Synthesis and Use of 1-O-β-D-Galactopyranosyl-D-mannitol as Source of D-Mannitol for *Escherichia coli**

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ABSTRACT: 1-O-β-D-Galactopyranosyl-D-mannitol has been synthesized by condensation of tetra-O-acetyl-α-D-galactopyranosyl bromide with either 1,2,3,4-tetra-O-acetyl-β-D-mannopyranose or 1,2,3,4-tetra-O-acetyl-6-O-trityl-β-D-mannopyranose, followed by de-O-acetylation and reduction with sodium borohydride. In a second route, 2,3,4,5-tetra-O-acetyl-D-mannitol, obtained by tritylation of D-mannitol, acetylation, and detritylation, was condensed with tetra-O-acetyl-α-D-galactopyranosyl bromide and the resulting product was de-O-acetylated. 1-O-β-D-Galactopyranosyl-D-mannitol was tested for use as a carbon source for several strains of *Escherichia coli*. It was shown that mutants constitutive for the lactose system can metabolize this disaccharide, while inducible strains cannot. Utilization of this compound depends upon the presence of the galactoside permease for entry, and β-galactosidase for hydrolysis.

The first step in the dissimilation of D-mannitol in *Escherichia coli* is its phosphorylation to D-mannitol 1-phosphate *via* the phosphoenolpyruvate-dependent phosphotransferase system (Tanaka *et al.*, 1967a). Free mannitol from the medium does not accumulate within the cell, but is phosphorylated during, or immediately after, its movement across the cell membrane. For the study of this process, a device was needed by which free, nonphosphorylated mannitol could be generated inside the cell. Accordingly, we synthesized 1-O-β-D-galactopyranosyl-D-mannitol and investigated the possibility that it could enter the cell by the galactoside permease system and be hydrolyzed internally by β-galactosidase to yield free mannitol. In this paper we describe this synthesis and experiments which show that this compound can indeed serve as an internal source of mannitol.

1-O-β-D-Galactopyranosyl-D-mannitol (**8**) was synthesized by two different routes, as shown in Scheme I. In the route starting from the mannose derivatives **2** or **3**, the final intermediate, 6-O-β-D-galactopyranosyl-β-D-mannose (**5**), can be reduced conveniently with sodium borotritide to give a ^{3}H-labeled substrate, while the second route starts from materials (D-mannose and D-mannitol) which are commercially available with a ^{14}C label.

Experimental Section

General Methods. Melting points were determined with a Mettler FP-2 thermometric apparatus and correspond to "corrected melting point." Optical rotations were determined in 1-dm semimicro tubes with a Perkin-Elmer polarimeter no. 141; the chloroform used was Analytical reagent grade and contained approximately 0.75% ethanol. Infrared spectra were recorded with a Perkin-Elmer Model 237 spectrophotometer, on potassium bromide disks. Evaporations were performed *in vacuo*, with an outside bath temperature kept below 45°. The microanalyses were performed by Dr. W. Manser, Zurich, Switzerland.

Chromatographies. Thin-layer chromatographies (ascending) were performed on plates coated with Merck silica gel F-254 in the solvent systems of 2–4% ethanol in chloroform. The components were detected by spraying with anisaldehyde–sulfuric acid and heating at 125°. Column chromatographies were performed on Merck silica gel no. 7734 (70–325 mesh) (Darmstadt, Germany) or on "Alumina Fluka" type 507C, neutral, activity stage 1, from the Fluka AG (Buchs SG, Switzerland). The paper chromatographies were performed, descending, on Whatman paper no. 1, and the spots were detected with the silver nitrate reagent. The gas–liquid partition chromatographies were performed with a gas–liquid partition chromatograph, Perkin-Elmer Model 900, equipped with a flame ionization detector, on a column of stainless steel (300 × 0.3 cm), packed with 3% OV-17 on Gas-Chrom Q (60–80 mesh) (Applied Science Laboratories, State College, Pa.), after per(trimethylsilyl)ation with Tri-Sil (Pierce Chemical Co., Rockford, Ill.). The compounds were injected at 120°, and the temperature was raised at the rate of 5°/min; the times of elution were compared to that of the per(trimethysilyl) ether of *myo*-inositol.

