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TRANSMEMBRANE EFFECTS OF β-GALACTOSIDES ON THIOMETHYL-β-GALACTOSIDE TRANSPORT IN *ESCHERICHIA COLI*

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SUMMARY

1. The transport rate in *Escherichia coli* (ML strain) of thiomethyl-β-[^{14}C]-galactoside was measured in the presence and absence of thiomethyl-β-galactoside (TMG), *o*-nitrophenyl-β-galactoside (ONPG), or thio-β-digalactoside (TDG) on the side of the cell membrane toward which [^{14}C]TMG transport was occurring.

2. Efflux rate of [^{14}C]TMG was markedly stimulated by the presence of non-radioactive TMG in the external medium, whereas preloading these cells with non-radioactive TMG caused little effect on the influx rate of low concentrations of [^{14}C]-TMG and only a small stimulation of influx rate at high concentrations.

3. ONPG in the external medium caused a smaller stimulation of [^{14}C]TMG efflux rate than did TMG. Preloading the cells with ONPG inhibited influx rate of low concentrations of [^{14}C]TMG, but had little effect on this rate for high concentrations.

4. TDG in the external medium caused only a slight stimulation of [^{14}C]TMG efflux rate, whereas preloading with TDG causes a marked inhibition of the influx rate of all concentations of [^{14}C]TMG.

5. These results are consistent with the hypothesis that TMG undergoes exchange diffusion with [^{14}C]TMG, ONPG has little exchange diffusion, and TDG has an inhibitory "exchange" effect; but that superimposed on these exchange effects are the effects of an external partial diffusion barrier.

INTRODUCTION

In 1954 HEINZ[1] observed that the entry rate of radioactively labeled glycine into mouse Ehrlich ascites tumor cells was stimulated by preloading the cells with unlabeled glycine. More recently MAWE AND HEMPLING[2] and LEVINE, OXENDER AND STEIN[3] found that the unidirectional-efflux rate of glucose from human erythrocytes was increased by the presence of glucose in the suspending medium. The stimulation of unidirectional-transport rate caused by the presence of a substrate for that transport system on the side of the membrane toward which transport is occurring (the *trans* side) has been termed "exchange diffusion"[4]. This phenomenon has commonly been attributed to a more rapid rate of movement across the membrane of carrier–

Abbreviations: TMG, thiomethyl-β-galactoside; TDG, thio-β-digalactoside; ONPG, *o*-nitrophenyl-β-galactoside.

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substrate complexes compared with free carriers[1–3,5]. Under these conditions, the rate-limiting step in unidirectional transport of a substrate would be the return of the empty carriers from the *trans* side of the membrane, and this return could be facilitated by a flux of substrate in this same direction.

The β-galactoside transport system in *Escherichia coli* has been investigated in several laboratories for exchange diffusion by examining for effects of preloading with substrate on the rate of uptake of β-galactosides, and in each instance no effect of preloading was observed[6–8]. However, in 1964 KOCH[9] found that β-galactoside efflux was markedly affected by substrate in the external medium. This apparent inconsistency is the subject of the present investigation. The data obtained are in agreement with the hypothesis that exchange diffusion does occur in these cells, but that superimposed on the exchange effects are the effects of an external partial diffusion barrier. It will be demonstrated that with substrate on the *trans* side of the membrane the exchange and barrier effects are additive for efflux, but tend to cancel each other for influx.

MATERIALS AND METHODS

Bacteria and media

The organisms used in this study were mutants derived from the Pasteur Institute strain ML–308 ($i^-z^+y^+a^+$). ML–308–831 ($i^-z^+y^+a^-$) is constitutive for both β-galactoside transport and β-galactosidase (EC 3.2.1.23), but lacks thiogalactoside transacetylase (unpublished work). This transacetylase-negative strain was utilized to eliminate the accumulation of acetylated sugar which occurs in ML–308 during prolonged incubation in thiogalactoside. ML–308–225 ($i^-z^-y^+a^+$) is constitutive for transport and transacetylase, but does not produce β-galactosidase[8]. This organism was utilized for studies with *o*-nitrophenyl-β-galactoside.

