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Membrane Transport of *p*-Nitrophenyl-α-Galactoside by the Melibiose Carrier of *Escherichia coli*

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Summary. *p*-Nitrophenyl-α-galactoside (α-*p*NPG) was found to be a substrate for the melibiose transport system of *Escherichia coli*. This sugar enters induced cells via the carrier and is split by α-galactosidase to galactose and *p*-nitrophenol. In mutant cells lacking the α-galactosidase [^{3}H]-α-*p*NPG accumulated to concentrations 15 times higher than the external medium. The transport of α-*p*NPG is inhibited by both Na^+ and Li^+. Na^+ (10 mM) reduced the K_m for α-*p*NPG from 0.45 to 0.18 mM and reduced the V_{max} from 6.7 nmoles/min/mg dry wt to a value of 3.0.

The melibiose transport system of *E. coli* shows several unusual features not found in other transport systems. As Prestidge and Pardee (1965) have shown, the transport system is temperature sensitive, being active at 30 °C but inactive at 37 °C (the temperature at which *E. coli* grows in nature). In spite of this apparent handicap cells grow readily on melibiose at 37 °C, because this disaccharide induces the lactose transport system which permits entry of melibiose (Beckwith, 1963). A second interesting feature of this system is the unusual spectrum of cation requirements for transport. The carrier can transport melibiose by cotransport with either protons or sodium ions but is inhibited by lithium (Tsuchiya, Lopilato & Wilson, 1978; Tsuchiya & Wilson, 1978). On the other hand, thiomethylgalactoside transport can occur by cotransport with lithium (Tsuchiya, Raven & Wilson, 1977; Lopilato, Tsuchiya & Wilson, 1978) or sodium ions (Stock & Roseman, 1971; Tokuda & Kaback, 1977; Tsuchiya et al., 1977) but not with H^+ (Tsuchiya & Wilson, 1978). In the present study, a further difference in cation specificity for substrate of this carrier is described. The transport of *p*-nitrophenyl-α-galactoside is inhibited by both sodium and lithium ions.

Materials and Methods

Bacterial Strains and Growth Conditions

Strains of *E. coli* used in this study are listed in Table 1. Strain W3133 (from Dr. Salvador Luria) is a derivative of K-12 which possesses a deletion through both *lac* Z and *lac* Y genes (which code for β-galactosidase and lactose transport, respectively). The melibiose transport carrier in this strain is temperature-sensitive; it is inactive at 37 °C, but active at 30 °C. Strain W3133-2, isolated from strain W3133 by growing at 37 °C, possesses a melibiose transport carrier which is stable at 37 °C. Strain RA11, a derivative of W3133-2, is α-galactosidase negative (mel A^-); and strain RE16, a derivative of W3133-2, is melibiose transport negative (mel B^-). Strain RA11/plC 16–37 was isolated by mating each of the Clarke-Carbon (1976) plasmid strains into RA11 and isolating a melibiose positive F-ductant. It was found that the plasmid was mel A^+B^-. The growth medium used was Medium 63 (Cohen & Rickenberg, 1956) which contains KH_2PO_4 (13.6 g), $(NH_4)_2SO_4$ (2.0 g), $MgSO_4 \cdot 7H_2O$ (0.2 g), $FeSO_4 \cdot 7H_2O$ (0.005 g), and H_2O (1 liter) adjusted to pH 7.0 with KOH. Unless otherwise stated, cells were grown in Medium 63 containing 0.2% casamino acids (Difco), 0.5 μg/ml B_1 and 10 mM melibiose (as an inducer).

Synthesis of [^{3}H]-Melibiose

[^{3}H]-Melibiose was obtained by hydrolysis of [^{3}H]-raffinose by invertase (Bacon, 1955). Yeast invertase solution was prepared by dissolving 0.01 g of the enzyme yeast invertase in 10 ml acetate

Table 1. Bacterial strains

Strain of *E. coli*	Melibiose genotype[a]	Source
W3133	mel A^+B^{ts}	Salvador Luria
W3133-2	mel A^+B^+	Lopilato et al., 1978
RA11	mel A^-B^+	Lopilato et al., 1978
RE16	mel A^+B^-	This paper
RA11/plC16-37	mel A^-B^+/F'	T.H. Wilson
	mel A^+B^-	T. Tsuchiya, *Unpublished*

[a] All strains carry a deletion through the *lac* Z and *lac* Y genes.

