0.4% XF-1150 silicon oil on a solid support of Gas-Chrom P (100 to 200 mesh). A second identical column was also used as a balancing column in order to correct for column bleeding.

Gas-Liquid Chromatography of Trimethylsilyl Sugar Derivatives

Sugars were derivatized to their trimethylsilyl (TMS) forms by the method of Musick and Wells (89). All sugar phosphates were converted to their acid forms by treatment with Dowex 50W-X8 H^+ before derivatization. A 1.0 mg sample of an ethanol mixture of the sample was dried at 30°C under nitrogen in a 0.5-dram vial. To the dried sample was added 80 µl of anhydrous pyridine, 200 µl of bis-trimethylsilyl-acetamide (BSA), and 20 µl of trimethylchlorosilane (TMCS).

The reaction vessels were then flushed with nitrogen, sealed with teflon screw caps, and heated at 85°C for 45 minutes. The derivatized samples were allowed to cool to room temperature after which they were immediately analyzed by gas chromatography. Gas chromatography was performed with a Hewlit Packard High Efficiency Gas Chromatograph utilizing a 6 foot x 2 mm internal diameter glass column containing 3°/o SE-30 on a solid support of Supelcoport 80 to 100 mesh. Instrumental settings were as follows: Nitrogen flow rate = 45.5 ml/minute, Flash heater temperature = 310°C, Detector temperature = 310°C, temperature program rate = 1°C/minute.

The temperature program range for the TMS sugars and TMS sugar phosphates was 150°C to 200°C and 200°C to 250°C respectively unless otherwise stated. Chart recordings were made at 1 cm/2 minutes.

Combined Gas-Liquid Chromatography—Mass Spectrometry.

The TMS derivatives of sugars and sugar phosphates were

synthesized as described previously. Mass spectrometry of samples was performed immediately after the TMS derivitization in order to avoid any stability problems of the derivatives. Mass spectra were recorded at 70 eV with an LKB 9000 mass spectrometer-gas chromatograph. The relative abundance of fragments was displayed as bar graphs by means of a data aquisition and processing program utilizing a pdp8/e computer (90). The instrument settings were as follows: Source temperature = 290°C, Accelerating voltage = 3.5 kV, Ionizing current = 60 μA. The samples were gas chromatographed through a 4 foot, 2 mm internal diameter glass coiled column packed with SP-2100 on Supelcoport 100 to 120 mesh. The temperature ranges used were as described before for the TMS derivatives and the program rates were carried out at 3°C/minute.

Determination of Acid Lability

Acid lability was determined according to the method of Wolff and Kaplan (91). The samples, usually 10 μmoles, were first converted to their acid forms by treatment with an excess of Dowex 50W-X8 H^+. The resin was washed with an equal volume of distilled water and the collected filtrates were combined. The resulting solution was then adjusted to 0.5 ml. With samples from the periodate oxidation, the excess periodate was destroyed by the addition of 1 ml of ethylene glycol per 5 ml of sample. The solutions were then transferred to test tubes (1.1 cm x 10 cm) which were placed in a water bath thermostated at 95°C. The solutions were immediately made

1 N in HCl by addition of 0.5 ml of 2 N HCl and then the test tubes covered with glass marbles. The course of hydrolysis was monitored by removing 50 µl to 200 µl aliquots and assaying for P_i.

Periodate Oxidation

Analytical periodate oxidation was conducted in the following manner, observing the considerations of Guthrie (92) and Bobbitt (93). To the reaction vessel, a 25-ml volumetric flask, was added 12 ml of a solution containing 45 to 66 µmoles of the salt form of the sugar phosphate. A volumetric pipette was then used to add 2.725 mmoles of $NaIO_4$ contained in 10.00 ml of distilled water. The solution was rapidly diluted to volume and the reaction vessel immediately sealed with a rubber stopped fitted with inlet and exit ports. A blank flask without sugar was treated in the same manner. Dry CO_2-free nitrogen was then continuously flushed through the inlet port allowing any evolved CO_2 to be displaced through the exit port into a collection flask containing 2.5 M NaOH such that the gases slowly bubbled into the alkaline solution. The reaction vessels were placed in a thermostated water bath at 20°C and the water bath was then covered with aluminum foil in order to protect the reaction mixture from light. It was found in a preliminary experiment under the same reaction conditions that fructose 6-phosphate consumed the expected quantity of periodate within 12 hours with the consumption of the first two molecular equivalents of periodate within one

hour. The periodate oxidations were therefore allowed to proceed for 12 hours. At the end of the oxidation procedure, the reaction solutions were analyzed for all products. Samples which could not be immediately analyzed were freed of periodate by the addition of ethylene glycol or sodium bisulfite followed by freezing at -20°C.

Enzyme Assays

In each of the following assays the enzyme concerned was present in rate-limiting quantities such that the enzyme concentration would be proportional to the rate of the reaction catalyzed. Furthermore, each assay was dependent upon the presence of the specific enzyme and substrate necessary to initiate the reactions involved. All assays were monitored for either the reduction of NAD^+ or the oxidation of NADH (except where stated) at 340 nm in micro-quartz cuvettes with 1-cm light paths using a Gilford absorbance-recording spectrophotometer. A conversion factor of 0.41 absorbance units per 0.01 μmole of NADH in 0.15 ml was used in determining the number of units of enzyme.

Assays for PTS Components

E_I, HPr, and E_{II}^{mtl}, activities were assayed using a modification of the "mannitol continuous assay" (94). Each activity was determined in the presence of saturating concentrations of the other two activities. The assay consisted of the following components in a final volume of 0.15 ml:

12 μmoles of 2-amino-2-methyl-1,3-propanediol buffer (pH 9.0), 0.2 μmole of $NADP^+$, 0.2 μmole of NAD^+, 0.01 μmole of $MgCl_2$, 1.0 μmole of PEP, 1.0 μmole of D-mannitol, 0.57 μmole of 2-mercaptoethanol, 2 units of phosphoglucoisomerase, 1 unit of D-glucose 6-phosphate dehydrogenase, 1 unit of mannitol 1-phosphate dehydrogenase (partially purified as described later), and variable amounts of E_I, E_{II}, or HPr depending upon which was being assayed. The reaction could be initiated with either PEP or one of the PTS constituents but initiation with PEP was the routine procedure. An absorbance change of 0.41 was equivalent of 0.01 μmole of NADPH formed since the E_{II} preparation still contained NADH oxidase activity (The NADH oxidase activity was not eliminated by inhibitors such as potassium cyanide or Quinacrine without incurring inhibition of the coupling enzymes.) In addition to the NADH oxidase activity, other competing reactions such as pyruvate kinase and phosphatases prevented assaying for the PTS constituents in crude extracts and ammonium sulfate fractionations. A unit of activity was defined as that quantity of enzyme required to produce 1 μmole of NADPH per minute.

Mannitol 1-Phosphate Dehydrogenase Assay

Since mannitol 1-phosphate was not readily available, this dehydrogenase was assayed in the reverse direction using fructose 6-phosphate and observing the decrease in absorbance at 340 nm. Interfering D-sorbitol 6-phosphate dehydrogenase was not present with the mannitol 1-phosphate dehydrogenase

since cells grown on mannitol due not induce the D-sorbitol 6-phosphate dehydrogenase (26). The assay consisted of the following components in a final volume of 0.15 ml: 10 μmoles of glycylglycine buffer (pH 7.5), 0.05 μmole of NADH, 1.0 μmole of fructose 6-phosphate, and limiting amounts of enzyme. The reaction was initiated with the addition of substrate. The rate of the reaction was measured within the first two to three minutes due to the gradual decrease in the catalysis rate after this period as was also observed by Liss, et al. (26). After subtracting the background rate contributed by NADH oxidase, if present, the amount of mannitol 1-phosphate dehydrogenase activity was calculated. A unit of activity was defined as the amount of enzyme required to catalyze oxidation of 1 μmole of NADH per minute under the reaction conditions employed.

L-Galactitol 1-Phosphate Dehydrogenase Assay

L-Galactitol 1-phosphate dehydrogenase was assayed using chemically synthesized L-galactitol 1-phosphate as the substrate. The assay consisted of the following amounts of components in a final volume of 0.15 ml: 8 μmoles of Tris-HCl buffer (pH 9.0), 0.57 μmole of 2-mercaptoethanol, 1.0 μmole of $MgCl_2$, 0.5 μmole of NAD^+, 0.5 μmole of L-galactitol 1-phosphate, and limiting amounts of enzyme. The reaction was initiated by the addition of substrate. Galactitol could not substitute for L-galactitol 1-phosphate as a substrate for the enzyme as shown in Table (1). Table (2) shows that

TABLE 1. Validity of the L-galactitol 1-phosphate dehydrogenase assay and activity with galactitol as substrate.

Assay Description				Absorbance change per minute
	Crude extract (µl)	L-Galactitol 1-phosphate (µmoles)	Galactitol (µmoles)	
Complete (see text)	5.0	1.2	0.00	2.4×10^{-3}
Complete plus 2 x (enzyme)	10	1.2	0.00	4.9×10^{-3}
Complete minus L-galactitol 1-phosphate	5.0	0.00	0.00	0.00
Complete minus enzyme	0.0	1.2	0.00	0.00
Complete plus galactitol minus L-galactitol 1-phosphate	5.0	0.00	1.0	0.00
Complete plus galactitol minus L-galactitol 1-phosphate and enzyme	0.0	0.00	1.0	0.00
Complete plus galactitol	5.0	1.2	1.0	2.4×10^{-3}

TABLE 2. Cofactor specificity of L-galactitol 1-phosphate dehydrogenase and apparent inducibility

	Assay Description				Absorbance Change per minute
	$NADP^+$ (μmoles)	NAD^+ (μmoles)	Galactitol extract (mg)	Glucose extract (mg)	
Complete plus NAD^+ minus $NADP^+$ (see text)	0.0	0.1	0.061	0.00	0.012
Complete plus $NADP^+$ minus NAD^+	0.1	0.0	0.061	0.00	0.00
Complete plus NAD^+ minus $NADP^+$	0.0	0.1	0.00	0.074	0.00
Complete plus $NADP^+$ minus NAD^+	0.1	0.0	0.00	0.074	0.00

the enzyme was specific for NAD^+ as the cofactor and does not use $NADP^+$ for either L-galactitol 1-phosphate or galactitol. Furthermore, the enzyme was apparently inducible since no activity could be seen in crude extracts of cells grown on D-glucose (Table 2). A unit of activity was defined as the amount of enzyme necessary to catalyze the reduction of 1 μmole of NAD^+ per minute under the conditions described.

Phosphoglycolate Phosphatase Assay

Phosphoglycolate phosphatase activity was assayed according to the method of Christeller (95). The assay mixture contained the following constituents in a final volume of 0.5 ml: 20 μmoles of cacodylate buffer (pH 6.3), 25 μmoles of sodium citrate, 2 μmoles of $MgCl_2$, 1 μmole of phosphoglycolate, and limiting amounts of enzyme. The reaction, equilibrated at 30°C in a thermostated water bath, was initiated by the addition of enzyme and terminated after 10 minutes by the addition of 0.2 ml of 10% TCA. An assay blank containing the complete reaction mixture incubated for zero time was also performed in order to account for background quantities of P_i. After centrifugation of the denatured protein in a table-top centrifuge at room temperature for 10 minutes, a 0.5-ml sample of the supernatant was assayed for release of P_i by the modified method of Fiske and SubbaRow (86) as described previously. One unit of activity was defined as the quantity of enzyme required to hydrolyze one μmole of phosphoglycolate into P_i and glycolate per minute at 30°C.

Partial Purification and Preparation of Enzymes

All of the following operations were performed at 0 to 5°C unless otherwise stated. All centrifugations except the ultracentrifugation were accomplished with a Servall refrigerated centrifuge. In the anion exchange and gel filtration steps only the fractions exhibiting the highest specific activity were pooled.

Preparation of E_{II}^{gltl} and E_{II}^{mtl}

E_{II}^{mtl} and E_{II}^{gltl} were prepared from crude extracts of bacteria grown on D-mannitol and galactitol respectively. The membrane fraction containing the E_{II} activity was isolated by centrifugation at 100,000 x g for four hours in a Spinco L-2 refrigerated ultracentrifuge. The resulting pellet containing the E_{II} activity was suspended in 20 to 30 ml of 0.02 M Tris-HCl buffer (pH 7.5) containing 0.028 M 2-mercaptoethanol by use of a syringe equipped with a 22 gauge needle. The supernatant from the ultracentrifugation was retained for purification of the soluble enzymes. The suspended membrane vesicles were sonicated for 20 minutes under the same conditions used for the preparation of the cell extract and then were ultracentrifuged as before at 100,000 x g. The resulting membrane pellet was allowed to drain free from the supernatant and was then resuspended in a minimum volume of the same buffer. This suspension was then applied to a small column (1.2 cm x 9 cm) of Biogel P-200 and the membrane fraction was

eluted in the void volume with the same buffer. The eluted membrane vesicles were then concentrated by ultracentrifugation as performed before and pellet was resuspended in a minimum volume of the same buffer after removal of the supernatant. This final suspension of membrane vesicles was then used as prepared E_{II}. All E_I and HPr activities were absent in this preparation as well as 80% of the NADH oxidase activity. When stored on ice the activity was stabile for approximately one week whereas freezing and unfreezing the preparation inactivated all of the E_{II} activity.

Partial Purification of E_I

The retained supernatant from the E_{II} preparation was diluted to approximately 10 mg of protein per ml using the same buffer. The solution was then brought to 35% ammonium sulfate saturation by the slow addition of powdered enzyme-grade $(NH_4)_2SO_4$ with constant stirring. The mixture was allowed to equilibrate for one hour followed by centrifugation for 15 minutes at 30,000 x g. The precipitate was discarded and the supernatant volume measured. The appropriate amount, calculated from a nomograph (96), of ammonium sulfate was added to the solution in the same manner as before to increase the saturation to 70%. The precipitate, containing the E_I activity, was centrifuged at 12,000 x g for 15 minutes. The resulting supernatant was saved for isolation of HPr activity and the precipitate was dissolved in a minimum of 0.02 M potassium phosphate buffer (pH 8.0) containing 0.028 M

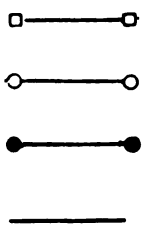
2-mercaptoethanol, and 10% (v/v) glycerol. The solution was desalted, using the same buffer, on a Biogel P-6 column (2.1 cm x 30 cm) eluting the E_I activity in the void volume. The activity was pooled and diluted with the same buffer to a protein concentration of 2 mg/ml. The protein solution was then applied to a DEAE cellulose column (1.7 cm x 17 cm) which had been equilibrated with the same buffer. The column was washed with three column volumes of the same buffer and then the E_I was eluted with a 0.0 to 0.5 M linear KCl gradient developed in 10 column volumes of the same buffer. The elution profile of E_I activity and protein is shown in figure (1). The activity was pooled and dialyzed for 24 hours against two changes of 20 volumes each of the same buffer. The dialyzed E_I activity was then applied to a second DEAE cellulose column (1.7 cm x 17 cm) which had been equilibrated with the same buffer containing 0.1 M KCl. The column was washed with the same buffer containing 0.1 M KCl and then a 0.1 to 0.3 M linear KCl gradient in 10 column volumes of the same buffer was used to elute the E_I activity. Figure (2) shows the elution of E_I and protein. The E_I activity was pooled and dialyzed in the same manner as before. To the dialyzed solution was added 0.10 M acetic acid drop-wise with continuous stirring until the pH had been lowered to 4.5. The turbid mixture was then centrifuged at 12,000 x g for 15 minutes. The resulting supernatant was poured off and immediately re-adjusted to pH 8.0 by the drop-wise addition of 0.5 M NaOH with constant stirring. The resulting solution was then concentrated

by pressure dialysis using an UM-10 ultrafilter membrane (Amicon) at 40 psi of nitrogen. The concentrate was then applied to a Biogel P-200 column (2.1 cm x 88 cm) and the E_I activity eluted with the same buffer. Figure (3) shows the elution profile of E_I activity and protein. The fractions containing activity were then pooled and frozen at -20°C. This preparation of E_I was stabile for over six months. Table (3) summarizes the purification data. Calcium phosphate gel and hydroxylapetite were also used for the purification of E_I but did not yield any further purification.

Partial Purification of HPr

The 70% ammonium sulfate saturated supernatant from the E_I preparation was adjusted to 100% saturation in the same manner used in the previous ammonium sulfate fractionations. After the solution had equilibrated for one hour the precipitate was isolated by centrifugation at 12,000 x g for 15 minutes. The pellet, containing the HPr activity, was allowed to drain free of the supernatant and was then dissolved in a minimum of 0.02 M potassium phosphate buffer at pH 7.5. The solution was then desalted by molecular seiving through a column of Biogel P-6 (2.1 cm x 30 cm), eluting HPr in the void volume using the same buffer. The fractions containing activity were pooled and diluted with the same buffer to a protein concentration of 2 mg/ml. Three volumes of DEAE cellulose, equilibrated in the same buffer, were then added batchwise to the solution with continuous stirring. The pH was

FIGURE 1. DEAE cellulose chromatography I of the 35 to 70 percent ammonium sulfate fraction from the Biogel P-6 protein pool.



Protein.

Mannitol 1-Phosphate Dehydrogenase.

Enzyme I.

Calculated KCl gradient.

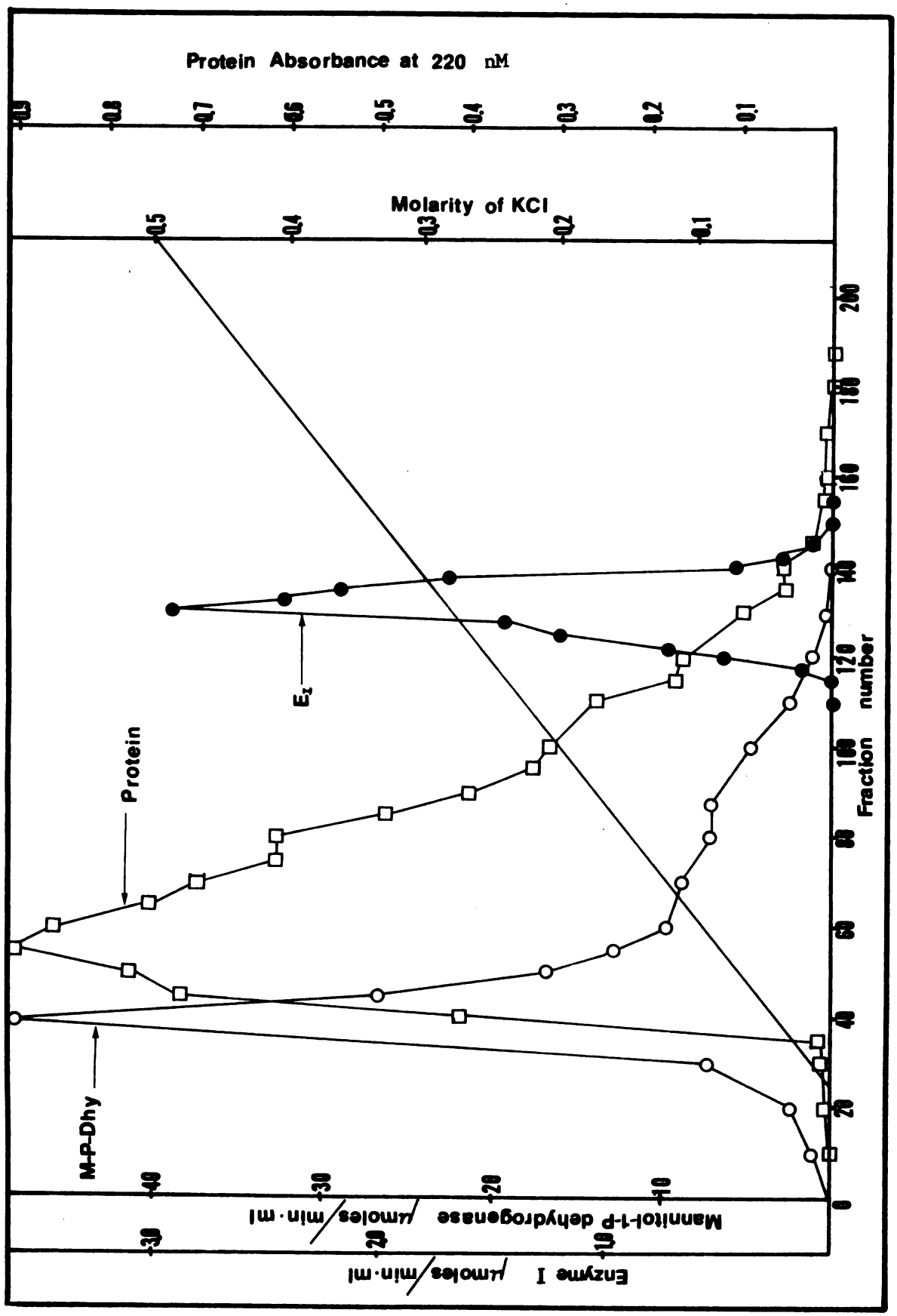
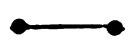

Protein Absorbance at 220 nM
Molarity of KCl
Fraction number
Protein
E_T
M-P-Dhy
Mannitol-1-P dehydrogenase μmoles/min·ml
Enzyme I μmoles/min·ml

FIGURE 2. DEAE cellulose chromatography II of the dialyzed E_I pool from DEAE cellulose chromatography I.

E_I activity.



Protein.

..... Calculated KCl gradient.

ENZYME I (UNITS/ML)
0.6
0.4
0.2
0
50
100
150
200
FRACTION
MOLARITY KCl
0.30
0.25
0.20
0.15
0.10
PROTEIN (ABSORBANCE AT 220 nM)
0.3
0.2
0.1
0.0

FIGURE 3. Biogel P-200 chromatography of the concentrated E_I pool from DEAE cellulose chromatography II.

E_I activity.

Protein.