Bacterial Strains. The genotypes of the strains used are shown in Table I. Strains X8018M and X8030M are spontaneous mannitol-positive revertants derived from strains X8018 and X8030, respectively. The latter strains (not shown) were obtained from J. R. Beckwith, ML308 and ML308-225 were obtained from T. H. Wilson; Hfr3300 from D. G. Fraenkel.

Chemicals for Biological Assay. D-Mannitol and α-lactose were obtained from Eastman Kodak Co. (Rochester, N. Y.); L-proline and L-tryptophan from Calbiochem (Los Angeles, Calif.), and thiamine hydrochloride from Sigma Chemical (St. Louis, Mo.).

Growth of Cells. The basal mineral medium (SM) has been previously described (Tanaka *et al.*, 1967b). Proline was added

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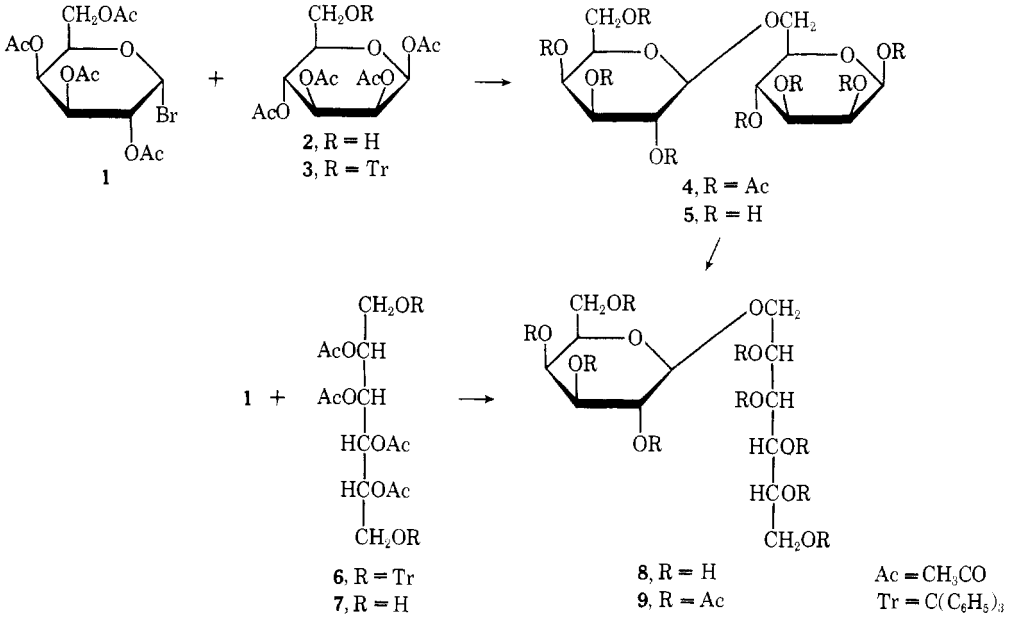
SCHEME I



to give a final concentration of 150 μg/ml; tryptophan, 20 μg/ml; thiamine, 20 μg/ml. Cells were pregrown as indicated in the figure legends. Cultures were grown at 37°, in 5 ml of medium, in 300-ml erlenmeyer flasks which were fitted with side arms. The flasks were shaken at about 240 cycles/min on a rotary shaker. Growth was monitored turbidimetrically with a Klett colorimeter (No. 42 filter). 1 Klett unit = 4×10^6 cells/ml of a logarithmically growing culture.

