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THE INTERACTION BETWEEN PERMEASES AS A TOOL TO FIND THEIR RELATIONSHIP ON THE MEMBRANE

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SUMMARY

When cells which are constitutive for galactoside permease are grown in glucose-containing media, permeases for both are synthesized. Substrates for the glucose permease now inhibit the entry and speed the exit of previously accumulated galactosides. The concentration of α-methylglucoside which half-inhibits *o*-nitrophenylgalactoside entry into the cell was 5 times lower than the concentration that supported half-maximal uptake of α-methylglucoside. When cells with only galactoside permeases are grown in a medium which induces glucose but not galactoside permease, the sites which had been laid down in the first phase never become sensitive to the action of α-methylglucoside. In the reverse situation, the galactoside permeases that are laid down in the first half generation do interact with the preexisting glucose permeases, but later ones show no interaction. These effects must be due to the spatial arrangement of the elements of the transport system in cell membrane. Although in glucose-grown cells the permease inactivation by *N*-ethylmaleimide is prevented by thiodigalactoside, α-methylglucoside does not protect the galactoside transport sites. However, it can greatly augment thiodigalactoside protection. All the observations lead to the idea that more than 1 permease molecule can interact with a common transport molecule in the cell membrane and that 2 permease molecules can only interact if they have been affixed close together on the growing membrane.

INTRODUCTION

Selective permeation of certain molecules across biological membranes has long been of general interest to the biologist. The study of transport in bacteria has been productive, contributing to the entire field the present concept of the permease system[1–5]. In the most recent model[5], the transport assembly consists of three

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parts: (1) a stereospecific component on the cell membrane, the permease; (2) a less specific carrier element called the transporter or carrier; and (3) an internal energy-dependent enzymatic assembly for the dissociation of the transporter–substrate complex.

A portion of the evidence for both a more specific permease and a less specific carrier element is the fact that glucose inhibits the transport of galactosides in constitutive galactoside permease, producing strain only if the cells have been grown on glucose[5]. This observation had been made by both our laboratory and by KESSLER AND RICKENBERG[6]. The present study expands upon this subject, to study in particular the interaction between galactosides and glucose permeases when the two permeases are introduced into the membrane in various ways so that the two types may be either incorporated into the membrane together or separately.

Through the use of the chromogenic substrate, *o*-nitrophenylgalactoside, the velocity of transport by galactoside permease can be quantitated. The rate of hydrolysis under a wide range of conditions is limited by the slower permeation and not by the amount of β-galactosidase inside the cells. Using this assay we report on the properties of transport system when the interaction with both substrates through different permeases occurs, and we present studies which show that interaction is only possible when the two permeases have been introduced into the membrane together or at least almost simultaneously.

MATERIALS AND METHODS

Conditions and techniques were those previously used in this laboratory[5,7–9]. The prototype *Escherichia coli*, strain ML 30, and one of its constitutive derivatives, ML 308, were used. The organisms were grown on medium M-9 with a designated carbon source under forced aeration at 37°. Hydrolysis of *o*-nitrophenyl-β-D-galactoside was measured in a thermostatted Gilford multiple-sample absorbance recorder. The reaction was generally carried out in a cuvette containing 2.5 ml cells and 0.2 ml substrate at 28°. Final concentration of *o*-nitrophenylgalactoside used in a standard assay for the permease was 3.7 mM. The interaction of glucose permease with galactoside permease was routinely measured by adding α-methyl-D-glucopyranoside to the assay system to a final concentration of 1.8 mM. The former value is about 4 times the half-saturation concentration for entry, whereas the latter is about 200 times its apparent K_m for uptake.

Enzyme was measured after destruction of the cell membrane by treatment of cells with 1 drop of 1 part 10% dodecylsulfate–1 part toluene and 1 drop mercaptoethanol per 10 ml of culture. The cell suspension was then briefly but vigorously agitated at room temperature. In order to measure the transporter capability of the cells, the permease was inactivated with formaldehyde at a final concentration of 9 mM. The results of such measurements include hydrolysis resulting from the breakdown of the cell membrane and exposure of β-galactosidase to the substrate, in addition to cryptic hydrolysis.

