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Efflux and the Steady State in α-Methylglucoside Transport in *Escherichia coli*

HERBERT H. WINKLER

Department of Microbiology, School of Medicine, University of Virginia, Charlottesville, Virginia 22903

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Efflux and the steady state in a group translocation system, the α-methylglucoside (αMG) transport system, were investigated. The maximum intracellular level of α-methylglucoside is a function of a steady state. There is no inhibition of αMG influx as the intracellular pool of αMG, and α-methylglucoside-6-phosphate (αMGP) rises. This steady state has three components: αMG influx, action of an αMGP phosphatase, and αMG efflux. The phosphatase is the rate-limiting step (half-time = 5.0 min); thus, the true efflux rate (half-time = 2.0 min) cannot be simply measured from the kinetics of αMG loss from the cell. Under our steady-state conditions the percentage of intracellular radioactivity present as αMGP was 71%. Under conditions of zero influx, after an efflux of 12 min the percentage present as αMGP fell to 55%. However, when fluoride was present during the efflux period, the percentage of the sugar as αMGP increased to about 85%. Fluoride greatly inhibits both influx and phosphatase activity (half-time = 50 min). The efflux of αMG from the cell is apparently also fluoride-sensitive but to a lesser extent (half-time = 4.1 min). These data are summarized in a model describing the three components of the steady-state and effect of fluoride.

A hallmark of carrier-mediated transport, whether active transport or facilitated diffusion, is that the carrier functions in both directions, i.e., in both efflux and influx. The efflux mechanism, in a transport system in which the physiological role of the system is to move a metabolizable substance into the cell, has a twofold role. First, efflux may permit a highly specific and controllable mechanism to prevent the concentration from building up in the cytoplasm to overly high levels. By means of the efflux mechanism, the cell can eliminate the transported compound and establish an appropriate steady-state intracellular level. Second, and more fundamental, the efflux mechanism provides for the return of the carrier (or a reorientation of the active site) to the location necessary for continued influx. Except in the case of obligatory exchange diffusion, the presence of the substrate on the carrier is not required for its return. An example of the role of efflux in the formation of a steady state is the case of the active transport of lactose by *Escherichia coli*. Here the energy coupling serves to diminish the affinity of the efflux system so that the lactose in the cell does not exit at a rate equal to its influx until the steady-state concentration in the cell is many times that in the medium (16).

The uptake of glucose and its analogue, α-methylglucoside (αMG), by *E. coli* occurs neither by facilitated diffusion nor by active transport, but by group translocation (9, 13). In group translocation the substrate is chemically altered by a vectorial enzyme system in the membrane during its passage across the membrane. Therefore, the transported species appearing in the cytoplasm is different from that in the medium. Thus, in the case of αMG transport, αMG is removed from the medium and α-methylglucoside-6-phosphate (αMGP) is delivered to the cytoplasm via the phosphoenolpyruvate (PEP)-phosphotransferase first described by Kundig et al. (9). Since in the absence of HPr~P or enzyme I, the enzyme II for galactose can function as a facilitated diffusion system (13), it seems appropriate to consider the PEP-phosphotransferase system for sugar transport in *E. coli* within the broad category of carrier-mediated transport. The relationship of efflux to enzyme II will be of key significance in this categorization.

This investigation concerns the nature of the steady state and the role of efflux in the trans-

port of αMG, a system in which the influx is by group translocation.

MATERIALS AND METHODS

Bacterial strains, media, and growth conditions. The strain used throughout this study was DF2000 (3). The strain received from D. Fraenkel was derived from *E. coli* K-10, a prototrophic Hfr strain. The mutant is missing the enzymes phosphoglucose isomerase and glucose-6-phosphate dehydrogenase. The strain was chosen for study so that glucose could eventually be used as a nonmetabolizable substrate. Our basal medium was the phosphate-based medium, Davis Medium A (1), supplemented with thiamine (1 μg/ml) and $FeSO_4 \cdot 7H_2O$ (0.0005%). Glycerol (0.4%) was used as carbon source. Cells were grown for three doublings to obtain an exponentially growing culture. The cells were washed once and resuspended in basal medium with the indicated additions.

Uptake assay. Cells were incubated with ^{14}C-αMG, filtered on membrane filters (Millipore Corp., Bedford, Mass.), and washed; radioactivity was determined as previously described (15). All assays were performed at 23 C.

