

## Melibiose Transport in *Escherichia coli*

KAZUMI TANAKA, SUMIKO NIIYA, AND TOMOFUSA TSUCHIYA\*

*Department of Microbiology, Faculty of Pharmaceutical Sciences, Okayama University, Tsushima-naka, Okayama 700, Japan*

Transport of [<sup>3</sup>H]melibiose, prepared from [<sup>3</sup>H]raffinose, was investigated in *Escherichia coli*. Na<sup>+</sup> stimulated the transport of melibiose via the melibiose system, whereas Li<sup>+</sup> inhibited it. Kinetic parameters of melibiose transport were determined. The *K<sub>t</sub>* values were 0.57 mM in the absence of Na<sup>+</sup> or Li<sup>+</sup>, 0.27 mM in the presence of 10 mM NaCl, and 0.29 mM in the presence of 10 mM LiCl. The *V<sub>max</sub>* values were 40 and 46 nmol/min per mg of protein in the absence and in the presence of NaCl and 18 nmol/min per mg of protein in the presence of LiCl. Melibiose transport via the melibiose system was temperature sensitive in a wild-type strain of *Escherichia coli* and was not inhibited by lactose. On the other hand, melibiose uptake via the lactose system was not temperature sensitive, was inhibited by lactose, and was not affected by Na<sup>+</sup> and Li<sup>+</sup>. Methyl-β-D-thiogalactoside, a substrate for both systems, inhibited the transport of melibiose via both systems.

Active transport of sugars, amino acids, and other nutrients is frequently associated with the cotransport of cations. In bacterial cells transport processes frequently use H<sup>+</sup> as a coupling ion (11, 23), whereas Na<sup>+</sup> is utilized in animal cells (3). In *Escherichia coli*, H<sup>+</sup>-lactose, H<sup>+</sup>-alanine and H<sup>+</sup>-lactate cotransport have been directly demonstrated (2, 23). The melibiose transport system of *E. coli* and *Salmonella typhimurium* is a unique system with respect to the coupling ion for substrate transport. Stock and Roseman (15) reported that the melibiose transport system in *S. typhimurium* is a Na<sup>+</sup>-dependent cotransport system. They observed a stimulation of <sup>14</sup>C-labeled methyl-β-D-thiogalactoside (TMG) transport by Na<sup>+</sup> and Li<sup>+</sup>. They also observed uptake of <sup>22</sup>Na in the presence of TMG, but not in its absence. Thus, the presence of Na<sup>+</sup>-substrate cotransport in bacterial systems was suggested.

Previously we have shown (21) that an artificially imposed electrochemical Na<sup>+</sup> gradient drives uptake of TMG mediated by the melibiose transport system in energy-starved cells of *E. coli* and that a TMG gradient elicits uptake of Na<sup>+</sup> (22). Tokuda and Kaback (17) reported the concomitant uptake of both TMG and <sup>22</sup>Na in membrane vesicles of *S. typhimurium*. Thus, there are several lines of evidence which support the notion of Na<sup>+</sup>-TMG cotransport via the melibiose system. Similarly, a glutamate transport system in *E. coli* B has also been reported to be a Na<sup>+</sup> symport system (9, 19), as are all amino acid transport systems in *Halobacterium halobium* (8). Many other transport systems in bacteria have been reported to be Na<sup>+</sup>-dependent

systems, although it is not clear whether or not such systems are Na<sup>+</sup>-substrate cotransport systems (11).

TMG is a synthetic substrate for the melibiose transport system, and radioactive TMG is commercially available. Thus, TMG has been used exclusively for the study of the melibiose transport and has been frequently used for the study of the lactose system. Although melibiose is a natural substrate for these systems, radioactive melibiose is not commercially available. Thus, it was difficult to measure active transport of melibiose. Saier and co-workers (12, 13) reported a method for the preparation of radioactive melibiose, an analog of melibiose. In this paper we report a simple and effective method for the preparation of [<sup>3</sup>H]melibiose. Using this substrate, we have characterized melibiose transport in *E. coli* and studied the effect of monovalent cations on this system.