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buffer, pH 4.6 (Lück, 1965). The enzyme (0.1 ml) was added to 0.9 ml of ^{3}H-raffinose (0.41 mg/ml, 1.0 mCi/ml) and incubated at 30 °C for 3 hr. The product of the reaction mixture was placed on Whatman No. 1 filter paper and developed by descending technique in *n*-butanol/ethanol/water (10:1:2 vol/vol) for periods ranging from 5 days to 24 days (Block & Zweig, 1958). Under these conditions melibiose was separated from raffinose and from fructose. The disaccharide was eluted from the paper.

Accumulation of Radioactive Galactosides

Induced cells of strain RA11 were washed twice with Medium 63 and resuspended in the same buffer to a concentration of 0.3–0.4 mg dry wt of cells per ml. The reaction, performed at room temperature, was initiated by the addition of the labeled substrate. At various intervals 0.2 ml aliquots were filtered, washed with 5 ml of buffer, and filters counted in a scintillation counter.

Entry of p-Nitrophenyl-α-Galactoside (α-pNPG)

Induced cells of W3133-2 and RE16 were washed twice with 0.1 M potassium phosphate buffer, pH 7.0, and resuspended in the same buffer containing chloramphenicol (0.1 mg/ml) plus dithiothreitol (1 mM) to a final concentration of 0.2 mg dry wt of cells per ml. After incubation at 30 °C for 10 min the reaction was initiated by addition of α-*p*NPG to give a final concentration of 0.5 mM, and the reaction vessels were shaken at 30 °C. At various intervals 3-ml samples were removed and placed in tubes containing 3 ml of 0.6 M Na_2CO_3. Tubes were then centrifuged to remove cells, and the yellow color of the *p*-nitrophenol in the supernatant was monitored with a Klett-Summerson colorimeter (No. 42 filter).

α-Galactosidase Assay

α-Galactosidase was assayed as follows (Burstein & Kepes, 1971; Schmitt & Rotman, 1966): cells were washed in 50 mM Tris-HCl, pH 7.5, and suspended in an assay mixture containing 50 mM Tris-HCl (pH 7.5), 5 mM $MnSO_4$, 50 mM 2-mercaptoethanol and 10 mM α-pNPG. After incubating at 37 °C for various time intervals, 1-ml samples were removed placed in 4.5 ml of 0.6 M Na_2CO_3. Tubes were centrifuged and the *p*-nitrophenol liberated was monitored as above. Under these conditions, (i.e., high substrate concentration) it is the enzyme and not the transport that is the rate-limiting step.

Chemicals

Melibiose, *p*-nitrophenyl-α-galactopyranoside (α-*p*NPG), methyl-β-thiogalactopyranoside (TMG), methyl-α-galactoside (α-MGal), and morpholinopropane sulfonic acid (MOPS) were purchased from Sigma Chemical Co. Yeast invertase was from Calbiochem. [^{14}C]-TMG and [^{3}H]-raffinose were purchased from New England Nuclear Co. [^{3}H]-α-*p*NPG was prepared by treatment of α-*p*NPG with galactose oxidase followed by reduction with [^{3}H]-$NaBH_4$ (Kennedy, Rumley & Armstrong, 1974). [^{14}C]-methyl-α-galactoside was prepared by Dr. Nasir-ud-Din from [^{14}C]-galactose and methanol.

Results

When cells of strain W3133-2 induced for the melibiose operon were exposed to *p*-nitrophenyl-α-galactoside (α-*p*NPG), there was hydrolysis of the galactoside to yield the yellow product *p*-nitrophenol. The sugar α-*p*NPG entered the cell on the melibiose carrier; it was split by the intracellular α-galactosidase to free galactose and *p*-nitrophenol, the latter diffusing out of the cell into the incubation medium. In order to determine whether the transport step was rate-limiting in this process, the α-galactosidase activity in disrupted cells was compared with the rate of the overall hydrolysis in the intact cells. Figure 1 shows that sonicated cells split α-*p*NPG three times faster than intact cells, indicating that the enzyme activity was greater than the transport rate. The activity of the α-galactosidase *in vivo* is probably far greater than that found in this experiment, since Burstein and Kepes (1971) have discovered that the enzyme of *E. coli* requires NAD^+ for full activity. In the presence of 0.1 mM NAD^+ α-galactosidase activity is stimulated at 10- to 20-fold. If we assume that the enzyme *in vivo* is fully activated by its coenzyme,