Isopropyl-β-D-thiogalactoside (Mann Chemical Co.) was used for induction of the galactoside system; the concentrations used depended on the carbon source and our purpose, and are given below for each specific experiment. In order to induce the glucose permease, cells were grown on glucose as a carbon source. The cells were

washed twice and resuspended in minimal medium without carbon source. Chloramphenicol at a concentration of 20 μg/ml was added to stop protein synthesis, and the cells were stored in the cold until a few minutes before use. They were then placed in the 28° water bath before transfer to a cuvette for use.

The permease system was inactivated with *N*-ethylmaleimide by the procedure we have used previously in studies of the nucleoside transport system[9].

RESULTS

Influence of growth in glucose on the galactoside transport system

In confirmation of our previous results[5], *E. coli* ML 308, constitutive for the β-galactoside permease system, shows differences in its galactoside transport depending on the carbon source on which it is grown. As can be seen from Table I, the maximum velocity of *o*-nitrophenylgalactoside hydrolysis is reduced when glucose instead of succinate is used. This reduction is in the range of 2–3-fold, since rates of 150–251 μmoles/g·min have occurred with different cultures on different days. Other work has shown that accumulation of gratuitous galactoside permease substrates is also decreased in glucose-grown cells[10]. The second difference elicited by the change in carbon source is that glucose and α-methylglucoside have become potent inhibitors of *o*-nitrophenylgalactoside hydrolysis *in vivo*; this inhibition is a membrane phenomenon since α-methylglucoside does not inhibit hydrolysis in the lysates. The inhibition is 4–13% in succinate-grown cells and 60–80% in glucose-grown cells. This inhibition is somewhat variable from cell batch to batch; but within a given batch, the inhibition is highly reproducible $\pm$ 2%. It should also be noted that the α-methylglucoside-inhibited rate of *o*-nitrophenylgalactoside hydrolysis *in vivo* never gets as low as the cryptic rate, as indicated by the color formation with formaldehyde-treated cells, even though the standard α-methylglucoside concentration is 300 times greater than the concentration that gives half-inhibition of *o*-nitrophenylgalactoside hydrolysis. Thus, the difference, which amounts to 10–20% of the uninhibited galactoside transport, appears to be independent of glucose inhibition in these glucose-grown ML 308 cells. The simplest interpretation of this is that some small parts of the galactoside permease systems are analogous to those in succinate-grown cells in that they do not interact with glucose or glucosides at any concentration. Increasing the concentration of *o*-nitrophenylgalactoside several fold does not overrule the inhibition by the high concentration of α-methylglucoside.

Various studies of the kinetics of the inhibition process were made. Fig. 1 shows the rates of cellular hydrolysis as a function of substrate concentrate—both in the absence and in the presence of 18 μM α-methylglucoside. This concentration is much lower than the standard concentration employed routinely and is slightly below the K_m for α-methylglucoside for the glucose permease (29 μM) as reported by KESSLER AND RICKENBERG[6]. The value 0.2 mM is quoted by KEPES AND COHEN[4] for unpublished experiments of MONOD, HALVORSON AND JACOB. At first glance, it would appear that the graph of the uninhibited control is a hyperbola. However, in previous studies[5], it was reported that while the curve is accurately a hyperbola with succinate-growing cells, it is not with glucose-grown cells. By growth in glucose, the amount of permease is decreased several fold while the rate of cellular *o*-nitrophenylgalactoside hydrolysis of genetic cryptic cells or of formaldehyde-treated cells does not

TABLE I

THE INFLUENCE OF GROWTH CONDITIONS ON β-GALACTOSIDASE, β-GALACTOSIDE PERMEASE AND GLUCOSE PERMEASE ON THE CONSTITUTIVE STRAIN ML 308

Growth conditions	*In vivo (μmoles/ g·min)*	*Lysate (μmoles/ g·min)*	*In vivo + α-methyl-glucoside (μmoles/ g·min)*	*Crypticity ratio of lysate : in vivo*	*Sites inhibited (%)*	*In vivo + formaldehyde (μmoles/ g·min)*
Succinate batch*	420	9240	390	26	8 (4–13)	46
Glucose batch*	210	2330	62	12	71 (60–80)	22
Chemostat						
Glucose limited**	470	12600	293	29	40	62
Nitrogen-limited glucose carbon**	18	200	5	11	72	4
Nitrogen-limited succinate carbon**	200	8370	172	42	14	60

* Values given are averages of several experiments of cultures. Ranges are given for some items in parentheses.