### MATERIALS AND METHODS

**Chemicals.** TMG, melibiose, and invertase (EC 3.2.1.26) were obtained from Sigma Chemical Co. Lactose, galactose, glucose, fructose, and sucrose were from Wako Pure Chemical Co. Carbonyl cyanide *m*-chlorophenylhydrazone was from Calbiochem. [<sup>3</sup>H]raffinose and L-[U-<sup>14</sup>C]glutamic acid were purchased from New England Nuclear Corp. Sephadex G-15 was from Pharmacia Fine Chemicals. All other chemicals were of reagent grade and were obtained from commercial sources.

**Chromatography.** Solutions containing [<sup>3</sup>H]raffinose, [<sup>3</sup>H]melibiose, and [<sup>3</sup>H]fructose were fractionated by application of 1.2-ml samples to a column (1.8 by 100 cm) of Sephadex G-15 equilibrated with water. The column was eluted with water at a flow rate of 5.6

ml/h, and 1.6-ml fractions were collected. The positions of raffinose, melibiose, and fructose were determined by prior chromatography of authentic sugars on the same column. Column chromatography was performed at room temperature. For paper chromatography, sample solutions were spotted onto filter paper (Toyo no. 51). Standards of melibiose, raffinose, fructose, sucrose, galactose, and glucose were also spotted separately. The paper was developed in *n*-butanol-acetic acid-water (26:6:25) for 23 h. Standard sugars were visualized by spraying with diphenylamine-aniline reagent (1). For the measurement of radioactivity, the paper was cut into 0.5- by 1.0-cm pieces, and the amount of radioactivity in each piece was determined.

**Bacteria and growth.** *E. coli* K-12 strain W3133 (6) is a lactose operon-deleted strain with a temperature-sensitive melibiose transport system. Strain RA11 (6) is an  $\alpha$ -galactosidase-negative, melibiose transport-positive strain and possesses a temperature-stable melibiose transport system; it was derived from W3133. *E. coli* ML308-225 lacks the melibiose operon. This strain possesses a constitutive lactose transport system but does not possess  $\beta$ -galactosidase (24). Cells were grown at 37°C, unless otherwise indicated, on minimal medium (16) supplemented with 1% tryptone (Difco). Sodium salts were replaced with potassium salts in the medium. Melibiose (1 or 10 mM, respectively) was added to the culture medium of strains RA11 and W3133. Cells were harvested in the late exponential phase of growth and washed with minimal medium.

**Transport assay.** Transport assays were performed by filtration (6) in minimal medium, with K<sup>+</sup> replacing Na<sup>+</sup>. The final concentration of melibiose in the assay medium was 50  $\mu$ M, unless otherwise stated.

**Protein assay.** Protein contents were determined by the method of Lowry et al. (7).

## RESULTS

**Preparation of [<sup>3</sup>H]melibiose.** The first step in the preparation of [<sup>3</sup>H]melibiose was cleavage of [<sup>3</sup>H]raffinose by invertase. [<sup>3</sup>H]raffinose (0.3 ml of a 1 mM solution) was mixed with an equal volume of 0.2 M sodium acetate buffer, pH 4.9, containing 3  $\mu$ g of invertase and incubated for 90 min at 50°C (4). Greater than 90% of raffinose was cleaved under these conditions. An equal volume (0.6 ml) of 0.5 M potassium phosphate buffer, pH 7.0, was added to the mixture, which was heated in a boiling-water bath for 3 min to inactivate the invertase. After cooling in an ice bath, the mixture was applied to a column of Sephadex G-15. Elution was performed with distilled water. Portions (1  $\mu$ l) of each fraction were counted in a liquid scintillation counter. One minor peak (I) and two major peaks (II and III) were obtained. The fractions containing peaks I, II, and III were pooled separately and concentrated by lyophilization. Figure 1 shows the results of paper chromatography of peak II, which was identified as melibiose.