PROTEIN (ABSORBANCE AT 220 nM)
0.30
0.20
0.10
ENZYME I (UNITS/ML)
0.15
0.10
0.05
0
10
20
30
40
50
60
70
80
90
100
110
120
130
FRACTION

readjusted to 7.5 and the mixture was allowed to equilibrate for 30 minutes. The aqueous phase was separated from the DEAE cellulose by light suction filtration through a Buchner funnel. The filtrate, containing HPr activity, was concentrated by pressure dialysis in the same manner as used for E_I. HPr can alternatively be concentrated by lyophilization at -80°C. The concentrate was then used as partially purified HPr. This preparation of HPr was free of all E_I and E_{II} activities. When frozen at -20°C, the HPr preparation was stabile for two weeks. A summary of the purification is shown in Table (4).

Partial Purification of Mannitol 1-Phosphate Dehydrogenase

Mannitol 1-phosphate dehydrogenase was partially purified by a modification of the method used by Liss, et al. (26) such that concomitant purification of the enzyme during the preparation of E_I could be accomplished. The activity eluting in the first anion exchange step for E_I (Figure 1) was pooled and dialyzed for 24 hours against two changes of 20 volumes each of 0.05 M $NaHCO_3$ buffer at pH 8.0. The pH of the dialyzed solution was lowered to 5.0 by the slow addition of 0.01 M acetic acid with constant stirring. Four volumes of calcium phosphate gel were then added to the solution and the pH was readjusted to 5.0. The mixture was gently stirred for 30 minutes and then centrifuged at 12,000 x g for 10 minutes. The resulting supernatant was discarded and the activity was eluted from the gel by four washings with equal

TABLE 3. Summary of purification of E_I. Activity was not determined in crude extracts and the 35 to 70% ammonium sulfate fraction due to interfering reactions described in Enzyme Assays.

Fraction	Total Units	Specific Activity (units/mg protein)	Percent Recovery	Fold Purification
Crude Extract	-	-	-	-
$(NH_4)_2SO_4$ (35 to 70%)	-	-	-	-
Biogel P-6	146	0.050	(100)	(1.0)
DEAE cellulose I	123	0.79	84	16.0
DEAE cellulose II	56.0	2.0	38	39.4
pH 4.5	43.8	6.0	30	116
Biogel P-200	39.6	6.4	27	123

volumes of 0.05 M $NaHCO_3$ buffer at pH 8.0. Each washing involved gentle stirring of the gel with buffer for 30 minutes at a final pH of 8.0 followed by centrifugation as before. The washings were combined and dialyzed as before except against 0.05 M Tris-HCl buffer (pH 7.5) containing 5×10^{-3} M 2-mercaptoethanol and 0.05 M NaCl. The dialyzed solution was then concentrated by pressure dialysis under identical conditions utilized for E_I. The concentrate was used as partially purified mannitol 1-phosphate dehydrogenase which was stabile stored frozen at -20°C for several weeks. A summary of the purification is shown in Table (5).

Partial Purification of L-Galactitol 1-phosphate Dehydrogenase.

A crude extract of bacteria grown on galactitol was fractionated with ammonium sulfate between 35% and 70% saturation as was done for the preparation of E_I. The precipitate, containing L-galactitol 1-phosphate dehydrogenase activity, was dissolved in a minimum volume of 0.02 M Tris-HCl buffer (pH 9.0) containing 0.028 M 2-mercaptoethanol. The resulting solution was desalted on a column of Biogel P-6 (1.5 cm x 11 cm) using the same buffer to elute the activity in the void volume. The fractions containing activity were pooled and diluted to a protein concentration of 2 mg/ml followed by application to a DEAE-cellulose column (1.7 cm x 17 cm) equilibrated with the same buffer. The column was washed with three column volumes of buffer and then the activity eluted using a 0.0 to 0.5 M linear KCl gradient

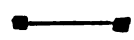
TABLE 4. Purification summary of HPr. Activities in crude extract and the ammonium sulfate fraction were not determined due to the interfering reactions stated in Enzyme Assays.

Fraction	Total Units	Specific Activity (units/mg protein)	Percent Recovery	Fold Purification
Crude Extract	-	-	-	-
$(NH_4)_2SO_4$ (70 to 100%)	-	-	-	-
Biogel P-6	2.0	0.005	(100)	(1.0)
DEAE cellulose	1.9	0.01	92	2.0

TABLE 5. Summary of purification of mannitol 1-phosphate dehydrogenase.

Fraction	Total Units	Specific Activity (units/mg protein)	Percent Recovery	Fold Purification
Crude Extract	201	0.36	100	1.0
$(NH_4)_2SO_4$ (35 to 70%)	200	0.39	99.6	1.1
DEAE cellulose	204	2.1	101	5.7
$CaPO_4$ Gel	133	4.1	66.2	11

FIGURE 4. DEAE cellulose chromatography of L-galactitol 1-phosphate dehydrogenase from the 35 to 70% ammonium sulfate fraction after desalting over Biogel P-6.


L-Galactitol 1-phosphate dehydrogenase.


Protein.

..... Calculated KCl gradient.

L-GALACTITOL 1-PHOSPHATE DEHYDROGENASE (UNITS/ML)
0.15
0.10
0.05
0
50
100
150
200
FRACTION
MOLARITY KCl
0.5
0.4
0.2
PROTEIN (ABSORBANCE AT 220 nM)
1.2
0.9
0.6
0.3

developed in 10 column volumes of the same buffer. Figure (4) shows the elution profile of activity and protein. The fractions containing L-galactitol-1 phosphate dehydrogenase were pooled and dialyzed against 20 volumes of fresh buffer for 12 hours. The dialyzed activity was used as partially purified L-galactitol 1-phosphate dehydrogenase which was stable for approximately one week when stored on ice. Table (6) summarizes the purification data obtained.

Partial Purification of Phosphoglycolate Phosphatase.

Phosphoglycolate phosphatase was partially purified according to the method of Christeller (95). A 350 g portion of field-grown Maryland Mammoth tobacco leaves which had been deveined, water-washed, and frozen at -20°C for 3 months were suspended in 700 ml of glass distilled water at 0°C to 4°C. The crude extract was prepared by homogenizing the suspension in a Waring blender using short bursts of homogenization over a period of 30 minutes. The homogenate was then strained through four layers of cheese cloth and the filtrate centrifuged at 10,000 x g for 25 minutes to remove cell debris. The resulting supernatant was adjusted to 25% (v/v) acetone saturation at 0°C to 4°C by the slow addition (2 ml/minute) with constant stirring of reagent grade acetone cooled to -20°C with a dry ice-ethanol bath, through a 1 mm (i.d.) teflon tube. The solution was allowed to equilibrate for 30 minutes after the addition of acetone and was then centrifuged at 10,000 x g for 15 minutes at 0°C. The pellet was discarded

and the supernatant increased to 40% acetone saturation in the same fashion as previously described. The resulting solution was allowed to equilibrate for 30 minutes and then the precipitate was removed by centrifugation at 10,000 x g for 15 minutes. The pellet, containing the phosphoglycolate activity, was dissolved in 0.02 M cacodylate buffer (pH 6.3). The second acetone fractionation was conducted in the same way as used for the first acetone fractionation, saving only the 25 to 40% acetone precipitate. This precipitate was dissolved in 0.02 M cacodylate buffer (pH 6.3) and fractionated with acetone for a third time. The precipitate formed from the 25 to 40% acetone fraction was allowed to dry at room temperature and pressure for 24 hours after which it was dissolved in sufficient 0.02 M cacodylate buffer (pH 6.3) containing 5 mM citrate to yield a protein concentration of 2 mg/ml. The last acetone fractionation had resulted in almost a complete loss of activity and was probably due to the addition of the cooled acetone by pipette rather than by a teflon tube thus allowing the acetone to become too warm. The diluted enzyme solution was applied to an eqilibrated DEAE cellulose column (1.2 cm x 9 cm) followed by washing with three column volumes of the same buffer. The activity was then eluted with a 0.0 to 0.5 M linear KCl gradient developed in 10 column volumes of the same buffer. Figure (5) illustrates the elution profile of both activity and protein. The fractions containing activity and the best separation from protein were pooled and dialyzed for 12 hours against

TABLE 6. Summary of purification of L-galactitol 1-phosphate dehydrogenase.

Fraction	Total Units	Specific Activity (units/mg protein)	Percent Recovery	Fold Purification
Crude Extract	9.9	0.0080	100	1.0
$(NH_4)_2SO_4$ (35 to 70%)	11	0.018	108	2.1
DEAE cellulose	8.1	0.067	81.0	8.0

FIGURE 5. DEAE cellulose chromatography of phosphoglycolate phosphatase from the third 25 to 40% acetone fraction.

▲—▲ Phosphoglycolate phosphatase.

●—● Protein.

.... Calculated KCl gradient.

PHOSPHOGLYCOLATE PHOSPHATASE (UNITS/ML)
0.4
0.3
0.2
0.1
0
25
50
75
100
FRACTION
MOLARITY KCl
0.6
0.4
0.2
PROTEIN (ABSORBANCE AT 220 nM)
0.06
0.04
0.02

TABLE 7. Summary of purification of phosphoglycolate phosphatase. The loss of enzyme in the third acetone step is explained in the text.

Fraction	Total Units	Specific Activity (units/mg protein)	Percent Recovery	Fold Purification
Crude Extract	1180	0.150	100	1.0
First Acetone Fractionation (25 to 40%)	1070	6.20	91	39.5
Second Acetone Fractionation (25 to 40%)	823	11.1	70	70.9
Third Acetone Fractionation (25 to 40%)	7.21	21.1	0.61	136
DEAE cellulose	6.77	27.3	0.57	180

20 volumes of 0.02 M cacodylate buffer (pH 6.3) containing 50 mM citrate. The dialyzed solution, which exhibited a specific phosphatase activity for only phosphoglycolate was then used as partially purified phosphoglycolate phosphatase. A summary of the purification data appears in Table (7).

Quantitative Analyses

Where applicable in the following determinations, a standard curve was prepared and the analysis of the sample made within its linear range. Spectrophotometric readings were made at the appropriate wave length using a Gilford 300-N spectrophotometer equipped with an automatic sampling system. Spectrophotometer readings made in the ultraviolet region were made with a Gilford 2400 spectrophotometer using micro-quartz cuvettes of 1-cm light paths.

Inorganic Phosphate Determination

P_i was determined by the method of Fiske and SubbaRow as modified by Clark (97). All determinations were performed using nitric acid-washed tubes free of P_i. When total organic phosphate analyses were performed, the method of Clark (98) was used. When P_i determinations were used for P_{org} acid lability experiments, the acid molybdate reagent was added last in order to avoid exposure of the sample to an initially high concentration of acid which could cause hydrolysis.

Protein Determination

The concentration of protein was determined by two methods, both of which use bovine serum albumin as a standard. The first procedure consisted of measuring the absorbance of the peptide bond at 210 or 220 nm (99) after dilution of an aliquot of the protein solution with distilled water to a final volume of 0.2 ml in micro-quartz cuvettes. All other protein determinations were conducted using the method of Lowry, et al. (100).

Ketohexose 6-Phosphate Determination

The method of Roe (101), which is specific for ketohexoses, was used in the determination of ketohexose 6-phosphate concentrations. The presence of the phosphate group does not change the specificity of the reaction but does decrease the intensity of the color produced by 40% (102). The standard curve was determined from fructose 6-phosphate.

Determination of Reducing Sugar

The submicro method of Park and Johnson (103) was used in the determination of loss of reducing sugar for the sodium borohydride reduction of D-galactose 6-phosphate. The procedure included the addition of 2 drops of 0.05 N H_2SO_4 to the sample in order to destroy the excess borohydride (additional acid negated the colorimetric reaction.) Concentrations were determined from a glucose 6-phosphate standard curve.

Enzymatic Determination of L-Galactitol 1-Phosphate

L-Galactitol 1-phosphate was quantitatively determined using the L-galactitol 1-phosphate dehydrogenase assay as previously described except for a modification in the amounts of enzyme and substrate used. An excess of enzyme (5 milliunits) and less than 0.05 µmole of substrate were used. The reaction was initiated by the addition of substrate after monitoring the solution for 5 minutes. Two controls, one missing enzyme and the other missing substrate, were also used in this analysis. The total absorbance change was determined after subtracting that change due to dilution by the added substrate. The number of µmoles of L-galactitol 1-phosphate was determined from the conversion factor stated in Enzyme Assays. This assay was also used for the determination of the purity of both chemically and enzymatically synthesized galactitol phosphate.

Determination of Phosphoglycolate

Phosphoglycolate was quantitatively determined by an enzymatic assay and by the total hydrolysis of P_{org} which was described previously. The enzymatic analysis was essentially the same as the assay used for phosphoglycolate phosphatase except that the enzyme concentration was increased to 0.46 unit and the substrate concentration was decreased to 0.1 to 0.8 µmole. Since the determinations were utilized for analysis of phosphoglycolate produced from the periodate oxidation previously described, assay samples were treated with ethylene

glycol in order to destroy the excess IO_4^- present (0.2 ml of ethylene glycol/ml of sample). The reaction mixture was initiated by the addition of enzyme and then incubated for 120 minutes at 30°C. The liberated P_i was determined as previously described, adding the acid molybdate reagent last in order to avoid potential acid hydrolysis of P_{org}.

Determination of Formaldehyde

Formaldehyde, produced in the periodate oxidation experiment, was assayed colorimetrically using the method of Frisell, et al. (104). Commercial formaldehyde was standardized gravimetrically using the reaction procedure of Horning and Horning (105). Excess periodate was destroyed by treatment with sodium bisulfite (4 ml of 10% $NaHSO_3$/ml sample). Ethylene glycol was not used to destroy the excess periodate since it would be oxidized to formaldehyde.

Determination of Glycolate

The method of Calkins (106) was used for the determination of glycolate. Since the glycolate samples were analyzed from the periodate experiment, they had to be freed of possible contamination from formaldehyde which was known to interfere in the determination. In addition to this, the organic phosphate compounds required removal since the assay was performed in concentrated H_2SO_4 which could cause the hydrolysis of phosphoglycolate to glycolate. The phosphate compounds were removed from 1 ml of sample by the addition of AG1-X2 Cl^- at pH 8.0. The resin was removed by suction

filtration and washed with a few milliliters of 0.001 M NH_4OH. The collected filtrate was then freed of formaldehyde by evaporation under reduced pressure at 30°C in a rotary evaporator. The sample was then diluted to 20 ml with 2 N H_2SO_4 and an aliquot containing 5 to 20 μg of glycolate was removed for analysis in a 15-ml conical centrifuge tube.

Phosphoglycoaldehyde Determination

The phosphoglycoaldehyde was not determined directly but was quantitated in terms of acid-labile phosphate. This assay was specific in the particular analysis to which it was applied (the periodate oxidation analysis of the ketohexose phosphates that yielded either the acid stabile phosphoglycolate or labile phosphoglycoaldehyde). The determination was made at the same time the acid lability was determined as described previously. The P_i liberated was determined using the modified Fiske and SubbaRow procedure previously described.

Determination of Carbon Dioxide

Evolved carbon dioxide from the periodate oxidation reaction mixtures was collected by slowly flushing the reaction vessels with dry CO_2-free nitrogen into flasks containing 5.00 ml of CO_2-free 2.5 M NaOH. A degradation train similar to that described by Bernstein and Wood (107) was utilized to bubble the gases into the NaOH solutions. The amount of CO_2 absorbed was then determined by the method of Pierce, Haenisch, and Sawyer (108).

Determination of Formate

The amount of formate produced in the periodate oxidation analysis was determined by titration with NaOH (109). The excess periodate was destroyed by the addition of 1.00 ml of ethylene glycol to a 5.00 ml aliquot of sample. The solution was then titrated to the phenophthalein end point.

Determination of Periodate Consumption

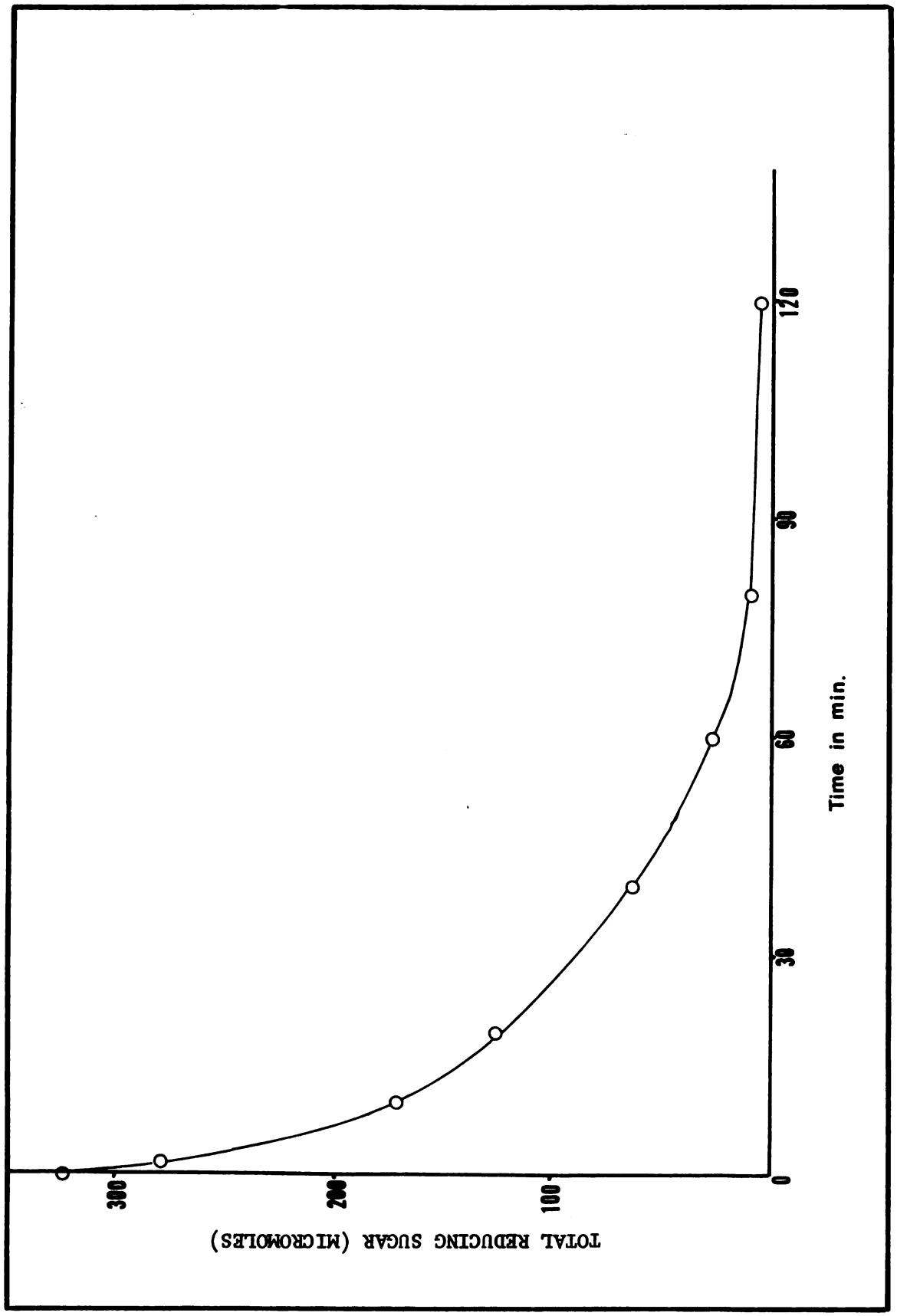
The reduction of periodate to iodate was determined titrimetrically with sodium thiosulfate according to the method of Pierce, Haenisch, and Sawyer (110).

Chemical Synthesis of L-Galactitol 1-Phosphate

L-Galactitol 1-phosphate was synthesized using sodium borohydride reduction according to the method of Liss, et al. (26). A 0.128-g sample of commercial anhydrous D-galactose 6-phosphate as the barium salt was dissolved in 5.0 ml of triply glass-distilled water. The sugar phosphate was converted to the acid form by the addition of an excess of clean Dowex 50W-X8 H^+ followed by gentile stirring for 20 minutes. The resin was removed by suction filtration and then washed with an equal volume of distilled water. The resulting filtrate was adjusted to pH 8.0 with 0.5 M NaOH in order to prevent acid oxidation of the $NaBH_4$ to be added. To the alkaline solution was added a solution of 0.012 g of fresh $NaBH_4$ dissolved in 0.5 ml of distilled water previously adjusted to

pH 8.0. The reaction mixture was constantly stirred and maintained at room temperature. The total loss of reducing sugar was monitored before and after the addition of $NaBH_4$ utilizing the submicro ferricyanide method of Park and Johnson (103) as described in Materials and Methods. Figure (6) delineates the course of the reaction and reveals that the reduction was essentially complete in 2 hours. The reaction was allowed to proceed for 4 hours in order to insure complete reduction or absence of a positive reducing-sugar test. The solution was then adjusted to pH 7.0 by the addition, with stirring, of powdered boric acid which oxidized part of the excess $NaBH_4$ left after reduction. The remaining $NaBH_4$ was oxidized with concomitant removal of cations by the addition of excess Dowex 50W-X8 H^+. The Dowex resin was not used in the initial oxidation due to its tendency to give off a reddish dye. The solution was stirred for 10 minutes after which the resin was removed by suction filtration. The resin was then washed with 3 volumes of distilled water and all the filtrates were combined into a round bottom flask. The solution was evaporated to dryness under reduced pressure at 30°C in a rotary vacuum evaporator. The solid residue of boric acid was converted into methyl borate by the addition of excess anhydrous methanol. The borate ester was removed by evaporation under reduced pressure in a rotary evaporator at 30°C. The methanol treatment was repeated nine more times after which only the product, a clear and extremely viscous oil, was left. The product was then dissolved in distilled water

FIGURE 6. Sodium borohydride reduction of commercially prepared D-galactose 6-phosphate. At the noted time intervals aliquots (0.5 to 5 µl) were removed and assayed for reducing sugar as described in Materials and Methods. Details of the reaction condition are given in the text.


