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Properties of the Entry and Exit Reactions of the Beta-Methyl Galactoside Transport System in *Escherichia coli*

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The K_m, V_{max}, and K_i of the entry reaction were determined for three substrates of the β-methyl galactoside transport system: D-galactose, D-glycerol-β-D-galactoside, and β-methyl-D-galactoside. Although the data for D-galactose and D-glycerol-β-D-galactoside followed simple Michaelis-Menten kinetics, the results for β-methyl-D-galactoside deviated from Michaelis-Menten kinetics in that the K_i for β-methyl-D-galactoside inhibition of both of the other two substrates was 10-fold greater than the K_m for β-methyl-D-galactoside entry. Furthermore, two partial *mgl*$^-$ strains retain 56% of the parental level of the β-methyl-D-galactoside entry reaction, but only 12% of the parental level of transport of the other two substrates. The exit reaction of β-methyl-D-galactoside was shown to be first order. It was stimulated sixfold when the cells were provided with an energy source. This stimulation required adenosine 5'-triphosphate or a related compound. The exit reaction was not altered by mutations in any of the three cistrons which inactivate the β-methyl-D-galactoside entry reaction, was not increased by growth in the presence of inducers of the entry reaction, and was not repressed by growth on glucose. The striking differences between the entry and exit reactions suggest that they either use different carriers or that none of the three cistrons which are currently known to code for components of the β-methyl galactoside transport system code for its membrane carrier.

The β-methyl galactoside transport system of *Escherichia coli*, whose properties have been recently reviewed by Boos (6), belongs to the class of transport systems that are associated with releasable binding proteins (15). This class is distinct from the class of membrane-bound transport systems, of which lactose permease is the best known example (19), and from the class of group translocation systems, of which the phosphotransferase systems are the most thoroughly studied (30).

Transport systems associated with binding proteins differ in three important ways from the membrane-bound systems. First, the two classes differ in their response to osmotic shock (14) and spheroplasting. Second, the two classes utilize different sources of energy to drive active transport. Third, the results of genetic studies suggest that membrane-bound systems may involve only a single protein (19), whereas binding protein systems appear to require several proteins (1, 23). The energy source-driving active transport by most binding protein systems is adenosine 5'-triphosphate (ATP) or a related compound (3, 4), whereas the class of membrane-bound transport systems appears to utilize the energized membrane state to drive active transport (16, 17, 27, 31-33). It was originally reported (25) that the energized membrane state drove active transport by the β-methyl galactoside transport system, but it now seems clear (35) that this system uses ATP or a related compound.

Conclusive evidence for multiple components in the β-methyl galactoside transport system was provided by Ordal and Adler (23, 24), who showed that mutants lacking the β-methyl galactoside transport system form three complementation groups, one of which represents the structural gene for the galactose-binding protein (5). In addition, Robbins and Rotman (29) have shown that strains with mutations in the binding protein cistron still grow on β-methyl-D-galactoside if its concentration is raised above 10^{-3} M. Strains with mutations in either of the other two cistrons do not grow even at a β-methyl-D-galactoside concentration of 5×10^{-3} M.

Despite the many studies of the β-methyl galactoside transport system, there have not been any detailed studies of the kinetics of the entry reaction and there has been only one study of the exit reaction (26). In this study, Parnes and Boos found that the exit reaction was not affected by mutations in the structural gene for the galactose-binding protein; how-

ever, since they used as their substrate D-galactose, which is also a substrate of the galactose-specific transport system (26), it is difficult to be certain which system is responsible for the exit they studied.

In this study, the kinetics of the entry reaction of the β-methyl galactoside transport system were measured for three substrates: D-galactose, β-methyl-D-galactoside, and D-glycerol-β-D-galactoside. In addition, the properties of the exit reaction were determined using the substrate β-methyl-D-galactoside, which is transported only by the β-methyl galactoside transport system under the conditions of this study. All the results of these kinetic studies fit simple Michaelis-Menten kinetics with one exception. The exception is that the K_i values for β-methyl-D-galactoside inhibition of both D-galactose and D-glycerol-β-D-galactoside are 10-fold greater than the K_m for β-methyl-D-galactoside entry. This result suggests that the mechanism of the entry reaction for β-methyl-D-galactoside is different from the mechanism for the other two substrates. This conclusion is supported by the finding that two different *mgl*$^-$ strains retain 12% of the parental level of the entry reaction for D-galactose and D-glycerol-β-D-galactoside and 56% of the parental level of the β-methyl-D-galactoside entry reaction.

The β-methyl galactoside exit reaction differed from the entry reaction in several important ways. First, the exit reaction was not affected by mutations in any of the three β-methyl galactoside cistrons even when they completely inactivated the entry reaction. Second, the rate of exit was the same whether the cells were uninduced, induced, or repressed for the entry reaction. Third, the exit reaction was not inhibited by 0.2 M KCl, which totally inhibited the entry reaction. Finally, the exit reaction exhibited first-order kinetics at every internal concentration tested, i.e., it showed no saturation.