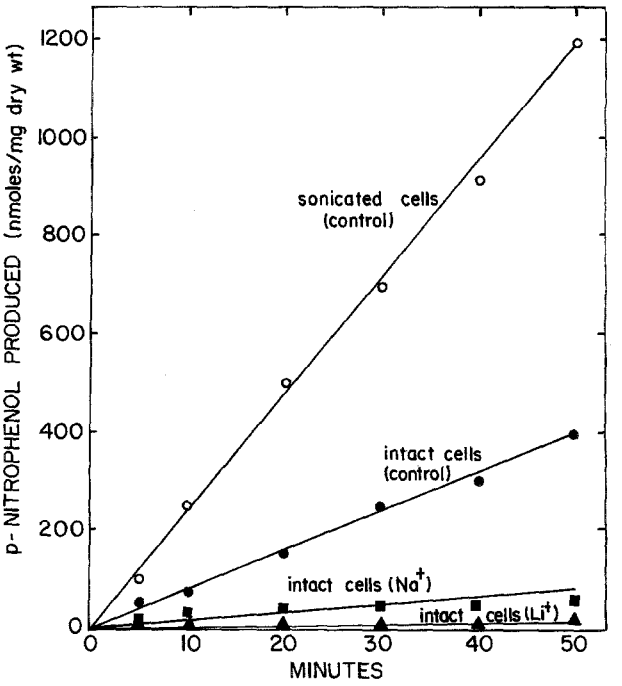

Fig. 1. Effect of Na^+ and Li^+ on α-*p*NPG transport. Cells of strain W3133-2 were grown at 37 °C in 10 mM melibiose. Cells were divided into two equal portions, harvested, washed twice with 100 mM potassium phosphate buffer pH 7, and resuspended in the same buffer. One portion of cells was sonicated, centrifuged at 30,000 × *g* for 30 min, and the supernatant was used in the assay. Intact cells or supernatant were incubated at 30 °C in 100 mM potassium phosphate (pH 7), 0.1 mg/ml chloramphenicol, 1 mM dithiothreitol, 0.5 mM α-*p*NPG and 10 mM NaCl or LiCl where indicated. Samples (3 ml) were removed at indicated times and placed in 3 ml of 0.6 M Na_2CO_3. *p*-Nitrophenol was determined colorimetrically. Symbols: Sonicated cells control, ○; intact cells control, ●; intact cells + 10 mM NaCl, ■; intact cells + 10 mM LiCl, ▲. NaCl and LiCl had no effect on sonicated cells (results not shown)

the true intracellular activity of α-galactosidase would be 30–60 times greater than the rate of transport by intact cells. Thus, entry of α-*p*NPG is clearly rate limiting in *p*-nitrophenol production by intact cells.

The addition of 10 mM Na^+ to the washed cells inhibited α-*p*NPG hydrolysis by 80% and Li^+ inhibited almost 95% (Fig. 1). Since these two cations had no effect on the α-galactosidase (legend to Fig. 1), it was assumed that these ions act directly on the melibiose carrier.

Evidence for the view that α-*p*NPG enters the cell on the melibiose carrier was provided by studies with the transport negative (but α-galactosidase positive) mutant RE16. Figure 2 indicates that the sugar enters the transport negative cell very slowly and is completely unaffected by the presence of Na^+ or Li^+. In subsequent experiments the slow entry rate found in RE16 was subtracted from the total rate to give entry via the melibiose carrier.

The effect of growth phase on enzyme and transport activities was next investigated. Figure 3 shows that α-galactosidase and transport activities rise in parallel to a maximum level at late logarithmic phase of growth. This is then followed by a fall in both activities as the cells enter stationary phase. In most subsequent experiments cells were harvested in late logarithmic phase of growth.

The effect of different concentrations of Na^+ on sugar transport was next investigated. The concentration of Na^+ producing half maximum inhibition of α-*p*NPG transport was 0.45 mM (Fig. 4).

The effect of Na^+ (10 mM) on the rate of transport of different concentrations of α-*p*NPG is shown in Fig. 5. In the absence of Na^+ the K_m for α-*p*NPG was 0.45 mM and the V_{max} was 6.7 nmol/mg dry wt/min. In the presence of sodium ion the K_m was reduced to 0.18 mM and the V_{max} reduced to a value of 3.0. This result suggests that sodium ion should inhibit the entry rate when the sugar concentration is high, but might stimulate the entry rate at very low concentrations of sugar. This prediction was borne out by experiments on the accumulation of [^{3}H]-α-*p*NPG in the α-galactosidase negative mutant RA11. Figure 6 shows that when cells lacking the α-galactosidase were exposed to 2 μM radioactive α-*p*NPG the sugar was accumulated within the cell to concentrations 15 times higher than that in the external medium. With this very low external concentration of sugar, sodium, and lithium ions stimulated transport. On the other hand, when the external concentration of sugar was raised to 500 μM (above the K_m value) the two cations inhibited transport. This differential effect of sodium ion depending on the concentration of sugar was also observed by Tanaka, Niiya and Tsuchiya (1980) for melibiose and Li^+ in this same cell.