TOTAL REDUCING SUGAR (MICROMOLES)
300
200
100
0
30
60
90
120
Time in min.

and neutralized by titration with 0.5 N NaOH. The resulting solution was evaporated under reduced pressure at 30°C in a rotary evaporator until a thick clear oil remained. The oil was then dissolved in a minimum volume of distilled water and suction filtered through a sintered glass-disk to remove any insoluble matter. The filtrate was raised to 95% (v/v) ethanol saturation by slowly adding, with stirring, 100% ethanol. The resulting precipitate was cooled at 0 to 4°C for a few hours and was then centrifuged out of solution in a table-top centrifuge for 15 minutes. The supernatant was discarded and the pellet was resuspended in a minimum volume of 100% ethanol by sonication for a few minutes in an ice-cooled 10-kHz Raytheon sonicator. The mixture was transferred to a tared amber vial and the solvent evaporated under nitrogen at 30°C. The anhydrous product, L-galactitol 1-phosphate (molecular weight = 306.5), was then stored desiccated at 0°C. The percent yield was 95%.

Reagents

Uracil, D-mannitol, galactitol, glucose, sorbitol, galactose, fructose, Tris-HCl, glycylglycine, alkaline phosphatase, phosphoenolpyruvate, 2-mercaptoethanol, phosphoglucose isomerase, glucose 6-phosphate dehydrogenase, fructose 1-phosphate, galactose 6-phosphate, glucose 6-phosphate, sodium citrate, phosphoglycolate, DEAE cellulose, hydroxylapetite, bovine serum albumin, lactate dehydrogenase, α-glycerophosphate dehydrogenase, triose phosphate isomerase,

fructose 6-phosphate kinase, and sodium pyruvate were purchased from Sigma Chemical Company, St. Louis, Missouri; NAD^+, NADH, $NADP^+$, phosphoglycoaldehyde, and ATP were purchased from Calbiochem, San Diego, California; 2-amino-2-methyl-1,3-propandiol, dimedon, thiourea, resorcinol, chromotropic acid, and 2,7-dihydroxynaphthalene were purchased from Eastman Organic Chemicals, Rochester, New York; enzyme-grade ammonium sulfate was from Schwarz/Mann, Orangeburg, New York; cacodylate and glycolate were purchased from Matheson Coleman Bell, New York, New York; sodium borohydride was obtained from Ventron Metal Hydrides, Beverly, Massachusetts; Biogel P-6, Biogel P-200, and AG1-X2 were purchased from Biorad, Richmond, California; ethylene glycol, formaldehyde, EDTA, and sodium periodate were purchased from Mallinckrodt Chemical Company, St. Louis, Missouri; Dowex 50W-X8, acetic anhydride, pyridine, and Dowex 1-X8 were purchased from J.T. Baker Chemical Company, Phillipsburg, New Jersey; polyethyleneglycol adipate, polyethyleneglycol succinate, XF-1150 silicon oil, and Gas-chrom P were purchased from Applied Science Laboratories Inc., State College, Pennsylvania; trimethylchlorosilane and bis-trimethylsilylacetamide were purchased from Pierce Chemical Company, Rockford, Illinois; fructose 6-phosphate was purchased from Boehringer Mannheim, New York, New York. Calcium phosphate gel was prepared by the method of Keilin and Hartree (111). D-tagatose was a gift from H.A. Lardy. Tagatose 6-phosphate, and tagatose 1,6-diphosphate aldolase

were gifts from D. Bisset. Rhamnitol, fucitol, arabitol, xylitol, and inositol were gifts from D.T. Lamport.

Other Analytical Procedures

DEAE cellulose was successively washed with 1 N NaOH, 1 N HCl, 1 N NaOH, and then with distilled water to neutrality (112). All Dowex ion-exchange resins were treated overnight with 1 N NaOH followed by washing with 1 N HCl and then with distilled water to neutrality. All Biogels were prepared as recommended by Biorad (13). Dialysis tubing was prepared by boiling in distilled water containing 10 mM EDTA for 15 minutes and then washing with room-temperature distilled water.

RESULTS

PTS-Catalyzed Phosphorylation of Galactitol

Experiments were conducted in order to determine if galactitol could be phosphorylated using PEP as the phosphoryl donor and PTS components as catalysts. Determinations employing crude extracts were unsuccessful, presumably due to the presence of competing enzymes such as L-galactitol 1-phosphate dehydrogenase and phosphatases. Consequently partially purified PTS components were utilized to demonstrate PTS-catalyzed phosphorylation of galactitol. The reactions were conducted in 6 mm x 50 mm Kimax culture tubes. The complete reaction mixture contained the following constituents in a final volume of 200 µl: 8.0 µmoles of Tris-HCl buffer (pH 7.5), 0.56 µmole of 2-mercaptoethanol, 20 µmoles of galactitol, 0.02 µmole of $MgCl_2$, 3.0 µmoles of sodium fluoride to inhibit phosphatase activity (114), 0.011 mg of prepared E_{II}^{gltl} membranes, 14.6 milliunits of E_I, 0.486 milliunit of HPr, and 1.0 µmole of PEP. The reaction mixtures were warmed to 30°C and then the reaction initiated by the addition of PEP. After the addition of PEP, the solutions were gently mixed on a Vortex mixer and then incubated for 45 minutes (unless otherwise stated) at 30°C. The reactions were stopped by

TABLE 8. PTS-dependent phosphorylation of galactitol. The reaction conditions are stated in the text. The incubation time was 45 minutes unless otherwise stated.

Assay Description				Galactitol phosphate formed (nmoles/45 min.
	E_I (units)	HPr (milli-units)	E^{gltl}_{II} (mg)	
Complete (see text)	0.015	0.216	0.011	9.30
Complete minus E_I	0.00	0.216	0.011	0.000
Complete minus E^{gltl}_{II}	0.015	0.216	0.00	0.000
Complete minus HPr	0.015	0.000	0.011	0.000
Complete minus galactitol	0.015	0.216	0.011	0.000
Complete minus PEP	0.015	0.216	0.011	0.000
Complete minus PEP plus 1.0 umole ATP	0.015	0.216	0.011	0.000
Complete plus 2 x (HPr)	0.015	0.432	0.011	20.9
Complete plus 2 x (E_I+E^{gltl}_{II})	0.030	0.216	0.22	9.10
Complete plus 2 x (HPr)	0.015	0.432	0.011	10.1 (nmoles/ 22 min.)

heating the culture tubes in a boiling water bath for 7 minutes after which they were cooled in ice. The denatured protein was removed by centrifugation at 5,000 x g for 10 minutes at 0°C and the resulting supernatants were then assayed for the quantity of galactitol phosphate produced by using galactitol phosphate dehydrogenase as described in Materials and Methods. Usually 90 µl were assayed.

Table (8) shows that the phosphorylation of galactitol was dependent upon each of the partially purified constituents of the PTS. The data also show that the reaction was dependent upon galactitol and PEP and that ATP could not substitute for PEP. Furthermore, the rate of formation of galactitol phosphate, under the reaction conditions, was constant with time and increased with a proportionate increase in the quantity of HPr present but not with an increase in the concentrations of E_{II}^{gltl} and E_I. This demonstrated that HPr was the rate-limiting component in the reaction and that the PTS serves as the phosphorylation system for galactitol.

Preparation and Isolation of the Product of the PEP-Dependent Phosphorylation of Galactitol

In order to prepare sufficient product of the PTS-catalyzed reaction for identification, it was necessary to scale up the reaction described in the above section. The reaction mixture contained the following quantities of components in a final volume of 100 ml: 11.7 units of partially purified HPr, 22 units of partially purified E_I, 152 mg of

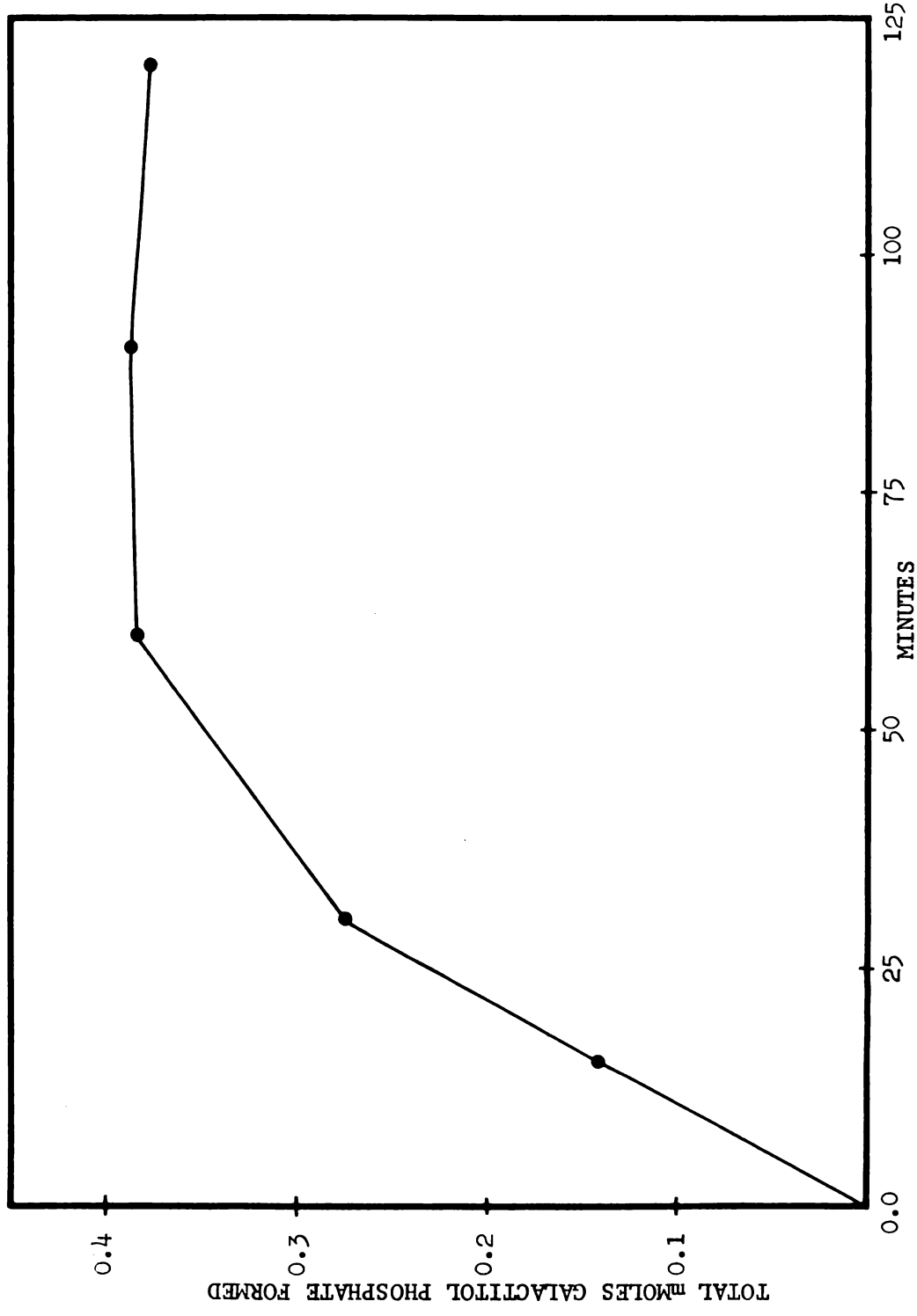
E_{II}^{gltl}, 1.5 mmoles of NaF, 0.4 mmole of Tris-Hcl buffer (pH 7.5), 0.28 mmole of 2-mercaptoethanol, 0.1 mmole of $MgCl_2$, 1.0 mmole of PEP, and 3.0 mmoles of galactitol. The reaction mixture without PEP was prepared in a stoppered 125-ml flask which was immersed in a water bath thermostated at 30°C. The reaction was initiated by the addition of the PEP when the contents of the flask reached 30°C. At various time intervals 200 µl samples were removed from the reaction vessel to culture tubes (6mm x 50 mm) which were then heated in a boiling water bath for 7 minutes to stop the reaction. The tubes were then cooled in ice and the denatured protein was removed by centrifugation at 10,000 x g for 15 minutes at 0°C in a Servall centrifuge. The resulting supernatants were then assayed for the amount of product formed using galactitol phosphate dehydrogenase as described in Materials and Methods. The course of the reaction is shown in Figure (7).

Under the reaction conditions employed, the formation of product was linear for 30 minutes and became constant after one hour. The reason for the cessation of product formation is not certain but it may reflect product inhibition, instability of the PTS constituents, or the presence of an L-galactitol phosphate phosphatase activity. It did not arise from limiting amounts of substrate since both PEP and galactitol were still present as evidenced by their elution from the Dowex 1-X8 bicarbonate column (see below).

When the incubation time had reached 90 minutes, 1 ml of the reaction mixture was removed for further monitoring

of the reaction while the remainder of the mixture was heated in a boiling water bath for 15 minutes and then cooled on ice. The precipitate was then removed by centrifugation at 10,000 x g for 30 minutes at 0°C. The resulting supernatant was saved and the precipitate was resuspended in a minimum volume of distilled water in order to recover more of the product. The suspension was centrifuged as before and the resulting supernatant combined with the first supernatant. The product was then isolated from the resulting supernatant pool by ion-exchange chromatography utilizing a 50-ml Dowex 1-X8 HCO_3^- column (14 cm x 2.2 cm) according to the procedure of Roseman, et al. (28). Two thirds of the sample was applied to the column followed by washing with three column volumes of distilled water. The washing removes cations, galactitol, buffer ions, and the 2-mercaptoethanol. The column was then washed with 300 ml of 0.4 M $NaHCO_3$. The PEP remained bound to the column until it was eluted with 1.0 M $NaHCO_3$. The $NaHCO_3$ was then removed from the product solution as CO_2 gas with concomitant removal of cations by adding an excess of Dowex 50W-X8. The resin was removed by suction filtration and then washed with one resin-volume of distilled water. The resulting filtrate was transferred to a round bottom flask and evaporated under reduced pressure at 30°C in a rotary evaporator. The clear oil that remained in the flask was dissolved in a minimum of distilled water and then titrated to neutrality with 0.5 M NaOH. The solution was

FIGURE 7. Enzymatic synthesis of galactitol phosphate. The reaction conditions employed and assay procedure used are described in the text.


TOTAL mMOLES GALACTITOL PHOSPHATE FORMED
0.4
0.3
0.2
0.1
0.0
25
50
75
100
125
MINUTES

then evaporated to an oil as before, followed by dissolving the oil in a minimum amount of distilled water. The product was then precipitated out of solution by slowly adding 100% ethanol to the solution with constant stirring at 0 to 4°C until the mixture was 95% (v/v) saturated in ethanol. The product was centrifuged from the solution and the isolation was continued in the same manner as described for the isolation of chemically synthesized L-galactitol 1-phosphate (see Materials and Methods). The percent recovery of the product synthesized was 96.8 with a yield of 252 μmoles.

Identification of the Enzymatically Synthesized Reaction Product as L-Galactitol 1-Phosphate and Characterization of Chemically Synthesized L-Galactitol 1-Phosphate

The isolated enzymatically synthesized reaction product was simultaneously analyzed for its purity and tested as a substrate for partially purified L-galactitol 1-phosphate dehydrogenase (Table 9). The reaction was dependent upon NAD^+, galactitol, and galactitol phosphate dehydrogenase. Furthermore, the complete reaction mixture yielded a total absorbance change of 0.452 whereas the theoretical value was 0.459 on the basis of the amount of the reaction product added. The purity of the synthesized compound was, therefore, calculated to be 98.4% and the identity of the compound tentatively established as galactitol phosphate.

Although there was little doubt as to the identity of the chemically reduced D-galactose 6-phosphate (see Materials and Methods) its characterization was still demanded to confirm

the structure as L-galactitol 1-phosphate and thereby establish it as a standard to which the enzymatically synthesized compound could be compared. Therefore the chemically synthesized compound was characterized with respect to gas-liquid chromatography, mass spectrometry, acid lability, and periodate oxidation. As with the enzymatically synthesized reaction product, the chemically synthesized L-galactitol 1-phosphate was first analyzed for its purity and tested as a substrate for L-galactitol 1-phosphate dehydrogenase. A 0.5 µl sample was removed from a 0.030 M stock solution of the compound and assayed accordingly. The absorbance change observed was 0.592 which was equivalent to 0.014 µmole of L-galactitol 1-phosphate. The theoretical value was 0.015 µmole and therefore the purity of the compound was calculated to be 93.3%. Furthermore, the reaction was dependent upon NAD^+, substrate, and L-galactitol 1-phosphate dehydrogenase as described in Materials and Methods (see Enzyme Assays).

Gas-Liquid Chromatography of Derivatives of Chemically and Enzymatically Synthesized Galactitol Phosphate Before and After Dephosphorylation

Chemically synthesized L-galactitol 1-phosphate was dephosphorylated with *E. coli* alkaline phosphatase and then derivatized to the hexaacetate form as described in Materials and Methods. GLC analysis of the derivative is shown in Figure (8) with comparisons to the acetate derivatives of commercially prepared galactitol and D-galactose. The product yielded only a single peak (44.5 minutes) which had nearly

TABLE 9. Purity of the enzymatically synthesized galactitol 1-phosphate and test as a substrate for l-galactitol phosphate dehydrogenase. A 13.7 mg sample of the disodium salt of the product was dissolved in 2.00 ml of distilled water and 0.5 µl was assayed with galactitol phosphate dehydrogenase as described in Materials and Methods.

Assay Description				Total absorbance change at 340 nm
	NAD^+ (µmoles)	L-Galactitol 1-phosphate dehydrogenase (milliunits)	Reaction product (µmoles)	
Complete (see text)	0.500	3.1	0.011	0.452
Complete minus NAD^+	0.000	3.1	0.011	0.000
Complete minus galactitol phosphate dehydrogenase	0.500	0.00	0.011	0.000
Complete minus reaction product	0.500	3.1	0.00	0.000

the same retention time as standard galactitol hexaacetate (44.4 minutes). This did not necessarily demonstrate that the dephosphorylated L-galactitol 1-phosphate was galactitol since TMS derivatives of other hexitols may exhibit identical retention times. Consequently, gas chromatography of the hexaacetate derivatives of commercially available hexitols was performed.

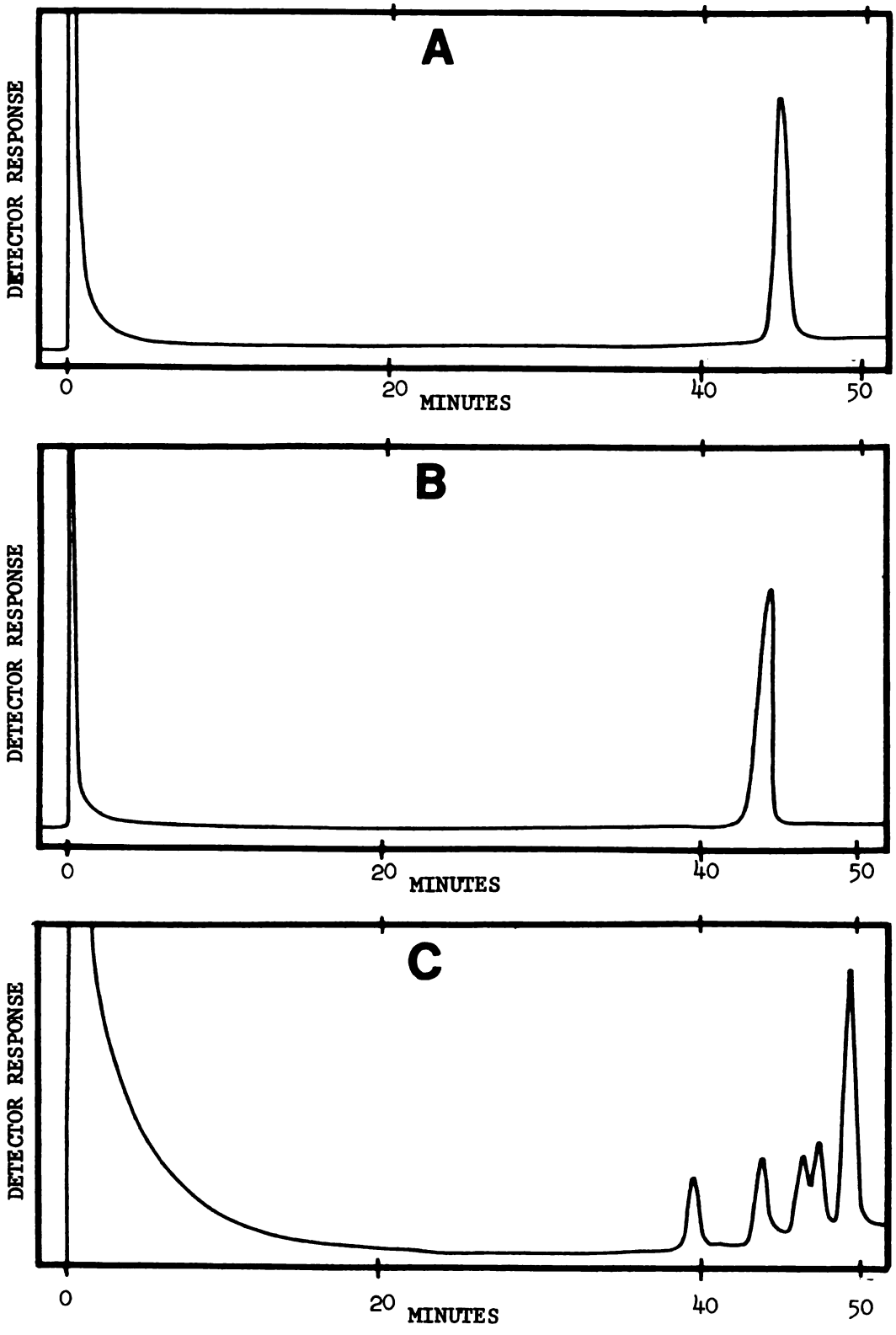
Figure (9) is the GLC tracing of a standard mixture of commercial polyols converted to their corresponding polyacetate derivatives. Table (10) lists the names and retention times of the peaks shown in Figure (9). Galactitol is clearly distinguishable from the other hexitol derivatives: inositol, mannitol, and sorbitol. Three other hexitols - D-talitol, D-iditol, and allitol - were not analyzed due to their unavailability, but have been previously shown to be clearly separable from galactitol under similar conditions (115). It could not be concluded, however, that dephosphorylation of chemically synthesized L-galactitol 1-phosphate yielded galactitol since the second isomer peak of the galactose acetate derivative had nearly the same retention time as did galactitol (44.0 minutes) and therefore allowed for a very remote possibility that the chemically synthesized L-galactitol 1-phosphate was still D-galactose 6-phosphate or extremely contaminated with it. In order to eliminate this possibility, a mass spectral analysis on all of the D-galactose isomers as their TMS derivatives and also the TMS derivatives of the dephosphorylated L-galactitol 1-phosphate

FIGURE 8. GLC characterization of the hexaacetate derivative of chemically synthesized L-galactitol 1-phosphate after dephosphorylation. The derivatization procedure and GLC conditions are described in Materials and Methods.