Efflux assay. Cells were incubated with isotopically labeled medium for 25 min and then diluted 200-fold into the indicated medium to allow efflux. A sample (20 ml) was filtered at the indicated times but was not washed, and radioactivity of the filtered cells was determined. When the intracellular pools were to be determined, the dilution was reduced to 10-fold, 5 ml was filtered and washed, and 47-mm filters were used instead of 25-mm filters. The changes were made to have adequate levels of αMG and αMGP in the extracts.

Cell water. Intracellular water was determined as previously described (16) on the parental strain, and this value was used to calculate the intracellular concentration. When αMG and αMGP were not separated, the intracellular concentration refers to the sum of the two radioactive species. A value of 0.5 μliters of cell water per 0.5 ml of cells at 100 Klett units at 420 nm was obtained. This corresponds to 2.5 μliters of cell water per mg (dry weight).

Determination of the intracellular pool. An example of cells corresponding to 1 to 2 μliters of cell water was filtered on a 0.45-μm pore size membrane filter of 47-mm diameter, and the filtered cells were washed with 5 ml of basal medium at room temperature. The filtered cells were then extracted with 3 ml of boiling water for 10 min, cooled, and centrifuged, and the volume was adjusted back to 3 ml. A 2-ml sample of the supernatant fluid was applied to a column (4 by 0.6 cm) of AG 1-X8 formate resin in a Pasteur pipette. The neutral and anionic forms were eluted, and the radioactivity was determined as previously described (14).

The efflux medium was analyzed by passing a 1-ml sample of the cell-free medium over the column described above. These values were corrected for the extracellular αMG and αMGP present in the sample of incubated cells that had been added to the efflux medium. The cells were removed from the medium either by centrifugation or by filtering the suspension through a dry membrane filter.

Theoretical simulations. First-order rate constants were assumed for both the phosphatase and efflux based on the closeness of fit of the experimental data to a straight line on a plot of log intracellular concentration versus time. Let $d[\alpha MGP]/dt = -[\alpha MGP]k_1$ and $d[\alpha MG]/dt = [\alpha MGP]k_1 - [\alpha MG]k_2$ represent the rate equations for the loss of intracellular αMGP and αMG, respectively. Integration of these equations gives:

$$[\alpha MGP] = [\alpha MGP]_0 e^{-k_1 t} \quad (1)$$

$$[\alpha MG] = [\alpha MG]_0 e^{-k_2 t} + (k_1/k_2 - k_1)[\alpha MGP]_0 (e^{-k_1 t} - e^{-k_2 t}) \quad (2)$$

$$[\alpha MG] + [\alpha MGP] = \{[\alpha MG]_0 + (k_1/k_1 - k_2)[\alpha MGP]_0\} e^{-k_2 t} - (k_2/k_1 - k_2)[\alpha MGP]_0 e^{-k_1 t} \quad (3)$$

Computer simulations for intracellular αMGP, αMG, and the total radioactivity with time were obtained by using a Hewlett-Packard 9100 calculator and plotter.

RESULTS

Steady state. The level of radioactivity within cells incubated with ^{14}C-αMG plateaus as a function of time is shown in Fig. 1. This plateau could be due to either a balance of influx and efflux, i.e., a steady state, or to a decreased rate of influx resulting from a negative feedback by the intracellular pool as has been suggested by work with membrane vesicles (7). That the steady-state model is the correct one is shown in Fig. 1. In this experiment, cells were incubated with ^{12}C-αMG for 30 min, at which time the extracellular concentration of ^{12}C-αMG had not changed, but the intracellular level plateaued at the level indicated by the uptake of radioactive αMG in the companion experiment. At 30 min, ^{14}C-αMG was added to the system, and the intracellular radioactivity was determined at the indicated times. The steady-state model would predict that the uptake in these preloaded cells would have the same time course as the control cells, whereas the feedback model would predict a greatly inhibited uptake by the preloaded cells. The time course of entry in these preloaded cells, including the early points as shown in the insert in Fig. 1, has the same time course that the control cells exhibited when not preloaded with ^{12}C-αMG. This supports the steady-state model in which the influx rate is not influenced by the intracellular pool of αMG or αMGP.