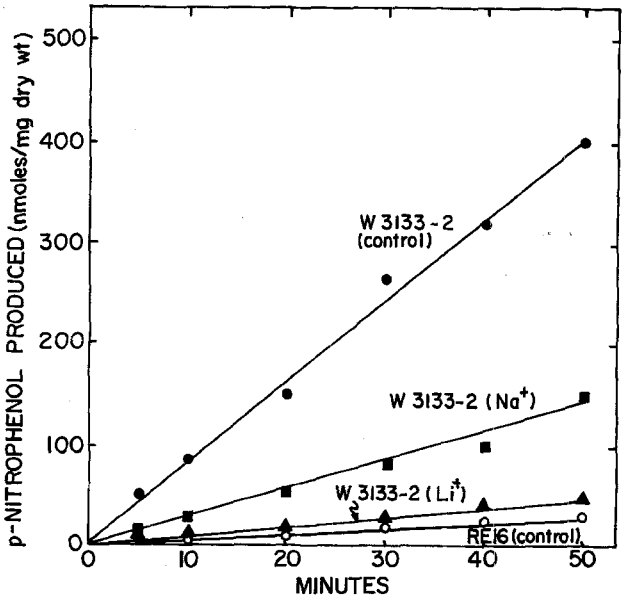

Fig. 2. Effect of Na^+ and Li^+ on α-*p*NPG transport in normal cell and transport-negative mutant. Cells of strains W3133-2 and RE16 (mel B$^-$) were grown at 37 °C in 10 mM melibiose. Washed cells were incubated in the presence of 0.5 mM α-*p*NPG at 30 °C and assayed for *p*-nitrophenol production as described under Fig. 1. Symbols: RE16 Control, ○; W3133-2 control, ●; W3133-2+10 mM NaCl, ■; W3133-2+10 mM LiCl, ▲. NaCl and LiCl had no effect on RE16 (results not shown)

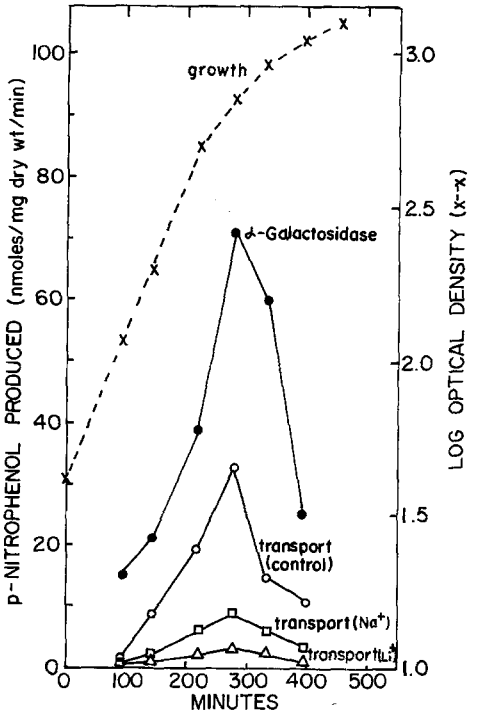

Fig. 3. Effect of growth on α-galactosidase and α-*p*NPG transport. Cells of strain W3133-2 were grown in 350 ml of medium 63 containing 0.2% casamino acids, 0.5 μg/ml B_1 and 10 mM melibiose at 37 °C. At the indicated intervals 25 ml portions were removed and cells assayed for α-*p*NPG transport and α-galactosidase activity. Symbols; Growth, ×---×; α-galactosidase, ●; α-*p*NPG transport control, ○; α-*p*NPG transport+10 mM NaCl, □; α-*p*NPG transport+10 mM LiCl, △