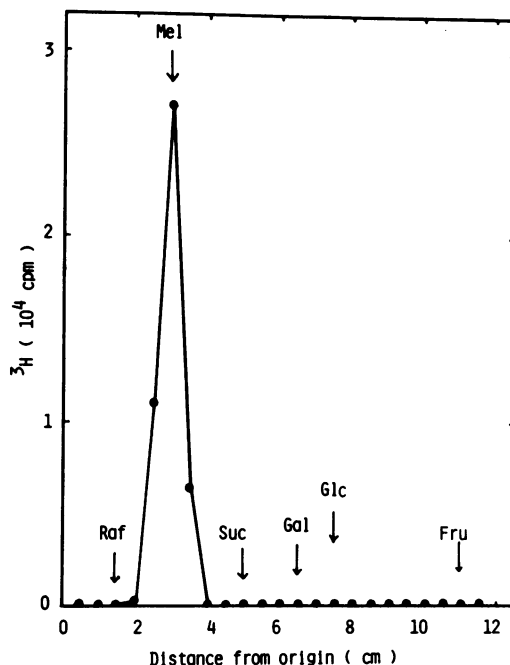


FIG. 1. Paper chromatography of the melibiose fraction (peak II) obtained from a Sephadex G-15 column. Concentrated sample and authentic sugars were developed on the paper as described in the text. Arrows indicate the positions of authentic sugars: Raf, raffinose; Mel, melibiose; Suc, sucrose; Gal, galactose; Glc, glucose; Fru, fructose.

Peak I was identified as raffinose, and peak III was identified as fructose (not shown). Contamination of other sugars was not detected in these fractions. Neither sucrose nor galactose was detected in peaks II and III, indicating that there was no  $\alpha$ -galactosidase activity in the invertase preparation used in this study. The peak II fraction was concentrated, diluted with cold melibiose, and used for the experiments described below.

**Transport of melibiose via the melibiose system.** Strain RA11 lacks both  $\alpha$ -galactosidase activity and a lactose transport system, making it suitable for the study of melibiose transport via the melibiose system. Cells of RA11 were grown in minimal medium containing 1% tryptone and 1 mM melibiose and tested for uptake of [<sup>3</sup>H]melibiose (Fig. 2). Induced cells of RA11 took up melibiose, and this uptake was inhibited by TMG but not by lactose. TMG transport via the melibiose system was shown to be strongly inhibited by the proton conductor carbonyl cyanide *m*-chlorophenylhydrazine and a sulfhydryl inhibitor, *N*-ethylmaleimide (6). These two compounds also strongly inhibited melibiose transport in RA11 (Table 1). Iodoa-

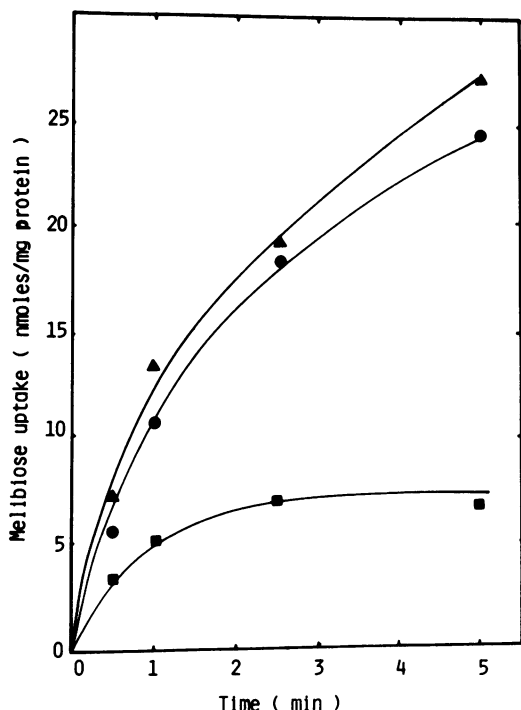


FIG. 2. Transport of melibiose in strain RA11. Induced cells (0.15 mg of protein per ml) were incubated in minimal medium containing NaCl (10 mM) and [ $^3\text{H}$ ]melibiose (50  $\mu\text{M}$ ). Symbols: (●) control; (▲) lactose, or (■) TMG added to 500  $\mu\text{M}$ .