A. Commercial galactitol.

B. Chemically synthesized L-galactitol 1-phosphate after dephosphorylation.

C. D-Galactose (fifth peak is an inositol standard).


A
DETECTOR RESPONSE
0
20
40
50
MINUTES
B
DETECTOR RESPONSE
0
20
40
50
MINUTES
C
DETECTOR RESPONSE
0
20
40
50
MINUTES

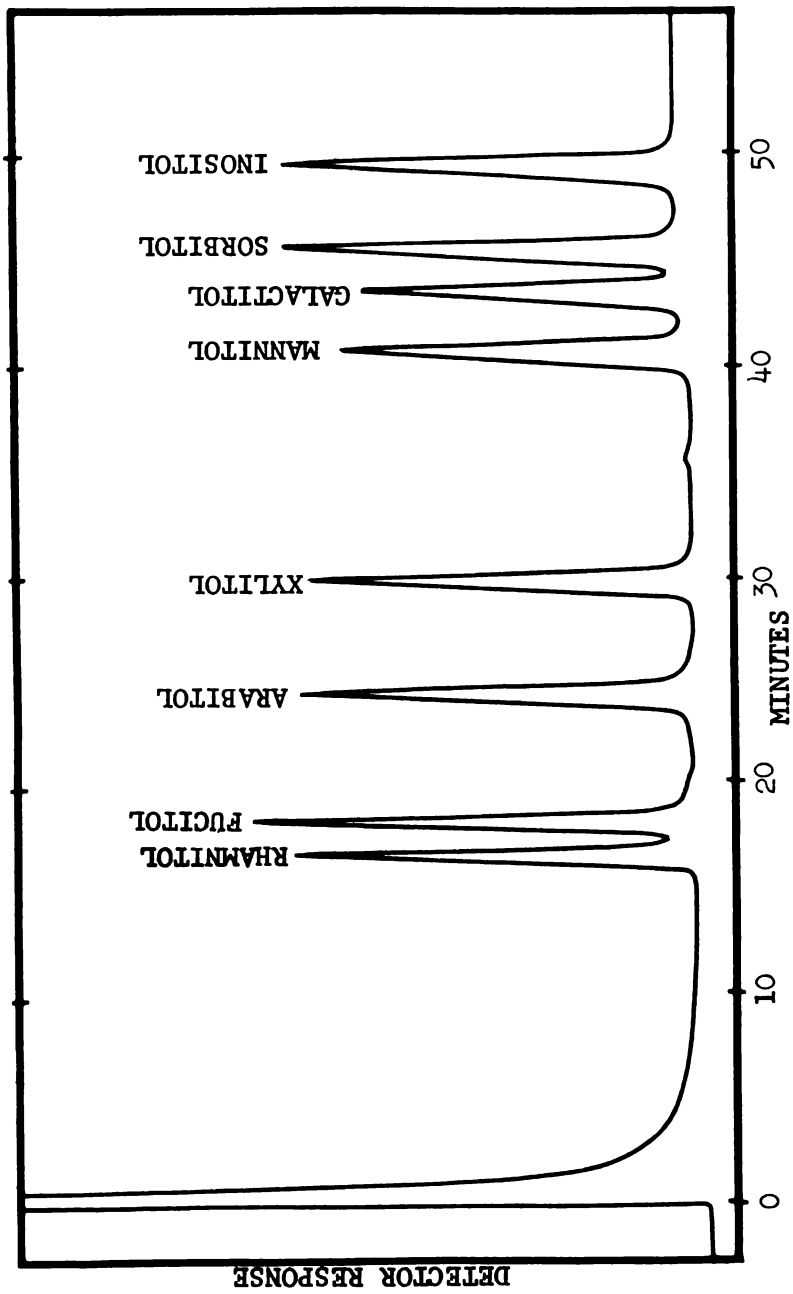


Figure 9. GCL of some polyols as their polyacetate derivatives. GLC conditions and the derivatization procedure are described in Materials and Methods.

TABLE 10. Retention times of the polyacetate derivatives of the polyols analyzed in Figure (9).

Sugar Alcohol	Retention Time in minutes
Rhamnitol	16.2
Fucitol	17.9
Arabitol	24.3
Xylitol	29.9
Mannitol	41.4
Galactitol	44.4
Sorbitol (D-Glucitol)	46.6
Inositol	50.7

and commercial galactitol was performed (presented in the following section).

The enzymatically synthesized galactitol phosphate was analyzed by gas-liquid chromatography as the TMS derivative before and after dephosphorylation. Figure (10) shows that the TMS derivatives of chemically synthesized L-galactitol 1-phosphate and the enzymatically synthesized compound exhibited single peaks and had identical retention times, 44.9 minutes. This also indicated that the compounds were pure by GLC criteria. The dephosphorylation of the enzymatically synthesized galactitol phosphate and its conversion to the TMS derivative yielded a compound which clearly chromatographed apart from the TMS derivative of the phosphorylated form under identical GLC conditions. But this compound had nearly the same retention time as the TMS derivative of commercial galactitol, 16.9 minutes and 17.0 minutes respectively (Figure 11). When the enzymatically synthesized galactitol phosphate was dephosphorylated and derivatized to the hexaacetate form it exhibited a single peak of essentially the same retention time as commercial galactitol, 44.3 minutes and 44.4 minutes respectively (Figure 12). The data strongly imply, therefore, that the product isolated from the PTS-catalyzed reaction was galactitol phosphate.

Mass Spectrometry of the TMS Derivative of Chemically Synthesized L-Galactitol 1-Phosphate After Dephosphorylation.

Should the mass spectrum of enzymatically synthesized

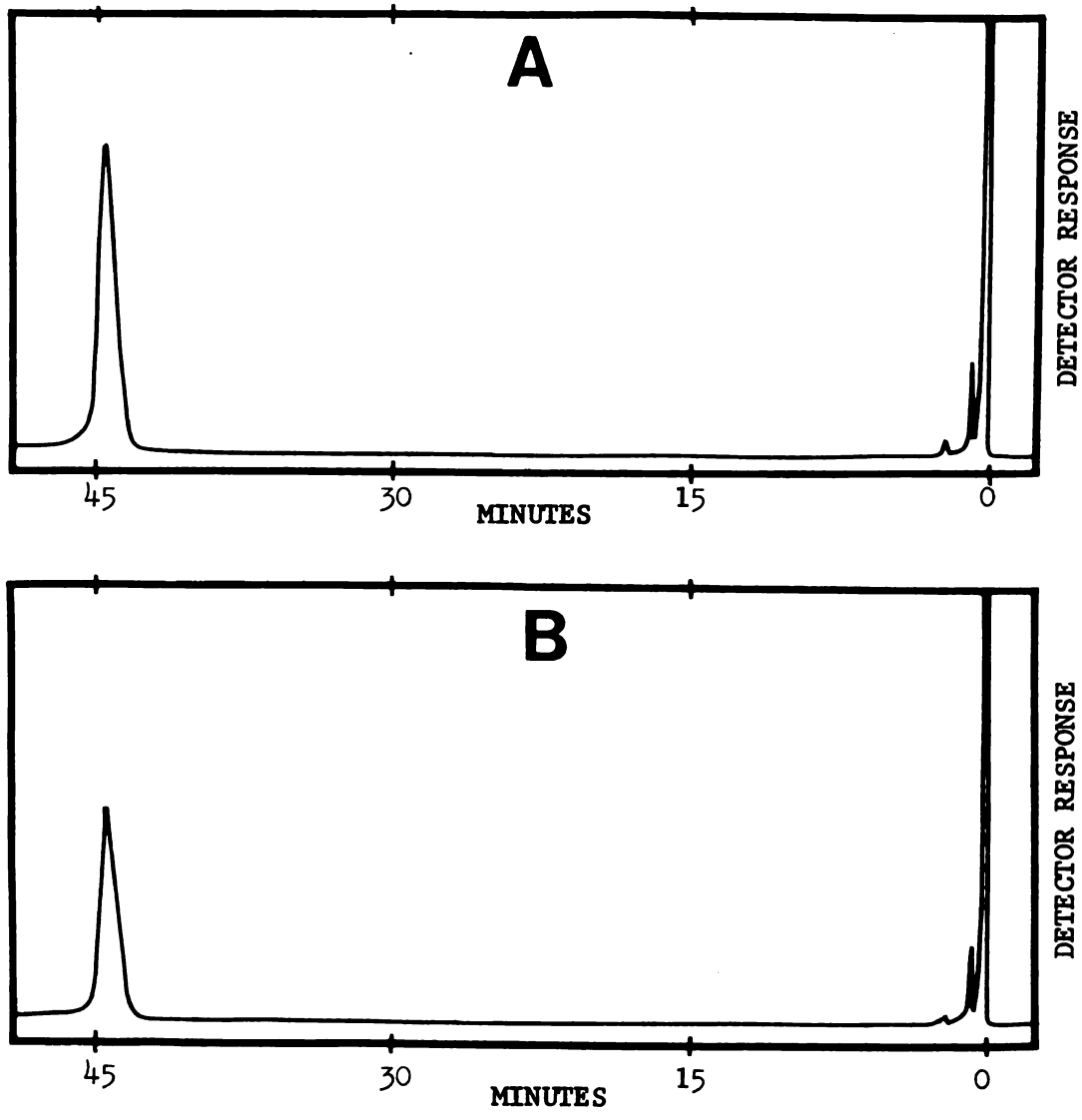


Figure 10. GLC analysis of the TMS derivative of enzymatically synthesized galactitol phosphate. TMS derivatization and general GLC conditions are described in Materials and Methods. The temperature program rate was 1°C/min. from 150°C to 250°C. The attenuation was 1600

A. TMS derivative of chemically synthesized L-galactitol 1-phosphate.

B. TMS derivative of enzymatically synthesized galactitol phosphate.

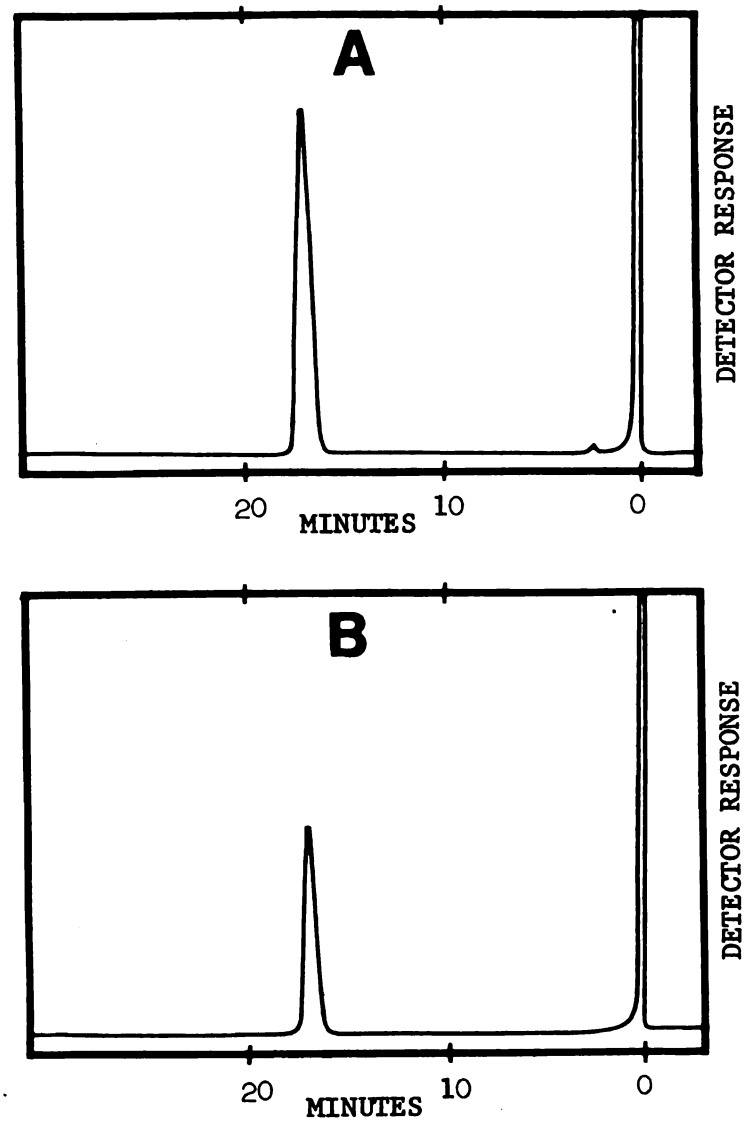


Figure 11. GLC of the TMS derivative of enzymatically synthesized galactitol phosphate after dephosphorylation. The GLC conditions were identical to those described for Figure (10). The dephosphorylation is described in Materials and Methods.

A. TMS derivative of commercial galactitol.

B. TMS derivative of enzymatically synthesized galactitol phosphate after dephosphorylation.

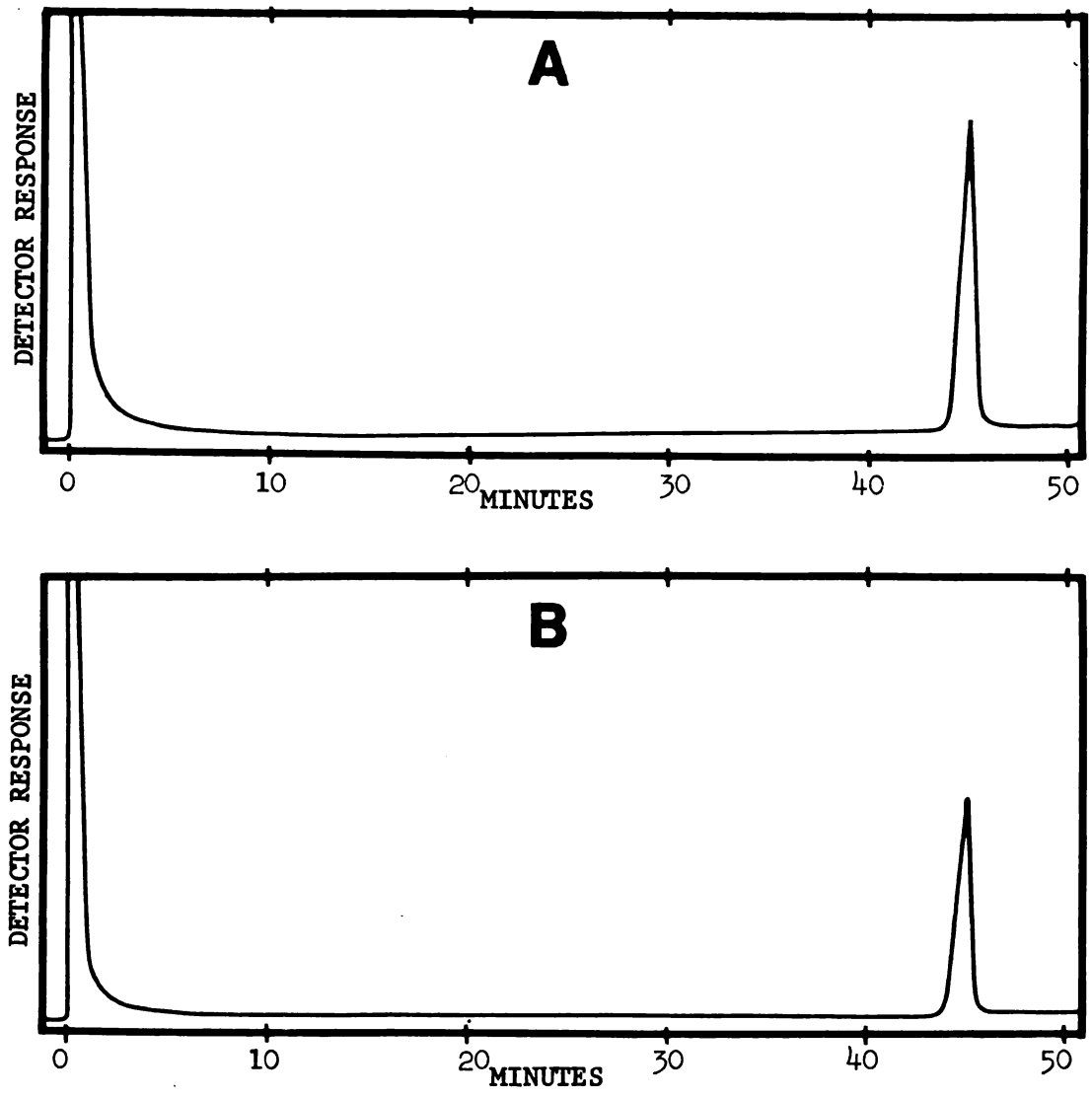


Figure 12. GLC of the hexaacetate derivative of enzymatically synthesized galactitol phosphate after dephosphorylation. The GLC conditions and derivatization procedure were identical to those described in Figure (9). Dephosphorylation was performed as described in Materials and Methods.

A. Standard galactitol hexaacetate.

B. Hexaacetate derivative of enzymatically synthesized galactitol phosphate after dephosphorylation.

galactitol phosphate be congruent with that of chemically synthesized galactitol phosphate then mass spectroscopy of the enzymatically synthesized compound after dephosphorylation would not be necessitated. Therefore the mass spectrum of the TMS derivative of only the chemically and not the enzymatically synthesized galactitol phosphate after dephosphorylation was obtained as one criterion for establishing the chemically synthesized compound as an L-galactitol 1-phosphate standard.

The chemically synthesized galactitol phosphate was dephosphorylated as before and converted to the TMS derivative as described in Materials and Methods. Figure (13) shows that the TMS derivative yielded a single peak which chromatographed as standard TMS-galactitol did. Figure (14) shows the mass spectra of each of the three TMS-galactose isomers, which agree with the spectra obtained by DeJongh, et al. (116). Figure (15) reveals the mass spectra of the TMS-dephosphorylated compound and the TMS-galactitol standard. Each of the three galactose isomers yielded a mass spectrum of different and salient m/e ratios than that by either the standard galactitol or the dephosphorylated compound. In all spectra the parent ions were not seen due to their low abundance. The data obtained were therefore consistent with the identification of chemically reduced D-galactose 6-phosphate as a hexitol phosphorylated at a primary alcohol position.

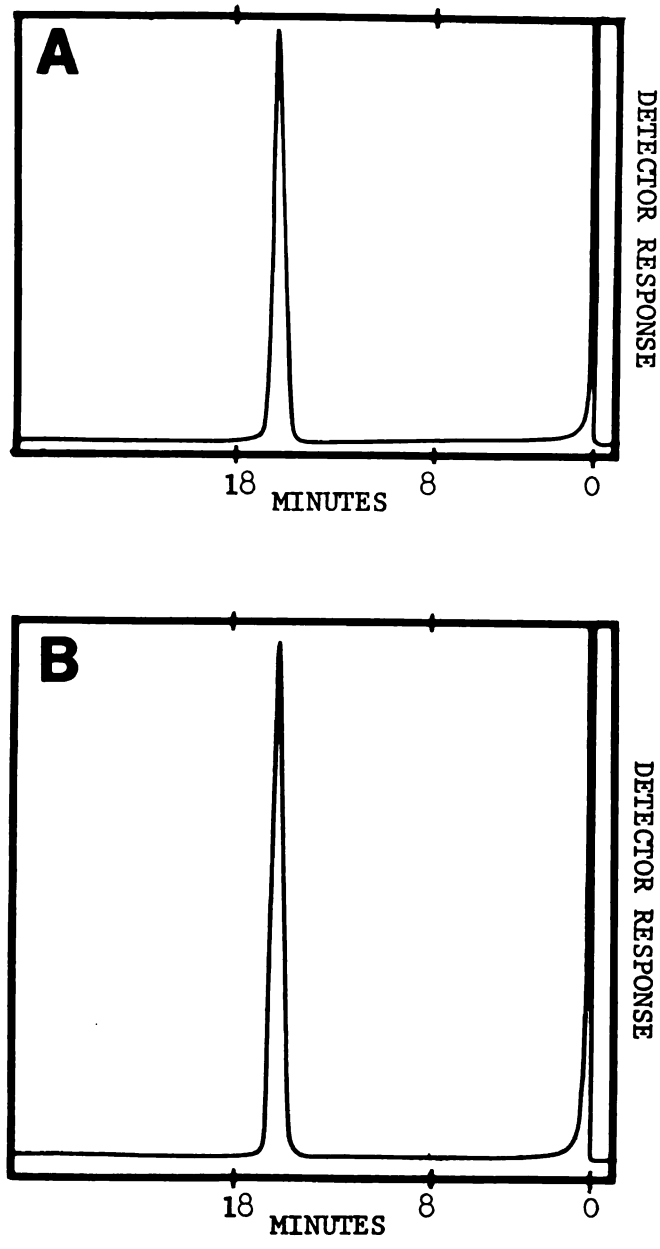


Figure 13. GLC of the TMS derivative of chemically synthesized L-galactitol 1-phosphate after dephosphorylation. The GLC conditions and derivatization procedure are described in Materials and Methods. The temperature program rate was 1°C/min. from 150 to 200°C.