Synthesis of 1-O-β-D-Galactopyranosyl-D-mannitol

1,2,3,4-Tetra-O-acetyl-6-O-(2,3,4,6-tetra-O-acetyl-β-D-galactopyranosyl)-β-D-mannopyranose (**4**). FROM 1,2,3,4-TETRA-*O*-ACETYL-β-D-MANNOPYRANOSE (**2**). A mixture of 1,2,3,4-tetra-*O*-acetyl-β-D-mannopyranose (Helferich and Leete, 1929; Reynolds and Evans, 1940) (3.48 g), anhydrous calcium sulfate (8 g), and silver oxide (2 g) in dry, alcohol-free chloroform (22 ml) was stirred in the dark for 1 hr. Iodine (0.5 g) was added and, under continuous stirring, a solution of 2,3,4,6-tetra-*O*-acetyl-α-D-galactopyranosyl bromide (4.2 g) in dry, alcohol-free chloroform (22 ml) was added progressively in 1 hr. The mixture was filtered through Celite, and the filtrate was evaporated to a syrup, which was dissolved in hot methanol. The solution was kept overnight at 4° to give long, prismatic crystals (5.33 g, 78%); mp 162–163°, $[\alpha]_D^{20}$ −18° (*c* 3.3, chloroform). *Anal.* Calcd for $C_{28}H_{38}O_{19}$: C, 49.55; H, 5.66; O, 44.79. Found: C, 49.62; H, 5.67; O, 44.95.

FROM 1,2,3,4-TETRA-*O*-ACETYL-6-*O*-TRITYL-β-D-MANNOPYRANOSE (**3**). Anhydrous calcium sulfate (1.5 g) was added to a solution of silver perchlorate (2.3 g) in dry nitromethane (20 ml). The suspension was stirred for 15 min to ensure complete absence of water. 1,2,3,4-Tetra-*O*-acetyl-6-*O*-trityl-α-D-mannopyranose (Helferich and Leete, 1929; Reynolds and Evans, 1940) (5.9 g) was added, the suspension was cooled to 0–5°, and 2,3,4,6-tetra-*O*-acetyl-α-D-galactopyranosyl bromide (4.5 g) was added with vigorous stirring. The mixture was allowed to warm to room temperature, when it became orange in color, and silver bromide and triphenylmethyl perchlorate precipitated. After 30 min, the mixture was filtered, and the filtrate was washed with cold, saturated sodium hydrogen carbonate solution, and water. The precipitated triphenylmethanol was filtered off, and the filtrate was diluted with chloroform and dried with anhydrous sodium sulfate. It was concentrated to a syrup, which was dissolved in chloroform and chromatographed on silica gel. The fractions containing a compound having an R_F value identical with that of the previously described preparation were concentrated, and crystallization from methanol gave 2.17 g (32%); mp 162–163°, $[\alpha]_D^{20}$ −18° (*c* 2.0, chloroform). The mixture melting point with **4** prepared from **2** was not depressed and the infrared spectra were identical: ν_{max}^{KBr} 1755, 1740, 1440–1430, 1373, 1250–1225, 1160, 1090, 1063, 970, and 950 cm^{-1}.

6-O-β-D-Galactopyranosyl-β-D-mannose (**5**). Compound **4** (3.39 g, 5 mmoles) was dissolved in 50 mM sodium methoxide in methanol (100 ml), and the solution was kept for 12 hr at 4°. An excess of dry methanol was added to dissolve the sodium complex which had formed (mp 110–114°, with dec), and the sodium ions were removed by filtration through Amberlite IR-120 (H^+). The solution was evaporated, and the residual syrup was crystallized from methanol–ethanol to give 1.46 g

TABLE I: Genotypes of *Escherichia coli* Strains Used.

Strain	Control	Galactoside Permease	β-Galactosidase
X8018M[a] (K12)	Constitutive, o^c	+	+
X8030M[a] (K12)	Constitutive, o^c	−	+
ML308	Constitutive, i^-	+	+
ML308-225	Constitutive, i^-	+	−
Hfr3300 (K12)	Constitutive, i^-	+	+
1 (Hfr Cavalli)	Inducible, i^+	+	+

[a] Requires proline, tryptophan, and thiamine.

(85%) of a microcrystalline product; mp 123–128° (softening at 112°), $[\alpha]_D^{20}$ +7.0 (initial) to +9.3° (after 72 hr, *c* 3.3, water). The compound gave a positive Benedict's test. *Anal.* Calcd for $C_{12}H_{22}O_{11}$; C, 42.10; H, 6.49; O, 51.41. Found: C, 42.00; H, 6.42; O, 51.20.