Effect of azide and fluoride on influx. Figure 2 shows a confirmation of the results of Hoffee, Englesberg, and co-workers obtained several years ago (2, 5, 6). When the uptake of αMG is measured in a medium containing a metabolizable substrate (in our case glycerol), azide stimulated the uptake whereas fluoride inhibited it. As can be seen, after 2 min of incubation, the cells poisoned with fluoride barely equilibrated with

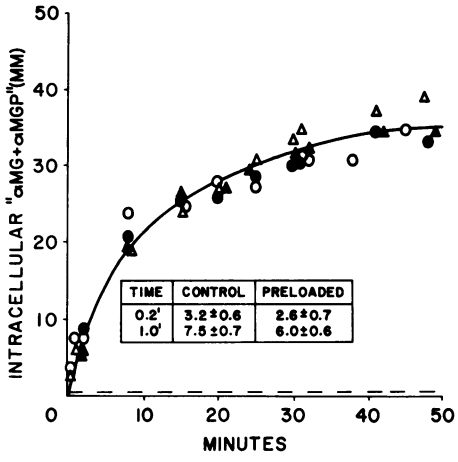

FIG. 1. *^{14}C-αMG uptake with and without preloading with ^{12}C-αMG. Glycerol-grown cells were harvested and resuspended in the basal medium plus NaN_3 (30 mM) and glycerol (0.01%). The cells indicated by the circles were incubated with ^{14}C-αMG (5×10^{-4} M, 0.1 μCi/ml) and filtered at the indicated times. Cells (△, ▲) were incubated with ^{12}C-αMG (5×10^{-4} M) for 30 min, and then ^{14}C-αMG (10 μCi/ml) was added (1% of the total volume) and the cells were filtered as before. Two experiments of each type are shown for the entire time course. The insert box shows the mean for five determinations of the early rates with standard deviations of the mean.*

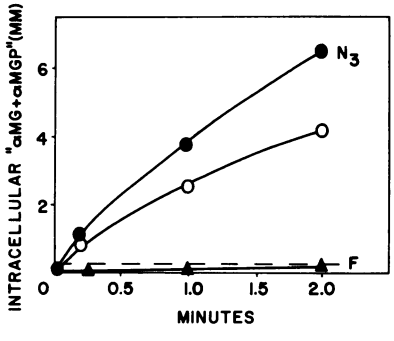

FIG. 2. *Effect of azide and fluoride on αMG uptake. Glycerol-grown cells were harvested and resuspended in basal medium plus glycerol (0.01%). NaN_3 (30 mM) was added to the cells in curve N_3, and NaN_3 (30 mM) plus NaF (20 mM) were added to curve F. The poisons were present for 5 min before the addition of ^{14}C-αMG (2.3×10^{-4} M, 0.1 μCi/ml). The dashed line indicates the concentration of αMG in the medium.*

the αMG in the medium, whereas those cells treated with azide had an intracellular concentration approximately 30 times that in the medium. The most probable mode for the inhibition of αMG influx by fluoride is an inhibition of enolase and a subsequent fall in the PEP pool needed for the operation of the phosphotransferase. The effect of azide has been rationalized by Roseman (13) as an antagonism between adenosine triphosphate formation and the synthesis of PEP.

αMGP remains in the cell. I confirmed for *E. coli* B and K strains my earlier report (14) that a cell of strain ML in the steady state contains both αMGP and αMG; approximately 70% of the total is αMGP. From these cells, at least 90% of the intracellular radioactivity can be lost from the cell under efflux conditions with no αMGP appearing in the medium. This experiment was performed in the presence and absence of substrate, azide, fluoride, and iodoacetate at 23 and at 15 C, and in no case was αMGP found in the medium. Thus, there must be a phosphatase present which dephosphorylates αMGP to αMG, the species which can exit from the cell. Furthermore, to maintain a steady-state level of radioactivity in which the percentage of the total radioactivity present as αMGP is constant, the phosphatase must be rate-limiting relative to the influx and efflux of αMG.