The exit reaction resembled the entry reaction in its requirement for energy, as it was stimulated sixfold by ATP or a related compound and was not stimulated by the energized membrane state.

MATERIALS AND METHODS

Bacterial strains. The strains used in this work are all derivatives of *E. coli* K-12 and are listed in Table 1.

TABLE 1. *Bacterial strains*

Strain[a]	Relevant properties
D115	Wild type (36)
D193	*uncA* (mutant lacking the Ca^{2+}, Mg^{2+} ATPase)
OW1	Wild type for galactose uptake
OW2	*mglB*$^-$ mutant with a defective galactose-binding protein
OW17	*mglA*$^-$ mutant with lowered activity of the β-methyl galactoside transport system
OW26[b]	*mglC*$^-$, properties similar to those of OW17
OW22	*mglA*$^-$, lacks the β-methyl galactoside transport system
OW31	*mglC*$^-$, properties similar to those of OW22
OW41	*mglA*$^-$*C*$^-$, properties similar to those of OW22

[a] D193 was isolated by transducing strain 7 (wild type [13]) to β-glucoside utilization by the method described in reference 21 using P1 phage grown on a β-glucoside-positive derivative of strain AN120 *uncA* (9). The β-glucoside-positive derivative of AN120 was isolated on plates containing the β-glucoside, salicin, after diethyl sulfate mutagenesis. The β-glucoside-positive transductants of strain 7 were restreaked to purify the colonies and then tested by spotting onto minimal succinate and minimal glucose plates. Strain D193 grew on glucose but not on succinate and lacked the Ca^{2+}, Mg^{2+} adenosine 5'-triphosphatase (ATPase) activity. All OW strains (obtained from George Ordal) are *thr*$^-$ *leu*$^-$ *his*$^-$ and were described in references 23 and 24.

[b] At present mutants in the *A* and *C* cistrons of the *mgl* locus have identical phenotypes by every known test.

Chemicals. D-[^{14}C]galactose, [^{14}C]glycerol, and β-[^{14}C]methyl-D-galactoside were purchased from New England Nuclear Corp., Boston, Mass. β-[^{14}C]glycerol-D-galactoside was synthesized from [^{14}C]glycerol by the procedure of Boos, Lehmann, and Wallenfels (8). Unlabeled D-glycerol-β-D-galactoside was the generous gift of Thomas Silhavy, a student of W. Boos. Other sugars and sugar analogues were purchased from Sigma Chemical Co., St. Louis, Mo.

Growth of cells. Cells were grown at 37 C with shaking in Erlenmeyer flasks containing less than 10% of their volume of phosphate-buffered minimal medium (34). Carbon sources were always added at a concentration of 0.2%. Growth was stopped when the culture reached a density of about 7×10^8 cells/ml (optical density at 600 nm of 0.80, 1-cm path length, in a Gilford spectrophotometer). The cells were centrifuged at room temperature ($7000 \times g$ for 7 min), resuspended in 5 ml of minimal medium, and recentrifuged. This wash was repeated, and the final cell pellet from 10 ml of original culture was resuspended in 1 ml of minimal medium lacking carbon source and stored at 0 C for, at most, 4 h before making the transport measurements.

Transport measurements. An amount of the above cell suspension which would take up from 1 to 25% of the substrate (usually 0.2 ml) was added to the volume of fructose minimal medium needed to give a 0.6-ml final volume in a tube (12 by 75 mm) and incubated for 10 min at room temperature

(21 C). The reaction was started by the addition of 10 μl of ^{14}C-labeled substrate; 0.2-ml portions of the cell suspension were removed and filtered onto 25-mm, 0.45-μm nitrocellulose filters (presoaked in 0.15 M NaCl) 30 and 60 s after the substrate was added. The filtered cells were washed with 4 ml of 0.15 M NaCl at room temperature. When a competing sugar was present, it was added immediately before the addition of substrate. The filters were dried in an 80 C oven and counted in a Packard Tri-Carb counter using a scintillation fluid containing 4 g of Omnifluor per liter of toluene. The efficiency was 65%. All data are reported as nanomoles per milligram of cell protein. Cell protein was determined according to Lowry et al. (20). β-[^{14}C]methyl-D-galactoside was used at a specific activity of 5.9×10^3 counts/min per nmol unless another value is given.

All transport rates were calculated from the average of the rates determined from the 30- and 60-s time points. The value of the 60-s time point never differed from twice the value for the 30-s time point by more than 20%.