The mineral medium used was Medium 63 (see ref. 10) *plus* NaCl, which consisted of 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), NaCl (2.9 g) added to 1 l of water and adjusted to pH 7.0 with KOH. For growth 1% casein tryptone digest was added as a carbon source. Cells in the logarithmic phase of growth were obtained by transferring 1.0 ml of an overnight cell suspension into a sidearm flask containing 30 ml of fresh growth medium and incubated on a rotary shaker at about 200 rev./min at 37° until the suspension reached an absorbance of 250 Klett units (No. 42 filter), which corresponds to 0.56 mg dry weight per ml. These cells were then centrifuged at 0°, washed by suspending in mineral medium, again centrifuged, and then suspended at a suitable density for subsequent experiments in medium containing 1.0 mg glucose* per ml as energy source and 0.1 mg chloramphenicol per ml to prevent further growth.

* The presence of glucose under these conditions had no measurable effect on β-galactoside transport. It was added to prevent the plasmolysis of the cell seen in its absence. If logarithmically growing cells were centrifuged, washed, and resuspended in mineral medium *plus* chloramphenicol with no carbon source, they appeared to rapidly plasmolyze (shrink and pull away from their cell wall). This shrinkage was indicated by a decrease of intracellular space with which thiomethyl-β-[^{14}C]galactoside ([^{14}C]TMG) equilibrates in *E. coli* ML–35 (which lacks the β-galactoside transport system), and was confirmed by an increase in sucrose space (sucrose over short periods of time cannot penetrate the plasma membrane). The fact that the cytocrit (a measure of the total volume within the cell walls) remained constant in spite of these changes indicated that the plasma membrane must be pulling away from the cell wall.

Chemicals

Thiomethyl-β-[^{14}C]galactoside ([^{14}C]TMG) was obtained from New England Nuclear Corp.; thiomethyl-β-galactoside (TMG), *o*-nitrophenyl-β-galactoside (ONPG), and thio-β-digalactoside (TDG) from Mann Research Laboratories, Inc.; D-glucose from Merck and Co.; casein tryptone digest from Difco Laboratories. Chloramphenicol was a gift of the Parke, Davis Co.

Measurements of [^{14}C]TMG fluxes

Initial influx rates of [^{14}C]TMG into nonpreloaded cells were measured by adding the radioactive galactoside to a cell suspension or pellet and at 6, 9, 12, 15, 18, and 21 sec thereafter filtering aliquots of this suspension on a Millipore filter (0.65 μ pore size). The volumes and cell densities were dependent upon the concentration of galactoside being used. In one type of experiment, for example, 2 ml of cells (A = 400) suspended in mineral medium containing glucose and chloramphenicol were added to 2 ml of radioactive TMG dissolved in this same medium. Aliquots of 0.5 ml were delivered to the Millipore filters at the times indicated above with a Becton, Dickinson and Co. (Rutherford, N.J.) automatic syringe. Immediately after filtration, the filters were washed by adding 15 ml mineral medium (23°). The filters were placed in counting vials and 15 ml of BRAY's liquid scintillation fluid[11] added. The vials were shaken vigorously and counted in a Nuclear-Chicago liquid scintillation counter. The value of 0.6 μl cell water/ml cell suspension of A = 100 Klett units (No. 42 filter) was used to calculate the intracellular concentrations of [^{14}C]TMG (see ref. 8). The slope of the internal galactoside concentration *versus* time for the 6 samples was taken as the influx rate. Since this plot is approximately linear for the first 30 sec this represents the initial rate.