Effect of fluoride and αMG on the efflux of radioactivity. The efflux of radioactivity from cells which were incubated with ^{14}C-αMG and then diluted 200-fold into the indicated medium is shown in Fig. 3. The half-time for exit into the same medium used to reach the steady state (basal medium with glycerol and azide), except that nonradioactive αMG (10 mm) was present instead of the ^{14}C-αMG, is shown in the trace

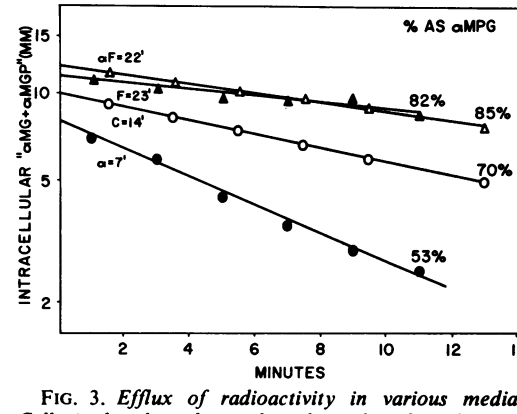

FIG. 3. *Efflux of radioactivity in various media. Cells in basal medium plus glycerol and azide were incubated with αMG (2.3×10^{-4} M, 0.1 μCi/ml) for 20 min and then diluted 200-fold into the indicated medium: (C) basal medium plus NaN_3 (30 mM) and glycerol (0.01%); (α) same as C plus αMG (1.0×10^{-2} M); (F) same as C plus NaF (20 mM); (αF) same as F plus αMG (1.0×10^{-2} M). At the indicated times 20 ml of the suspension was filtered, and at the conclusion of the trace 100 ml was filtered onto 47-mm filters and extracted, and the per cent of the radioactivity that was αMGP was determined.*

marked α. A value of 7 min was obtained. When the nonradioactive αMG was omitted (trace C), the half-time was now doubled to a value of 14 min. This is probably due to the recapture of exiting ^{14}C-αMG from the periplasmic space. Recapture was first suggested for efflux via the lactose transport system by Winkler and Wilson (16) and later confirmed and described in detail by Robbie and Wilson (10).

As shown in Fig. 3, fluoride (20 mM) slowed the rate of exit of radioactivity from the cell. The rate was essentially independent of the presence of nonradioactive αMG in the exit medium (traces αF and F, Fig. 3), since the recapture (influx) was blocked by fluoride (Fig. 2). Extraction of the cellular pool of αMG and αMGP after 11 to 13 min of efflux showed little change from the original steady state in the percentage of the total radioactivity present as the phosphorylated species when recapture was maximal (trace C; 70%). However, there was a fall in percentage of αMGP in the α trace (53%) and an increase in percentage of αMGP in those efflux measurements made in the presence of fluoride (82 and 85%). Since these results showed an effect of fluoride on the composition of the intracellular pool, experiments were performed in which the composition of this pool was followed as a function of time.

Alteration in the intracellular pool during efflux. To study the intracellular pool as a function of time, cells were incubated with ^{14}C-αMG for 20 to 30 min in the basal medium with glycerol and azide. These cells were then diluted 10-fold into basal medium with glycerol, azide, ^{12}C-αMG (10 mM), and with or without fluoride (20 mM). Samples of the cells were then filtered, washed, and extracted to determine the amount of ^{14}C-αMG and ^{14}C-αMGP within the cells. Similarly, the filtrate from unwashed cells was analyzed to determine the nature and amount of material which had exited from the cell.

Figure 4 shows the amount of ^{14}C-αMGP within the cell as a function of time. Since αMGP cannot leave the cell, the fall in the level of αMGP is due to the action of a phosphatase. The αMGP converted can be quantitatively accounted for by the increase of αMG in the cell and medium. In the absence of fluoride, the fall in the level of ^{14}C-αMGP appears to be a first-order process with a half-time of about 5 min. The presence of fluoride in the efflux medium inhibits the phosphatase, and a half-time of 50 min was approximated for the loss of ^{14}C-αMGP (Fig. 4). The trace shown by the dashed line in Fig. 4 depicts an experiment in which efflux was determined in the presence of fluoride but the inhibitor did not act immediately. The fall in the level of ^{14}C-αMGP, in this case, was biphasic, demonstrating the time constants for both the control and fluoride-inhibited phosphatases.

