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Membrane Translocation of Mannitol in *Escherichia coli* Without Phosphorylation

ELLEN SOLOMON,[1] KENJI MIYAI,[2] AND E. C. C. LIN

Department of Biological Chemistry and the Department of Microbiology and Molecular Genetics, Harvard Medical School, Boston, Massachusetts 02115

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Galactosyl-mannitol can be transported into cells of *Escherichia coli* by β-galactoside permease and can be hydrolyzed rapidly to mannitol and galactose by β-galactosidase. When a mutant strain lacking enzyme I of the phosphoenolpyruvate phosphotransferase system and constitutive in the lactose system was presented with galactosyl-mannitol in which the mannitol moiety was labeled with ^3H, the liberated mannitol remained inside the cell if the Enzyme II complex of the phosphoenolpyruvate phosphotransferase system for mannitol was uninduced. It is postualted that one of the enzyme II proteins can still catalyze translocation of mannitol across the cell membrane even when phsophorylation is not possible.

The phosphoenolpyruvate (PEP) phosphotransferase system (2, 11), ubiquitous among bacterial species except strict aerobes (18), is responsible for the capture and phosphorylation of a number of carbohydrates which are utilized as sources of carbon and energy (9, 14, 15, 19, 21, 24-26). In this system a protein of small molecular weight, HPr, acts as an intermediate in the transfer of a high-energy phosphate from PEP to the carbohydrate substrate:

$$\text{HPr} + \text{PEP} \underset{}{\overset{\text{enzyme I}}{\rightleftharpoons}} \text{P-HPr} + \text{pyruvate} \quad (1)$$

$$\text{P-HPr} + \text{sugar} \xrightarrow{\text{enzyme II complex}} \text{sugar} - \text{P} + \text{HPr} \quad (2)$$

A single HPr and enzyme I appear to serve a family of enzyme II complexes, each specific for a different carbohydrate. The enzyme II complex is membrane bound and consists of at least two proteins and one phospholipid (12). When there is a failure to form P-HPr due to a genetic deficiency of either enzyme I or HPr, there can be neither accumulation of the free sugar against a gradient nor concentration of the sugar in phosphorylated form.

It has been suggested that, under those circumstances, the enzyme II complex might still be able to catalyze facilitated diffusion of the substrate across the cell membrane (26). In this report we describe a means to test this hypothesis by generating free mannitol, a substrate of the phosphotransferase system, in cells lacking enzyme I and its retention under conditions in which the enzyme II complex for mannitol was or was not induced.

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MATERIALS AND METHODS

Bacteria. *Escherichia coli* K-12 strain 279 (*ptsI*$^-$, *lac* $z^+y^+i^-$) was constructed as follows. Phage P1 was grown on strain Hfr3300 (*ptsI*$^+$, *lac* $z^+y^+i^-$) and used to infect strain 223 (*ptsI*$^-$, *lac* $z^-y^+l^+$). Lactose-positive transductants were selected on lactose minimal plates. Fifty such transductants were then spotted on tryptone plates and tested for *lac* constitutivity by spraying the patches with ONPG (0.013 M *o*-nitrophenyl-β-D-galactopyranoside in 0.25 M potassium phosphate buffer, pH 7.0). Strain 279 was one of these constitutive transductants. It was tested further on MacConkey plates in which mannitol was substituted for lactose to insure that the strain was still enzyme I negative.

Strain 278 (*ptsI*$^+$, *lac* $z^+y^+i^-$) was constructed similarly from strain 11 (*ptsI*$^+$, *lac* $z^-y^+i^+$), the parent of strain 223 (5). Strains 278 and 279 are auxotrophic for leucine and thiamine.

Culture media and growth conditions. The basal mineral medium used for growth of cells has been described previously (25). Casein acid hydrolysate (Nutritional Biochemicals Corp.; salt-free, vitamin-free) was added to mineral medium to give a final concentration of 1%. Leucine was added to give a final

[1] Present address: Service Génétique Cellulaire, Institut Pasteur, Paris 15, France.

[2] Present address: ICN Nucleic Acid Research Institute, 2727 Campus Drive, Irvine, Calif. 92664.

concentration of 50 μg/ml, and thiamine was added to 20 μg/ml. Growth was monitored turbidimetrically with a Klett colorimeter (no. 42 filter; 1 Klett unit = 4×10^6 cells of a exponentially growing culture per ml).

Assays. The enzyme II $complex_{mtl}$ assay has been previously described (5).