** Results of a single experiment.

change. When a correction for the cryptic hydrolysis was made, it was concluded that the K_m for the galactoside site is the same in glucose-grown cells as with succinate-grown cells. This is because the cryptic transport takes place through the remaining glucose permease sites, so that a first-order process must be added to the saturating transport through the specific galactoside permease site in calculating the total transport of the cells.

Comparison of the α-methylglucoside-inhibited and -uninhibited transport would suggest that the effective K_m is smaller for the inhibited system. This is a novel result*, but is probably not real. Large corrections for the cryptic rate for the α-methyl-glucoside-treated cells, similar to those discussed in the last paragraph, would need to be made. The rate measured in the presence of formaldehyde is shown by the *plus* symbol in Fig. 1. The correction is large since the rate in the presence of formaldehyde is a much larger fraction of the total α-methylglucoside-inhibited rate than it is of the total transport. Because the cryptic process is first order, this correction would tend to make the K_m of the transport by α-methylglucoside-inhibited cells larger than it appears from casual inspection of the figure. Furthermore, a portion of the hydrolysis in the presence of formaldehyde is due to enzyme liberated from autolyzed cells or from cells broken during centrifugation. Since the enzyme has tighter binding to the substrate *o*-nitrophenylgalactoside (K_m = 0.3 mM) than does the permease system (K_m = 1 mM), hydrolysis from such cause, although a minor fraction of the uninhibited rate, would be saturated at all the concentrations tested and would not

* Most types of inhibition give either no change or an increase for the apparent K_m measured in the presence of the inhibitor. The exception is that of coupling or uncompetitive inhibition. Such effects require that the binding of the inhibitor causes a conformational or allosteric alteration at the catalytic site. The fact that various types of heterogeneity of the enzymatic capabilities can simulate some of the more esoteric types of inhibition has not been generally appreciated.

be inhibited by α-methylglucoside. Correction for this exposed enzyme would also tend to bring the apparent K_m for the α-methylglucoside-inhibited curve toward that of the control.

Because of these corrections for cryptic transport and enzyme leakage and because of the correction for influence of α-methylglucoside-resistant sites which must be made on the basis of detailed assumptions of several kinds, we would only wish to conclude that α-methylglucoside at least does not increase the apparent K_m and, therefore, is not a competitive inhibitor of *o*-nitrophenylgalactoside transport.

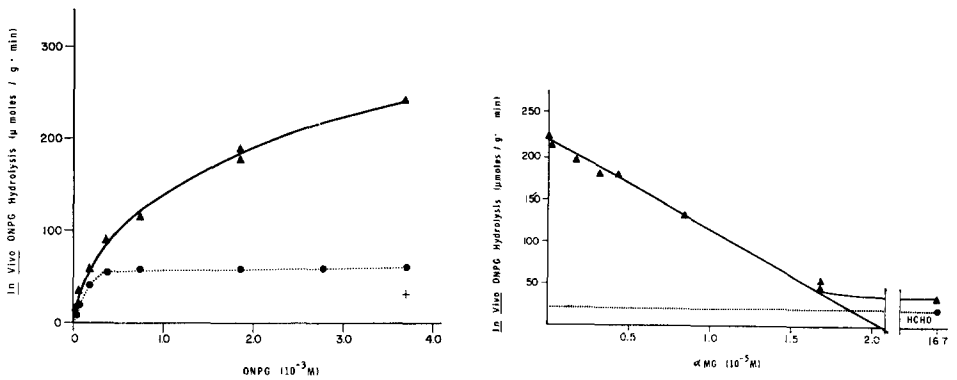


Fig. 1. The inhibition of cellular *o*-nitrophenylgalactoside (ONPG) hydrolysis by α-methylglucoside in glucose-grown organism. ▲, uninhibited; ●, 18 μM α-methylglucoside; +, 9 mM formaldehyde.