Exit measurements. Cells prepared as above (0.6 ml) were added to 1.2 ml of fructose minimal medium containing 5×10^{-6} M β-[^{14}C]methyl-D-galactoside and incubated for 30 min at room temperature. They were then centrifuged, and the pellet was resuspended in 18 ml of 30 C fructose minimal medium in a 150-ml beaker (time zero) and kept at 30 C. Aliquots of 3 ml were removed, filtered, washed, and counted exactly as for the uptake assay. Normally samples were taken at 1, 2, 3, 5, and 10 min after the final resuspension. The $t_{1/2}$ for the exit reaction was determined from the initial linear slope. When indicated, unlabeled sugars or inhibitors were present in the 18 ml of medium used to resuspend the preloaded cells. When arsenate inhibition was studied, the preloaded cells were resuspended in medium B (4) containing arsenate, but otherwise the exit assay was run as described above.

RESULTS

Specificity of uptake. An experimental difficulty encountered in studying the β-methyl galactoside transport system is that most of its substrates can be transported by other systems (11). This problem was avoided with the substrates D-glycerol-β-D-galactoside and β-methyl-D-galactoside by growing the cells at 37 C in the absence of inducers of lactose permease. Under these conditions the cells contain undetectable levels of lactose permease and melibiose permease, the two other systems which can transport these substrates (11). This was tested by measuring the uptake of thiomethyl-D-galactoside, which is a substrate of both lactose and melibiose permeases. The results in Table 2 show there was no uptake of thiomethyl-D-galactoside. Furthermore, all β-methyl-D-galactoside uptake in both D115 and the OW strains was completely inhibited by 8×10^{-5} M D-glucose, which is the substrate of the β-methyl galactoside transport system with the highest affinity and is not a substrate of any of the other systems that can transport β-methyl-D-galactoside (lactose or melibiose permease), although glucose is transported by two phosphotransferase systems. Other evidence that uptake of the two β-galactosides is due only to the β-methyl galactoside transport system is that their uptake showed only a single K_m in each case, and the K_m values (Table 3) are in excellent agreement with the values in the literature (6) for this system.

TABLE 2. *Specificity of β-methyl-D-galactoside uptake*

Strain	Transport substrate (uptake rate, nmol/min/mg)[a]		
	TMG	β-Methyl-D-galactoside	β-Methyl-D-galactoside plus unlabeled glucose
D115	0.05	5.9	0.2
OW1	0.02	5.2	0.1
OW17	0.02	2.9	0.2
OW26	0.02	2.9	0.1

[a] Cells were grown, harvested, and assayed for uptake as described in the Material and Methods section, except that when thiomethyl-D-galactoside (TMG) was the substrate samples were taken at 20 and 40 s. TMG was added to give a concentration of 1.7×10^{-5} M and had a specific activity of 2.2×10^{-4} counts/min per nmol. β-Methyl-D-galactoside was added to give a concentration of 3×10^{-5} M, and unlabeled D-glucose was added at a concentration of 8×10^{-5} M in the assays shown in the last column.

In the case of D-galactose, which is transported by many systems, the K_m for the β-methyl galactoside transport system of 5×10^{-7} is far lower than the next higher K_m, which is 1.7×10^{-4} M for the galactose-specific transport system (36). The V_{max} values for these two systems are nearly the same, and so, by using concentrations of D-galactose below 5×10^{-6} M, more than 90% of the measured uptake will be due to the β-methyl galactoside transport system.

Kinetic properties of the entry reaction. The K_m and V_{max} values for the rates of uptake of the three substrates D-galactose, β-methyl-D-galactoside, and D-glycerol-β-D-galactoside, were calculated from plots of S/V versus S using data obtained with strain D115. Figure 1 shows the results for β-methyl-D-galactoside as an example of the data obtained. The calculated values of K_m and V_{max} are given in Table 3 along with the values of K_i obtained when each substrate was used to inhibit the uptake of one of the other substrates. The K_i values were obtained from plots of I/V versus I. The plots for

TABLE 3. *Kinetic constants for the β-methyl galactoside transport system*

Substrate[a]	K_m (M)	V_{max} (nmol/min/mg)	K_i		
			D-Galactose	D-Glycerol-β-D-galactoside	β-Methyl-D-galactoside
D-Galactose	5.3×10^{-7}	12		1.0×10^{-6}	1.8×10^{-4}
D-Glycerol-β-D-galactoside	7.5×10^{-7}	20.2	3.8×10^{-7}		2.8×10^{-4}
β-Methyl-D-galactoside	1.8×10^{-5}	20.8			

[a] Strain D115 was grown, harvested, and assayed for transport as described in the Materials and Methods section. The values of K_m, K_i, and V_{max} were determined in experiments such as those shown in Fig. 1 and 2. The values for D-galactose and D-glycerol-β-D-galactoside inhibition of β-methyl-D-galactoside uptake could not be measured at the concentrations of inhibitor that are appropriate to determine K_i, as the inhibitor was removed by transport before the assay was complete. The other two substrates of the β-methyl galactoside transport system, D-glucose and D-fucose, were not studied, as they can be taken up by other transport systems present in strain D115.