Compound **5** showed $R_{maltose}$ 0.8 on Whatman No. 1 paper in 1-butanol–ethanol–water (3:1:1, v/v). After methanolysis with 0.5 M hydrogen chloride in methanol for 16 hr at 65°, followed by per(trimethylsilyl)ation, gas–liquid partition chromatography indicated the presence of equal amounts of galactose and mannose.

1-O-β-D-Galactopyranosyl-D-mannitol (**8**). To a solution of **5** (1.71 g) in water (25 ml) was added a solution of sodium borohydride (0.8 g) in water (10 ml). The mixture was kept for 5 hr at room temperature, and excess of Amberlite IR-120 (H^+) was added to destroy the remaining sodium borohydride and to remove the sodium ions. The suspension was filtered off, the filtrate was evaporated, and methanol was added several times to the residue and evaporated to remove the boric acid. The resulting syrupy product, which gave a negative Benedict's test, was crystallized from methanol–ethanol to give a microcrystalline product (1.51 g, 88%): mp 105–108° (softening at 83°), $[\alpha]_D^{20}$ +2.2° (*c* 4.3, water). *Anal.* Calcd for $C_{12}H_{24}O_{11}$: C, 41.85; H, 7.04; O, 51.11. Found: C, 42.03; H, 7.43; O, 50.89.

Compound **8** showed $R_{maltose}$ 0.68 on Whatman no. 1 paper in 1-butanol–ethanol–water (3:1:1, v/v).

1-O-(2,3,4,6-Tetra-O-acetyl-β-D-galactopyranosyl)-2,3,4,5,6-penta-O-acetyl-D-mannitol (**9**). FROM **8**. To a solution of **8** (0.34 g) in dry pyridine (5 ml), acetic anhydride (2 ml) was added, and the mixture was kept for 24 hr at room temperature. It was poured into ice–water, and the product was extracted with chloroform. The extract was dried with anhydrous sodium sulfate and concentrated *in vacuo*. The syrupy residue was chromatographed on neutral alumina (Fluka), in chloroform, to give an amorphous solid (0.55 g, 77%): $[\alpha]_D^{20}$ +8° (*c* 2.0, chloroform). *Anal.* Calcd for $C_{30}H_{42}O_{20}$: C, 49.85; H 5.87; O, 44.28. Found: C, 49.82; H, 5.90; O, 44.35.

FROM 2,3,4,5-TETRA-O-ACETYL-D-MANNITOL. Syrupy 2,3,4,5-tetra-*O*-acetyl-D-mannitol (**7**) was obtained from 2,3,4,5-tetra-*O*-acetyl-1,6-di-*O*-trityl-D-mannitol (**6**) by the procedure of Micheel and Micheel (1932). A suspension of **7** (3.5 g, 10 mmoles), anhydrous calcium sulfate (4 g), and silver oxide (1 g) in dry, alcohol-free chloroform (20 ml) was stirred in the dark for 1 hr. Iodine (0.25 g) was added, and under continuous stirring, a solution of 2,3,4,6-tetra-*O*-acetyl-α-D-galactopyranosyl bromide (2.1 g, 5 mmoles) in dry, alcohol-free chloroform (11 ml) was added progressively in 1 hr. The mixture was stirred for 24 hr, and then filtered through Celite. The filtrate was evaporated to a syrup which was dissolved in dry pyridine (25 ml), and acetic anhydride (10 ml) was added. The mixture was kept for 24 hr at room temperature, then poured into ice–water, and the product was extracted with chloroform. The extract was dried with anhydrous sodium sulfate and concentrated. The syrupy residue was chromatographed on neutral alumina (Fluka) with chloroform elution to give an amorphous solid (1.33 g, 37% based on 2,3,4,6-tetra-*O*-acetyl-α-D-galactopyranosyl bromide): $[\alpha]_D^{20}$ +8° (*c* 2, chloroform).

This product (0.5 g) was deacetylated with sodium methoxide in methanol to give 0.22 g (89%): mp 105–108° (softening at 87°), $[\alpha]_D^{20}$ +2.3° (*c* 1.5, water). The mixture melting point with the product prepared from **5** was not depressed and the infrared spectra were identical: 1740, 1435, 1375, 1230–1220, 1175, 1137, 1050, and 955 cm^{-1}.