Fig. 2. Dependence of cellular *o*-nitrophenylgalactoside (ONPG) hydrolysis on the concentration of α-methylglucoside (α-MG). *o*-Nitrophenylgalactoside concentration was 8.33 mM. The K_m for cellular hydrolysis is 1 mM (see ref. 5). When the *o*-nitrophenylgalactoside concentration is increased 10 times, the rate in the presence of 1.67 mM α-methylglucoside is also not altered.

Fig. 2 shows the results of varying the α-methylglucoside but maintaining the *o*-nitrophenylgalactoside at a constant concentration. Again a simple interpretation of kinetics cannot be given, since even at very high α-methylglucoside concentrations the inhibited rate of *o*-nitrophenylgalactoside hydrolysis never decreases to the rate obtained in the presence of formaldehyde. Roughly the concentration that inhibits to one-half the processes that can be inhibited is 8 μM α-methylglucoside. This is surprisingly low in view of the K_m for α-methylglucoside of 29 μM for steady-state accumulation[6]. It should be noted that at 20 μM the inhibition is as complete as it is at very large concentrations of glucosides. In other experiments where the substrate concentration varied over 25-fold, the half-inhibitory concentration is constant at 9 μM.

Short-time kinetic experiments were performed to find out how long it took to establish the inhibition. For these experiments the Gilford was operated as a single-trace recording spectrophotometer at a rate of 10 sec/inch, full scale 0.200 A. During the course of the experiment, the blank was not read; but as soon as the rate became constant after introducing the inhibitor, the blank was remeasured. The instrument

was sufficiently stable so that there was no change observable. For the experiments reported in Table II, inhibition by α-methylglucoside is fast with less than 1 sec being required for the full establishment of the inhibition.

Accumulation of α-methylglucoside in glucose-grown ML 30 to a steady-state level takes approx. 4 min (ref. 6); therefore, the inhibition observed here does not require a full accumulation of α-methylglucoside, nor is it affected at the plateau of accumulation where the non-utilizable α-methylglucoside is leaving as fast as it is entering. It thus appears that α-methylglucoside acts directly on the entry mechanism. Another point to be made from these experiments is that they provide additional evidence that β-galactosidase is present in great excess. The delay times represent the sum of the time it takes the inhibitor to enter and the time for the previously accumulated *o*-nitrophenylgalactoside to be cleaved in excess of the new steady-state concentration.

Effect of metabolic energy supply

In order to rule out the possibility that the inhibition by glucose analogues is a result of competition for the same source of metabolic energy needed for galactoside

TABLE II

RESPONSE TIME OF HYDROLYSIS *in vivo* TO α-METHYLGLUCOSIDE

Initial reaction mixture	*Inhibitor added*	*Control rate (μmoles/g·min)*	*Inhibited rate (μmoles/g·min)*	*Per cent inhibition by α-methylglucoside*
Cells of glucose-grown ML 308 *plus* 3.7 mM *o*-nitrophenylgalactoside *plus*				
—	1.8 mM α-methylglucoside	192	42.9	77
1.8 mM α-methylglucoside	0.01 M N_3^-	39.6	39.6	
0.03 M N_3^-	1.8 mM α-methylglucoside	99	20.4	79

transport, energy poisons were introduced into the reaction mixtures. From Table II it is seen that the same degree of inhibition by α-methylglucoside results in the presence or absence of azide. In experiments not shown, it was found that at a concentration of 2 mM *o*-nitrophenylgalactoside and 0.03 M azide the hydrolysis *in vivo* of ML 308 succinate-grown cells is 76% of that rate without azide; while with glucose-grown bacteria, *o*-nitrophenylgalactoside hydrolysis *in vivo* is 53% of the control rate*. Thus, it would appear that in the glucose-induced cells more sites appear to be coupled to the cell's energy resources. However, the more likely interpretation is that the absolute excess of β-galactoside-hydrolyzing capability over the capacity to transport galactosides is greater in succinate-grown cells than in the glucose-grown cells. Therefore, metabolic coupling is less necessary in the former case since the steady-state level of internal *o*-nitrophenylgalactoside is smaller. Thus,