To measure influx of [^{14}C]TMG into preloaded cells, the cells were first equilibrated with nonradioactive TMG, TDG, or ONPG for 30 min at 23°, then either the suspension was diluted into [^{14}C]TMG to the appropriate final external concentration of [^{14}C]TMG, or the suspension was centrifuged at 0° and resuspended in the appropriate concentration of [^{14}C]TMG. The dilution technique was used when cells had been preloaded with TMG, and the centrifugation was used for TDG and ONPG. The sampling and analysis procedures were identical with those used for nonpreloaded cells. In every case where a comparison between preloaded and nonpreloaded influx was made, both measurements were obtained on the same day with the same batch of cells using the same general procedure.

Efflux rates of [^{14}C]TMG were obtained by equilibrating a cell suspension of absorbance about 500 Klett units (No. 42 filter) with a 20 mM solution of this compound for 30 min at 23°, then diluting this equilibrated suspension 1:400 into mineral medium containing 1 mg/ml D-glucose with or without added β-galactosides. 4-ml samples were added to Millipore filters at 10, 20, 40, 60, 90 and 120 sec, washed and prepared for scintillation counting as above.

Theoretical

For use in this paper exchange diffusion will be defined, operationally, as any phenomenon whereby the unidirectional-flux rate of a transport substrate across the plasma membrane is affected by a substrate on the opposite (*trans*) side of the membrane. This general definition allows for both a "positive exchange diffusion" where

substate on the *trans* side of the membrane stimulates unidirectional-flux rate and "negative exchange diffusion" where it inhibits this rate.

The evidence presented in this paper suggests that there exists a barrier external to the plasma membrane which retards the movement of β-galactosides between the plasma membrane and the bulk external medium. If cells were equilibrated with [^{14}C]TMG, then diluted into a very large volume of medium containing no galactoside, efflux would occur rapidly across the plasma membrane, but the resistance of the partial diffusion barrier would result in a local concentration of the [^{14}C]galactoside near the membrane, which is higher than that in the bulk external medium. This locally high concentration adjacent to the external surface of the plasma membrane would result in the recapture of some of the molecules by the β-galactoside transport system (see Fig. 1A). Thus, the measurable efflux rate would not be that of the true unidirectional efflux, but would instead be a net rate composed of unidirectional efflux *minus* recapture.

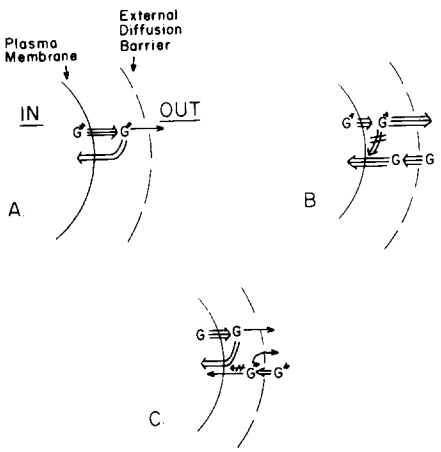


Fig. 1. Effects of an external diffusion barrier on transport fluxes. G*, a radioactively labeled galactoside; G, an unlabeled galactoside; arrows represent fluxes; ⊣⊢ represents a flux blocked by competition. A. Efflux into medium containing no galactoside (showing recapture). B. Efflux into medium containing a high concentration of unlabeled galactoside (showing blockage of recapture by competition). C. Influx into cells preloaded with unlabeled galactoside (showing inhibition of influx by competition with unlabeled galactoside).

If, however, efflux of [^{14}C]TMG were measured into a medium containing a nonradioactive substrate for the β-galactoside transport system, this substrate would compete with the recapture of [^{14}C]galactoside (Fig. 1B). The result would be a stimulation of the net ^{14}C-efflux rate by the nonradioactive substrate outside the cell. This, however, would not be exchange diffusion as defined above because it would not be a stimulation of the unidirectional-flux rate across the plasma membrane, but rather a stimulation of the net-flux rate across the entire complex existing between the cell water and the bulk external medium.

The partial diffusion barrier would also affect the initial influx rates. If the cells are preequilibrated with a nonradioactive galactoside, then transferred to a medium containing only [^{14}C]TMG, the nonradioactive galactoside would efflux and be recap-

tured as above, this recapture competing with the influx of the ^{14}C substrate (Fig. 1C).