A. TMS derivative of standard galactitol (attenuation = 3200).

B. TMS derivative of chemically synthesized L-galactitol 1-phosphate after dephosphorylation (attenuation = 160).

FIGURE 14. Mass spectrometry of the isomers of D-galactose $(TMS)_5$. The molecular weight of all isomers is 540. Absence of parent ions was due to their low abundance. Procedural details are described in Materials and Methods.

A. Isomer number 1.

B. Isomer number 2.

C. Isomer number 3.

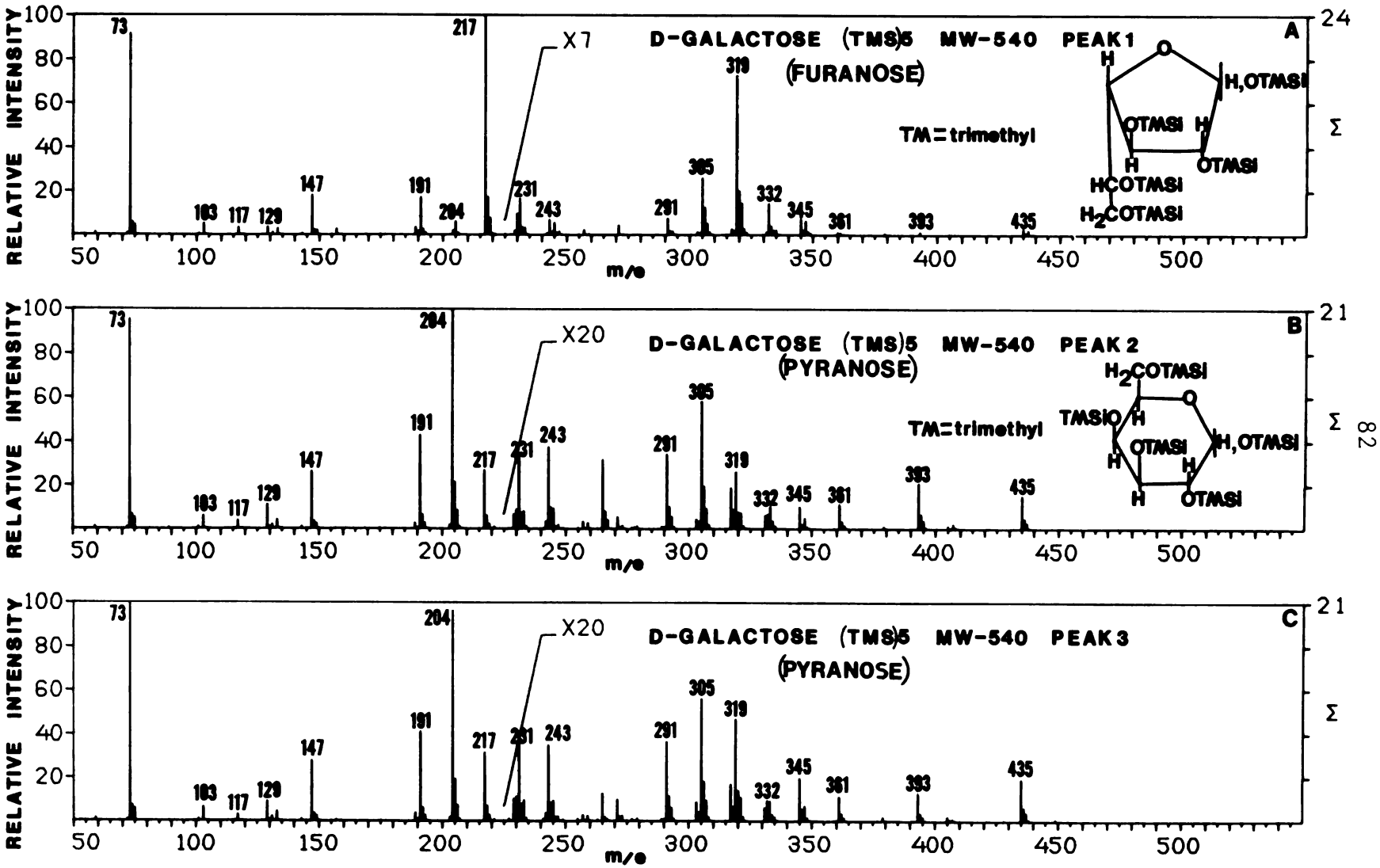
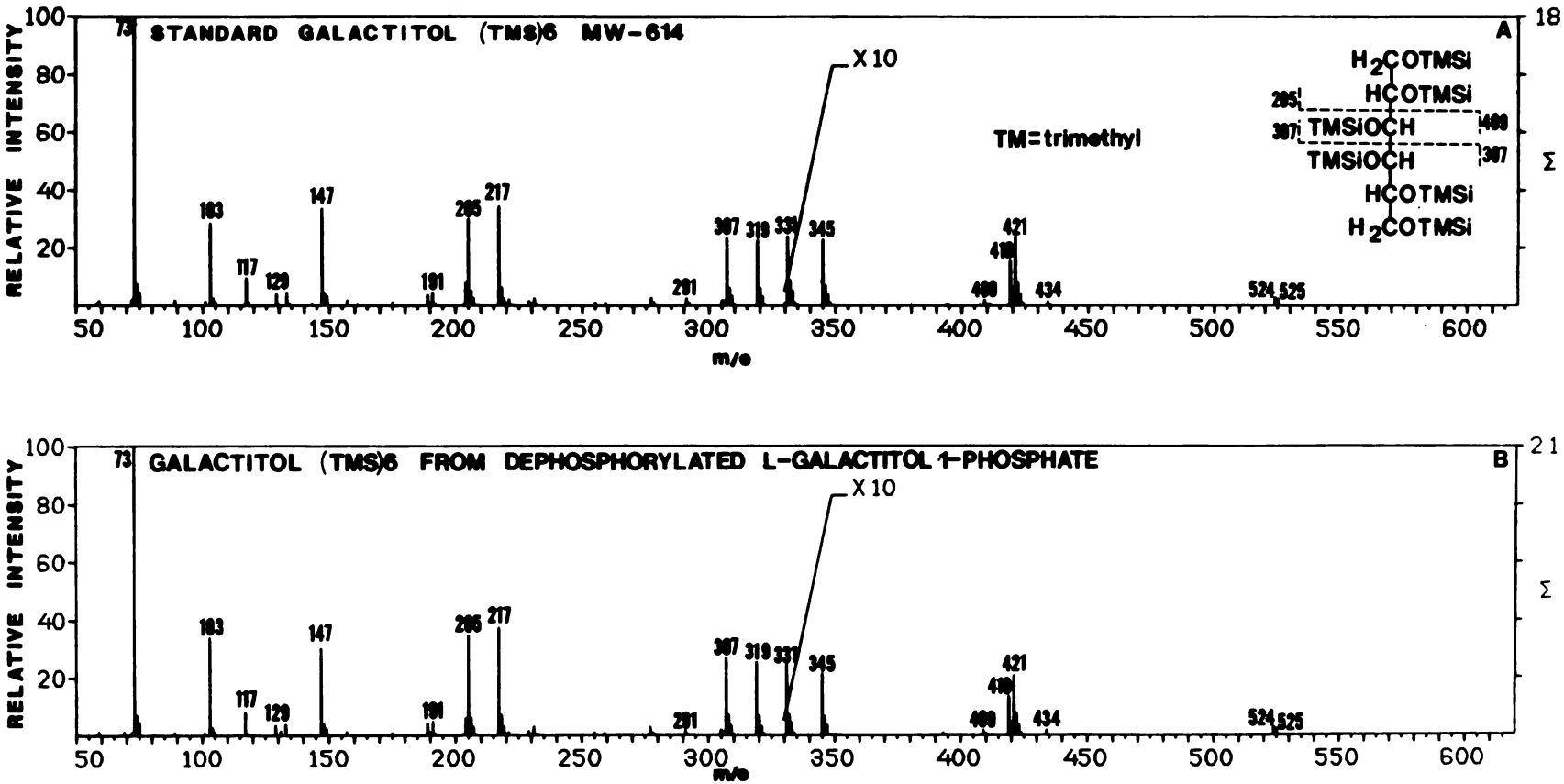

RELATIVE INTENSITY
100
80
60
40
20
73
217
X7
D-GALACTOSE (TMS)5 MW-540 PEAK1
(FURANOSE)
A
24
Σ
319
305
147
191
231
243
183
117
129
204
291
332
345
361
393
435
TM=trimethyl
H,OTMSI
OTMSI
HCOTMSI
H2COTMSI
50
100
150
200
250
300
350
400
450
500
m/e
RELATIVE INTENSITY
73
204
X20
D-GALACTOSE (TMS)5 MW-540 PEAK2
(PYRANOSE)
B
21
Σ
305
191
147
243
217
231
291
319
183
117
129
332
345
361
393
435
TM=trimethyl
H2COTMSI
TMSIO
H,OTMSI
OTMSI
m/e
RELATIVE INTENSITY
73
204
X20
D-GALACTOSE (TMS)5 MW-540 PEAK3
(PYRANOSE)
C
21
Σ
305
319
191
147
217
231
243
291
183
117
129
332
345
361
393
435
m/e

FIGURE 15. Mass spectrometry of chemically synthesized L-galactitol 1-phosphate as the TMS derivative after dephosphorylation. Procedural details are described in Materials and Methods. Parent ions were absent due to their low abundancies. The molecular weight of galactitol $(TMS)_6$ is 614.

A. Standard galactitol $(TMS)_6$.

B. TMS derivative of chemically synthesized L-galactitol 1-phosphate after dephosphorylation.


A
73
STANDARD GALACTITOL (TMS)6 MW-614
X10
TM=trimethyl
H2COTMSi
HCOTMSi
TMSiOCH
TMSiOCH
HCOTMSi
H2COTMSi
RELATIVE INTENSITY
m/e
B
GALACTITOL (TMS)6 FROM DEPHOSPHORYLATED L-GALACTITOL 1-PHOSPHATE

Mass Spectrometry of Enzymatically and Chemically Synthesized Galactitol Phosphate as TMS Derivatives

To confirm that both the enzymatically and chemically synthesized galactitol phosphate were hexitol monophosphates, they were subjected to combined GLC-mass spectrometry. Figure (16) displays the mass spectra for both the chemically synthesized L-galactitol 1-phosphate and the enzymatically synthesized galactitol phosphate. The two spectra are not exactly identical as is evidenced by the absence of some of the less abundant peaks, especially in the larger mass regions. This is most likely due to the difference in the quantity of compound fragmented since more of the enzymatic product was subjected to analysis than was the chemically synthesized compound. The most salient characteristic of both spectra is the diagnostic M-15 peak (m/e = 751) which corresponds to a loss of one methyl group from a hexitol phosphate (117). A parent ion was not seen in this analysis due to the absence or undetectable amounts of this ion. These mass spectra are different than the mass spectra of TMS-galactitol and TMS-galactose (Figures 14 and 15). The mass spectra of the four TMS-D-galactose 6-phosphate isomers, which correspond to those obtained by Horning and Horning (117), are shown in Figure (17). These spectra were also significantly different than that of the TMS-hexitol phosphates. The mass spectral data are therefore consonant with the contention that the enzymatically synthesized compound is galactitol phosphate. However, this was not definitive proof that L-galactitol

1-phosphate was the product of the PTS-catalyzed reaction since both the D and L isomers of any hexitol phosphorylated at either the one or six position would yield identical spectra as the TMS derivative. Consequently, it was decided to characterize the phosphorylated reaction product by chemical analysis after oxidation by L-galactitol 1-phosphate dehydrogenase.

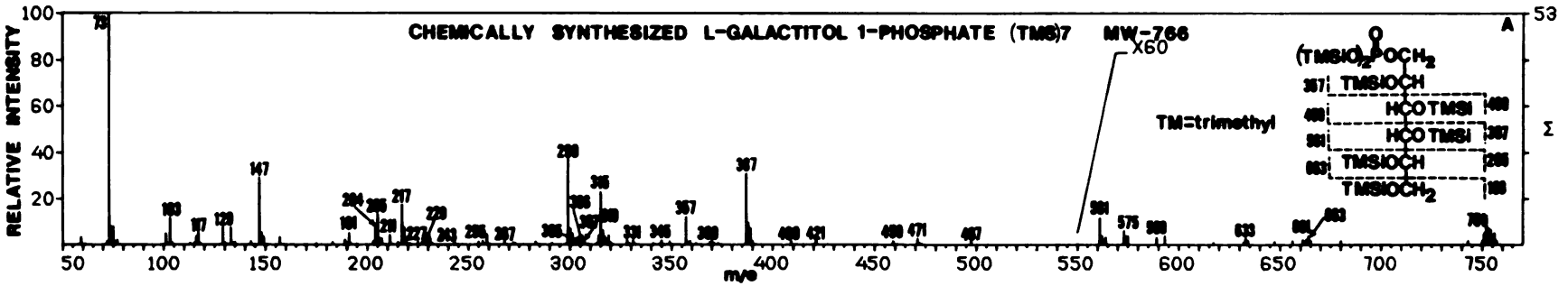
Periodate Oxidation of Chemically Synthesized L-Galactitol 1-Phosphate.

The chemically synthesized compound was analyzed by periodate oxidation in order to firmly establish the structure as L-galactitol 1-phosphate. The oxidation and analysis of products formed are described in Materials and Methods. Table (11) reveals the results obtained from the periodate oxidation in comparison to the theoretical values obtained from the degradation scheme shown in Figure (18). Since the molecule was a hexitol phosphorylated at a primary alcohol position, it was expected that only 1 mole of formaldehyde should be produced for every mole of compound and that further degradation would yield 3 moles of formic acid for every mole of compound. The nonoxidizable two-carbon moiety remaining would be the acid-labile phosphoglycoaldehyde and thus the phosphate function changes from stabile to labile as was found (graphs shown later). In addition the P_i, determined from phosphogycoaldehyde, to formaldehyde molar ratio was 1:1 in accordance with a hexitol that was monophosphorylated. Table (11)

FIGURE 16. Mass spectrometry of chemically synthesized L-galactitol 1-phosphate and enzymatically synthesized galactitol phosphate. Combined GLC-mass spectrometry was performed on the TMS derivatives as described in Materials and Methods. Three times more of the enzymatically synthesized compound than chemically synthesized compound was used in order to detect impurities during the GLC analysis. Mass numbers are assigned to the characteristic peaks.

A. Chemically synthesized L-galactitol 1-phosphate.

B. Enzymatically synthesized galactitol phosphate.


CHEMICALLY SYNTHESIZED L-GALACTITOL 1-PHOSPHATE (TMS)7 MW-766
A
X60
TM=trimethyl
RELATIVE INTENSITY
m/e
Σ
53

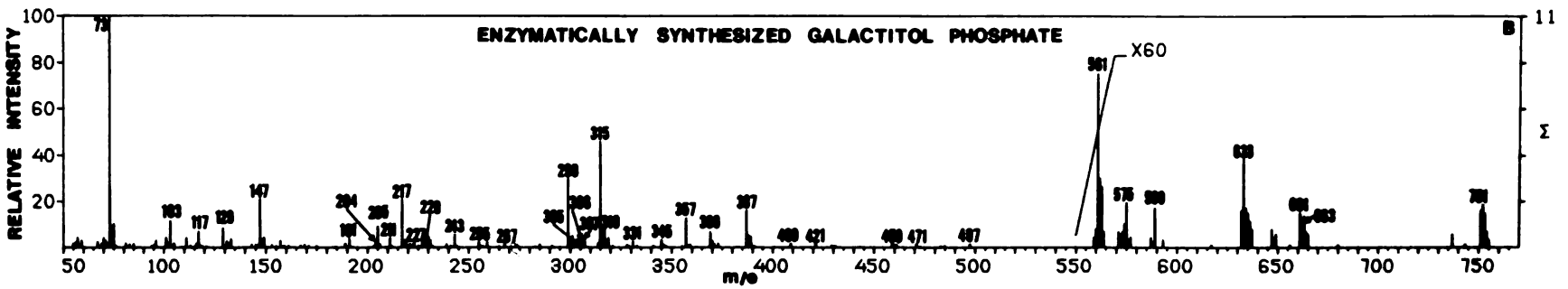

ENZYMATICALLY SYNTHESIZED GALACTITOL PHOSPHATE
B
X60
RELATIVE INTENSITY
m/e
Σ
11

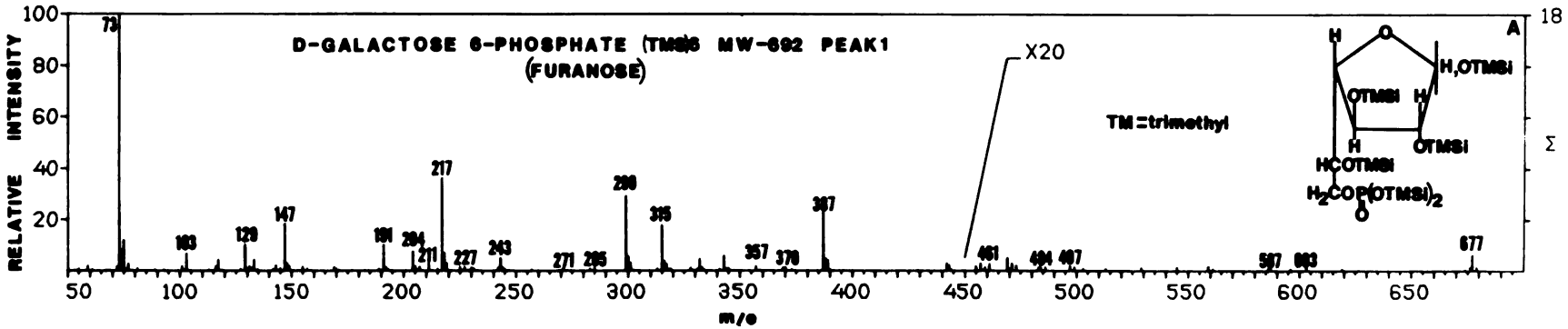
FIGURE 17. Mass spectrometry of standard D-galactose 6-phosphate $(TMS)_6$. All four isomers, molecular weight = 692, were examined. Details of analysis are described in Materials and Methods. The parent ions were absent due to their low abundancies.

A. Isomer number 1.

B. Isomer number 2.

C. Isomer number 3.

D. Isomer number 4.


D-GALACTOSE 6-PHOSPHATE (TMS)6 MW-692 PEAK1
(FURANOSE)
X20
TM=trimethyl
A
RELATIVE INTENSITY
m/e
Σ
18

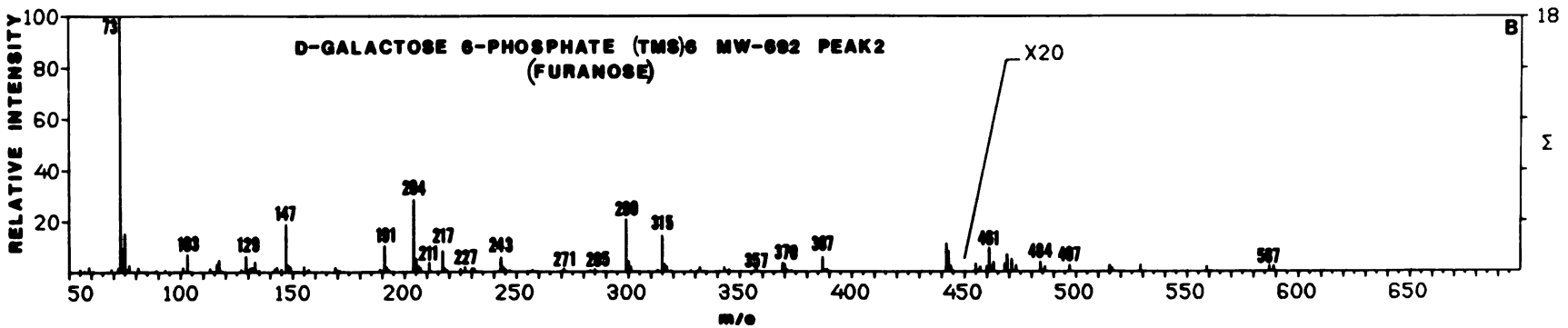
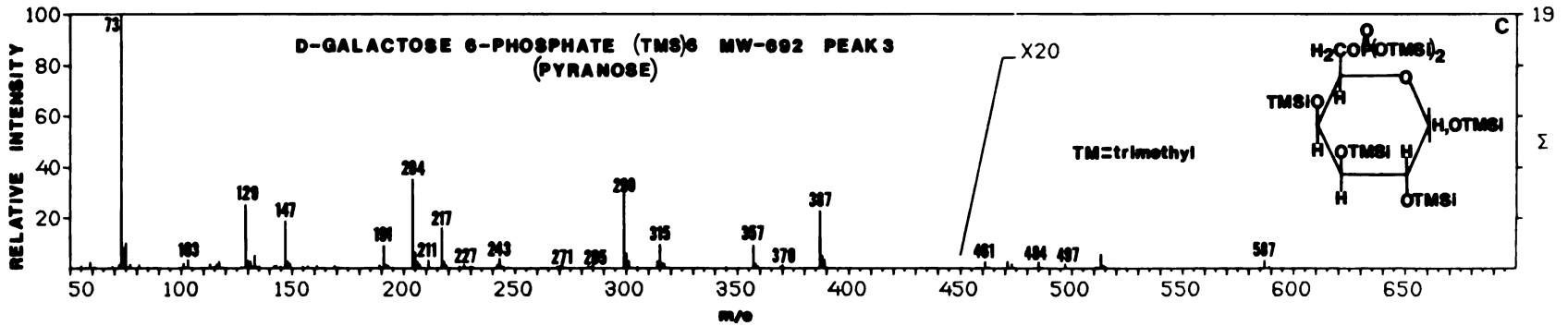
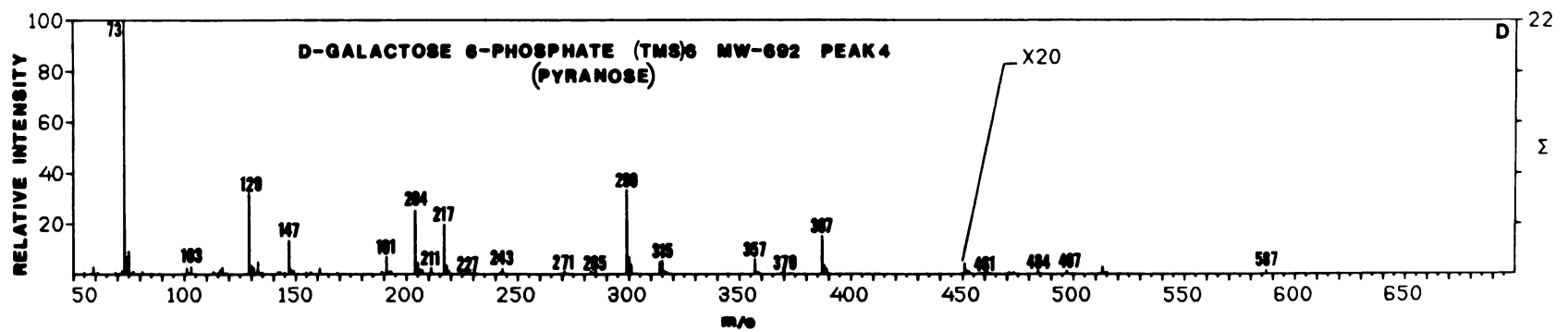

D-GALACTOSE 6-PHOSPHATE (TMS)6 MW-692 PEAK2
(FURANOSE)
X20
B
RELATIVE INTENSITY
m/e
Σ
18

FIGURE 17. (Continued . . . .)