* It is of interest to compare these numbers with results obtained 4 years previously with the same organism grown on a slightly different minimal medium in a completely different country[6]. There the 53% number was 59% and the 76% number was 72%.

from inspection of Table I, it is seen that the difference in the first two columns is 8820 for succinate-grown cells and 2120 for glucose-grown cells. The effect of energy poisons was also tested on the competition of α-methylglucoside for *o*-nitrophenylgalactoside hydrolysis (see Table II). Azide had no further effect on the transport process remaining in the presence of α-methylglucoside. Presumably, this absence of even the effect shown with succinate-grown cells is because of the even greater excess of β-galactoside hydrolytic capability over the transport capability in this situation. Of import is the fact that α-methylglucoside still inhibits *o*-nitrophenylgalactoside hydrolysis in azide-treated cells, and the change in rate occurs rapidly after the addition of α-methylglucoside. This is conclusive evidence that α-methylglucoside competition is not just a competition for some energy reserve.

Effect of N-ethylmaleimide and p-chloromercuribenzoate

It has been known for some time[3,11] that *p*-chloromercuribenzoate and other sulfhydryl-binding reagents inhibit the permease of the galactoside system, in the sense that the rate of entry is reduced to that characteristic of permeaseless cells[5]. *N*-Ethylmaleimide was chosen for further study since its action can be prevented by specific substrates of the galactoside permease[12,13]. *N*-Ethylmaleimide has also been used in differentiating nucleoside transport systems[9]. In an experiment not shown, it was found that when cells were incubated with 50 μM *N*-ethylmaleimide for 15 min at 28° the transport process was irreversibly inactivated 58%. The apparent K_m of the remaining galactoside transport process was 0.8 mM, identical to the value for the non-*N*-ethylmaleimide-treated portion of cells. This suggests, even more strongly than the straight-line nature of the corrected Lineweaver–Burk plots, that the majority of the transport sites have the same affinity for *o*-nitrophenylgalactoside.

As a control, succinate-grown ML 308 cells were tested for *N*-ethylmaleimide inactivation and for the protection of the transport assembly against this inactivation by thiodigalactoside and/or α-methylglucoside. It was found under conditions identical to those given in Table III that thiodigalactoside protected, as FOX AND KENNEDY[12] had found, while α-methylglucoside did not. In a particular experiment where there was a 64% inactivation in the control, there was a 67% inactivation in the presence of 0.08 M α-methylglucoside. The latter result is expected, since there is little interaction of α-methylglucoside on the galactoside permeases in such cells. In these control experiments, it was noted that the proportion of sites sensitive to α-methylglucoside was the same in the thiodigalactoside-protected cells as in the control cells, and that *N*-ethylmaleimide under our standard conditions did not inhibit the free-enzyme hydrolysis of *o*-nitrophenylgalactoside.

Table III shows the result of *N*-ethylmaleimide on ML 308 cells which were induced for the glucose permease by growth on glucose. Again thiodigalactoside protects and α-methylglucoside still does not appear to protect. Thus, α-methylglucoside can inhibit galactoside transport but can not protect the site, as do some substrates for the permease. There are three unexpected, although repeatedly reproducible, results shown in this table. First, there is a very marked synergism of thiodigalactoside protection against *N*-ethylmaleimide inactivation by α-methylglucoside. While this synergism is easily demonstrated, we have not been able to find conditions by varying the concentration of either *N*-ethylmaleimide or α-