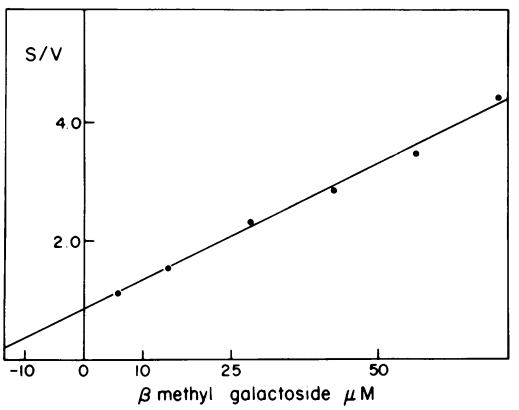

FIG. 1. *Determination of the K_m for β-methyl-D-galactoside transport in strain D115. S, substrate concentration in molar units; V, rate of uptake in millimoles per minute per milligram.*

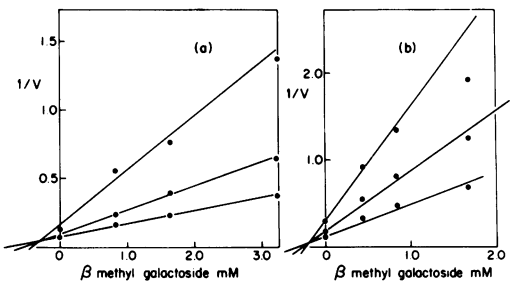

FIG. 2. *Determination of the K_i for β-methyl-D-galactoside inhibition of D-glycerol-β-D-galactoside uptake (a) or D-galactose uptake (b) in strain D115. The substrate concentrations tested were 0.57, 1.42, and 2.84 $\times 10^{-6}$ M in (a) and 1.6, 3.2, and 6.4 $\times 10^{-7}$ M in (b).*

β-methyl-D-galactoside inhibition are shown in Fig. 2.

All of the S/V versus S plots were linear; the plots of I/V versus I were reasonably linear, and they indicated that the inhibition is competitive. The K_m for D-galactose uptake is equal to the K_i for galactose inhibition of D-glycerol-β-D-galactoside uptake. Similarly, the K_m for D-glycerol-β-D-galactoside uptake is equal to the K_i for D-glycerol-β-D-galactoside inhibition of D-galactose uptake. All of these are the expected results if the β-methyl galactoside transport system fits the Michaelis-Menten model. The unexpected finding that the V_{max} value for D-galactose uptake by the β-methyl galactoside transport system is lower than the V_{max} values for the other two substrates may result from the interaction that occurs between the galactose-specific and β-methyl galactoside transport systems (36), as D-galactose is the only substrate transported by both systems.

The one result which cannot be fitted to the Michaelis-Menten model is the fact that the K_i values for β-methyl-D-galactoside inhibition of the other two substrates (2×10^{-4} M) are significantly larger than the K_m for β-methyl-D-galactoside uptake (2×10^{-5} M). This result suggests that the mechanism of β-methyl-D-galactoside transport differs in part from the mechanism for the other two substrates.

Additional evidence for such a difference comes from studies of the uptake of the three substrates by certain mutants altered in the genes encoding the components required for the β-methyl galactoside transport system (Table 4). Strains OW2, OW22, OW31, and OW41 completely lack uptake of all three substrates, but strains OW17 and OW26, which retain about 12% of the wild-type level of D-galactose and D-glycerol-β-D-galactoside transport, have over 50% of the wild-type level of β-methyl-D-galactoside transport. The K_m for β-methyl-D-galactoside transport and its sensitivity to inhibition by other sugars are not altered in OW17 or OW26 (Table 2).

Inhibition of the β-methyl galactoside entry reaction by high osmolality. The β-methyl-D-galactoside entry reaction was strongly inhibited by every salt tested and by

sucrose and glycylglycine when they were present at concentrations greater than 0.15 M (Table 5). This inhibition was completely reversible and rapid. This inhibition is not a general property of transport systems or of releasable binding protein transport systems, since uptake by the glutamine-, proline-, and galactose-specific transport systems showed much less inhibition. In fact the galactose-specific transport system showed very little inhibition even with 0.25 M KCl, which caused at least 50% inhibition for all other systems tested.