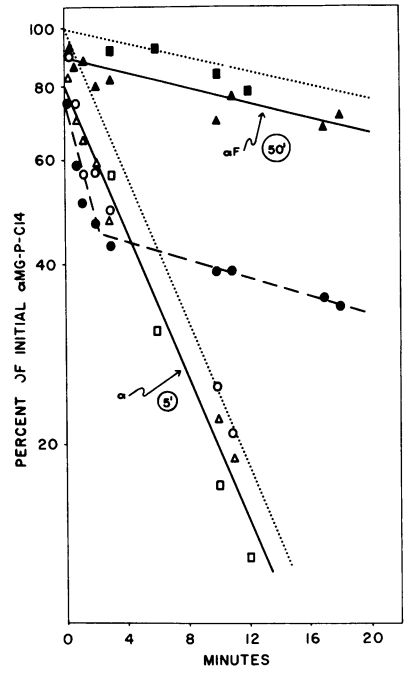

FIG. 4. *Effect of efflux on the intracellular pool of ^{14}C-αMGP. Glycerol-grown cells were washed and resuspended in basal medium plus glycerol and NaN_3 at about 800 Klett units. The suspension was incubated for 20 min with ^{14}C-αMG (initially 5 × 10^{-5} M, 0.2 μCi/ml). The suspension was then diluted-10 fold into the same medium without ^{14}C-αMG plus NaF (20 mM) and αMG (10 mM) in the trace marked "αF." The control exit trace, "α," was the same except that NaCl was substituted for NaF. A 5-ml amount of the diluted suspension was filtered over 47-mm membrane filters (type HA) at the times indicated and washed with basal medium. Samples (1 ml) of the original suspension were filtered similarly to obtain the initial values. The filters were immediately extracted with boiling water, and the radioactive αMG and αMGP were determined as described above. The dotted lines show the theoretical curves.*

Figure 5 shows the fall in the amount of total intracellular radioactivity (αMG + αMGP) in the same cells used in the experiments in Fig. 4. These time constants for the loss of total intracellular radioactivity are a measure of the rate-limiting phosphatase superimposed upon the exit of the neutral ^{14}C-αMG from the cell. The effect of fluoride on the exit of the total radioactivity is marked (Fig. 5); however, it is not inhibited to the extent of the phosphatase (Fig. 4). Again as in Fig. 4, the dashed line is a biphasic trace obtained in the presence of fluoride, suggesting a delayed inhibition by fluoride in this experiment.

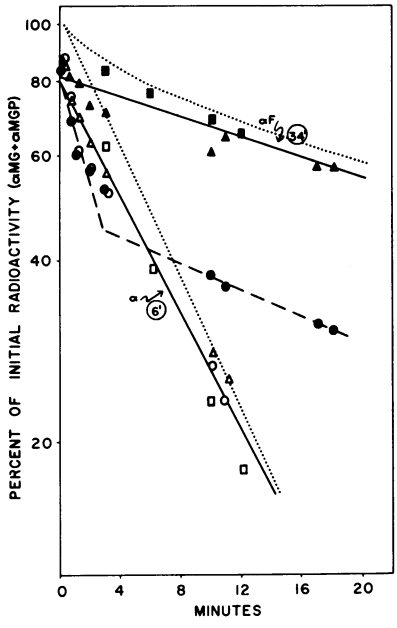

FIG. 5. *Effect of efflux on the total radioactive pool. Experimental design as Fig. 4. The dotted lines show the computer simulations.*

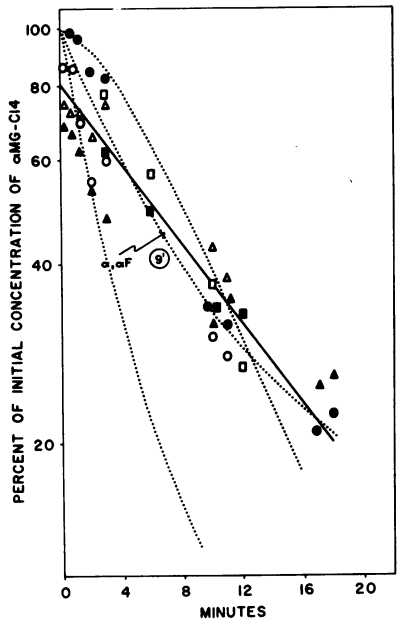

FIG. 6. *Effect of efflux on the intracellular concentration of ^{14}C-αMG. Experimental design as Fig. 4. Two theoretical curves are shown for the fluoride-inhibited cells. In one, the half-time of efflux is 2 min and in the second it is 4.1 min (the former does not fit the data).*

This trace shows both the control and inhibited half-times. The dotted lines are the computer simulations (see Table 1 and Discussion) for efflux in which the half-times for efflux were 2.0 and 4.1 min and the half-times for the phosphatase were 5.0 and 50 min for the control and fluoride-inhibited cases, respectively.