For assays of galactosyl-mannitol uptake by whole cells, strain 279 was grown in 1% Casamino Acids with and without mannitol to a density equivalent to about 125 Klett units. The cells were then harvested, washed twice with mineral medium, and resuspended in mineral medium to a density equivalent to 100 Klett units. Uptake was measured at 25 C in mineral medium containing 40 μg of lactose-free chloramphenicol (Parke, Davis and Co.) per ml. The incubation mixture contained 4.5 ml of cells, at 100 Klett units, and 0.5 ml of ^{3}H-galactosyl-mannitol (final concentration, 5×10^{-6} M). The reaction was terminated by delivering 1 ml of the incubation mixture onto a membrane filter (0.45 μm, Millipore Corp.) which had been wetted previously with mineral medium. The filter was then washed with 10 ml of mineral medium and dried, and the radioactivity was determined by scintillation counting.

Hydrolysis of ONPG was measured in whole cells from exponentially growing cultures. The cells were harvested, washed with mineral medium, and resuspended to a cell density of about 50 Klett units. The assay was performed as follows. ONPG (0.2 ml; 0.013 M in 0.25 M sodium phosphate buffer, pH 7.0) was added to 1 ml of the cell suspension in a 1-ml cuvette, and the rate of hydrolysis was followed spectrophotometrically at 420 nm. Toluenized cells were prepared by incubating 1 ml of the bacterial suspension with 1 drop of toluene at 37 C with shaking for 30 min (16). The suspension was then incubated at room temperature for 5 min, and the assay for ONPG hydrolysis was performed as above.

^{3}H-Galactosyl-mannitol. (Mannitol-6-^3H) 1-*o*-β-D-galactopyranosyl-D-manntiol was prepared as previously described (23) from non-radioactive galactosyl-mannose by reduction with sodium borotritide. The final specific radioactivity was about 4,300 counts per min per μmol. The structure of this compound is shown in Fig. 1.

Extraction of cell contents and chromatography. The cells to be used for extraction were collected by filtration and washed with mineral medium, as in the substrate uptake assay. The membrane filter was immediately placed in 3 ml of hot water in a large test tube in a boiling-water bath. Boiling was continued for 20 min. The filter was then removed and washed with an additional 1 ml of boiling water. (The radioactivity remaining on the filter, measured by counting the filter in 5 ml of Bray scintillation fluid, was found to be negligible.) At this point, the extracted cell contents were in a total volume of 4 ml of water which was stored frozen. The samples were later thawed and evaporated to dryness in conical centrifuge tubes, and the residues were resuspended in 0.1 ml of water. This 0.1 ml was applied to the chromatograms in 10-μliter samples over a distance of 2.5 cm along the origin. The tubes were then washed with an additional 0.1 ml of water, and this was also applied, at the same place, in 10 μliter portions.

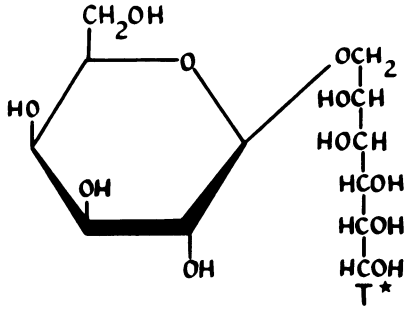

FIG. 1. *Structure of mannitol-6-^3H)1-o-β-D-galactopyranosyl-D-mannitol.*

Chromatography was carried out on Whatman no. 1 filter paper in a solvent system of 1-butanol:ethanol:water (3:1:1, vol/vol) (23). Chromatograms were run in a descending system at room temperature for 42 h. After drying, the chromatograms were cut into 0.5- or 1-cm-wide strips, and these were counted in 5 ml of 2,5-bis-[2-(5-tert-butylbenzoxasolyl)]-thiophene scintillation fluid.

To determine whether recovery from the chromatograms was stoichiometric, 5 and 10 μliters of a solution of ^{3}H-galactosyl-mannitol and 5 and 10 μliters of a solution of ^{3}H-mannitol were all run separately on the same chromatogram and counted as above. For both the galactosyl-mannitol and the mannitol, the ratio of the counts recovered from 5 and 10 μliters was 1.0:2.1.