TABLE 4. *Rates of uptake of the β-methyl galactoside transport system in mutants*

Strain[a]	Transport substrate (uptake rate, nmol/min/mg)		
	D-Galactose	β-D-Glycerol-β-D-galactoside	β-Methyl-D-galactoside
OW1	5.2	5.2	5.2
OW2	0.15	0	0
OW22	0.26	0	0
OW31	0.06	0	0
OW41	0.20	0	0
OW17	0.78	0.58	2.9
OW26	0.66	0.58	2.9

[a] Cells grown in minimal fructose medium containing 10^{-3} M D-fucose and 10 μg each of threonine, leucine, and histidine per ml were harvested, and uptake was assayed. The substrates used were 1.7×10^{-6} M D-[^{14}C]galactose, 1.5×10^{-6} M D-[^{14}C]glycerol-β-D-galactoside, and 2.8×10^{-5} M β-[^{14}C]-methyl-D-galactoside.

TABLE 5. *Inhibition of some transport systems by high osmolality*[a]

Compound added	Transport substrate (uptake rate, nmol/min/mg)			
	β-Methyl-D-galactoside	Gln	Pro	Gal
None	7.1	3.7	1.7	14.2
0.25 M KCl	0.1	1.6	0.6	9.7
0.15 M K_2SO_4	0.4	2.05	1.2	11.0
0.25 M glycylglycine	0.65	1.8	1.1	11.4
0.25 M sucrose	0.0	3.3	2.0	11.2
0.25 M KCl then removed	7.3			

[a] Strain D115 was grown, harvested, and assayed for transport as described in the Materials and Methods section, except that samples were taken at 20 and 40 s when glutamine (Gln) and proline (Pro) uptake was measured. Inhibitors were added 10 s before uptake was initiated by the addition of labeled substrate. The concentrations of substrates used were 2×10^{-5} M for β-methyl-D-galactoside, 4×10^{-5} M for Gln and Pro, and 2×10^{-4} M for Gal. In the last line, cells were incubated with KCl for 30 min, and then centrifuged, resuspended in fructose minimal medium, and assayed for transport.

The steady state level of β-methyl-D-galactoside. To determine the steady state level of β-methyl-D-galactoside uptake, it is necessary to show that β-methyl-D-galactoside is not metabolized in strain D115. This was tested in two ways. In the simplest test, equal aliquots of the total sample from the last point in Fig. 3 were counted with or without being dried down. If there were metabolism, the dried sample would have a lower value since β-[^{14}C]methyl-D-galactoside is hydrolyzed to [^{14}C]methanol, which is volatile; however, the values for the two samples were the same. In the second test an aliquot of cells was incubated with β-[^{14}C]methyl-D-galactoside for 90 min and filtered. The accumulated β-methyl-D-galactoside was released by washing the filter with 10 ml of cold distilled water. This procedure removed 94% of the radioactivity, and all of the released radioactivity co-chromatographed with β-methyl-D-galactoside in butanol–ethanol–water (50:32:18).

The data presented in Fig. 3 demonstrate that the uptake of β-methyl-D-galactoside reached a steady state and that the initial rate and steady state level of uptake were not low-

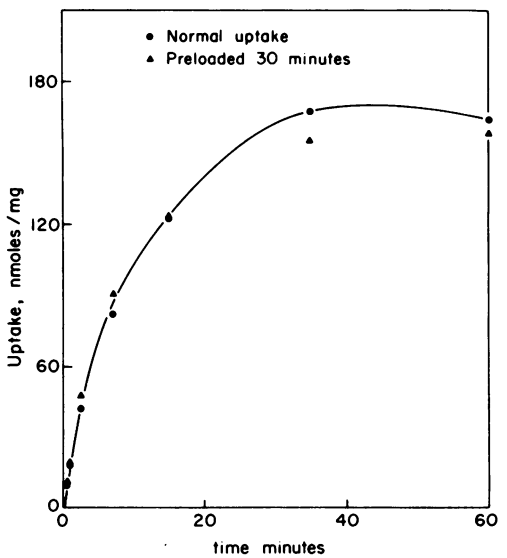

FIG. 3. *Effect of preloading with β-methyl-D-galactoside on its uptake. Cells of strain D115 (0.6 ml) were added to 1.2 ml of minimal fructose medium containing 50 μg of chloramphenicol per ml. They were incubated 10 min at 21 C, and then 1.0 μmol of unlabeled β-methyl-D-galactoside and 0.10 μmol of β-[^{14}C]methyl-D-galactoside were added. Samples (0.2 ml) taken at the indicated times were filtered, washed, and counted. The preloading experiment was run identically, except that the unlabeled substrate was added to initiate the reaction and labeled substrate was added 30 min later.*

ered by the presence of a pool of β-methyl-D-galactoside in the cells. This result indicates that the steady state level of β-methyl-D-galactoside is due to a dynamic equilibrium between uptake and exit. Whereas this result has been found for most *E. coli* active transport systems that have been studied, there are transport systems, e.g., phosphate uptake in *E. coli* (21) and amino acid transport in yeast (10), where the steady state level results from the fact that the uptake reaction is inhibited by the pool of free substrates in the cell.