D-GALACTOSE 6-PHOSPHATE (TMS)6 MW-692 PEAK 3
(PYRANOSE)
RELATIVE INTENSITY
m/e
X20
TM=trimethyl
C
19
Σ


D-GALACTOSE 6-PHOSPHATE (TMS)6 MW-692 PEAK 4
(PYRANOSE)
RELATIVE INTENSITY
m/e
X20
D
22
Σ

summarizes the periodate oxidation data and is consistent with the identity of the compound as a hexitol phosphorylated at a primary alcohol position.

Identification of the Phosphorylated Product as L-Galactitol 1-Phosphate by Enzymatic Oxidation to D-Tagatose 6-Phosphate.

To determine the position of the phosphate function and the enantiomeric configuration of the PTS-catalyzed reaction product, it was necessary to oxidize the compound with L-galactitol 1-phosphate dehydrogenase and then characterize the resulting product with respect to: GLC analysis, acid lability, periodate oxidation, and enantiomeric configuration. This characterization would also be expected to corroborate the identification of the hexitol moiety of the PTS-catalyzed reaction product as galactitol.

Preparation and isolation of D-tagatose 6-phosphate:

The enzymatically synthesized galactitol phosphate was enzymatically oxidized to a ketohexose phosphate by using NAD^+ as the cofactor and L-galactitol 1-phosphate dehydrogenase as the catalyst. In order to regenerate NAD^+ from the reduced form it was necessary to couple the oxidation reaction with a reduction reaction, i.e. pyruvate to lactate using lactate dehydrogenase. To insure complete conversion of the galactitol phosphate, an excess of pyruvate relative to galactitol phosphate and an excess of lactate dehydrogenase relative to galactitol phosphate dehydrogenase was used. In addition to these considerations, the reaction was buffered

TABLE 11. Periodate oxidation analysis of chemically synthesized L-galactitol 1-phosphate. A sample of 65.0 μmoles was oxidized and the products produced were analyzed as described in Materials and Methods.

Product (formed from periodate oxidation)	Total μmoles of product	Determined molar ratio of product: hexitol phosphate	Theoretical ratio of product: hexitol phosphate
IO_4^- Consumption	265	4.08	4
Theoretical IO_4^- Consumption	260		
Formaldehyde formed	64.8	0.99	1
Theoretical value	65.0		
Glycolate formed	0.0	0.00	0
Theoretical value	0.0		
Phosphoglycolate formed	0.0	0.00	0
Theoretical value	0.0		
Acid lability	No		
Theoretical lability	No		
Phosphoglycoaldehyde Formed	65.0	1.00	1
Theoretical value	65.0		
Formate Formed	196	3.02	3
Theoretical value	195		
Carbon dioxide formed	0.0	0.01	0
Theoretical value	0.0		

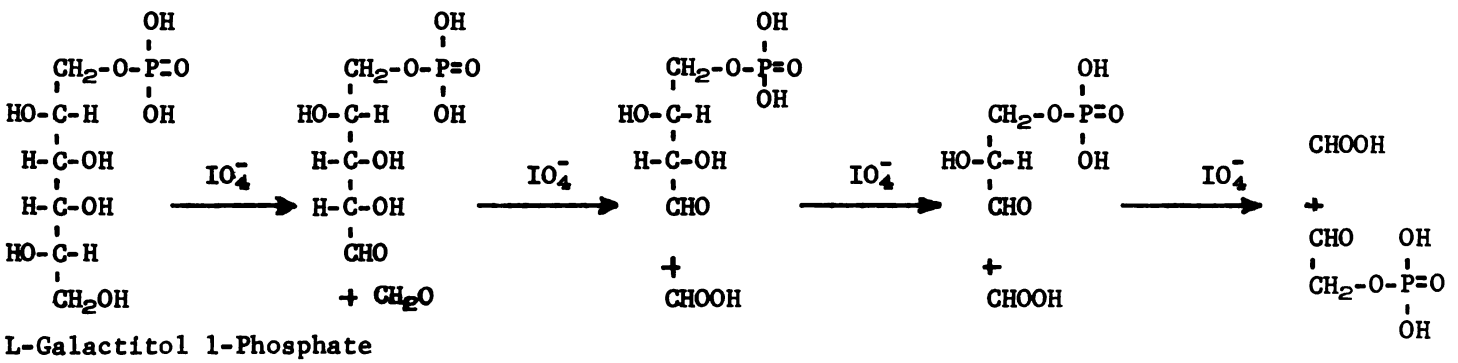


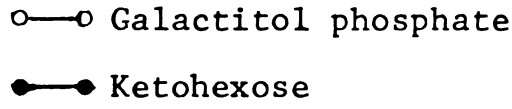
Figure 18. Periodate oxidation scheme for L-galactitol 1-phosphate

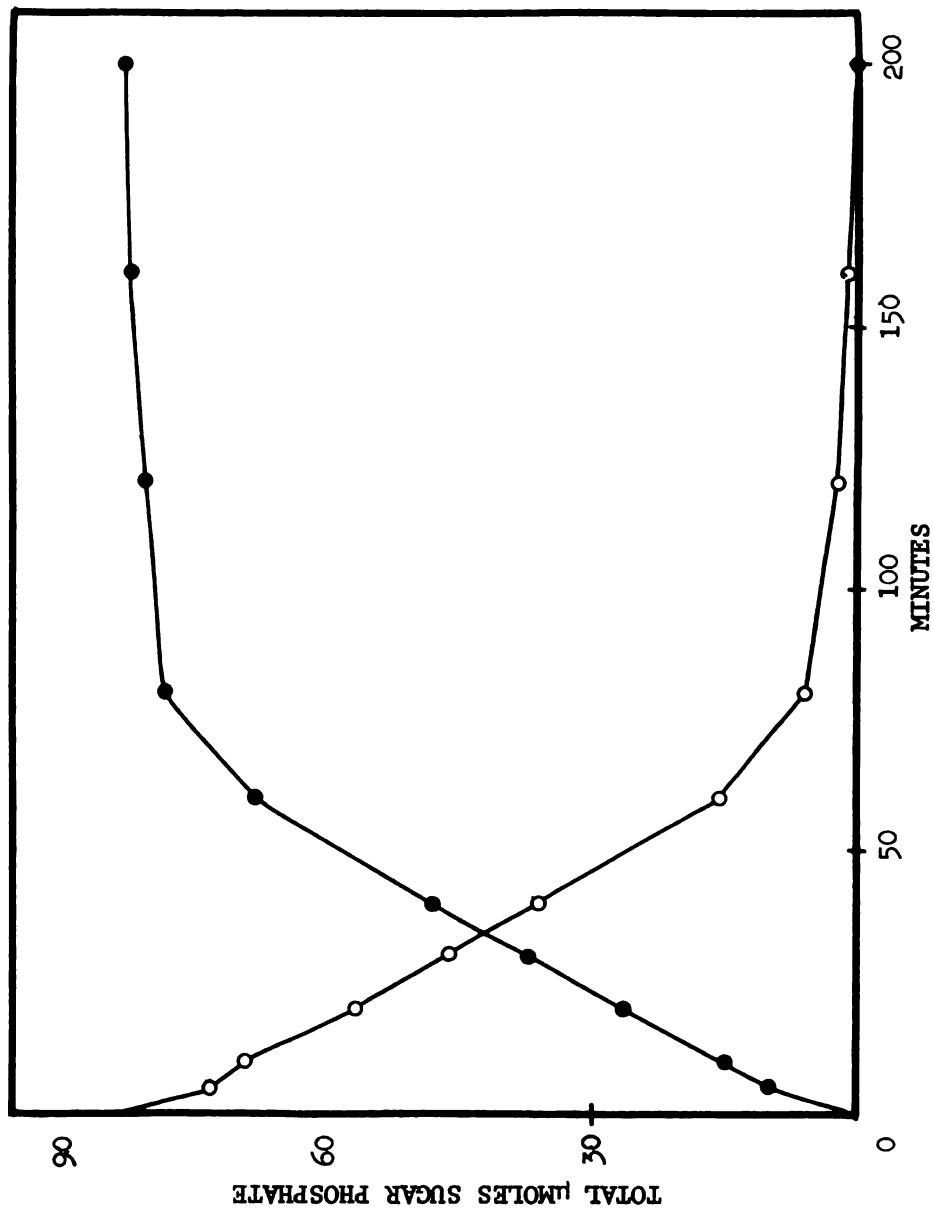
with phosphate. This was because the oxidation was carried out at pH 9.0 at which alkaline phosphatase activity, present with the partially purified galactitol phosphate dehydrogenase, is known to be inhibited by high phosphate concentrations (118). The reaction mixture contained the following constituents in a final volume of 3.10 ml: 20 μmoles of $MgCl_2$, 0.2 mmole of K_2HPO_4 (pH 9.0), 0.136 mmole of sodium pyruvate, 2.95 units of partially purified galactitol phosphate dehydrogenase, 56 units of lactate dehydrogenase which has been previously desalted on a small Biogel P-6 column, 50 μmoles of NAD^+, and 84 μmoles of the enzymatically synthesized galactitol phosphate. The reaction mixture was warmed to 30°C in a stoppered test tube placed in a thermostated water bath. The reaction was initiated by the addition of galactitol phosphate. At specific time intervals, 5 μl to 30 μl was removed to small culture tubes and then heated for a few minutes at 90°C. After heating, the tubes were cooled on ice and then the denatured protein removed by centrifugation at 5,000 x g for 10 minutes at 0°C. The resulting supernatants were then assayed for ketohexose and galactitol phosphate. The course of the reaction is shown in Figure (19). When the presence of galactitol phosphate was no longer detectable the reaction mixture was heated for 10 minutes at 100°C and then cooled on ice. The precipitate was removed by centrifugation at 10,000 x g for 15 minutes at 0°C. The supernatant was retained and the pellet resuspended in a minimum volume of distilled water in order to wash the product from the

denatured protein. The suspension was centrifuged as before and the supernatant combined with the first supernatant. The solution was then adjusted to pH 8.0 and the ketohexose phosphate product purified by the ion-exchange method of Khym (119).

To a column (0.78 sq. cm. x 14 cm) containing Biorad AG1-X2 Cl^- was applied the adjusted solution. The column was washed with 100 ml of 0.001 M NH_4OH to remove pyruvate, lactate, and cations. The column was then washed with 1.0 liter of 0.02 M NH_4Cl containing 0.01 M $K_2B_4O_7$ in order to remove phosphate ions. The presence of borate serves to complex with the hydroxyl functions of the sugar molecule and form a negatively charged complex which will strongly bind to the resin. The column was then washed with 1.0 liter of 0.025 M NH_4Cl containing 0.0025 M NH_4OH and 0.001 M $K_2B_4O_7$. The product was finally eluted with 2.0 liters of 0.025 M NH_4Cl containing 0.0025 M NH_4OH and 0.01 mM $K_2B_4O_7$. The eluate was collected in 10 ml fractions using a column flow rate of 1.3 ml/minute. A 2.0-ml aliquot was removed from the fractions and assayed for ketohexose. The elution profile of product is shown in Figure (20). The peak was asymmetrical but since it was found to consist of a single compound, the asymmetry may have been to abberations in the column packing or sample application. The fractions containing ketohexose were pooled into a large round bottom flask and then evaporated to 100 ml under reduced pressure at 30°C in a rotary evaporator. To

FIGURE 19. Enzymatic oxidation of enzymatically synthesized galactitol phosphate. The oxidation was performed using partially purified galactitol phosphate dehydrogenase as described in the text.

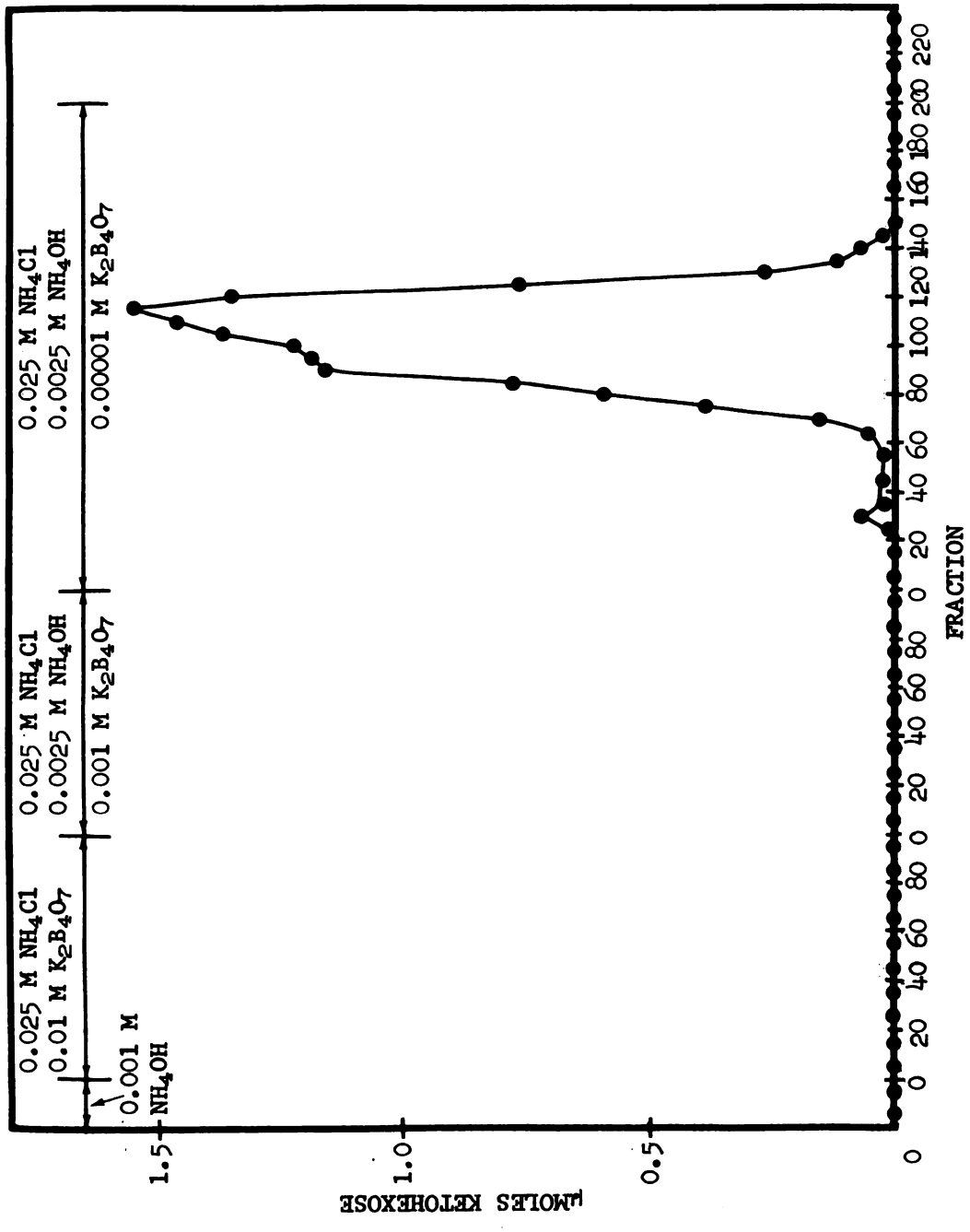



TOTAL μMOLES SUGAR PHOSPHATE
90
60
30
0
50
100
150
200
MINUTES

the concentrated solution was added an excess of Dowex 50W-X8 H^+ in order to remove NH_4^+ and other cations. The resin was removed by suction filtration followed by a wash with one resin-volume of distilled water. The filtrate was then freed of HCl by evaporation to a syrup under reduced pressure at 30°C in a rotary evaporator. The resulting syrup was dissolved in a minimum volume of distilled water and then tested for the absence of P_i and chloride ions. The solution was then freed of borate ions by the addition of anhydrous methanol and removal of the resulting methyl borate by evaporation to dryness under reduced pressure at 30°C in a rotary evaporator. The methanol treatment was repeated three more times after which the oil was dissolved in distilled water and titrated to neutrality using 0.1 M $Ba(OH)_2$. The resulting solution was evaporated to dryness under reduced pressure as described before and the solid residue suspended in 100% ethanol. The suspension was transferred to a tared amber vial and the solvent evaporated under nitrogen at 30°C. The percent recovery of synthesized ketohexose phosphate was 86.9% with a yield of 73 μmoles of the barium salt. The anhydrous salt was then stored desiccated at 0°C.

Figure (20) reveals a one to one stoichiometry between the production of ketohexose and the loss of starting compound. It can also be seen that quantitative conversion of the reactant to ketohexose was accomplished under the described conditions. After the ketohexose product was isolated it was

FIGURE 20. AG1-X2 Cl^- Ion-exchange chromatography of the enzymatically synthesized ketohexose phosphate. The μmoles of ketohexose are expressed in terms of the total amount present in the fraction (10 ml) assayed. Other details are given in the text.


0.025 M NH4Cl
0.01 M K2B4O7
0.001 M NH4OH
0.025 M NH4Cl
0.0025 M NH4OH
0.001 M K2B4O7
0.025 M NH4Cl
0.0025 M NH4OH
0.00001 M K2B4O7
μMOLES KETOHEXOSE
0.5
1.0
1.5
0 20 40 60 80 0 20 40 60 80 0 20 40 60 80 100 120 140 160 180 200 220
FRACTION

characterized with respect to GLC analysis as the TMS derivative before and after dephosphorylation, acid lability, ketohexose/P_i ratio, periodate oxidation, and purity and enantiomeric configuration.

GLC of the TMS derivative of the oxidized reaction product before and after dephosphorylation: The unidentified ketohexose phosphate was derivatized to the TMS form and analyzed by GLC utilizing D-tagatose 6-phosphate as a standard. Figure (21) shows that the TMS derivative of chemically synthesized D-tagatose 6-phosphate had the same retention time as the TMS derivative of the unidentified ketohexose phosphate (i.e. 3.13 minutes). The shoulder seen after the peak was presumably the α or β isomer of the derivatized compounds. When the sample was dephosphorylated and derivatized to the TMS form it exhibited only a single peak which had the same retention time as authentic D-tagatose (Figure 22). It had been previously established that tagatose is separable from the other ketohexoses under the same GLC conditions as used in this analysis (120). It was therefore concluded that the product of the L-galactitol 1-phosphate dehydrogenase-catalyzed oxidation was tagatose phosphate, probably tagatose 6-phosphate.