To determine the extent of quenching of tritium on the chromatograms, the radioactivity from 5 μliters of ^{3}H-mannitol, which was run in the chromatographic system as described above was compared with the same amount of ^{3}H-mannitol placed directly on a membrane filter. The same was done with 5 μliters of ^{3}H-galactosyl-mannitol. In the case of mannitol, the chromatograms contained 15% of the counts which were present on the membrane filter, and in the case of galactosyl-mannitol, 17%. These figures were used to determine the recovery of counts from the cell extracts compared with those present in the cells on membrane filters, as measured in uptake assays. Virtually 100% of the radioactivity from the cells was recovered on the chromatograms, although the actual number of counts was quite low.

Non-radioactive spots were detected as follows. Chromatograms were dried, sprayed with silver nitrate reagent (0.5 ml of saturated $AgNO_3$ added to 100 ml of acetone, plus sufficient water to dissolve the resulting precipitate), dried again, and sprayed with a solution consisting of 2.5 ml of saturated NaOH in 100 ml of 95% ethanol. Spots were preserved by dipping the chromatogram in a 4% solution of sodium thiosulfate.

RESULTS

Uptake of galactosyl-mannitol. The mutant (strain 279) used in the experiments lacks enzyme I (less than 2% of activity observed in wild-type cells) and, in addition, is constitutive

for the expression of the genes in the *lac* operon.

It has been shown previously (23) that strains of *E. coli* which are *lac* constitutive can grow on galactosyl-mannitol, an analogue of lactose (galactosyl-glucose). It has also been shown that this growth depends upon the action of β-galactosidase on this compound, indicating that the galactosyl-mannitol is split to give galactose and mannitol. Therefore a *lac* constitutive strain which is presented with ^{3}H-galactosyl-mannitol (with the tritium label in the mannitol moiety) should be able to generate free, internal ^{3}H-mannitol. The fate of such ^{3}H-mannitol could then be examined to see whether the presence of the enzyme II complex$_{mtl}$ has any effect on the ability of the cells to retain the non-phosphorylated carbohydrate.

When strain 279 is grown with mannitol present in the medium, although growth on mannitol does not occur, the enzymes of the *mtl* system are nonetheless induced (22). For these experiments it was therefore possible to utilize two cultures of strain 279, one of which had been induced for enzyme II complex$_{mtl}$ and the other not (Table 1).

Uptake of ^{3}H-galactosyl-mannitol was measured in two such parallel cultures (Fig. 2). Those cells which had been induced for the enzyme II complex$_{mtl}$ showed considerably less retention of radioactivity than the noninduced cells. This observation is interpreted to mean that the presence of the enzyme II complex$_{mtl}$ facilitated the exit of ^{3}H-mannitol from the cells, since the entry process of the galactosyl-mannitol via the *lac* permease was expected to be the same in both cases.

However, it was necessary to demonstrate that the difference between the two test cultures was solely that of induction of the *mtl* system and not due to a difference in expression of the *lac* system. The latter possibility was tested by measuring ONPG hydrolysis in intact cells (measure of β-galactoside permease activity) and toluenized cells (measure of β-galactosidase activity) (Table 2). The rates of hydrolysis of ONPG in nontoluenized cells of both cultures are the same, indicating that both populations have equal amounts of β-galactosidase permease and therefore transport the same amount of galactosyl-mannitol. In toluenized cells the rate of ONPG hydrolysis is slightly lower in the cells grown with mannitol, indicating that these cells have somewhat less β-galactosidase activity. However, this rate is seven times the rate in the nontoluenized cells, so that the limiting step is the permeation of galactosyl-mannitol, for both cultures.

It should also be mentioned that when uptake

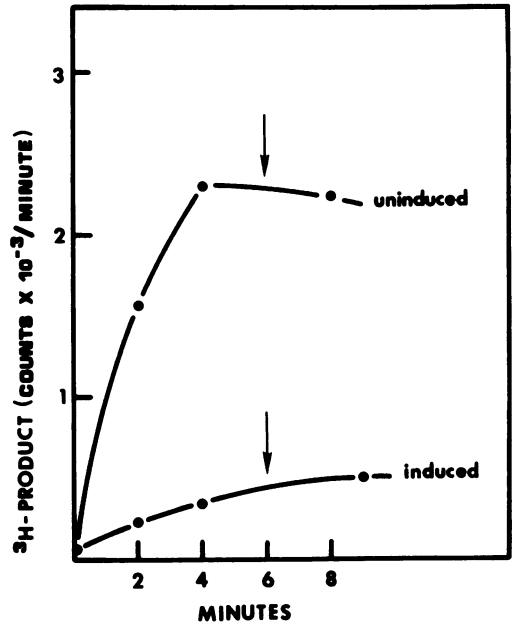

FIG. 2. *Uptake of ^{3}H-galactosyl-mannitol by cells of strain 279 uninduced (grown in 1% Casamino Acids) or induced (grown in 1% Casamino Acids plus 0.2% mannitol) in the enzyme II complex$_{mtl}$. The assays were performed as described in Materials and Methods with ^{3}H-galactosyl-mannitol (4,000 counts per min per nmol) added to a final concentration of 5 × 10^{-6} M. At the time indicated by arrows, samples were withdrawn for chromatographic analysis of the radioactivity in the cellular contents.*