Properties of the exit reaction. The exit of β-methyl-D-galactoside followed first-order kinetics (Fig. 4 and 5) even at the highest internal concentration tested, 3×10^{-2} M. The internal concentration was calculated using a value of a 5.4-μl internal volume per mg of protein (37). As shown in Fig. 5, the exit was first order at every temperature tested from 12 to 37 C.

The presence in the external medium of substrates of the β-methyl galactoside transport system did not change the initial rate of exit, although they did allow exit to follow first-order kinetics for a longer time (Fig. 4). This was probably due to the inhibition of the reentry of the material which had left the cells.

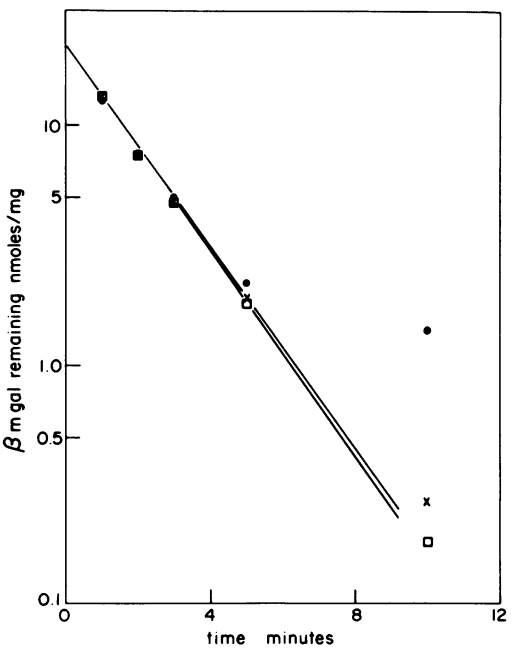

FIG. 4. *Lack of an effect of transport substrates on the initial rate of exit of β-methyl-D-galactoside in strain D115. Strain D115 was grown, harvested, preloaded, and assayed for exit at 21 C as described in Materials and Methods. Symbols: (●) No addition to the exit medium; (×) 1.0 × 10^{-3} M D-galactose present during exit; (□) 1.0 × 10^{-3} M D-glucose present during exit.*

An unexpected finding was that the exit reaction appeared to require ATP or a related compound, as is shown in Table 6 and Fig. 6. In a strain which carries a mutation which inactivates the Ca^{2+}, Mg^{2+} adenosine 5'-triphosphatase, exit was strongly stimulated by a glycolytic substrate, fructose, but was only slightly stimulated by an oxidative substrate, D-lactate. Furthermore, arsenate strongly inhibited exit

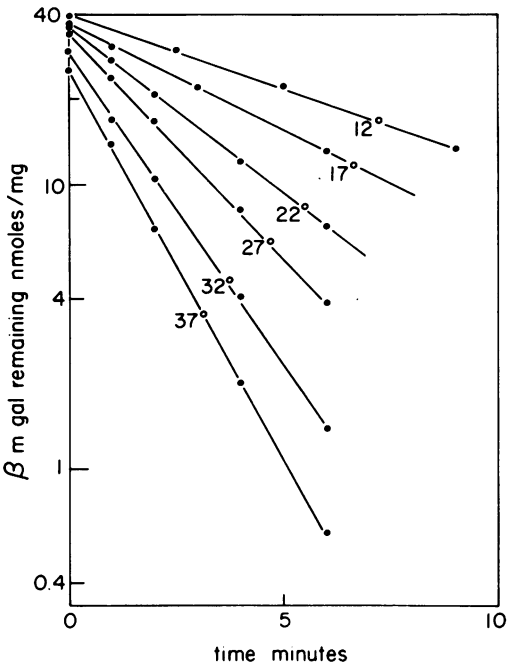

FIG. 5. *Exit of β-methyl-D-galactoside at various temperatures. The exit medium contained 1.0 × 10^{-3} M D-glucose and was maintained at the indicated temperature.*

TABLE 6. *Energy requirement of the exit reaction*[a]

Energy source	Inhibitor	Exit t_i (min)
None		13
D-Lactate		11
Fructose		2.0
Fructose	FCCP	5.4
Fructose	Arsenate	8.4

[a] Strain D193 was grown, harvested, and preincubated with β-[^{14}C]methyl-D-galactoside as described in the Materials and Methods section. The t_i for the exit reactions were measured as described in the Materials and Methods section, except that the exit media contained 1.0 × 10^{-3} M unlabeled β-methyl-D-galactoside, the indicated carbon source at a concentration of 0.2%, and, where indicated, 0.5 mM arsenate or 8.3 μM carbonyl cyanide-*p*-trifluoromethoxyphenylhydrazone (FCCP). When the exit medium lacked a carbon source or contained D-lactate or arsenate, samples were taken at 1, 3, 7, 13, and 20 min.