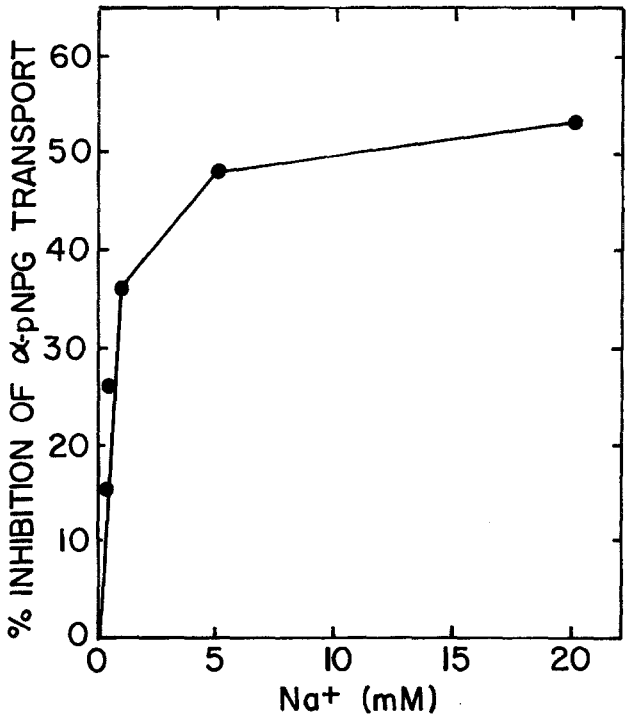

Fig. 4. Effect of Na^+ concentration on α-*p*NPG transport. Cells of strain W3133-2 were grown at 37° in Medium 63 containing 0.5 μg/ml B_1, 2% glycerol and 10 mM melibiose. Cells were harvested, washed twice with 100 mM MOPS (morpholinopropane sulfonic acid, pH adjusted to 6.9 with tetramethyl ammonium hydroxide). Cells were incubated at 30 °C in 100 mM MOPS (pH 6.9) 0.1 mg/ml chloramphenicol, 1 mM dithiothreitol, 0.5 mM α-*p*NPG and various concentrations of NaCl. The production of *p*-nitrophenol was determined as described under Fig. 1. The K_m for Na^+ was 0.45 mM.

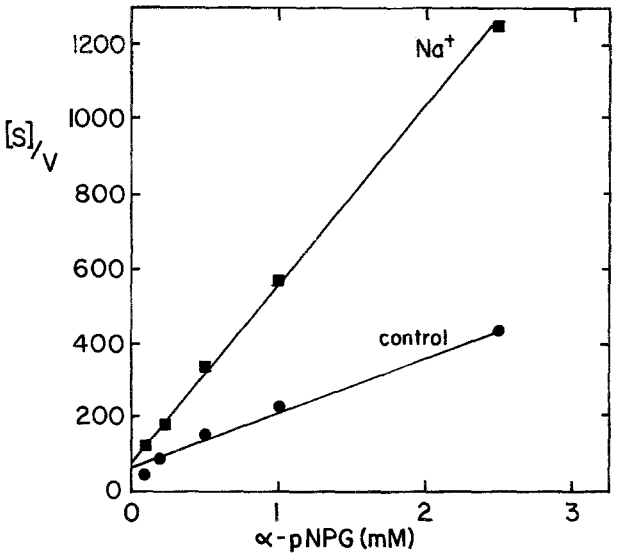

Fig. 5. Kinetics of Na^+ inhibition of α-*p*NPG transport. Washed cells of strain W3133-2 were incubated in 100 mM Tricine buffer, pH 8, 3 mM $MnSO_4$, 100 mM 2-mercaptoethanol, 0.1 mg/ml chloramphenicol and 10 mM NaCl where indicated. Reaction was initiated by addition of various concentrations of α-*p*NPG (*S*) and the rate of *p*-nitrophenol production (*V*) was determined in nmol/mg dry wt/min. The rates of entry of these same concentrations of α-*p*NPG into RE16 (mel B^-), similarly determined, were assumed to be due to diffusion and were subtracted from the values obtained with W3133-2 to give the carrier-mediated rate of transport