Determination of the acid lability of the oxidation product and chemically synthesized L-galactitol 1-phosphate and the determination of the ketohexose/P_i molar ratio of the oxidation product: The acid lability of the unidentified

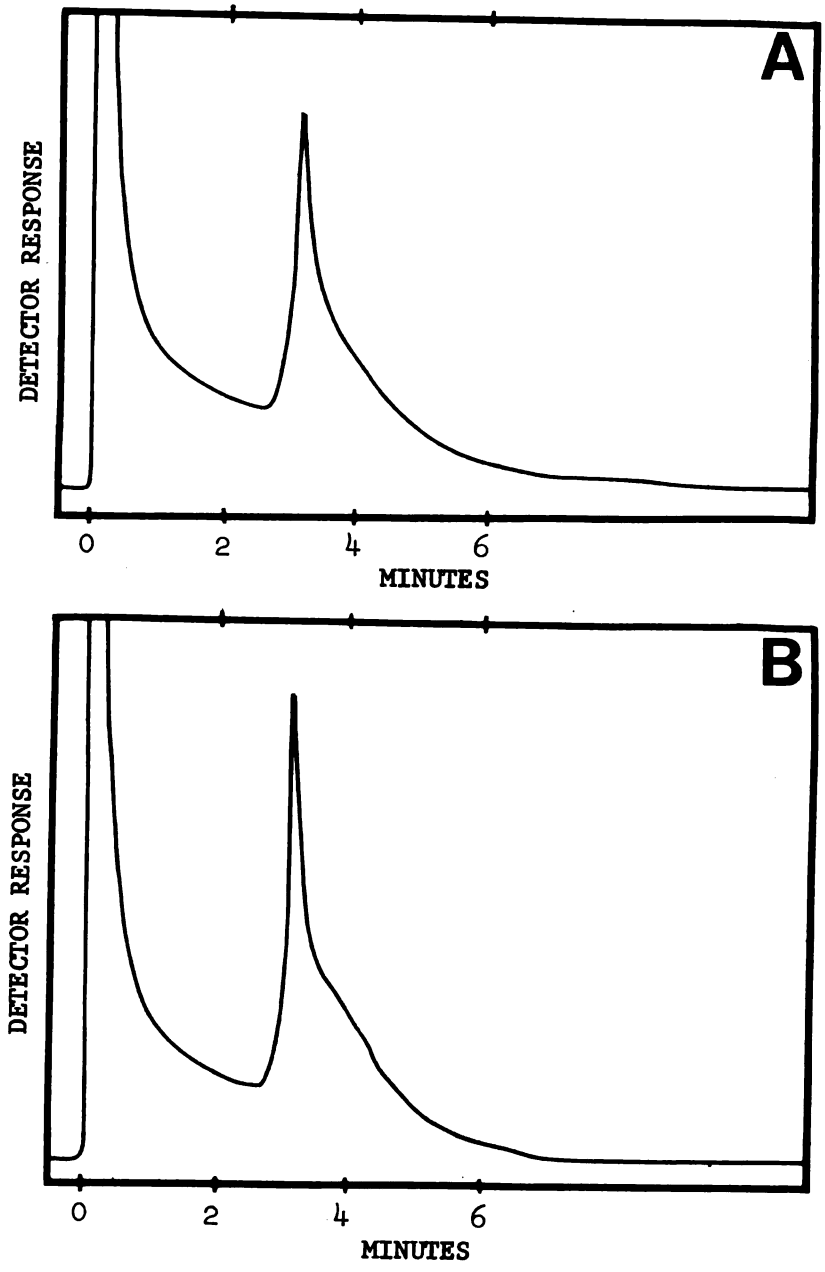


Figure 21. GLC of TMS derivatives of the unidentified ketohexose phosphate and authentic D-tagatose 6-phosphate. The analysis was conducted as described in Materials and Methods. The GLC temperature was set to isothermal at 200°C and the attenuation at 320.

A. Chemically sythesized D-tagatose 6-phosphate.
B. Enzymatically synthesized ketohexose phosphate.

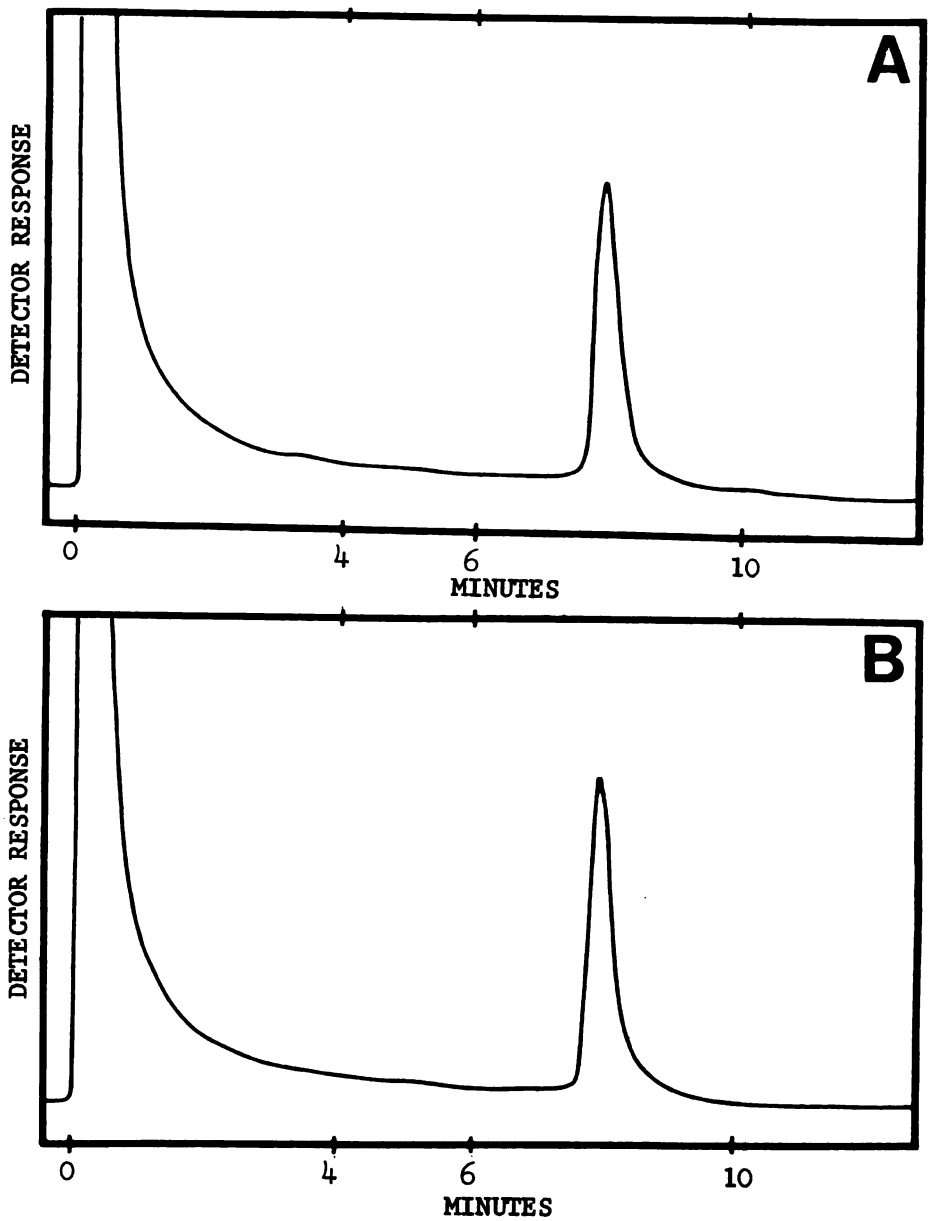


Figure 22. GLC of TMS derivatives of authentic D-tagatose and the unidentified ketohexose phosphate after dephosphorylation. The dephosphorylation and analysis was accomplished as described in Materials and Methods. The GLC was set to isothermal at 155°C and the attenuation at 80.

A. Standard D-tagatose TMS derivative.
B. TMS derivative of the unidentified ketohexose phosphate after dephosphorylation.

ketohexose phosphate was differentiated from that of fructose 1-phosphate, fructose 6-phosphate, and chemically synthesized L-galactitol 1-phosphate according to the procedure in Materials and Methods. Figure (23) delineates the hydrolysis rates of each compound. The rate of liberation of P_i from the unidentified ketohexose phosphate closely paralleled the rate of fructose 6-phosphate, which was comparable to that established previously (24), while the hydrolysis rates of fructose 1-phosphate and chemically synthesized L-galactitol 1-phosphate were the most labile and stabile respectively. The acid stability of the chemically synthesized L-galactitol 1-phosphate was as predicted for a hexitol phosphorylated at a primary alcohol position. This was consistent with the other characterization data of the chemically synthesized L-galactitol 1-phosphate (shown previously). The unidentified ketohexose phosphate was determined to be acid-stabile and the number one position was eliminated as a possible location of the phosphate group since the hydrolysis rate was significantly less than that of fructose 1-phosphate. However, the position of the phosphate function cannot be conclusively assigned to the C-6 position but can be bonded only at an acid-stabile location. The position was definitively established through periodate oxidation analysis of the compound (see below).

The ketohexose/P_i molar ratio was determined by use of the Roe assay and total P_i analysis as described in Materials and Methods. Table (12) shows the results for the unidentified

ketohexose phosphate as well as the values for fructose 6-phosphate and fructose 1-phosphate. The values were essentially those expected, 1:1. Therefore the unidentified ketohexose phosphate was monophosphorylated.

Periodate oxidation of the unidentified ketohexose phosphate: Periodate oxidation of the unidentified ketohexose phosphate was conducted in the classical manner as described in Materials and Methods. Both fructose 6-phosphate and fructose 1-phosphate were also used in the same analysis as standards. The expected reaction scheme is delineated in Figure (24). The ketohexose phosphates are shown to react in their ring forms since this has been established previously (121). The rate-limiting step in the degradation of these sugar phosphates is the hydrolysis of the ester bond generated from the consumption of the first two molar equivalents of periodate. The hydrolysis rate is slow due to the mild acid pH of the reaction and production of additional acid equivalents (122). From Figure (24) it is evident that a ketohexose 1-phosphate would yield two different products and different molar ratios of products than would a ketohexose 6-phosphate. This difference in periodate oxidation characteristics was therefore used as a criterion for identifying the ketohexose phosphate.

Table (13) reveals the total amount of periodate consumed by each compound as determined by titration with sodium thiosulfate. The empirical values agree very closely

FIGURE 23. Acid Lability of the unidentified ketohexose phosphate. Acid hydrolysis of samples was conducted in 1 N HCl at 95°C and the P_i liberated was determined as described in Materials and Methods.

●—● D-Fructose 1-phosphate.

■—■ D-Fructose 6-phosphate.

○—○ Unidentified ketohexose phosphate.

□—□ Chemically synthesized L-galactitol 1-phosphate.

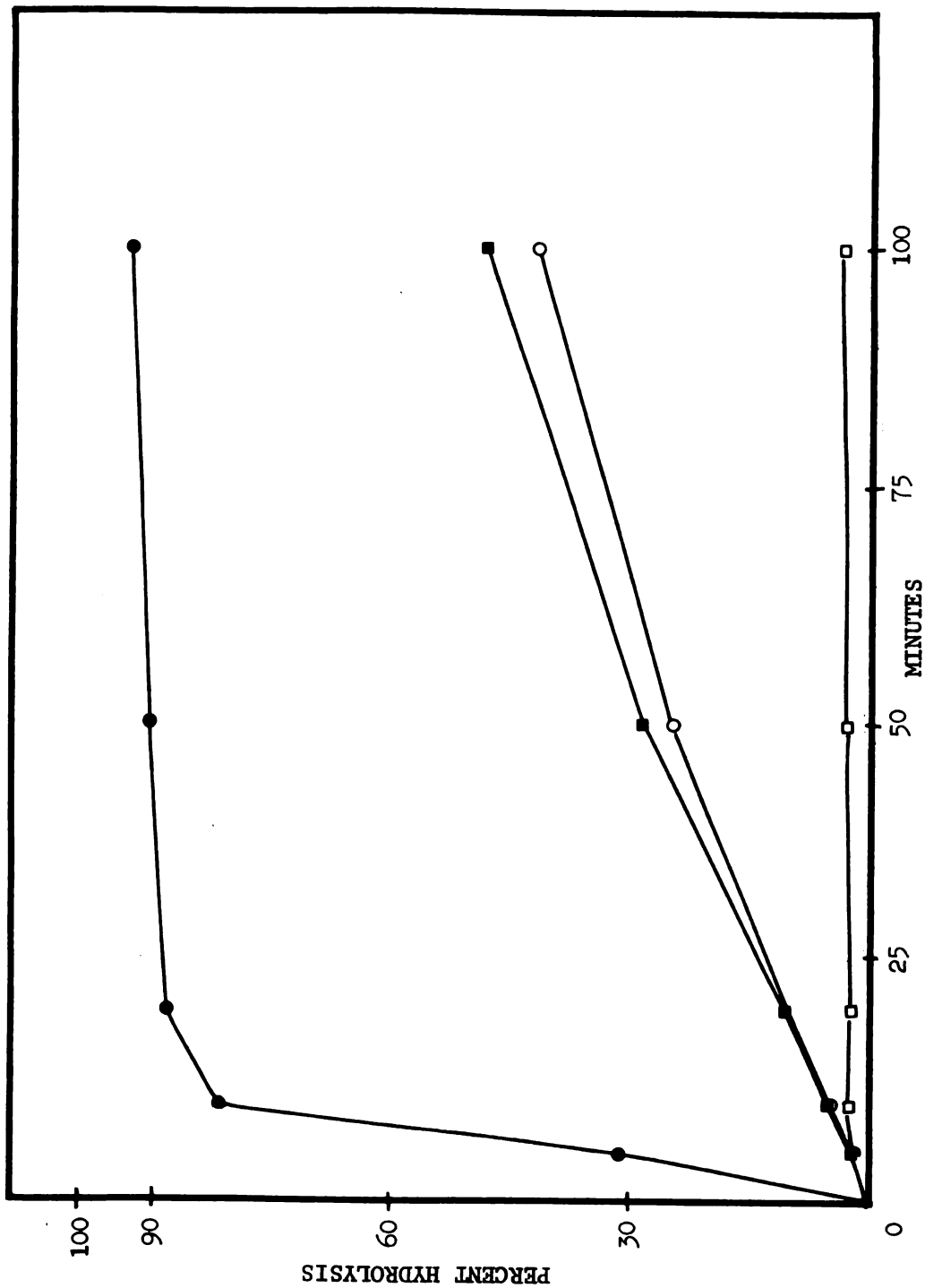

PERCENT HYDROLYSIS
0
30
60
90
100
MINUTES
25
50
75
100

TABLE 12. P_i/ketohexose molar ratio of the unidentified ketohexose phosphate. Details of the analysis are given in Materials and Methods.

Ketohexose phosphate	P_i (μmoles)	Ketohexose (μmoles)	P_i/Ketohexose (molar ratio)	Theoretical P_i/Ketohexose (molar ratio)
Fructose-6-P	0.098	0.100	0.98	1.00
Fructose-1-P	0.098	0.098	1.00	1.00
Unidentified ketohexose phosphate	0.102	0.098	1.04	1.00

with the theoretical values as determined from the reaction scheme in Figure (24). For each mole of ketohexose 6-phosphate three moles of periodate were reduced to IO_3^- and every mole of ketohexose 1-phosphate consumed four moles of periodate. The unidentified ketohexose 6-phosphate consumed three moles of periodate for each mole of the ketohexose phosphate suggesting that it was very similar in structure to D-fructose 6-phosphate.

The quantity of formaldehyde produced by each sample was quantitatively analyzed using chromotropic acid (Table 14). As expected, D-fructose 1-phosphate yielded a stoichiometric amount of formaldehyde, one mole of formaldehyde for every mole of sample, while D-fructose 6-phosphate and the unidentified ketohexose phosphate produced no detectable amounts of formaldehyde. The results were in concert with the phosphorylation of the unidentified ketohexose phosphate at position number six because only D-fructose 1-phosphate can furnish an oxidizable primary alcohol function during the course of periodate oxidation. D-Fructose 6-phosphate as the dialdehyde intermediate hydrolyzes yielding a two-carbon moiety (glycolate) containing a primary alcohol group which cannot be oxidized to formaldehyde due to the adjacent carboxyl function (123).

Glycolate was colorimetrically determined utilizing 2,7-dihydroxynaphthalene. Table (15) shows that D-fructose 6-phosphate and the unidentified ketohexose phosphate produced the expected amount of glycolate while D-fructose

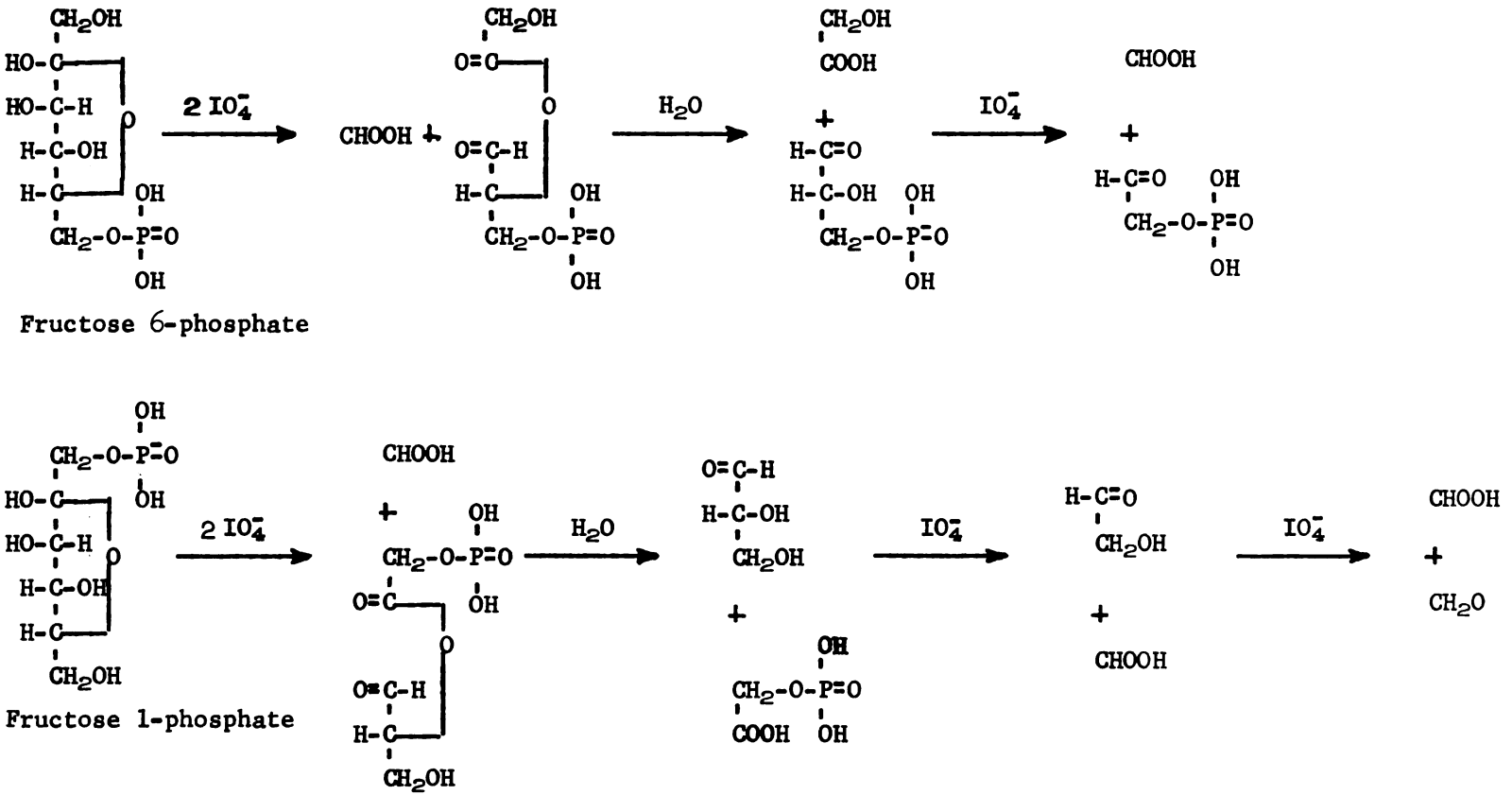


FIGURE 24. Theoretical periodate oxidation scheme of ketohexose phosphates (121).

TABLE 13. Consumption of periodate by ketohexose phosphates. The reaction conditions and titrimetric determination of IO_4^- consumption are described in Materials and Methods.

Ketohexose phosphate	Total sample (μmoles)	Total IO_4^- consumption (μmoles)	Theoretical IO_4^- consumption (μmoles)
Fructose-6-P	65.2	195	195
Fructose-1-P	65.2	256	260
Unidentified ketohexose phosphate	45.5	139	136

1-phosphate did not produce this end product. Examining the reaction scheme outlined in Figure (24), fructose-1-P cannot produce glycolate since carbon atoms one and two are constituents of phosphoglycoaldehyde while the remaining carbons are further oxidized to one carbon units. The glycolate produced from ketohexose 6-phosphates is not further oxidized due to the poor complexing behavior of the carboxyl group with the periodate ion (93).

The amount of formic acid produced was determined by titration with standardized sodium hydroxide. In this case it was expected that all of the compounds would produce formic acid. However, fructose 1-phosphate would produce three moles of formic acid per mole of compound and the ketohexose 6-phosphates would produce two moles of formic acid per mole of compound. Table (16) shows that the observed values agreed very closely with the theoretical values (see Figure 24).

The three-carbon moiety of fructose 1-phosphate is degraded completely into three one-carbon units whereas the analogous moiety from fructose 6-phosphate can only be broken down to formic acid and phosphoglycoaldehyde due to the presence of the phosphate group at carbon number six.

The production of carbon dioxide was determined since a possibility existed that the number one position of the ketohexose 6-phosphates could be oxidized first leaving carbon number two available for oxidation to CO_2. This possibility was not likely since the data accumulated indicated

TABLE 14. Production of formaldehyde after periodate oxidation of the unknown ketohexose phosphate. Details of the determination are given in Materials and Methods.

Ketohexose phosphate	Total sample (μmoles)	Total formaldehyde produced (μmoles)	Theoretical formaldehyde produced (μmoles)
Fructose-6-P	65.2	0.0	0.0
Fructose-1-P	65.2	65.7	65.2
Unidentified ketohexose phosphate	45.5	0.0	0.0

TABLE 15. Formation of glycolate after periodate oxidation of the unidentified ketohexose phosphate. Details of the determination are described in Materials and Methods.

Ketohexose phosphate	Total sample (μmoles)	Total glycolate produced (μmoles)	Theoretical glycolate produced (μmoles)
Fructose-6-P	65.2	60.0	65.2
Fructose-1-P	65.2	0.0	0.0
Unidentified ketohexose phosphate	45.5	45.6	45.5

TABLE 16. Formic acid production from the unidentified ketohexose phosphate after periodate oxidation. Details of the determination are described in Materials and Methods.

Ketohexose phosphate	Total sample (μmoles)	Total formic acid produced (μmoles)	Theoretical formic acid produced (μmoles)
Fructose-6-P	65.2	135	130
Fructose-1-P	65.2	195	195
Unidentified ketohexose phosphate	45.5	91.4	91.0

initial cleavage between carbons two and three or carbons three and four leaving no intermediate which would be oxidizable to CO_2. The formation of CO_2 was determined using D-fructose as a standard. Table (17) shows that fructose produced carbon dioxide in the expected quantity while the phosphorylated sugars produced essentially no CO_2. The small amount produced by fructose 1-phosphate and the unidentified ketohexose phosphate represented only 5% and 3% of the total μmoles of the compounds respectively. These values most likely rose from errors in titration and/or absorption of CO_2 from the air. The results were those expected since the phosphorylated sugars react as though they are in the ring form and the free ketohexoses react as though they are in the open chain form and are able to yield carbon dioxide (123). The absence of CO_2 production, furthermore, was not due to absorption of CO_2 into the reaction mixture as carbonic acid since the solution was acidic and consequently caused the carbonic acid to be broken down to CO_2 and H_2O.