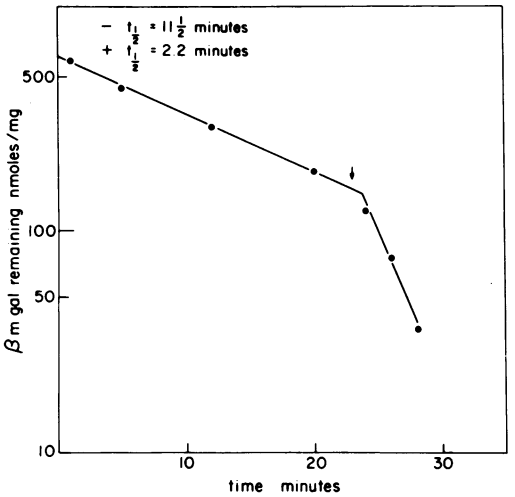

FIG. 6. *Stimulation of β-methyl-D-galactoside exit by an energy source. Strain D115 was prepared and assayed for exit as described in Materials and Methods, except that the cells were preloaded in the absence of an energy source with 2 × 10^{-3} M β-[^{14}C]methyl-D-galactose (specific activity, 340 counts/min per nmol) for 60 min, and the pelleted cells after preloading were resuspended in 24 ml of minimal medium lacking an energy source. At 26 min D-fructose was added to give a final concentration of 0.2%.*

even in the presence of fructose, whereas the uncoupler carbonyl cyanide-*p*-trifluoromethoxyphenylhydrazone gave an intermediate inhibition. These results are all very similar to those found for the uptake reaction (35).

The rate of exit was determined separately for cells carrying mutations in each of the three cistrons (*mglA, B,* and *C*) required for a functional β-methyl galactoside transport system (Table 7). The rate of exit was similar to the wild type in every strain even when the mutations caused a complete loss of the entry reaction.

It was necessary to use a high external concentration (2 × 10^{-3} M) for preloading the strains mutated in the *A* and *C* cistrons to obtain an internal concentration near 2 × 10^{-3} M used for the other strains. Since the exit from these mutants was inhibited in the absence of an energy source (data not shown) exactly as was the exit of a wild-type strain, it is likely that the process seen in the mutants is exit and not an artifact such as the release of externally bound material.

The regulation of the exit reaction was studied by determining the rate of exit (i) in cells of strain D115 grown in minimal medium with fructose as a carbon source (uninduced cells), (ii) in cells grown in the same medium containing 1 × 10^{-3} M D-fucose (induced cells), and (iii) in cells grown in minimal medium containing D-glucose as a carbon source (repressed cells). Glucose-grown cells have extremely low levels of both the β-methyl galactoside entry reaction and the galactose-binding protein (18, 35). The exit rate was the same in induced and uninduced cells and was slightly faster in repressed cells (Table 7). An additional difference between the uptake and exit reactions of the β-methyl galactoside transport system is that exit was not inhibited at all (Table 7) by a concentration of KCl which almost completely inhibited uptake.

DISCUSSION

The unusual kinetics (K_i 10 times greater than K_m) found for the β-methyl-D-galactoside entry reaction are extremely puzzling. One possible explanation would be the existence of two or more binding sites for β-methyl-D-galactoside which interact with each other. However, such a model would predict cooperativity in β-methyl-D-galactoside transport, and this is not observed. Furthermore, the galactose-binding

TABLE 7. *β-Methyl-D-galactoside exit and uptake*

Strain[a]	Uptake (nmol/min/mg)	Exit rate $t_{\frac{1}{2}}$(min)	Initial concn in cell (mM) prior to exit measurements
D115 uninduced	0.85	1.8	0.17
D115 induced	7.3	1.8	0.4, 2.0
D115 repressed	0.20	1.0	0.28
D115 induced + 0.2 M KCl	0.3	2.0	0.45
OW1 induced	2.8	2.4	0.22
OW2 induced	0.05	2.2	0.22
OW22	0	3.1	2.0
OW31	0	3.3	0.9
OW41	0	2.1	1.4
OW26	2.9	2.7	0.8

[a] Cells were grown in minimal fructose medium to which 10^{-3} M D-fucose was added to obtain induced cells. Repressed cells were grown in minimal glucose medium. For the assays of exit rate, the cells were preloaded with β-[^{14}C]methyl-D-galactoside at a concentration of 10^{-5} M for induced D115 and OW1 and 6 × 10^{-5} M for uninduced and repressed D115 and for OW2. The β-methyl-D-galactoside used for preloading OW22, OW31, OW41, and OW26 had a specific activity of 340 counts/min per nmol and was used at a concentration of 2 × 10^{-3} M. Exit assays carried out with induced D115 cells using β-methyl-D-galactoside solutions of either specific activity gave identical rates of exit. In the experiments reported in line 4, exit and uptake assays were run normally except that 0.2 M KCl was present during the assays.

protein, which is the component most likely to have such binding sites, is a single polypeptide chain which has never been shown to aggregate. In my experiments the galactose-binding protein has a single binding site for galactose as was reported by Anraku (2), although Boos has reported two moles of galactose bound per mole of binding protein (7). The fact that β-methyl-D-galactoside uptake did not behave in the same way as the uptake of the other two substrates of this system in certain mutants of the *mglA* and *mglC* cistrons provides further evidence that the mechanism of the β-methyl-D-galactoside entry reaction differs from that of the other two substrates and suggests that this difference may involve the components encoded by the *mglA* and *mglC* cistrons.