TABLE III

PROTECTION OF THE GALACTOSIDE PERMEASE IN GLUCOSE-GROWN ML 308 AGAINST *N*-ETHYLMALEIMIDE INACTIVATION

Treatment	*Rate of o-nitrophenylgalactoside hydrolysis in vivo (μmoles/g·min)*		
	o-Nitrophenylgalactoside	*o-Nitrophenylgalactoside + formaldehyde*	*o-Nitrophenylgalactoside + α-methylglucoside*
None	203.0	13.0	39.3
N-Ethylmaleimide	61.3	10.5	15.2
N-Ethylmaleimide* + 5 mM thiodigalactoside	98.0	29.4	42.9
N-Ethylmaleimide* + 5 mM α-methylglucoside	56.0	13.6	57.9
N-Ethylmaleimide* + thiodigalactoside + α-methylglucoside	160.0	33.2	127.0

* *N*-Ethylmaleimide at a final concentration of 0.5 mM was added to the cell suspension, which was aerated at 28° for 15 min. Cells were then centrifuged, washed once with Medium 9 with mercaptoethanol (0.01 M) and resuspended in Medium 9 without carbon source. In some cases Millipore filtration was substituted for centrifugation. When additional sugars were present, the cells were preincubated 3 min in their presence before the addition of *N*-ethylmaleimide. Although these results were from a single experiment, the salient features were observed in more than three trials.

methylglucoside so that a protective effect by α-methylglucoside alone could be demonstrated. This synergism could be interpreted, perhaps, by saying that when both α-methylglucoside and thiodigalactoside are present the transport of thiodigalactoside is slowed by the α-methylglucoside so that those elements in the galactoside transport system unique to galactoside transport are complexed to a greater degree or for a larger fraction of the time with the thiodigalactoside, and thus are not available for reaction with *N*-ethylmaleimide. The second finding is that cells treated with *N*-ethylmaleimide in the presence of α-methylglucoside no longer are inhibited by α-methylglucoside. This is indeed surprising; had the sites been more easily inhibited by the glucoside, instead of being less easily inhibited, the observation could have been explained. The results require the assumption of very special properties of the transport system, and these are under further study and will be discussed below. The third point is that thiodigalactoside protection renders the transport system less sensitive to formaldehyde inhibition of function. Together these findings imply that the transport system is more complex than had been evident from experimental work obtained so far.

Shifts of growth conditions

In this section, the interaction between "old" and "new" permease systems will be presented. In the first type of experiment *E. coli* ML 308 was grown in such a manner as to induce glucose permeases into cells that previously had only galactoside sites and no glucose sites. Aliquots were taken and the standard assay performed in the presence and absence of fully inhibitory concentrations of α-methylglucoside and of formaldehyde. Fig. 3 is the result of this experiment, which is pre-