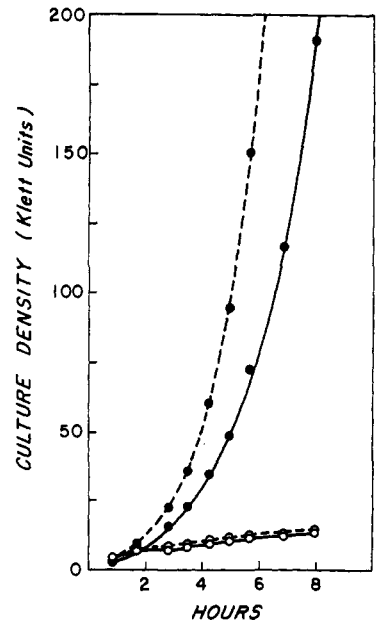


FIGURE 1: Growth of galactoside permease-positive and -negative strains on 1-*O*-β-D-galactopyranosyl-D-mannitol and lactose. Strains X8018M (●) and X8030M (○) were grown overnight in a 0.2% solution of mannitol, washed once with SM, and resuspended in solutions of 0.1% 1-*O*-β-D-galactopyranosyl-D-mannitol (——) and 0.1% lactose (-----), respectively, at a dilution corresponding to about 5 Klett units of absorbance.

Results

Synthesis of 1-O-β-D-Galactopyranosyl-D-mannitol (**8**). Since biochemical experiments would require the use of radioactively labeled compound **8**, the modes of synthesis and the starting material was selected with this requirement in mind. In a first route, 2,3,4,6-tetra-*O*-acetyl-α-D-galactopyranosyl bromide (**1**) was condensed with 1,2,3,4-tetra-*O*-acetyl-β-D-mannopyranose (**2**) (Helferich and Leete, 1929; Reynolds and Evans, 1940) to give in 78% yield the fully acetylated derivative **4** of 6-*O*-β-D-galactopyranosyl-β-D-mannose (**5**). The same derivative **4** was obtained in 32% yield by condensing **1** with the 6-*O*-trityl derivative **3** (Helferich and Leete, 1929; Reynolds and Evans, 1949) of **2** in presence of silver perchlorate according to Bredereck *et al.* (1959, 1960). Deacetylation of **4** gave **5**, which was reduced with sodium borohydride into crystalline 1-*O*-β-D-galactopyranosyl-D-mannitol (**8**), the C-6 of D-mannitol being equivalent to the C-1. All intermediates were obtained in crystalline form. In a second route, 1 equiv of the bromide **1** was directly condensed with 2,3,4,5-tetra-*O*-acetyl-D-mannitol (**7**), which had been obtained from the 1,6-di-*O*-trityl derivative **6** (Micheel and Micheel, 1932), to give in 37% yield the syrupy fully acetylated derivative **9** of **8**. Deacetylation of **9** gave crystalline **8**.

In the reduction of **5** into **8**, sodium borotritide can be used to introduce a tritium labeling, whereas ^{14}C labeling can be obtained by starting from the commercially available ^{14}C-labeled D-galactose and D-mannitol.

Metabolism of 1-O-β-D-Galactopyranosyl-D-mannitol by E. coli. In order to test whether the entry of 1-*O*-β-D-galactopyranosyl-D-mannitol (**8**) into the cell is dependent on the galactoside transport system, permease-positive and -negative strains were compared for their ability to grow on this compound. As seen in Figure 1, strain X8018M (y^+) grew normally on both galactosylmannitol (**8**) and lactose, whereas strain