TABLE 1. Effect of various inhibitors on melibiose transport<sup>a</sup>

Inhibitor	Melibiose uptake	
	nmol/mg of protein at 30 s	% of control
Control	5.75	100
+20 $\mu\text{M}$ CCCP	0.21	4
+1 mM <i>N</i> -ethylmaleimide	0.34	6
+1 mM iodoacetic acid	5.56	97

<sup>a</sup> Cells of RA11 were preincubated with inhibitors for 10 min in minimal medium containing 10 mM NaCl. [ $^3\text{H}$ ]melibiose was added to give a final concentration of 50  $\mu\text{M}$ . CCCP, Carbonyl cyanide *m*-chlorophenylhydrazone.

cetic acid at 1 mM had little effect, as reported for TMG transport (6).

$\text{Na}^+$  stimulated the transport of TMG via the melibiose system in both *E. coli* and *S. typhimurium* (15, 21). On the other hand,  $\text{Na}^+$  inhibited the transport of *p*-nitrophenyl- $\alpha$ -D-galactopyranoside via the same system in *E. coli* (20) and *S. typhimurium* (18). Thus, it was of interest to test the effect of  $\text{Na}^+$  (and  $\text{Li}^+$ ) on melibiose transport.  $\text{Na}^+$  stimulated the transport of mel-

ibiose in RA11 (Fig. 3). This observation is consistent with the previous result that an artificially imposed melibiose gradient elicited influx of  $\text{Na}^+$ , which suggested  $\text{Na}^+$ -melibiose cotransport (22). The effect of  $\text{Li}^+$ , however, was less clear.  $\text{Li}^+$  reproducibly inhibited the initial rate of melibiose transport, but cells accumulated more melibiose in the presence of 10 mM LiCl than in the absence of  $\text{Li}^+$  at the steady-state level. Previously we reported that melibiose transport measured by the glucose production method was markedly inhibited by  $\text{Li}^+$  (20). Because of the limitations of the colorimetric determination of glucose, the concentration of melibiose had to be high (10 mM). Interestingly, the inhibitory effect of  $\text{Li}^+$  on [ $^3\text{H}$ ]melibiose transport was more obvious at higher concentrations of melibiose.  $\text{Li}^+$  inhibited both the initial rate and the final extent of melibiose transport at 10 mM melibiose (Fig. 4).

The effect of  $\text{Na}^+$  and  $\text{Li}^+$  on the kinetic parameters of melibiose transport was investigated (Fig. 5). Addition of 10 mM NaCl to the

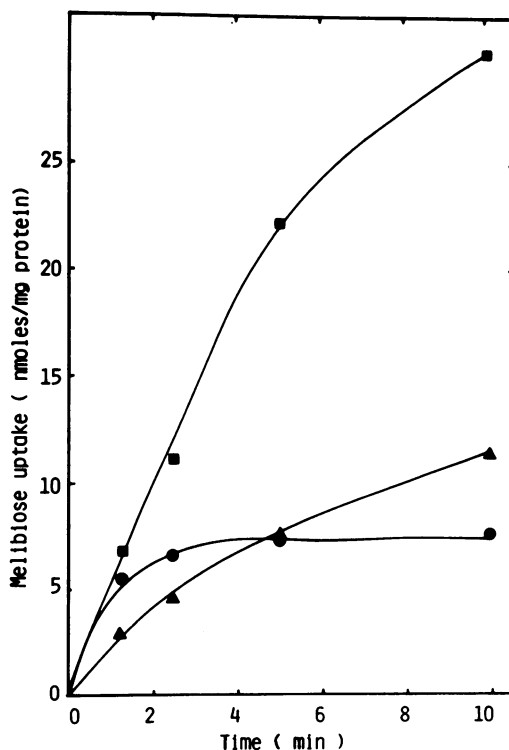


FIG. 3. Effect of  $\text{Na}^+$  and  $\text{Li}^+$  on melibiose transport in strain RA11. Induced cells (0.15 mg of protein per ml) were incubated in minimal medium containing [ $^3\text{H}$ ]melibiose (50  $\mu\text{M}$ ). Symbols: (●) control; (■) NaCl or (▲) LiCl added to the assay mixture to 10 mM and incubated for 10 min before initiation of the reaction.