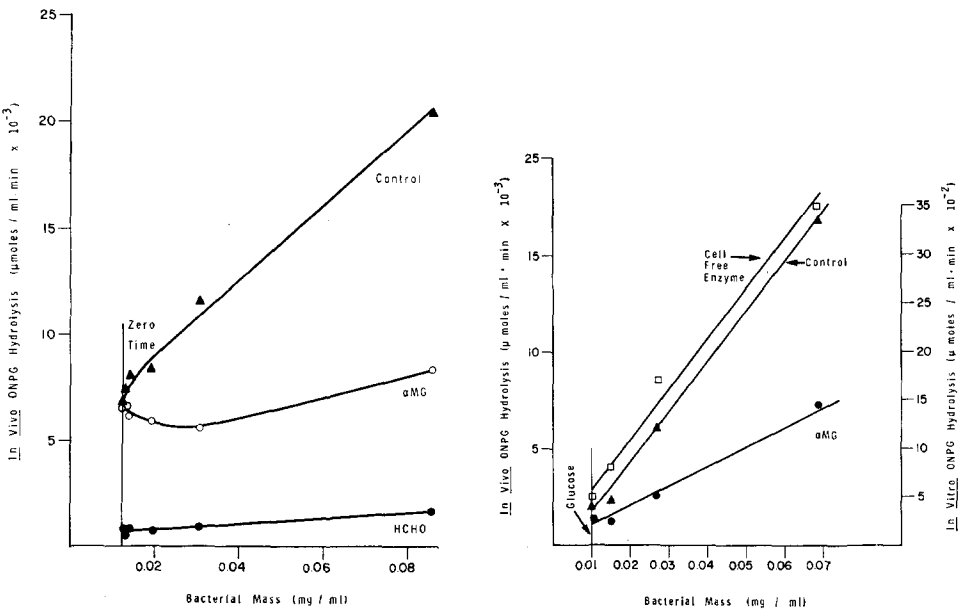


Fig. 3. Differential plot of permease production on shift of constitutive bacteria from succinate to glucose. The glucose concentration after the shift was 0.5 mM. The doubling time before shift was 70 min; the doubling time after shift was 46 min. There was no lag in turbidity increase. The slope of straight lines through experimental points corresponds to differential rates of: control, 179; α-methylglucoside (α MG), about 23; formaldehyde, 13 μmoles/g·min. These values are comparable with the values of cultures grown indefinitely at higher glucose concentrations. ONPG, *o*-nitrophenylgalactoside.

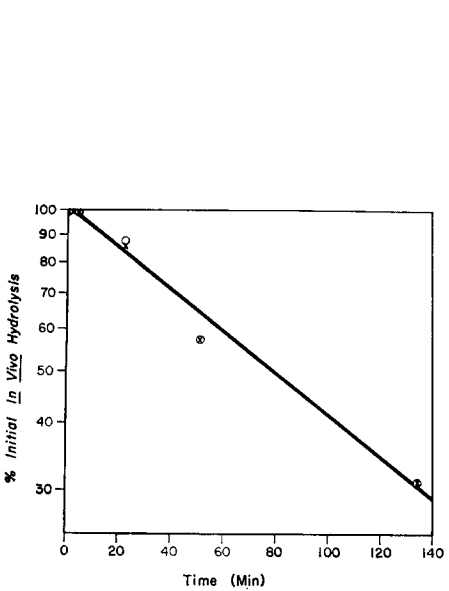
Fig. 4. Differential plot of permease production on shift of induced bacteria from succinate to glucose. IPTG present, 0.5 mM preshift and 5 mM postshift. Carbon sources as for Fig. 3. Doubling times were 146 min and 58 min, respectively. The differential rates are: *in vitro*, 2600; *in vivo*, 258; *in vivo plus* α-methylglucoside (α MG), 100 μmoles/g·min. These values are consistent with those of the constitutive bacteria under these conditions. ONPG, *o*-nitrophenylgalactoside; IPTG, Isopropylthiogalactoside.

sented as an isometric "Monod" plot. At time zero, the inhibition of α-methylglucoside is that of the succinate-grown ML 308, *i.e.* 0–13% inhibition. As growth and production of both permeases continue, the new galactoside sites on the membrane appear to be capable of interaction with the new glucose permeases, because essentially all the increment in hydrolysis of *o*-nitrophenylgalactoside is α-methylglucoside sensitive. Thus, all new sites formed throughout the entire 8-fold increase in bacterial mass interact effectively with the new glucose permeases. During this time the specific activity of the cells decreased from 570 μmoles/g·min, a value characteristic of succinate-grown bacteria, to 237 μmoles/g·min, a value typical of glucose-grown ML 308. The inducible *E. coli* strain ML 30 shows similar response if conditions are properly chosen, *i.e.* if inducer concentrations are such as to overcome the effects of catabolite repression[10], the result (Fig. 4) is similar to that of the constitutive ML 308 in the presence of the inducer glucose (Fig. 3), except that under these conditions there are some α-methylglucoside-independent sites being laid down. The total amount of α-methylglucoside-sensitive sites increases with time when both glucose and galactoside sites of transport are being placed in the membrane. Thus, only these

new sites appear to be acting through a common element, interacting with both kinds of permease. This suggests that the older permeases do not interact with the newer permease systems.

This can be shown more clearly with the inducible organism than with the constitutive one. When fully induced, succinate-grown ML 30 cells are placed into a glucose medium with no inducer and the level of permease and its inhibitors are all followed as previously described, the results shown in Fig. 5 were obtained. The important observation is that at no stage in the growth on glucose are the preexisting β-galactoside permease systems transformed or altered in such a way that they become inhibitable by α-methylglucoside. Thus, when the glucose permease is induced and the galactoside permease is not, there appears to be no interaction between the "new" glucose and the "old" galactoside transport systems.