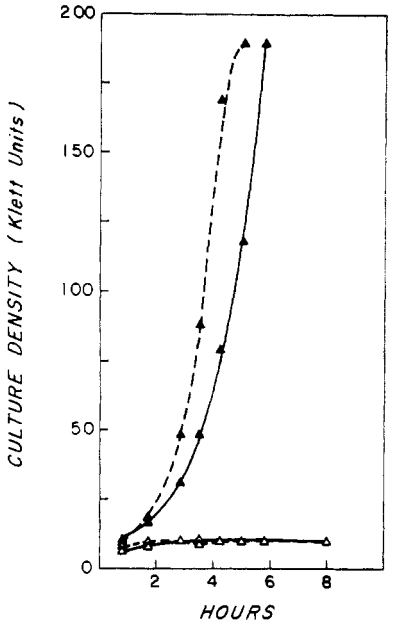


FIGURE 2: Growth of β-galactosidase-positive and -negative strains on 1-*O*-β-D-galacopyranosyl-D-mannitol and lactose. Strains ML308 (▲) and ML308-225 (△) were grown overnight in a 0.2% solution of mannitol, washed once with SM, and resuspended in solutions of 0.1% 1-*O*-β-D-galacopyranosyl-D-mannitol (——) and 0.1% lactose (-----), respectively, at a dilution corresponding to about 10 Klett units of absorbance.

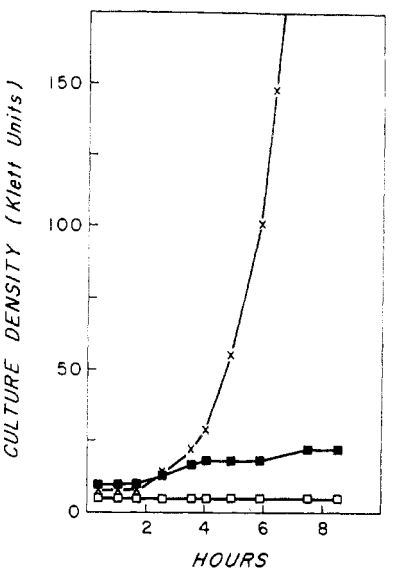


FIGURE 3: Growth of *lac* constitutive and *lac* inducible strains on 1-*O*-β-D-galacopyranosyl-D-mannitol. Strain Hfr3300 (×) was pregrown in a solution of 0.2% mannitol until an absorbancy of about 100 Klett units was reached, washed once with SM, and resuspended in a solution of 0.1% 1-*O*-β-D-galactopyranosyl-D-mannitol. Strain 1 was pregrown in solutions of 0.2% mannitol (■) and 0.2% lactose (□), respectively, until an absorbance of about 100 Klett units was reached, washed once with SM, and resuspended in a solution of 0.1% 1-*O*-β-D-galacopyranosyl-D-mannitol.

X8030M (y^-) grew on neither. Both strains are constitutive for the lactose system so that the inability of strain X8030M to grow on these compounds was due solely to the absence of the permease, and not to the lack of induction of β-galactosidase.

Similarly, in order to test whether β-galactosidase is necessary for the metabolism of galactosylmannitol (**8**) once it has entered the cell, β-galactosidase-positive and -negative strains were compared for their ability to grow on the test compound. Figure 2 shows that β-galactosidase is, in fact, necessary. Strain ML308 (z^+) grew on both galactosylmannitol (**8**) and lactose, whereas ML308-225 (z^-) grew on neither. These strains are also *lac* constitutive, so that the galactoside permease was present in both.

Finally, galactosylmannitol (**8**) was examined as an inducer of the *lac* system. Here, the response of an inducible strain, 1 (i^+), was compared with that of a constitutive strain, Hfr3300 (i^-). Both strains are galactoside permease and β-galactosidase positive. Figure 3 shows that galactosylmannitol (**8**) was utilized only by the constitutive strain. It will not serve as an inducer. When strain 1 was pregrown on mannitol, no growth on this disaccharide was seen. However, when it was pregrown on lactose, thereby inducing the lactose system, some growth did occur. As expected, this growth was linear, rather than exponential, due to the dilution of this system as the culture divided.

Discussion

The usefulness of a D-mannitol derivative for the study of mannitol permeation depends on the ability of *E. coli* to transport it, and then to hydrolyze it internally to yield D-mannitol. 1-*O*-β-D-Galactopyranosyl-D-mannitol (**8**) was selected because the hydroxyl group at C-1 of D-mannitol is protected, because it could be easily synthesized, and because it was a possible substrate for β-galactosidase. The present findings that the galactoside permease and β-galactosidase can act on galactosylmannitol (**8**) are in agreement with numerous previous studies which indicate broad substrate specificity for these two proteins.