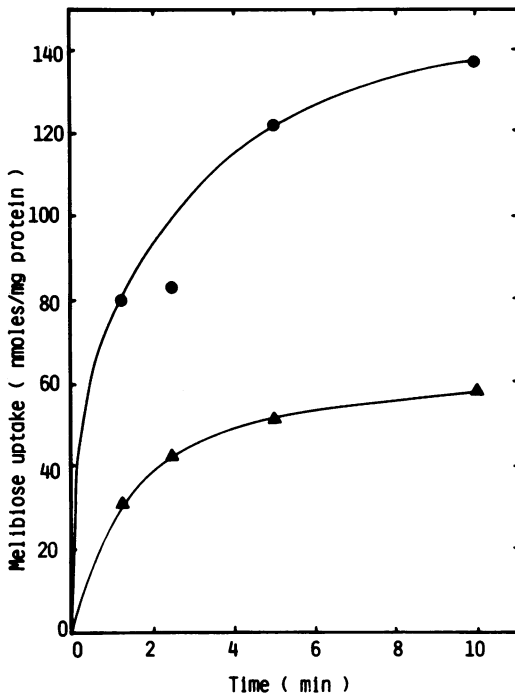


FIG. 4. Inhibition of melibiose transport by  $\text{Li}^+$ . Induced cells of RA11 (0.15 mg of protein per ml) were incubated in minimal medium containing [ $^3\text{H}$ ]melibiose (10 mM). Symbols: (●) control; (▲) LiCl added to 10 mM.

assay system lowered the  $K_t$  of melibiose transport from  $0.57 \pm 0.04$  to  $0.27 \pm 0.03$  mM (average of four determinations). Addition of 10 mM LiCl gave almost the same  $K_t$  value ( $0.29 \pm 0.04$  mM) as addition of 10 mM NaCl. The  $V_{\max}$  values were  $40 \pm 5$ ,  $46 \pm 6$ , and  $18 \pm 3$  nmol/min per mg of protein in the absence or in the presence of 10 mM NaCl or 10 mM LiCl.

One of the striking characteristics of the melibiose transport system of *E. coli* K-12 strains such as W3133 is its temperature sensitivity (6, 10), although strain RA11 possesses a temperature-resistant melibiose carrier (6). W3133 grown at  $30^\circ\text{C}$  exhibited transport of both melibiose and glutamate (Fig. 6). When cells were grown at  $37^\circ\text{C}$ , however, melibiose transport activity was markedly reduced, but glutamate transport was normal. Thus, the decrease of melibiose transport activity in cells grown at  $37^\circ\text{C}$  was not due to general damage of the cell membrane, but rather to a temperature-labile character of the melibiose carrier (6, 10, 20). Uninduced cells did not show any significant uptake of melibiose (not shown).

**Transport of melibiose via the lactose system.** Melibiose is also a substrate of the lactose transport system (5). Figure 7 shows the

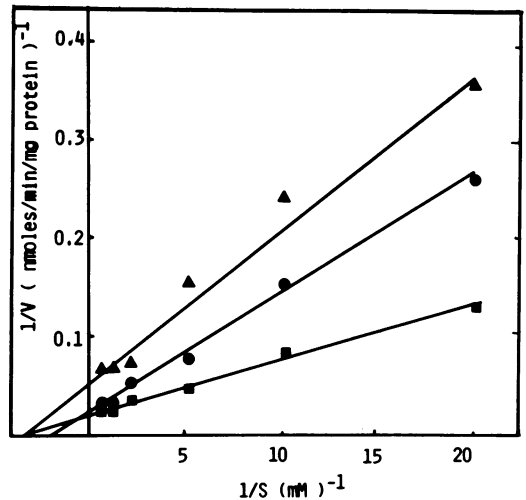


FIG. 5. Determination of kinetic parameters of melibiose transport. Induced cells of strain RA11 (0.15 mg of protein per ml) were added to minimal medium containing various concentrations of [ $^3\text{H}$ ]melibiose. Symbols: (●) control; (■) NaCl or (▲) LiCl (10 mM) added to the assay mixture. A 30-s point was taken to measure the initial velocity.

transport of melibiose in strain ML308-225, which possesses a constitutive lactose transport system but lacks the melibiose system. Melibiose was accumulated in this strain, and TMG and lactose, both of which are substrates of this system, were both inhibitory.