Thus the effect of an external partial diffusion barrier when nonradioactive substrate is present on the *trans* side of the membrane would be to increase measurable efflux rate of ^{14}C substrate from the cell, and to decrease initial influx rate of ^{14}C substrate into the cell. If, in addition, there were a positive exchange diffusion, the exchange-diffusion stimulation by *trans* substrate would be additive with the diffusion-barrier stimulation of efflux, but would tend to cancel the barrier inhibition of influx. Conversely, if there were a negative exchange diffusion, the inhibitions would be additive for influx, but the effects would tend to cancel for efflux. These relationships are presented in Table I.

TABLE I

EFFECT OF *trans*-MEMBRANE GALACTOSIDE ON FLUX RATE OF [^{14}C]GALACTOSIDE

	Influx	*Efflux*
Positive exchange diffusion		
Exchange diffusion effect	Stimulates	Stimulates
Diffusion barrier effect	Inhibits	Stimulates
Net effect	Little or no effect	Strongly stimulates
Negative exchange diffusion		
Exchange diffusion effect	Inhibits	Inhibits
Diffusion barrier effect	Inhibits	Stimulates
Net effect	Strongly inhibits	Little or no effect

RESULTS

Effect of external TMG on efflux

Fig. 2 gives an experiment showing efflux of [^{14}C]TMG from ML-308-831 into media containing various concentrations of nonradioactive TMG. It can be seen that

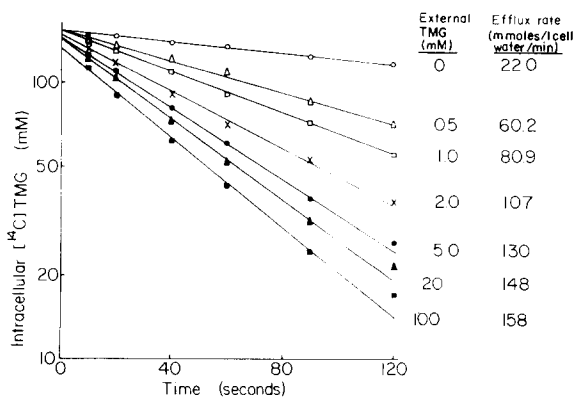


Fig. 2. Efflux of [^{14}C]TMG into media containing various concentrations of nonradioactive TMG. ML-308-831 were equilibrated for 30 min at 23° with 20 mM [^{14}C]TMG, then diluted 400-fold into media containing various concentrations of nonradioactive TMG. Samples were taken at 10, 20, 40, 60, 90, and 120 sec for measurement of intracellular [^{14}C]TMG. The concentration to the right of each curve is that of the nonradioactive TMG in the efflux medium, and the rate is the corresponding efflux rate determined from the curve.

even rather low external concentrations caused a large stimulation of efflux, and that the magnitude of the stimulation is a saturable function of the concentration. This saturation is consistent with either exchange diffusion or with competition with the recapture of the radioactive molecules. The maximally stimulated efflux rate was 7 2 times the efflux when no external substrate was present

Similar experiments were carried out with ML-308. The exit of [^{14}C]TMG from preloaded cells was stimulated to a similar degree by the presence of TMG in the external medium.

Effects of preloading with TMG on influx

[^{14}C]TMG at a concentration of 0.075 mM enters nonpreloaded cells of ML-308-831 at an initial rate of 12.8 ± 1.2 mmoles/l cell water per min (mean ± S.E. of mean of 9 observations). If, however, these cells are preequilibrated with 20 mM nonradioactive TMG, then diluted into the [^{14}C]galactoside such that the final external concentration is again 0.075 mM, the initial rate of [^{14}C]galactoside entry is 14.4 ± 0.7 mmoles/l cell water per min. These two rates are not significantly different.