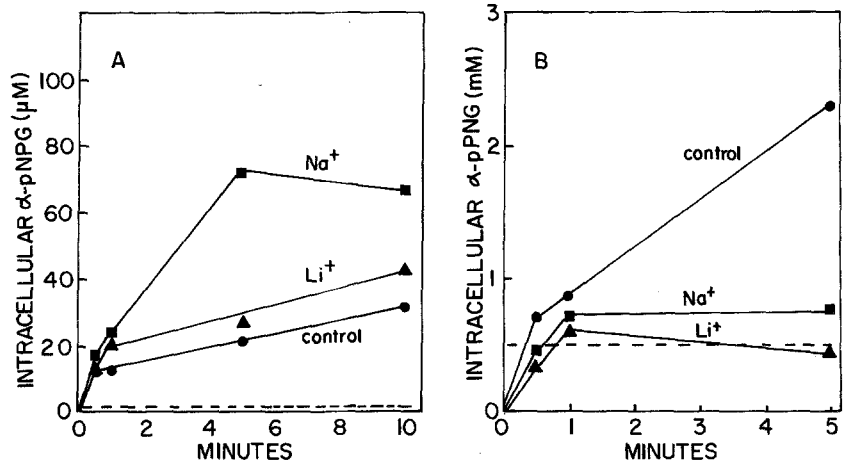

Fig. 6. Effect of Na^+ and Li^+ on α-*p*NPG accumulation in RA11. Washed cells of strain RA11 (mel A^-) were incubated in Medium 63 and tested for [^{3}H]-α-*p*NPG uptake at (*A*) low concentration (2 μM) and (*B*) high concentration (0.5 mM) of α-*p*NPG. Symbols (*A*): control, ●; +5 mM NaCl, ■; +5 mM LiCl, ▲. Symbols (*B*): control, ●; +10 mM NaCl, ■; +10 mM LiCl, ▲. Dotted lines show the concentration of [^{3}H]-α-*p*NPG in the medium

To confirm the fact that the melibiose carrier in RA11 was indeed the same as its parent and had not been altered during mutagenesis experiments were carried out with this cell into which had been inserted a plasmid carrying mel A^+B^-. In this cell which contained α-galactosidase it was possible to measure the kinetics of α-*p*NPG entry with a technique which involves entry of the sugar and hydrolysis to *p*-nitrophenol. Experiments with such a cell showed that the kinetics and cation inhibition were identical to those of W3133-2, indicating that the membrane carrier in RA11 was normal.

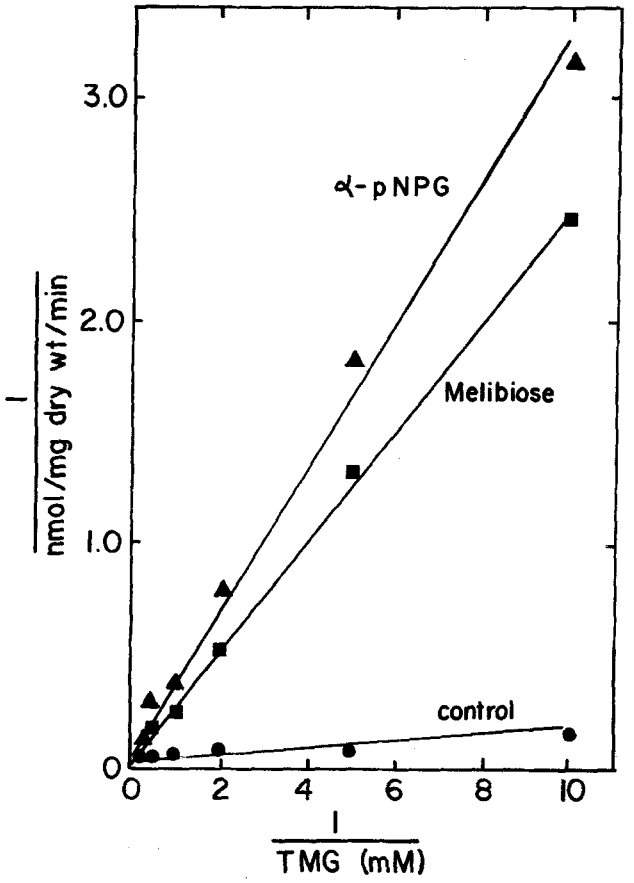

Fig. 7. Effect of melibiose and α-*p*NPG on TMG transport. Washed cells of strain W3133-2 were incubated in Medium 63 containing 0.2 mM NaCl and 5 mM melibiose or 5 mM α-*p*NPG where indicated. Reaction was initiated by addition of various concentrations of [^{14}C]-TMG. Samples were taken at 30 sec (initial rate) and TMG transport was determined in nmol/mg dry wt/min for the various concentrations. Data were plotted in a double-reciprocal plot. Symbols: Control, ●; +5 mM melibiose, ■; +5 mM α-*p*NPG, ▲