## DISCUSSION

To study the transport of melibiose in *E. coli*, we have developed a method for the preparation of radioactive melibiose. [ $^3\text{H}$ ]raffinose, a trisaccharide consisting of galactose, glucose, and fructose, was digested by invertase. The reaction mixture containing uncleaved raffinose, melibiose, and fructose was subjected to column chromatography of Sephadex G-15, resulting in a pure preparation of [ $^3\text{H}$ ]melibiose. The overall yield of radioactive melibiose was 75% of the theoretical value. This method is very simple and the yield is high.

Studies of melibiose transport in *E. coli* revealed the following points. (i) Cells induced with melibiose, which lack both  $\alpha$ -galactosidase and the lactose transport system, took up melibiose. Thus, active transport of radioactive melibiose was measured for the first time. (ii) Transport was sensitive to a proton conductor, carbonyl cyanide *m*-chlorophenylhydrazone, and to a sulfhydryl inhibitor, *N*-ethylmaleimide. (iii) Melibiose transport through the melibiose system was inhibited by TMG but not by lactose. This effect of lactose is consistent with the pre-

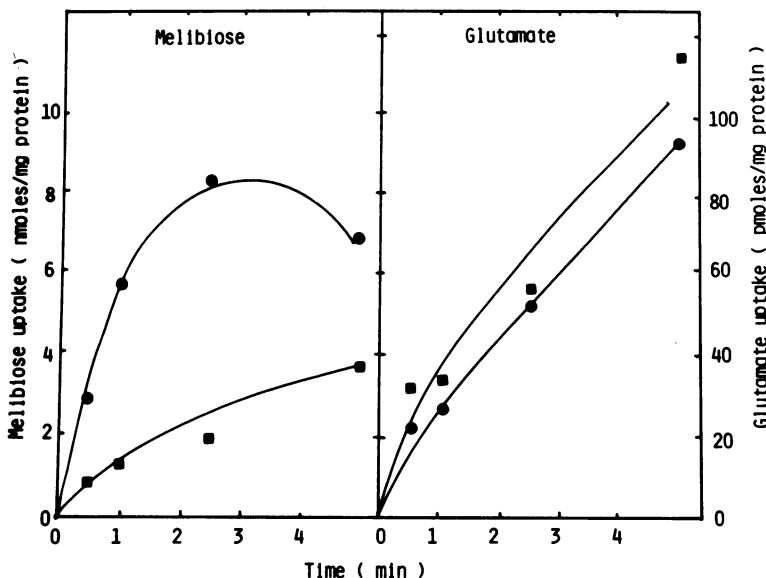


FIG. 6. Temperature sensitivity of the melibiose transport system. Cells of strain W3133 were grown in the presence of 10 mM melibiose at 30°C (●) or 37°C (■). Melibiose uptake was measured in minimal medium containing 10 mM NaCl and 50 μM [<sup>3</sup>H]melibiose at room temperature (20 to 22°C). Glutamate uptake was measured as described (19) at 1 μM [<sup>14</sup>C]glutamate.

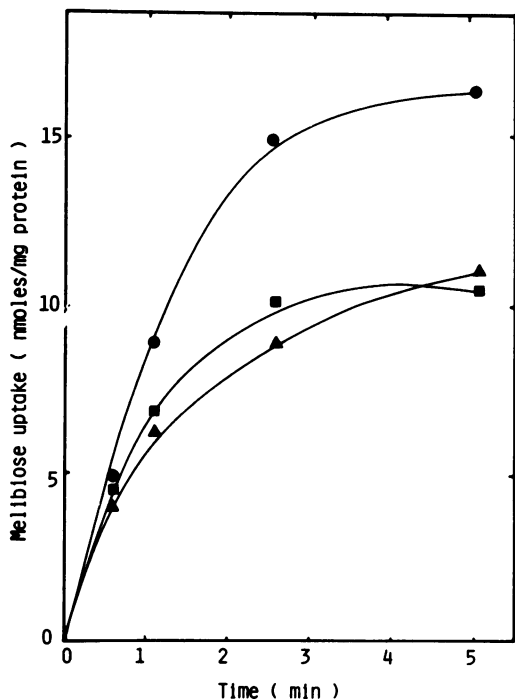


FIG. 7. Melibiose transport via the lactose system. Cells of strain ML308-225 were grown in the absence of melibiose. Melibiose uptake was measured in minimal medium containing [<sup>3</sup>H]melibiose (50 μM). Symbols: (●) control; (▲) lactose or (■) TMG added to the assay mixture to 500 μM.

vious observation suggesting that lactose has no affinity for the melibiose transport system (6, 10). (iv) Na<sup>+</sup> stimulated the transport of melibiose, and Li<sup>+</sup> inhibited it. Our recent results suggested that K<sup>+</sup> was essential for the inhibitory effect of Li<sup>+</sup> (manuscript in preparation).