The marked stimulation of efflux with lack of effect on influx is consistent with the hypothesis that TMG on the *trans* side of the membrane causes a positive exchange diffusion, but that superimposed on this are the effects of an external partial diffusion barrier (see Table I). Based on this hypothesis, it follows that if the concentration of external [^{14}C]galactoside available for entry into preloaded cells were increased, the competition for influx by the nonradioactive molecules being recaptured would be less effective. If a high enough [^{14}C]galactoside concentration were used, it should completely eliminate the effect of the partial diffusion barrier, leaving the stimulation due to the positive exchange diffusion. Thus, as the external concentration is increased, preloading with nonradioactive TMG should cause a greater and greater stimulation of initial influx rate relative to the corresponding nonpreloaded influx rate. Table II shows the observed effects of concentrations of external [^{14}C]TMG. The results are expressed as the ratio of the initial entry rate into preloaded cells to that into nonpreloaded cells. As the concentration of external [^{14}C]galactoside is increased, the stimulation caused by preloading is also increased. Initial entry rate of 20 mM

TABLE II

EFFECT OF TMG PRELOADING ON [^{14}C]TMG INITIAL INFLUX

Initial influxes of various concentrations of [^{14}C]TMG were measured into ML-308-831 which had either been preequilibrated with 20 mM nonradioactive TMG, or had been left nonpreloaded. The results are expressed as the ratio of the initial entry rate into preloaded cells over that into nonpreloaded cells. Each value is given as mean ± S.E. of the mean of 9 observations, each of which is the ratio of preloaded over nonpreloaded entry rates measured on the same day from the same batch of cells.

External [^{14}C]TMG concentration (mM)	*Preloaded influx rate / Nonpreloaded influx rate*
0.075	1.16 ± 0.07
0.20	1.46 ± 0.06
1.0	1.38 ± 0.03
5.0	1.73 ± 0.04
20.0	1.84 ± 0.07

[^{14}C]TMG was 72.0 ± 3.2 mmoles/l cell water per min into nonpreloaded cells and 131 ± 2.9 mmoles/l cell water per min into cells preloaded with 20 mM TMG. This stimulation is highly significant statistically ($P<0.001$). Since the stimulation was not much greater for entry of 20 mM than for 5 mM, 20 mM [^{14}C]TMG appears to give a near maximal effect. Preloading with 100 mM TMG caused no significant further stimulation of influx. This data is consistent with the hypothesis discussed above.

TABLE III

EFFECTS OF *trans* TMG ON [^{14}C]TMG FLUXES

Initial entrance rate of 20 mM [^{14}C]TMG was measured into ML-308-831 which had either been preequilibrated with 20 mM nonradioactive TMG, or had remained nonpreloaded. Efflux rate was measured from cells which had been preequilibrated with 20 mM [^{14}C]TMG then diluted into media containing either no TMG or 20 mM nonradioactive TMG. Numbers are the mean value of 3 observations. All observations were made on the same day from the same batch of cells.

Transmembrane nonradioactive TMG concentration	*Influx (mmoles/l cell water per min)*	*Efflux (mmoles/l cell water per min)*
None	75.9	23.1
20 mM	155	150

Table III compares influx and efflux rate in an experiment in which all measurements were made on the same batch of cells. At equilibrium influx must be equal to efflux. For the influx for equilibrium experiments cells preloaded by exposure to 20 mM TMG were transferred to 20 mM radioactive TMG and uptake of radioactivity measured; in the efflux experiments cells preequilibrated with 20 mM radioactive TMG were transferred to 20 mM nonradioactive TMG and the loss of radioactivity from the cells followed. It can be seen that experimentally determined influx was in good agreement with the efflux rate under equilibrium conditions. This rate is a twofold increase over the influx rate into nonpreloaded cells. Since the stimulation due to preloading is maximal with the high external concentration of 20 mM [^{14}C]TMG, this stimulation must be due entirely to the positive exchange diffusion as the recapture has been completely obliterated. If the stimulation due to exchange diffusion is twofold, then this phenomenon cannot be entirely responsible for the 6.5-fold stimulation observed for efflux into 20 mM TMG and an additional process, such as blocking of the recapture caused by a partial diffusion barrier, must be postulated.