Those compounds which are substrates for the galactoside permease include a large number of sugars, in addition to lactose. For example, β-D-galactosides, β-D-thiogalactosides, D-galactose itself, and certain α-D-galactosides, such as melibiose, are all transported by this permease (Buttin, 1968; Kepes and Cohen, 1964).

The specificity of β-galactosidase is more stringent than that of the permease, but it also includes a wide variety of compounds. The strictest part of the specificity involves the glycon part of the substrate, or D-galactose. Only changes at C-5 are compatible with hydrolyzability, as in β-D-fucosides (6-deoxy-D-galactosides) or in α-L-arabinosides; although use of even these compounds results in decreased rates of hydrolysis. Methylation at O-2, O-3, O-4, or O-6 results in complete loss of hydrolyzability. Also, the galactopyranoside ring is essential for activity; *o*-nitrophenyl β-D-galactofuranoside cannot be hydrolyzed. The oxygen atom in the galactosidic linkage of the substrate is also necessary for activity and cannot be replaced by a sulfur atom. On the other hand, many substitutions may be made in the aglycon part of the substrate, which result in a large number of compounds still hydrolyzable by β-galactosidase. These substitutions do, for the most part, however, affect the rate of hydrolysis and also the K_m of the enzyme. These changes may include linkage with another sugar residue, or an alkyl or aryl group. Among the compounds which have been tested and found to be substrates are *O*-β-D-galactopyranosyl-(1→3)-D-galactose, -(1→3)-D-fructose, -(1→3)- and -(1→5)-D-arabinose, and -(1→3)-, -(1→4)-, and -(1→6)-2-acetamido-2-deoxy-D-glucoses, and also methyl, ethyl, and *n*-butyl *O*-β-D-galactosides. Finally, the compounds

closest in structure to 1-*O*-β-D-galactopyranosyl-D-mannitol (**8**), which have also been found to be hydrolyzed by β-galactosidase, are 1-*O*-β-D-galactopyranosyl-D-glycerol and 2-*O*-β-D-galactopyranosyl-D-erythritol (Burstein *et al.*, 1965; Boos *et al.*, 1967; Wallenfels *et al.*, 1960; Wallenfels and Malhotra, 1961).

In wild-type *E. coli*, the components of the *lac* system (galactoside permease, β-galactosidase, and thio-β-galactoside transacetylase) are synthesized only in the presence of an inducer. The inducer specificity of this system has been examined in great detail. Lactose itself will serve as an inducer only if it is acted upon by β-galactosidase. It seems likely that the actual inducer is a product of a transglycosylation reaction between lactose and another acceptor molecule, since β-galactosidase does function as a transgalactosidase, as well as a hydrolase.

Small changes in either the glycon or aglycon part of the molecule can greatly alter its activity as an inducer. Any substitutions in the galactose portion generally eliminate its inductive ability, while certain substitutions in the aglycon portion, such as in 1-*O*-β-D-galactopyranosyl-D-glycerol, result in greatly enhanced inductivity. Generally, the inductive property of a compound is unrelated to its susceptibility to hydrolysis by β-galactosidase, for example, 2-propyl 1-thio-β-D-galactopyranoside. The same is true for the α-D-galactoside melibiose (Boos *et al.*, 1967; Muller-Hill *et al.*, 1964; Monod *et al.*, 1951).

Since there is no obvious common structural feature among these inducer molecules, it was not possible to predict whether 1-*O*-β-D-galactopyranosyl-D-mannitol would serve as an inducer itself. Fortunately, its utility for our purposes did not depend on its having this function.

Finally, it should be mentioned that these results do not actually demonstrate that free D-mannitol is released into the cytoplasm. Chromatographic identification of the cell contents is presently underway, using [*mannitol*-6-^{3}H]1-*O*-β-D-galactopyranosyl-D-mannitol.

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