As reported previously (6, 20, 22), Na<sup>+</sup> stimulated the transport of all substrates of this system tested except *p*-nitrophenyl- $\alpha$ -D-galactopyranoside. Li<sup>+</sup>, on the other hand, had no effect on *p*-nitrophenyl- $\beta$ -D-galactopyranoside or raffinose transport, although it stimulated TMG uptake. One of the most striking features of the Li<sup>+</sup> effect on this system was that Li<sup>+</sup> inhibited the transport of melibiose (20). Because of the limitation of the method used at that time, it was impossible to test the effect of Li<sup>+</sup> (and Na<sup>+</sup>) on the kinetic parameters of melibiose transport. As reported here, Li<sup>+</sup> actually inhibited the transport of melibiose at all concentrations of this substrate tested, lowering both *K<sub>t</sub>* and *V<sub>max</sub>* values. However, we are not sure whether or not such inhibition is the sole cause of the complete inhibition by added Li<sup>+</sup> of growth of cells on melibiose (20). Since Li<sup>+</sup> is a substrate of H<sup>+</sup>/Na<sup>+</sup> antiporter (14), which extrudes Na<sup>+</sup> and Li<sup>+</sup> from cells, the intracellular concentration of Li<sup>+</sup> would be very low even if Li<sup>+</sup> is added to the culture medium. However, if the melibiose transport carrier functions also as an Li<sup>+</sup> carrier to some extent in the presence of melibiose, the intracellular Li<sup>+</sup> concentration will reach a cer-

tain level which is determined by the efficiencies of the influx and efflux systems. Intracellular  $\text{Li}^+$  may be poisonous for cells. Our preliminary results support this possibility (unpublished observation). In the case of TMG transport via the melibiose system,  $\text{Li}^+$  decreased the  $K_t$  and increased the  $V_{\max}$  (6). The effect of  $\text{Na}^+$  on melibiose transport was similar to that of  $\text{Li}^+$  on TMG transport. At present, we are far from a good explanation of how these differences in cation-substrate coupling take place. It would be important to evaluate these differences in a purified system. For this, it is necessary to purify the carrier. Such investigations are now under way.

## ACKNOWLEDGMENTS

We thank B. P. Rosen, T. H. Wilson, and M. Futai for critical reading of the manuscript.

This work was supported in part by a grant-in-aid for scientific research from the Ministry of Education, Science and Culture of Japan.