TABLE 1. *Enzyme II complex$_{mtl}$ levels in E. coli strains 11 and 279*

Strain	Enzyme II complex$_{mtl}$[a]	
	CAA	CAA + mannitol
11	0.28	6.3
279	0.22	7.32

[a] Nanomoles per minute per milligram. CAA, Casamino Acids.

TABLE 2. *ONPG hydrolysis in strain 279*

Toluene	Rate[a]	
	CAA	CAA + mannitol
−	0.031	0.034
+	0.335	0.235

[a] Change in absorbancy per centimeter at 420 nm. CAA, Casamino Acids.

of ^{3}H-mannitol is measured in strain 279, grown either with or without mannitol in the medium, extremely little radioactivity is seen in the cells. Therefore, if hydrolysis of ^{3}H-galactosyl-mannitol were occurring through the activity of externalized β-galactosidase, no retention of tritium in these cells would be seen.

Determination of the rates of disappearance of galactosyl-mannitol from the incubation mixtures was attempted. However, the amounts of galactosyl-mannitol removed from the solutions by the cells were too small, relative to the total amount, for successful measurements.

Extraction and chromatography of cell contents. To demonstrate that the tritium which was retained in the cells, uninduced in the enzyme II complex$_{mtl}$, was indeed in ^{3}H-mannitol and not in ^{3}H-galactosyl-mannitol for a metabolic product, the radioactive contents of both induced and uninduced cells were examined. At the point shown by the arrows in Fig. 2, samples were taken, and the cellular contents were extracted and chromatographed as described in Materials and Methods.

As an internal standard, uninduced cells used for the actual experiment were incubated in mineral medium alone instead of with ^{3}H-galactosyl-mannitol. After the filters which contained these cells were placed in boiling water, ^{3}H-galactosyl-mannitol and ^{3}H-mannitol were added to separate samples. This was to insure that the galactosyl-mannitol and mannitol could be recovered intact from the extraction and chromatography procedures (Fig. 3). (Recovery of counts from the membrane filters was essentially 100%.) Figure 3A shows the positions of non-radioactive mannitol-1-P, mannitol, and galactosyl-mannitol, run in the same chromatography system at the same time as the actual experiment.

Figure 3B demonstrates that both ^{3}H-galactosyl-mannitol and ^{3}H-mannitol are recoverable in this system.

Figure 3C shows the results of the chromatography of the extracted cell contents. Neither set of cells retained any radioactivity as galactosyl-mannitol, confirming that the activity of β-galactosidase was indeed high enough to hydrolyze all of the galactosyl-mannitol brought into the cell. Of the radioactivity recovered from the uninduced cells, 87% was found in free mannitol. By contrast, no free ^{3}H-mannitol was found in the induced cells.

In both cases, some radioactivity was retained at the origin. As may be seen in comparing Fig. 3A and B, the galactosyl-mannitol and mannitol recovered from cells ran somewhat faster than the same compounds applied as