Figure 6 shows the decline in concentration of ^{14}C-αMG in the cell. The continued formation of ^{14}C-αMG during the course of the measurement prevents determination of the true efflux rate constant based on the change in intracellular concentration of ^{14}C-αMG. Hence, the half-time of 9 min shown is not the true half-time for the efflux of αMG; the true value is, of course, much faster. Similarly, measurements of the amount of ^{14}C-αMG appearing in the medium (not shown) do not provide a means for measuring the efflux half-time since, in that case, the driving force for efflux, the intracellular concentration, is not known. The important fact shown in Fig. 6 is that the fall in ^{14}C-αMG concentration is much more similar in the experiments with control cells and fluoride-inhibited cells (9-min half-times) than either the half-time for the conversion of αMGP to αMG or that for the loss of total radioactivity from the cell. Indeed, the half-times for the control and fluoride-inhibited cells are indistinguishable. This suggests that the efflux of αMG proceeds at an appreciable rate (half-time less than 9 min) even in fluoride-inhibited cells when the influx via group translocation is completely blocked and the αMGP-phosphatase is inhibited by 90%. Since the contribution to the ^{14}C-αMG pool by the ^{14}C-αMGP should be much less in the fluoride-inhibited cells than in the control cell, a faster half-time for the efflux of ^{14}C-αMG from these inhibited cells would be expected. The observation (Fig. 6) that the half-times of the control and fluoride cells are about the same raises the possibility that fluoride might be inhibiting, to some degree, the efflux per se of αMG. The computer simulations are shown by the dotted lines for the control cells ($t_{1/2}$ = 5 min for the phosphatase and 2.0 min for efflux) and the fluoride-inhibited cells ($t_{1/2}$ = 50 min for the phosphatase and both 2.0 min and 4.1 min for efflux). The $t_{1/2}$ combination of 50 min for the phosphatase and 2.0 min for the efflux is the curve with the steepest slope and does not approximate the experimental data well (Fig. 6).

The change in the percentage of the radioactive pool present as the phosphorylated sugar, αMGP, during efflux in the presence and absence of fluoride is shown in Fig. 7. From the initial steady-state level of 71% as αMGP, the percentage falls to a new steady-state of about 55% during the control efflux. On the other hand, the percentage rises in the case of the fluoride-inhibited cells to about 85%.

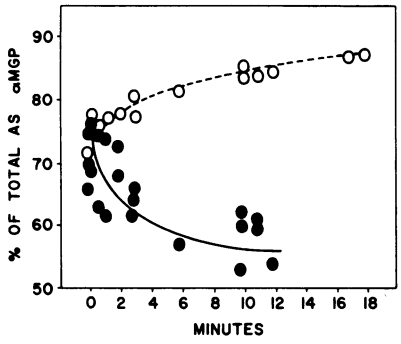

FIG. 7. *Effect of efflux on the percentage of the total radioactivity present as αMGP. Experimental design as Fig. 4.*

DISCUSSION

The transport of glucose and αMG has been well studied; however, the early work of Rogers and Yu (11), Hagihara et al. (4), and Hoffee and co-workers (2, 5, 6) was published several years before the application of the PEP-phosphotransferase system to glucose transport. Although phosphorylation of glucose during its passage across the membrane had been suggested earlier for *E. coli* (12), the formation of the phosphorylated derivative was assumed by most to be an intracellular trap rather than a permeation mechanism. The interaction of glucoside and galactoside transport was also extensively examined (17) and the lack of uptake of αMGP was documented (14). All of these studies, it is now fair to say, suffered from treating a group translocation system as an active transport system. Principally through the work of Kundig, Roseman, and co-workers (see reference 13 for review), the nature of transport via group translocation from a biochemical and genetic standpoint has become increasingly clear. Furthermore, Kaback and his group (reviewed in reference 8) studied αMG permeation in membrane vesicles.

Based on the data presented in this paper as well as previous investigations, a model can be constructed to summarize these findings and provide a hypothesis for further experimentation. Such a scheme is shown diagramatically in Fig. 8. Here αMG is present in the extracellular medium at unit concentration, whereas the total intracellular concentration in the steady-state is 100 times higher than that in the medium and αMGP represents 71% of the total intracellular pool. Since the half-time of the αMGP-phosphatase could be most accurately measured, this 5-min half-time was used as the basis of the subsequent calculations. If there are initially 71 units of αMGP and a half-time of 5 min for the phosphatase, 9.1 units of αMGP per min will be converted to αMG by this phosphatase. To have a steady-state, all three fluxes must be equal; therefore, the influx and the efflux must also be 9.1 units per min. The concentration of αMG driving the steady-state efflux would be the steady-state intracellular αMG concentration, 29 units. From this can be calculated an efflux half-time of 2.0 min.