## LITERATURE CITED

1. Bailey, R. W., and E. J. Bourne. 1960. Colour reactions given by sugars and diphenylamine-aniline spray reagents on paper chromatograms. *J. Chromatogr.* **4**:206-213.
2. Collins, S. H., A. W. Jarvis, R. J. Lindsay, and W. A. Hamilton. 1976. Proton movements coupled to lactate and alanine transport in *Escherichia coli*: isolation of mutants with altered stoichiometry in alanine transport. *J. Bacteriol.* **126**:1232-1244.
3. Crane, R. K. 1965.  $\text{Na}^+$ -dependent transport in the intestine and other animal tissues. *Fed. Proc.* **24**:1000-1006.
4. Goldstein, A., and J. O. Lampen. 1975.  $\beta$ -D-Fructofuranoside fructohydrolase from yeast. *Methods Enzymol.* **42**:504-511.
5. Kennedy, E. P. 1970. The lactose permease system of *Escherichia coli*, p. 49-92. In J. R. Beckwith and D. Zipser (ed.), *The lactose operon*. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
6. Lopillato, J., T. Tsuchiya, and T. H. Wilson. 1978. Role of  $\text{Na}^+$  and  $\text{Li}^+$  in thiomethylgalactoside transport by the melibiose transport system of *Escherichia coli*. *J. Bacteriol.* **134**:147-156.
7. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**:265-275.
8. MacDonald, R. E., R. V. Greene, and J. K. Lanyi. 1977. Light-activated amino acid transport system in *Halobacterium halobium* envelope vesicles: role of chemical and electrical gradients. *Biochemistry* **16**:3227-3235.
9. MacDonald, R. E., J. K. Lanyi, and R. V. Green. 1977. Sodium-stimulated glutamate uptake in membrane vesicles of *Escherichia coli*: the role of ion gradients. *Proc. Natl. Acad. Sci. U.S.A.* **74**:3167-3170.
10. Prestidge, L. S., and A. B. Pardee. 1965. A second permease for methyl-thio- $\beta$ -D-galactoside in *Escherichia coli*. *Biochim. Biophys. Acta* **100**:591-593.
11. Rosen, B. P., and E. R. Kashket. 1978. Energetics of active transport, p. 559-620. In B. P. Rosen (ed.), *Bacterial transport*. Marcel Dekker Inc., New York.
12. Saier, M. H., and C. E. Ballou. 1968. The 6-O-methylglucose-containing lipopolysaccharide of *Mycobacterium phlei*. *J. Biol. Chem.* **243**:4319-4331.
13. Saier, M. H., B. U. Feucht, and L. J. Hofstadter. 1976. Regulation of carbohydrate uptake and adenylate cyclase activity mediated by the enzyme II of the phosphoenolpyruvate:sugar phosphotransferase system in *Escherichia coli*. *J. Biol. Chem.* **251**:883-892.
14. Schuldiner, S., and H. Fishkes. 1978. Sodium-proton antiport in isolated membrane vesicles of *Escherichia coli*. *Biochemistry* **17**:706-711.
15. Stock, J., and S. Roseman. 1971. A sodium-dependent sugar cotransport system in bacteria. *Biochem. Biophys. Res. Commun.* **44**:132-138.
16. Tanaka, S., S. A. Lerner, and E. C. C. Lin. 1967. Replacement of a phosphoenolpyruvate-dependent phosphotransferase by a nicotinamide adenine dinucleotide-linked dehydrogenase for the utilization of mannitol. *J. Bacteriol.* **93**:642-648.
17. Tokuda, H., and H. R. Kaback. 1977. Sodium-dependent methyl 1-thio- $\beta$ -D-galactopyranoside transport in membrane vesicles isolated from *Salmonella typhimurium*. *Biochemistry* **16**:2130-2136.
18. Tokuda, H., and H. R. Kaback. 1978. Sodium-dependent binding of p-nitrophenyl  $\alpha$ -D-galactopyranoside to membrane vesicles isolated from *Salmonella typhimurium*. *Biochemistry* **17**:698-705.
19. Tsuchiya, T., S. M. Hasan, and J. Raven. 1977. Glutamate transport driven by an electrochemical gradient of sodium ions in *Escherichia coli*. *J. Bacteriol.* **131**:848-853.
20. Tsuchiya, T., J. Lopillato, and T. H. Wilson. 1978. Effect of lithium ion on melibiose transport in *Escherichia coli*. *J. Membrane Biol.* **42**:45-59.
21. Tsuchiya, T., J. Raven, and T. H. Wilson. 1977. Cotransport of  $\text{Na}^+$  and methyl- $\beta$ -D-thiogalactopyranoside mediated by the melibiose transport system of *Escherichia coli*. *Biochem. Biophys. Res. Commun.* **76**:26-31.
22. Tsuchiya, T., and T. H. Wilson. 1978. Cation-sugar cotransport in the melibiose transport system of *Escherichia coli*. *Membrane Biochem.* **2**:63-77.
23. West, I. C. 1970. Lactose transport coupled to proton movements in *Escherichia coli*. *Biochem. Biophys. Res. Commun.* **41**:655-661.
24. Winkler, H. H., and T. H. Wilson. 1966. The role of energy coupling in the transport of  $\beta$ -galactosides by *Escherichia coli*. *J. Biol. Chem.* **241**:2200-2211.