The studies of the exit reaction show that it differs from the entry reaction in several important ways. First, the exit reaction followed first-order kinetics at every internal concentration tested and thus shows no sign of saturation. The highest internal concentration obtained in these studies was 3×10^{-2} M, so that if the exit reaction does saturate, its K_m must be greater than 0.1 M. The lack of saturation means that the exit reaction will be faster than the V_{max} of the reaction entry if the internal concentration is high enough. The lack of saturation also means it is not possible to study the specificity of the exit reaction by trying to inhibit the exit of β-methyl-D-galactoside with other substrates.

Second, the exit reaction does not appear to require the products of the β-methyl galactoside operon which are all essential for the entry reaction. The fact that the exit reaction occurred at the wild-type rate in every *mgl*⁻ strain tested is strong evidence for the above conclusion; however, it is not conclusive evidence, since the OW mutants are not chain-termination mutants or deletion mutants. The strongest evidence that the products of the β-methyl galactoside operon are not required for the exit reaction is the fact that exit was not repressed by growth on glucose. Cells grown on glucose have both an extremely low level of the galactose-binding protein (18, 36) and a very low rate of the β-methyl-D-galactoside entry reaction. Robbins (28) has shown that all three β-methyl galactoside cistrons are under the control of a single operator gene and each cistron must be translated at the induced rate for the β-methyl-D-galactoside transport system to function normally. This indicates that all three β-methyl galactoside cistrons are probably repressed by growth on glucose. These experiments confirm the report of Parnes and Boos (26) that exit does not require the galactose-binding protein. A similar result has been reported for glutamate transport in *E. coli* (12).

Third, the exit reaction is not under the control of either of the two regulatory systems which regulate the entry reaction. Growth in the presence of D-fucose induced the entry reaction ninefold but did not change the exit reaction. Growth in glucose minimal medium repressed the entry reaction to 25% of the uninduced rate, whereas the exit reaction was increased slightly.

Finally, the exit reaction was not inhibited by 0.2 M KCl which caused more than 95% inhibition of the entry reaction.

The one similarity between the exit and entry reactions is that they both require ATP or a related compound. The stimulation of the exit reaction by an energy source is a surprising finding, since exit is proceeding along a concentration gradient. However, the results show only that ATP or a related compound must be present in the cell for exit to occur; they do not show that energy is consumed during exit.

Parnes and Boos (26) had reported that the exit reaction of the β-methyl galactoside transport system was increased by the presence of its substrates in the external medium. The results in Fig. 4 show that when an energy source is present there is no significant stimulation of the initial rate of exit by the addition of any substrate to the exit medium. The stimulation of efflux by glucose observed by Parnes and Boos is probably attributable to its role as an energy source, since they supplied no energy source during their exit measurements. The small stimulation of exit they found by adding external galactose may have resulted from inhibition of the re-entry of labeled galactose which had left the cell.

The experiments of Robbins and Rotman (29) suggest that the *mglA* and *mglC* cistrons code for the membrane carrier of the β-methyl galactoside transport system. The results reported in this paper show that the products of these two cistrons do not function in the exit reaction. This could indicate that the exit and entry reactions utilize different membrane carriers. However, there is another explanation of the data. The *A* and *C* gene products may not be the carrier but rather have another function, possibly to catalyze the energy-dependent release of substrate from the binding protein to another protein which would be the actual carrier. Since all of the known mutations affecting the β-methyl galactoside transport system are in either the *A*, *B*, or *C* cistrons, this new protein would probably have to have an essential function for the cell to explain why mutations in it have not been found. The inability of mutants

in the *mglA* and *mglC* cistrons to grow on β-methyl-D-galactoside found by Robbins and Rotman (29) would then be explained not by the loss of the carrier function, but by the fact that these strains are unable to concentrate β-methyl-D-galactoside. The binding protein mutant studied here was able to concentrate β-methyl-D-galactoside several fold. Since the K_m for hydrolysis of β-methyl-D-galactoside by β-galactosidase is very high, the rate of hydrolysis would be significantly faster in the *B* mutants than in the *A* and *C* mutants. Further experiments are required to distinguish between this possibility and the possibility that separate carriers function in exit and uptake.

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