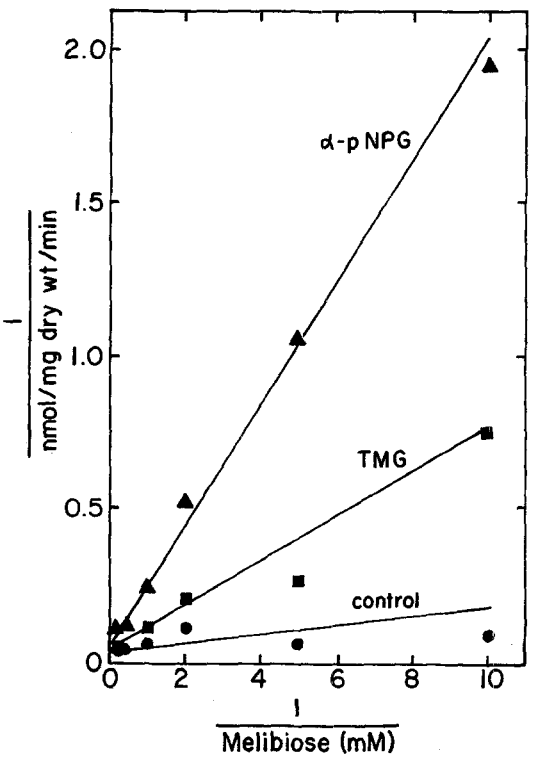

Fig. 8. Effect of TMG and α-*p*NPG on melibiose transport. Washed cells of strain RA11 were incubated in Medium 63 and 5 mM TMG or 5 mM α-*p*NPG where indicated. Reaction was initiated by addition of various concentrations of [^{3}H]-melibiose. Samples were taken at 30 sec (initial rate) and melibiose transport was determined in nmol/mg dry wt/min for the various concentrations. Data were plotted on a double-reciprocal plot. Symbols: Control, ●; +5 mM TMG, ■; +5 mM α-*p*NPG, ▲

Interaction Between α-pNPG, Melibiose and Thiomethylgalactoside

The interaction between different sugars with the melibiose carrier was next investigated. If two chemically related sugars each have affinity for the same active site of a transport system, one would expect that the K_m (measured directly) for a sugar would equal the K_i for that same sugar when tested as an inhibitor against the second sugar. This type of simple competitive inhibition was observed when melibiose or α-*p*NPG was tested as an inhibitor for TMG transport (Fig. 7). Similarly α-*p*NPG and TMG were found to competitively inhibit melibiose transport (Fig. 8). Very unexpectedly, neither TMG nor melibiose were found to have any inhibitory effect on the transport of 0.5 mM α-*p*NPG in W3133-2, even when the inhibitor concentration was raised to 20 mM (Table 2).

Table 2. Kinetic parameters of the melibiose transport system

Sugar	Na^+ (mM)	V_{max}	K_m (mM)	K_i (mM)[a]		
				vs. TMG	*vs.* Melibiose	*vs.* α-*p*NPG
TMG	0	25	0.91	–	1.2	>20
TMG	0.2	25	0.40			>20
Melibiose	0	26	0.30	0.32[b]	–	>20
α-*p*NPG	0	6.7	0.45		0.41	
α-*p*NPG	0.2		0.32	0.43		–
α-*p*NPG	10	3.0	0.18			

[a] The three sugars were tested as inhibitors of each other.
[b] With 0.2 mM Na^+.

Furthermore, the accumulation of 2 μM[^{3}H]-α-*p*NPG by RA11 was not inhibited by TMG or melibiose at an inhibitor concentration of 2 mM.

These unexpected findings prompted a consideration of the possibility that melibiose and TMG might be transported by one carrier and α-*p*NPG by another. Several lines of evidence suggest that this is