Phosphosphoglycoaldehyde was determined quantitatively as acid-labile phosphate since no convenient and direct assay of this compound was available. The acid-labile ketohexose 1-phosphate, which is expected to yield phosphoglycolate after periodate oxidation, would produce an acid-stabile phosphate compound while the acid-stable fructose 6-phosphate and the unidentified ketohexose phosphate would yield the acid-labile phosphoglycoaldehyde. Thus, the analysis would

TABLE 17. Analysis for carbon dioxide production after periodate oxidation of the unidentified ketohexose phosphate. The method of collecting CO_2 and the titrimetric determinations are described in Materials and Methods.

Sample	Total sample (μmoles)	Total carbon dioxide produced (μmoles)	Theoretical carbon dioxide produced (μmoles)
Fructose-6-P	65.2	0.0	0.0
Fructose-1-P	65.2	3.5	0.0
D-Fructose	65.0	61.5	65.0
Unidentified ketohexose phosphate	45.5	1.5	0.0

be diagnostic for phosphoglycoaldehyde under the conditions described in Materials and Methods. Figure (25) shows that the acid lability of the phosphate compounds from fructose 6-phosphate and the unidentified ketohexose phosphate closely paralleled the hydrolysis rate of authentic phosphoglycoaldehyde. The acid stability of the phosphoglycolate from fructose 1-phosphate was nearly identical with that of commercial phosphoglycolate. Therefore, the qualitative analysis was consistent with the evidence that the unidentified ketohexose phosphate must be phosphorylated at position number six. Table (18) represents the quantitative data obtained from the analysis. The total amount of acid-stabile phosphate, as determined by complete acid hydrolysis, for phosphoglycolate from fructose 1-phosphate also agreed very well with the theoretical value.

Phosphoglycolate was quantitatively analyzed utilizing highly purified phosphoglycolate phosphatase. Table (19) shows that only fructose 1-phosphate produced phosphoglycolate as predicted while the other two compounds yielded no such product. The quantity of phosphoglycolate detected agreed very closely with the theoretical value. The reaction scheme in Figure (24) predicts that ketohexose 6-phosphates cannot form phosphoglycolate but rather phosphoglycoaldehyde and therefore the unidentified ketohexose phosphate was determined to be phosphorylated at carbon atom number six.

The periodate oxidation data are summarized in Table (20). It can be seen that the experimentally obtained values

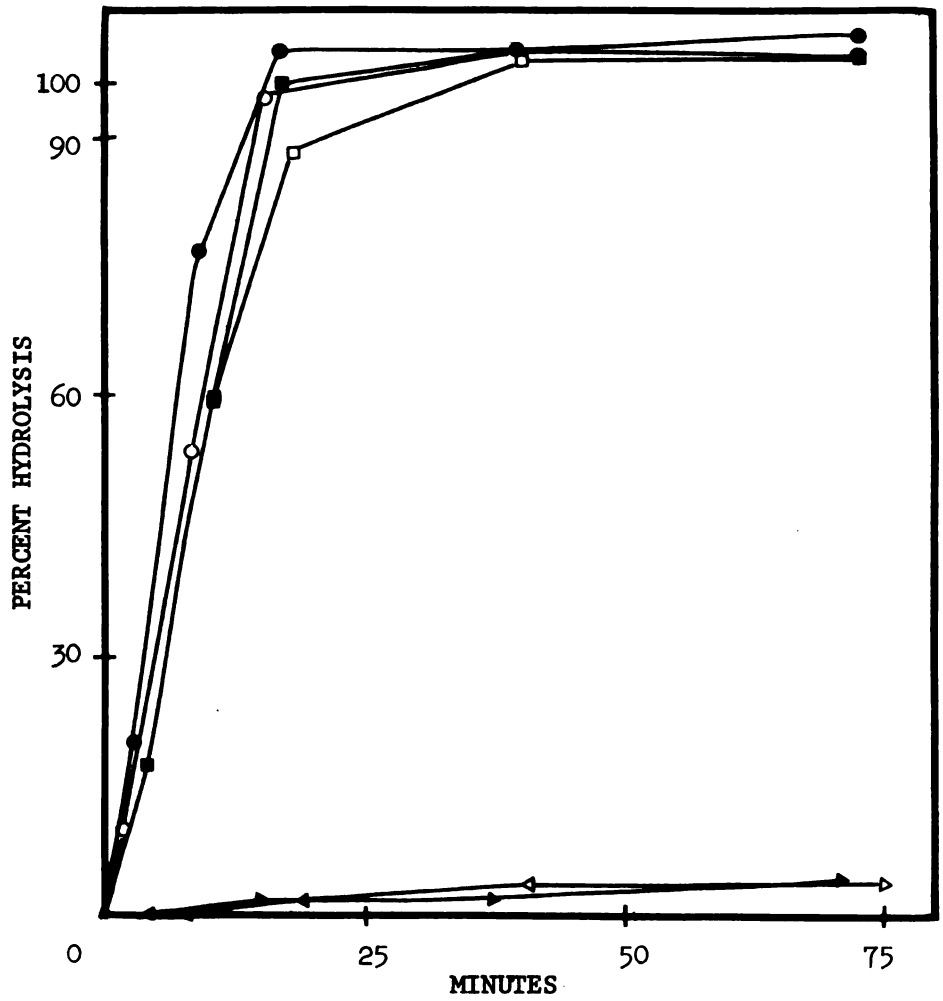


Figure 25. Acid lability of the organic phosphate produced after periodate oxidation of the unidentified ketohexose phosphate. The acid lability was determined as was done before periodate oxidation.

- ●—● Organic phosphate from the unidentified ketohexose phosphate.
- ■—■ Organic phosphate from the chemically synthesized L-galactitol 1-phosphate.
- ○—○ Organic phosphate from fructose 6-phosphate.
- □—□ Phosphoglycoaldehyde.
- △—△ Phosphoglycolate.
- ▲—▲ Organic phosphate from fructose 1-phosphate.

TABLE 18. Production of phosphoglycoaldehyde after periodate oxidation of ketohexose phosphates. The quantity of phosphoglycoaldehyde was determined as the amount of acid-labile phosphate formed as calculated from Figure (25). P_i determinations are described in Materials and Methods.

Ketohexose phosphate	Total sample (μmoles)	Total acid-labile P_i produced (μmoles)	Theoretical acid-labile P_i produced (μmoles)	Total acid-stabile P_i produced (μmoles)	Theoretical acid-stabile P_i produced (μmoles)
Fructose-6-P	65.2	66.0	65.2	0.0	0.0
Fructose-1-P	65.2	0.0	0.0	64.7	65.2
Unidentified ketohexose phosphate	45.5	45.0	45.5	0.0	0.0

are nearly identical with the theoretical figures. Since the data for the unidentified ketohexose phosphate yielded essentially the same results as the D-fructose 6-phosphate standard, it was concluded that the phosphate function was located in the number six position of the molecule and that there was no doubt of its identity as a ketohexose 6-phosphate. With the identification of the sugar moiety as tagatose by previous GLC analysis (Figure 22), it was now concluded that enzymatic oxidation of the galactitol phosphate from the PTS-catalyzed synthesis must have yielded tagatose 6-phosphate.

The above data afford no information as to the enantiomeric form of the enzymatically synthesized tagatose 6-phosphate. It was therefore necessary to determine the enantiomeric configuration in order to establish the position of the phosphate function on the galactitol phosphate molecule. The appropriate experiment was then conducted as described in the following section.

Purity and enantiomeric determination of the enzymatically synthesized tagatose 6-phosphate: Both the purity and the enantiomer configuration were established simultaneously by use of a coupled enzyme assay. The reaction mixture contained the following components in a micro-quartz cuvette (1-cm light path) in a final volume of 0.15 ml: 25 μmoles of glycylglycine buffer (pH 7.5), 4.0 μmoles of $MgCl_2$, 1.5 μmoles of ATP, 0.06 μmole of NADH, 13 units of rabbit muscle α-glycerophosphate dehydrogenase containing triose phosphate isomerase, 0.075 unit

TABLE 19. Formation of phosphoglycolate after periodate oxidation of the unidentified ketohexose phosphate. Assay of phosphoglycolate using phosphoglycolate phosphatase is described in Materials and Methods.

Ketohexose phosphate	Total sample (μmoles)	Total phosphoglycolate produced (μmoles)	Theoretical phosphoglycolate produced (μmoles)
Fructose 6-P	65.2	0.0	0.0
Fructose 1-P	65.2	63.3	0.0
Unidentified ketohexose phosphate	45.5	0.0	0.0

TABLE 20. Summary of the periodate oxidation data. All emperical values were calculated from the preceding tables and the theoretical values determined from the reaction scheme of Figure (24).

Oxidation product	μmoles Oxidation product/μmoles Sample		
	Fructose 6-phosphate	Fructose 1-phosphate	Unidentified ketohexose phosphate
IO_4^- Consumed	2.97	3.93	3.04
Theoretical Ratio	3.00	4.00	3.00
Formaldehyde	0.00	1.01	0.00
Theoretical Ratio	0.00	1.00	0.00
Glycolate	0.92	0.00	1.02
Theoretical Ratio	1.00	0.00	1.00
Phosphoglycolate	0.00	0.97	0.00
Theoretical Ratio	0.00	1.00	0.00
Acid Lability	Labile	Stable	Labile
Theoretical Lability	Labile	Stable	Labile
Formate	1.99	2.99	2.00
Theoretical Ratio	2.00	3.00	2.00
Carbon dioxide	0.00	0.04	0.03
Theoretical Ratio	0.00	0.00	0.00

of tagatose 1,6-diphosphate aldolase from Staphylococcus aureus, 10 units of rabbit muscle fructose 6-phosphate kinase, and 0.010 or 0.025 μmole of the tagatose 6-phosphate. The reaction was initiated by the addition of the substrate and the decrease in absorbance at 340 nm was monitored at 30°C with a Gilford 2400 recording spectrophotometer. The total absorbance change was then determined after correcting for that change due to dilution from addition of the substrate. The μmoles of sample or NADH oxidized was then calculated using the conversion factor given in the enzyme assay section (see Materials and Methods).

If the product was D-tagatose 6-phosphate then it was expected that the coupled enzyme assay would demonstrate a loss of two moles of NADH for every mole of D-tagatose 6-phosphate present. The L-isomer would yield a one to one stoichiometry between the ketohexose phosphate and loss of NADH due to the D-isomer specificity of triose phosphate isomerase (124). Table (21) shows that a one to two stoichiometry, moles ketohexose to moles NADH oxidized, exists rather than a one to one mole ratio. The analysis was performed at two different concentrations of the tagatose 6-phosphate and also in the absence of either fructose 6-phosphate kinase, tagatose 1,6-phosphate aldolase, or the tagatose 6-phosphate in order to insure the validity of the determination. From the results it was concluded that only the D-isomer was formed from the oxidation of the PTS-catalyzed reaction product. It was also concluded, then, that

TABLE 21. Purity and enantiomeric configuration of the enzymatically synthesized tagatose 6-phosphate. The details of the analysis are described in the text.

Assay Description			NADH oxidized (μmoles)	Expected NADH oxidized (μmoles)
	Tagatose-6-P (μmoles)	Total absorbance change at 340 nm		
Complete (see text)	0.025	2.050	0.050	0.050
Complete (see text)	0.010	0.800	0.019	0.020
Complete minus fructose-6-P kinase	0.025	0.000	0.000	0.000
Complete minus tagatose-1,6-diphosphate aldolase	0.025	0.000	0.000	0.000
Complete minus tagatose-6-P	0.000	0.000	0.000	0.000

L-galactitol 1-phosphate must have been formed from the PTS-catalyzed reaction. Calculation of the percent purity of the isolated oxidation product from Table (21) yielded a value of 100%.

DISCUSSION

The pathway by which certain hexitols such as mannitol and sorbitol are metabolized have been established previously (see Literature Review) but the pathway of galactitol catabolism has not been elucidated for any organism except for a Pseudomonas species (see Introduction). The work reported in this thesis conclusively demonstrated that an initiation of galactitol metabolism in Aerobacter aerogenes was dependent upon the phosphoenolpyruvate-dependent phosphotransferase system. Furthermore, the product of the PTS-catalyzed reaction was established as L-galactitol 1-phosphate by chemical, physical, and enzymatic methods.

Figure (26) delineates the reaction scheme by which galactitol is presumably phosphorylated by the PTS as based upon PTS-catalyzed phosphorylation of other sugars (35). Each of the participating catalysts involved was required for the biosynthesis of the phosphorylated product. In addition, the rate of synthesis of product was dependent upon the concentrations of the enzymatic constituents. The figure also reveals that PEP is the phosphoryl donor which is contrary, assuming that the same system is functional in both E. coli and A. aerogenes, to the implications of Wolff and Kaplan (25) who suggested that a kinase, and therefore ATP, was committed to the phosphorylation of galactitol. Their experiments

with crude extracts of E. coli grown on galactitol demonstrated a dependence upon ATP for the formation of a substrate utilized by partially purified L-galactitol 1-phosphate dehydrogenase. However, the experiments did not preclude the possibility that the crude extract catalyzed formation of PEP from ATP and endogenes levels of pyruvate present and thereby allowing the PEP to serve as the true phosphoryl donor. It is not surprising that Wolff and Kaplan implicated the role of a kinase for the formation of galactitol phosphate since they were not cognizant of the PTS which was discovered 8 years later in 1964 by Roseman and coworkers (27).

The position of the phosphoryl function on the reaction product was also of importance in this study since the PTS is known to phosphorylate certain sugars in the number one or number six positions (31, 34, 40). If the possibilities are circumscribed to the terminal positions of the hexitol then only two contingencies prevail for the identity of the PTS-catalyzed reaction product as shown in Figure (27). The figure also presents four possible products that can arise after subsequent oxidation of galactitol phosphate. By using product characterization methods, the identities of the products from the PTS-catalyzed reaction and the L-galactitol 1-phosphate dehydrogenase-catalyzed reaction were unequivacably established.

Chemically synthesized L-galactitol 1-phosphate from sodium borohydride reduction of commercially prepared D-galactose 6-phosphate exhibited the same physical, chemical,

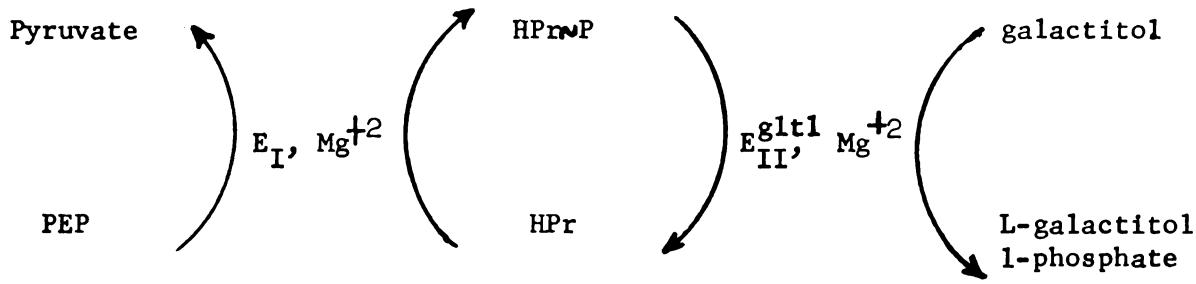
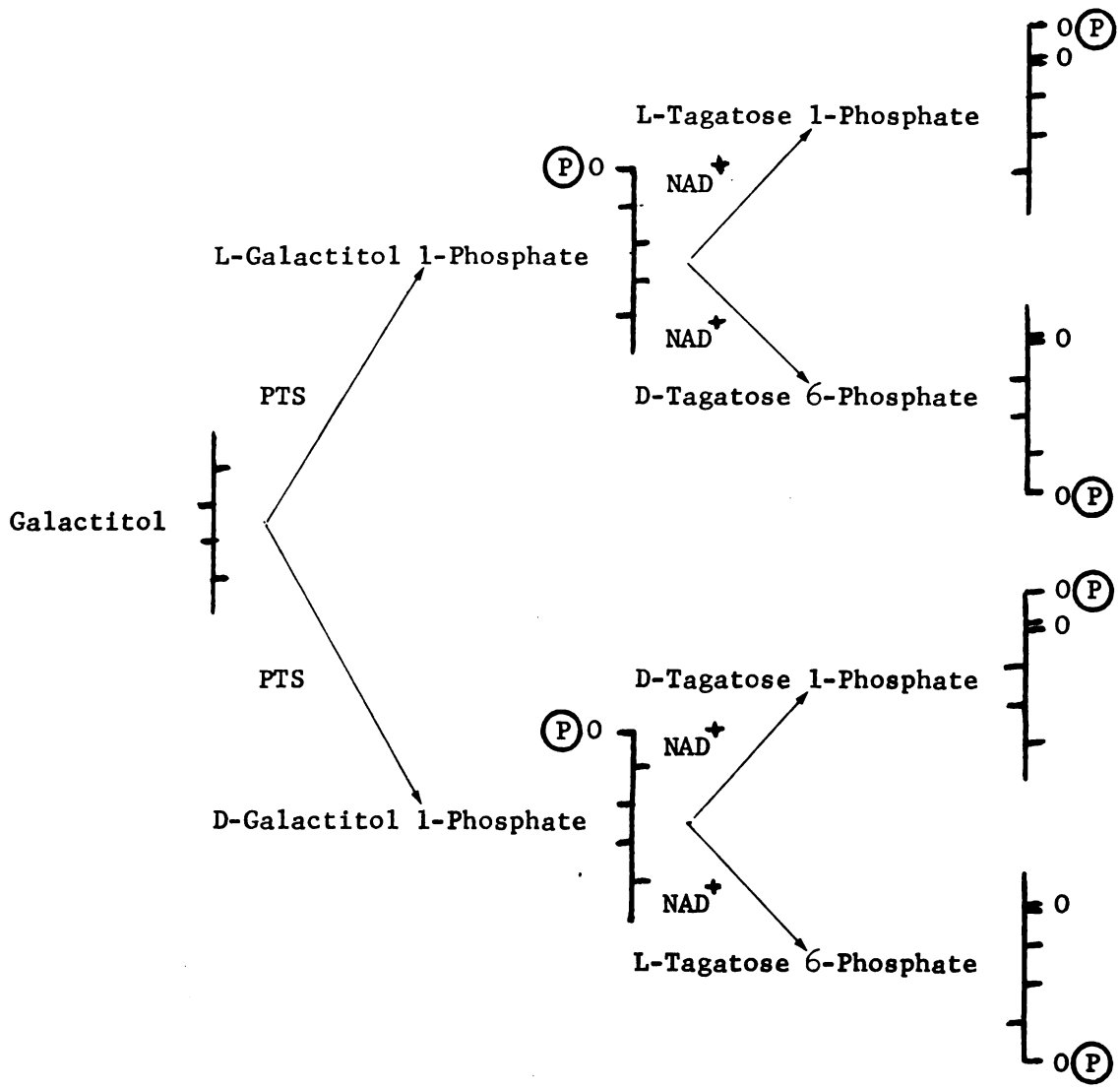


Figure 26. Initiation of galactitol metabolism by the PTS.

and enzymatic properties as did the enzymatically synthesized galactitol phosphate. Since both components yielded the same mass spectrum, had identical retention times in the GLC analysis, had the same retention time by GLC as did authentic galactitol after their dephosphorylation with alkaline phosphatase, and served as substrates for partially purified L-galactitol 1-phosphate dehydrogenase, it was almost conclusive that the product of the PTS-catalyzed reaction was L-galactitol 1-phosphate. When the compound was enzymatically oxidized utilizing L-galactitol 1-phosphate dehydrogenase, a ketohexose phosphate was formed which had the D configuration. When the ketohexose phosphate was analyzed by GLC it had the same retention time as did authentic D-tagatose 6-phosphate or D-tagatose upon dephosphorylation. This eliminated the possibilities of L-tagatose 1-phosphate and L-tagatose 6-phosphate as oxidation products. Upon periodate oxidation of the acid-stabile D-ketohexose phosphate, quantitative amounts of products were formed which were consistent with the periodate oxidation of a ketohexose 6-phosphate. The periodate oxidation also caused the formation of an acid-labile phosphate and therefore supported the conclusion that the phosphate function was located at C-6. The identity of the oxidized compound must, therefore, be D-tagatose 6-phosphate, which in turn proves that the PTS catalyzed phosphorylation of galactitol to produce L-galactitol 1-phosphate and not L-galactitol 6-phosphate.

FIGURE 27. Possible products of the PTS-catalyzed phosphorylation of galactitol. The respective products of the oxidation of each possible galactitol phosphate are also shown.


Galactitol
PTS
L-Galactitol 1-Phosphate
NAD+
L-Tagatose 1-Phosphate
NAD+
D-Tagatose 6-Phosphate
PTS
D-Galactitol 1-Phosphate
NAD+
D-Tagatose 1-Phosphate
NAD+
L-Tagatose 6-Phosphate
P
O

The data have implied that the second step in the biodegradation of galactitol is the oxidation of the phosphorylated derivative to D-tagatose 6-phosphate. This reaction would be catalyzed by L-galactitol 1-phosphate dehydrogenase as was shown to occur in E. coli (25). This hypothesis can be substantiated by examining the phenotypic characteristics of mutants in this enzyme and then determine whether the appropriate metabolite accumulates. Such genetic investigations were out of the scope of this study. But the implications stated are criteria for asking how galactitol is further metabolized in Aerobacter aerogenes.

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