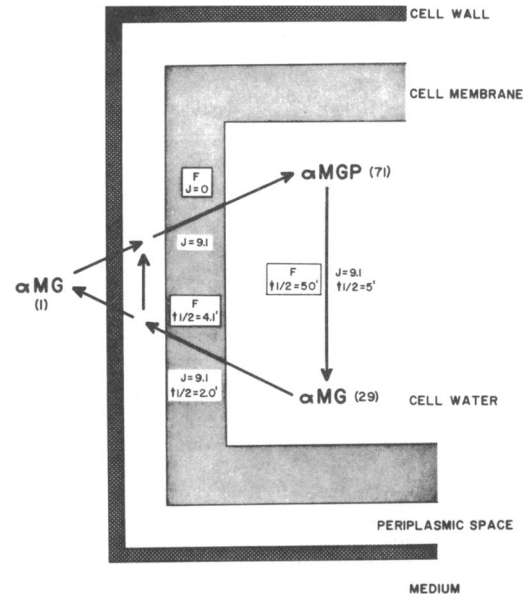

FIG. 8. *Model for the steady-state of αMG transport. The concentrations are shown in parentheses, flux is indicated by J, and the first-order rate constants are expressed as half-times (t ½); half-times in the presence of fluoride are indicated by F.*

To determine the effect of efflux on the intracellular pool in the model, the influx was made zero and the phosphatase and efflux assumed to have the same rates as in the steady-state, 5.0 and 2.0 min, respectively. Similarly, the effect of efflux in the presence of fluoride was examined. Again, the influx was set at zero. The phosphatase in the presence of fluoride had a half-time of 50 min. To determine the efflux rate in the fluoride-treated cells, the efflux of αMG was allowed to be equal to either 2.0 min (control) or 4.1 min (inhibited). The results of these calculations are shown in Table 1. The values generated in the model are very close to those found experimentally for the control efflux. The computer-generated values for efflux in the presence of fluoride are closer to the experimental values when the efflux of αMG is assumed to be inhibited (4.1-min half-time), as was suggested by the data in Fig. 6. An energy-requiring exit system was earlier suggested by Hoffee et al. (6); however, further experimentation will be necessary to docu-

TABLE 1. *Numerical values for the steady-state, efflux and theoretical simulation of αMG transport*[a]

Exit medium	Time (min)	Total radioactive pool (mM)	Total efflux	αMGP pool (mM)	Per cent of total as αMGP
αMG + Cl	0	3.4	0	2.4	71
αMG + Cl	12	0.8	71%	0.5	58
Theoretical (5, 2.0 min)	12	—	78%	—	60
αMG + F	0	2.4	0	1.7	71
αMG + F	12	1.7	32%	1.4	85
Theoretical (50, 2.0 min)	12	—	37%	—	95
Theoretical (50, 4.1 min)	12	—	32%	—	88

[a] Experimental values are the averages of the experiments shown in Fig. 4-7. The theoretical values were generated by using the half-times shown in parentheses for the phosphatase and efflux, respectively. The 0 time values are those for the steady-state; those at 12 min represent the situation after 12 min of efflux under the indicated conditions. All assays were performed at 23 C.

ment this as fact.

It is felt that the model predicts the behavior of the system very well considering the uncertainty of the time constants assumed. It can thus be concluded that the steady-state level of αMG and αMGP in the cell is determined by influx, efflux, and a phosphatase. The efflux proceeds at an appreciable rate but the rate-limiting phosphatase limits the ease of these measurements; i.e., a simple exit experiment such as performed with thiomethyl-galactose (16) will give an artifactual half-time for efflux which is much greater than the true half-time.

I am continuing to investigate the phosphatase and its relationship to the PEP-phosphotransferase. Similarly, the efflux mechanism is being studied with these factors in mind. In Fig. 8, I separated the influx, phosphatase, and efflux mechanisms for the sake of clarity; investigations in glucose-transport-negative mutants should disclose the actual relationship of these components.

ACKNOWLEDGMENTS

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