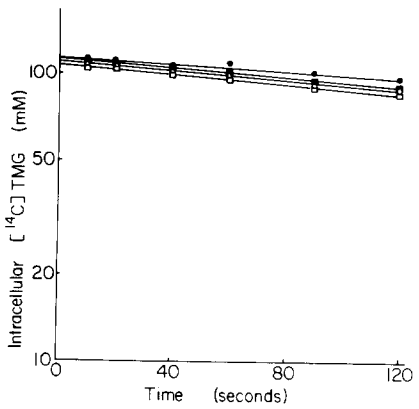
Effects of TDG on TMG fluxes

TDG is a very potent competitive inhibitor of TMG transport with an affinity for the transport system 25 times that of TMG[12]. Though both are substrates for the same transport system, TDG on the *trans* side of the membrane has quite different effects on the flux rates of [^{14}C]TMG than those reported above for TMG. When cells of ML-308-831 are preequilibrated with 20 mM [^{14}C]TMG, then transferred to media containing various concentrations of TDG, the efflux is approximately the same into all concentrations (see Fig. 3). Actually the digalactoside causes a very slight stimulation of efflux, but even when its concentration is made 100 times its half saturation level, the efflux rate is only 50% greater than efflux into medium with no substrate.

This is quite different from the 7.2-fold effect observed with TMG. KOCH[9] found that TDG caused an inhibition of [^{14}C]TMG efflux under the conditions of his experiments.

Preequilibrating the cells with 2 mM TDG, instead of causing a stimulation of influx as observed with TMG, caused a very marked inhibition of influx of all concentrations of [^{14}C]TMG. Even at a high external concentration of TMG (20 mM) the influx rate into preloaded cells was only 40 % of that into nonpreloaded cells.

According to Table I the effects observed for TDG are those expected for a system with a partial diffusion barrier and for a compound which causes a negative exchange diffusion.



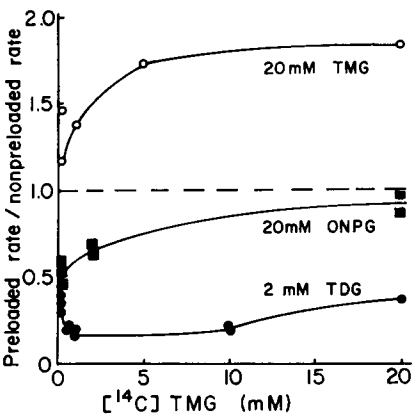


Fig. 3. Efflux of [^{14}C]TMG into media containing various concentrations of TDG. ML-308-831 were equilibrated with 20 mM [^{14}C]TMG, then diluted into media containing various concentrations of TDG. Samples were taken at 10, 20, 40, 60, 90, and 120 sec for measurement of intracellular [^{14}C]TMG. Efflux into medium with no TDG (●); 0.02 mM TDG (■); 0.05 mM TDG (○); 2.0 mM TDG (□).

Fig. 4. Effects of preloading on initial influx rate of [^{14}C]TMG. Cells were preequilibrated with 20 mM TMG, 2 mM TDG, or 20 mM ONPG and the initial rate of [^{14}C]TMG influx was measured and divided by the initial rate of influx into nonpreloaded cells. The preequilibrating compound is indicated on each curve.