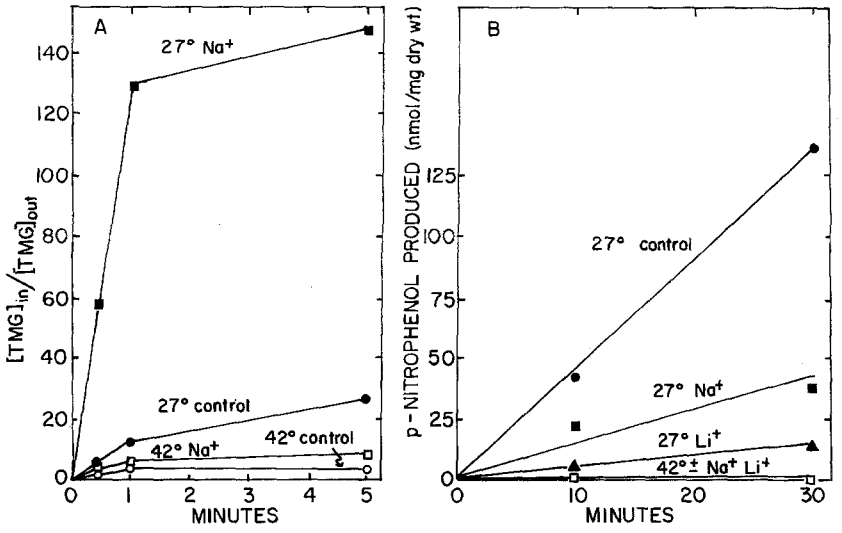

Fig. 9. Temperature sensitivity of the melibiose carrier. Cells of strain W3133 (mel B^{ts}) were grown in Medium 63 containing 0.2% casamino acids, 0.5 μg/ml B_1 and 10 mM methyl-α-galactoside (as inducer) at 27 °C and 42 °C. TMG uptake was measured by incubating cells in Medium 63 with or without 5 mM NaCl and taking samples (0.2 ml) at various times. Transport of α-*p*NPG was assayed as described in Fig. 1 and data corrected for entry by diffusion as described in Fig. 5. Symbols (*A*): TMG transport; 27 °C control, ●; 27° + 5 mM NaCl, ■; 42° control, ○; 42° + 5 mM NaCl, □. Symbols (*B*): α-*p*NPG entry; 27° control, ●; 27° + 10 mM NaCl, ■; 27° + 10 mM LiCl, ▲; 42° ± 10 mM NaCl or LiCl, □

Table 3. Effects of Na^+ and Li^+ on kinetic parameters

Sugar	Na^+		Li^+		Reference
	K_m	V_{max}	K_m	V_{max}	
TMG	Decreased	No effect	Decreased	No effect	Lopilato et al., 1978
Melibiose	Decreased	No effect	Decreased	Decreased	Tanaka et al., 1980
α-*p*NPG	Decreased	Decreased	ND[a]	ND[a]	
Methyl-α-galactoside[b]	Decreased	No effect	ND[a]	ND[a]	

[a] Not determined.
[b] [^{14}C]-Methyl-α-galactoside uptake into RA11 was measured in the presence and absence of Na^+.

not the case. Growth of cells in the presence of melibiose induces transport for all of these substrates (Tsuchiya et al., 1978). The transport negative mutant RE16 fails to transport any of the three substrates. Strong confirmatory evidence for a single carrier comes from the effect of temperature on transport of the three substrates. As discovered by Prestidge and Pardee (1965), the melibiose transport carrier of wild-type K12 strains is active at 30 °C but not at 42 °C. If a second carrier were present in these cells, it was unlikely that it should show the same temperature sensitivity as the melibiose carrier. Strain W3133 was grown at 27 °C or at 42 °C and TMG and α-*p*NPG transport were tested. The transport of TMG and α-*p*NPG were equally temperature sensitive (Fig. 9), which suggests that both substrates are transported by the same membrane carrier. Previous experiments (Tsuchiya et al., 1978) showed that the transport of melibiose was temperature sensitive in these cells. Thus, the conclusion seems inescapable that all three sugars are substrates for a single transport system, the melibiose carrier.

Discussion

Stock and Roseman (1971) demonstrated that both Na^+ and Li^+ stimulated TMG accumulation by the melibiose carrier of *Salmonella typhimurium* and suggested cation sugar cotransport. These studies were extended by Tokuda and Kaback (1977) who showed that Na^+ uptake was stimulated by TMG in membrane vesicles of *S. typhimurium*. A similar Na^+-TMG or Li^+-TMG cotransport was demonstrated in *E. coli* (Tsuchiya et al., 1977; Lopilato et al., 1978). Additional studies indicated that methyl-α-galactoside could be cotransported by this carrier with Li^+, Na^+ or H^+ (Tsuchiya & Wilson, 1978). It therefore came as a surprise to find that Li^+ strongly inhibited the uptake of melibiose, the natural substrate for the carrier (Tsuchiya et al., 1978). More recently Tanaka et al. (1980) have found that Li^+ (10 mM) stimulates melibiose uptake at low sugar concentrations. Results presented in this paper demonstrate that yet another substrate, α-*p*NPG, exhibits still another cation specificity. At high substrate

concentrations both Na^+ and Li^+ inhibit α-*p*NPG transport, whereas at low substrate concentrations both Na^+ and Li^+ stimulate α-*p*NPG accumulation.

An analysis of the effects of the cations, Na^+ and Li^+, on the kinetic parameters of sugar transport reveals a consistent pattern (Table 3). For the four sugars studied, Na^+ and Li^+ appear, in every case, to lower the K_m of the carrier for the sugar. However, in some instances these ions have a dual effect in that they also lower the V_{max}. When the ion exerts a dual effect, lowering both K_m and V_{max} the ion stimulates the rate of transport with low substrate concentrations and inhibits with high substrate concentration.

The failure of melibiose and TMG to inhibit α-*p*NPG transport is not yet understood and will require further investigation.

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