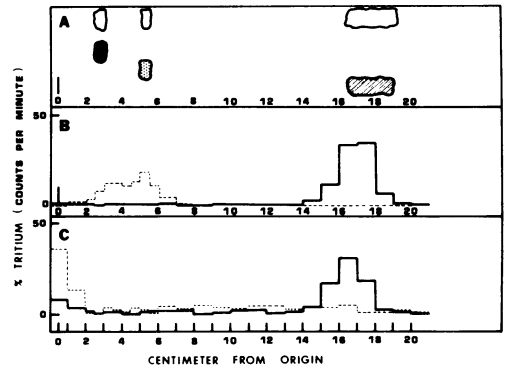

FIG. 3. *Chromatography of cell contents after uptake of ^{3}H-galactosyl-mannitol. The chromatography conditions and details of the experiment are described in the text. A, Separation of non-radioactive standards (20 μg of each): mannitol-1-phosphate (dark), galactosyl-mannitol (stippled), and mannitol (cross-hatched). Spots were developed by the procedure given under Materials and Methods. B, Demonstration of recovery of unaltered ^{3}H-galactosyl-mannitol (-----) or ^{3}H-mannitol (——) added to cell extracts. (As described in the text, 1,100 counts/min of ^{3}H-galactosyl-mannitol or 1,500 counts/min of ^{3}H-mannitol were subjected to the extraction procedure.) C, Chromatography of the cell contents of strain 279 after 6 min of uptake of ^{3}H-galactosyl-mannitol (4,000 counts per min per nmol, 5 × 10^{-6} M). Cells were grown on 1% Casamino Acids, uninduced (——), or on 1% Casamino Acids plus 0.2% mannitol, induced (-----). For B and C the ordinate (percent ^{3}H counts per minute) represents the percentage of total counts detected on the chromatogram.*

standards. Therefore it is possible that, if any mannitol-1-P had been formed due to some leakiness in the mutation in strain 279, this compound would be close to the origin in Fig. 3C, although it did move away from the origin in the test system (Fig. 3A). Alternatively, some other metabolite may have been formed which does not move in this chromatography system.

DISCUSSION

The question of whether an enzyme II complex can catalyze the translocation of the carbohydrate in the absence of phosphorylation has been addressed to in several previous studies. The results are not all in accord. Enzyme I-negative mutants of *E. coli* and *Salmonella typhimurium* normally are not able to grow on glucose. They can be made to grow on this sugar if the methyl-galactoside permease, which has good affinity for glucose (1, 3, 6) and can concentrate its substrate in unaltered form (13), is induced by D-fucose or galactose (4, 10, 21). Once glucose is brought into the cytoplasm by this alternative entry, it can be converted to

glucose-6-phosphate by a soluble adenosine triphosphate-dependent hexokinase (7). The conditional growth on glucose has been interpreted to mean that the enzyme II complex is inoperative for translocation of the substrate in the absence of P-HPr. The enzyme II complex for glucose is apparently constitutive in certain bacterial strains and inducible in others. The presence of adequate levels of this complex in the enzyme I-negative mutants has not been established in all cases, and thus it is not always clear that the impermeability to glucose is due to the lack of the membrane complex or to its inactivity.

In apparent contradiction to the first finding is the result from another study in which it was shown that glucose and its analogue, α-methylglucoside, even at low concentrations, remained effective in exerting catabolite repression on β-galactosidase synthesis in mutants of *E. coli* lacking enzyme I or HPr, but not in mutants lacking the enzyme II complex for glucose. It would thus appear that glucose and its analogue can still enter the cell via the enzyme II complex even though no phosphorylation takes place (17). It must be remembered, however, that enzyme I and HPr mutants are particularly sensitive to catabolite repression, whereas enzyme II mutants are not, and that this difference in sensitivity may, in part, account for the results.

From the results of a more recent study on the uptake of α-methylglucoside, it was proposed that phosphorylation of the substrate by the phosphotransferase system is preceded by a facilitated diffusion step (8). A critical point might be raised regarding the particular strain of *E. coli* with which this work was done. Even when enzyme I is missing, Hfr3300 produces high, uninduced levels of methylgalactoside permease (13) which is insensitive to *N*-ethylmaleimide under the conditions used in these experiments (J. Lengeler, personal communication). The intervention of this permease could thus account for the postulated unmasking of a facilitated diffusion system for α-methylglucoside which was found to be competitively inhibited by galactose (8). A subsequent study (G. Gachelin, personal communication), however, indicated that this permease was not present at sufficient level to alter the original conclusion.

Results of the present study again suggest that an enzyme II complex can mediate the passage of its substrate across the cell membrane without concomitant phosphorylation. However, the possibility that exit of mannitol was catalyzed by a phosphoenolpyruvate phosphotransferase-independent permease which is both active on mannitol and is inducible by it can only be excluded by the use of a double mutant missing enzyme I activity and is deleted in the enzyme II complex.

Up to now, the only criterion for defining enzyme II mutations is by phosphorylation. To study the role of the individual components of enzyme II complexes, an assay such as that of monitoring cytoplasmic volume changes during plasmolysis (20) is needed to measure the translocation process. The method developed in the present study provides another means for testing the activity of the enzyme II complex in catalyzing translocation and has the advantage of giving a high signal level when the process is blocked.

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