Effects of ONPG on TMG fluxes

ONPG is of special interest in that it appears to have very little exchange diffusion effect. When cells of ML-308-225 are pre-equilibrated with 20 mM ONPG and are tested for [^{14}C]TMG uptake, the initial influx rate of 20 mM [^{14}C]TMG is 92 % of its rate into nonpreloaded cells. Since, as was shown above, the effect of preloading on initial influx rate of 20 mM [^{14}C]TMG is due almost entirely to exchange diffusion, this would indicate that ONPG has little exchange effect. Therefore, any effects of ONPG on the *trans* side of the membrane on [^{14}C]TMG fluxes must be due primarily to the partial diffusion barrier. Thus, the finding that influx rate of 0.2 mM [^{14}C]TMG is 48 % inhibited by preloading with ONPG must be due to the competition caused by the recapture of ONPG. [^{14}C]TMG efflux is stimulated 3.2-fold by 20 mM ONPG in the external medium. This stimulation is clearly less than the 6.5-fold stimulation caused by 20 mM external TMG, which has the additive effect of a positive exchange diffusion.

Fig. 4 summarizes the effects of preloading with TMG, TDG, or ONPG on the

initial influx rate of [^{14}C]TMG. The results are expressed as the ratio of preloaded to nonpreloaded rates *versus* the external concentration of [^{14}C]TMG. It illustrates the effects on entrance of a positive exchange diffusion (TMG), of little exchange diffusion (ONPG), and of a negative exchange diffusion (TDG) superimposed on the effects of a partial diffusion barrier.

DISCUSSION

The evidence presented in this paper indicates that the β-galactoside transport system is far more complex than generally assumed. Both exchange diffusion[6–8] and external diffusion barriers[6] have in the past been declared either nonexistent or insignificant. However, the evidence presented here supports the existence of both, and demonstrates that under certain circumstances they can cause very substantial effects on flux rates.

Implications regarding kinetics

In a system possessing exchange diffusion and an external diffusion barrier, the transport of β-galactosides would not be expected to follow Michaelis–Menten kinetics. LEVINE AND STEIN[13], using as an exchange diffusion system glucose transport in the human erythrocyte, have demonstrated that neither K_m nor v_{max} is a constant, but rather they are functions of the substrate concentration on the *trans* side of the membrane. They pointed out that K_m and v_{max} are meaningless unless the *trans* concentration is specified and an additional kinetic parameter which indicated the magnitude of the exchange diffusion for that substrate is determined. Kinetics for β-galactoside transport in *E. coli* must be two stages more complex than those given for glucose transport in the human erythrocyte in that accumulation of β-galactosides occurs against a concentration gradient, and there exists an external diffusion barrier. That β-galactoside transport does not follow standard Michaelis–Menten kinetics has been demonstrated in this paper. As in the erythrocyte, the initial flux rates are functions of the substrate concentration on the *trans* side of the membrane.

The external diffusion barrier has the effect of creating a compartment adjacent to the external surface of the plasma membrane in which, during nonequilibrium conditions, the concentration of β-galactoside is different from either that within the cell or that in the bulk external medium. That this can result in recapture during exit has already been demonstrated. During influx into nonpreloaded cells, the substrate concentration in this compartment would be less than that in the bulk external medium because the galactoside pump is directed inward from the compartment and a concentration gradient must exist across the diffusion barrier for galactosides to enter from the external medium. Thus, the rate of transport across the plasma membrane would not correspond to the substrate concentration placed in the external medium, but rather to the lower concentration in the compartment with which the plasma membrane is actually in contact. This would create a further deviation from Michaelis–Menten kinetics, since the discrepancy between compartment and bulk external medium concentrations would vary, being greater at low substrate concentrations. Thus, the kinetics of the β-galactoside transport system of *E. coli* can only be usefully evaluated with the full knowledge of the complexities of the system.

Partial diffusion barrier

Anatomically, the most likely site for a diffusion barrier external to the plasma membrane would be the cell wall. MITCHELL AND MOYLE[14] have shown quite convincingly that it is the plasma membrane which acts as an osmotic barrier to small molecules while the cell wall functions as a mechanical support. However, this does not imply that the cell wall cannot offer a substantial resistance to the flow of a compound which is rapidly transported across the plasma membrane.

BAYER AND ANDERSON[15] in their detailed electron-microscopic study of the *E. coli* cell wall, have examined negatively stained, frozen preparations of intact cell walls and walls from which various layers had been removed. In evaluating the implications of their results it should be kept in mind that the approximate molecular diameter of TMG is 8 Å (estimated from ref. 16), while those of ONPG and TDG are somewhat greater. BAYER AND ANDERSON concluded that the following layers exist within the cell wall of *E. coli* (proceeding outward from the plasma membrane).

(1) The innermost layer is a rigid glucose-aminopeptide layer which is quite thin, but which is responsible for the cell shape. This is the layer which is attacked by lysozyme. This layer, after isolation and negative staining, showed no structural details, but it was pointed out that, because of the thinness of the layer, this did not preclude the existence of small holes. MARTIN[17] gives 17–25 Å as a maximum estimate for the thickness of this layer whose ultrastructure consists of a completely covalently linked lattice with rectangularly arranged repeating units every 30–100 Å.

(2) The next layer consists of round proteinaceous particles, 60–250 Å in diameter, packed together with much smaller elements of different shapes. Some irregularly shaped gaps, usually no more than 200 Å wide, were observed in this layer.

(3) Next is a layer of lipopolysaccharide. This appears to form a continuous layer except for a series of 40–60 Å wide channels which appear to penetrate the layer. These channels comprise only a very small portion of the total surface area.

(4) The outermost material is lipoprotein which appears to be distributed in patches and "sausagelike structures" over the cell surface.

With such a structure completely enclosing the cells, it hardly seems surprising that the flux of β-galactoside encounters some resistance outside the plasma membrane.

It is appreciated that the present hypothesis rests heavily on kinetics and that these are often open to more than one interpretation. We are searching, therefore, for more direct approaches, including experiments with cells whose cell walls are partially or completely removed.

Negative exchange diffusion

With the hypothesis that exchange diffusion is due to a difference in rate of reorientation across the membrane of carrier and carrier–substrate complex[1–3,5], it is no more difficult to imagine the substrate decreasing this rate than it is to imagine an increase. Thus, a negative exchange diffusion should be no more improbable than a positive exchange diffusion.

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REFERENCES

1 E. HEINZ, *J. Biol. Chem.*, 211 (1954) 781.
2 R. C. MAWE AND H. G. HEMPLING, *J. Cell. Comp. Physiol.*, 66 (1965) 95.
3 M. LEVINE, D. L. OXENDER AND W. D. STEIN, *Biochim. Biophys. Acta*, 109 (1965) 151.
4 H. LEVI AND H. H. USSING, *Acta Physiol. Scand.*, 16 (1948) 232.
5 J. A. JACQUEZ, *Am. J. Physiol.*, 200 (1961) 1063.
6 G. N. COHEN AND J. MONOD, *Bacteriol. Rev.*, 21 (1957) 169.
7 A. KEPES, *Biochim. Biophys. Acta*, 40 (1960) 70.
8 H. H. WINKLER AND T. H. WILSON, *J. Biol. Chem.*, 241 (1966) 2200.
9 A. L. KOCH, *Biochim. Biophys. Acta*, 79 (1964) 177.
10 L. A. HERZENBERG, *Biochim. Biophys. Acta*, 31 (1959) 525.
11 G. A. BRAY, *Anal. Biochem.*, 1 (1960) 279.
12 A. KEPES AND G. N. COHEN, in I. C. GUNSALUS AND R. Y. STANIER, *The Bacteria*, Vol. 4, Academic Press, New York, 1962, p. 179.
13 M. LEVINE AND W. D. STEIN, *Biochim. Biophys. Acta*, 127 (1966) 179.
14 P. MITCHELL AND J. MOYLE, *Symp. Soc. Gen. Microbiol.*, 6 (1956) 150.
15 M. E. BAYER AND T. F. ANDERSON, *Proc. Natl. Acad. Sci. U.S.*, 54 (1965) 1592.
16 S. G. SCHULTZ AND A. K. SOLOMON, *J. Gen. Physiol.*, 44 (1961) 1189.
17 H. H. MARTIN, *Ann. Rev. Biochem.*, 35 (1966) 457.