It is known that permease systems are unstable; we found previously that the half-time for the inactivation of permease in ML 30 (fully induced) with inducer removed to be 320 min, during growth in succinate medium[7]. However, the initial dis-



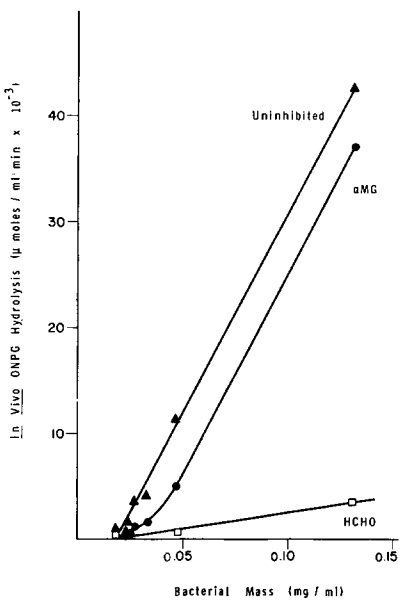


Fig. 5. Loss of preexisting galactoside permease during growth in glucose. ML 30 cells growing with a doubling time of 72 min in succinate medium and 1 M isopropylthiogalactoside were washed twice and resuspended in glucose and no isopropylthiogalactoside. Growth was immediate with a doubling time of 52 min. The activity of samples taken as before was followed. ×, without α-methylglucoside; ○, with α-methylglucoside. The results are presented as a semilog plot of the rates after correction for the formaldehyde "cryptic" rate. 100%, corresponding to time zero, was 435 μmoles/g·min. In a previous publication[7], exactly this experiment was performed except that succinate was the carbon source throughout. In that experiment the half-time was 320 min, instead of the 77 min observed here.

Fig. 6. Differential plot of galactoside permease production on cells previously induced to form glucose permease. ML 30 cells grown on glycerol were transferred to glucose for 2.5 generations. Then the cells were washed two times and resuspended in warmed Medium 9 containing 0.1% glycerol and 2.5 mM isopropylthiogalactoside. Growth continued with a doubling time of 62 min. At intervals, aliquots were chilled. Subsequently, the samples were washed and assayed as described. The last point corresponds to the 3-h growth sample. ONPG, *o*-nitrophenylgalactoside; α-MG, α-methylglucoside.

appearance rate after shift into glucose is such that the apparent half-time of the permease inactivation is 77 min. Since this is a greater fraction than the ratio of growth rates in the two media, this suggests that there is a positive inactivation or displacement of the galactoside permease by the glucose permease. A possible explanation is that the less specific carrier element is being displaced in the membrane to accommodate the new glucose permeases which are being built into the membrane. This can not be explained by the usual catchall definition of the glucose effect, since elements of the cell membrane which are already made are lost or inactivated.

In other experiments the same observation was made without centrifugation or other manipulation, simply by adding a glucose to the induction medium. Catabolite repression greatly decreased the inducibility caused by the level of isopropylthiogalactoside (0.2 mM). Again, at no stage was the galactoside transport sensitive to α-methylglucoside.

The inverse experiment, where glucose permeases are induced first and then galactoside permeases are introduced into the growing membranes, yields different kinds of information. *E. coli* strain ML 30, previously grown in succinate, was grown on glucose for approximately two generations. Then the cells were transferred into medium containing isopropylthiogalactoside and succinate but no glucose, and induction of the galactoside system followed. The results shown in Fig. 6 were obtained. The important new fact to be derived from this figure is that the galactoside permease sites placed on the membrane in the initial phase are inhibited by α-methylglucoside. At 10 min after induction, if correction is made for the cryptic rate, all the new sites are inhibitable. However, as growth continues, the ones introduced later cease to interact with α-methylglucoside. As late as 80 min after introduction of the gratuitous inducer isopropylthiogalactoside, the α-methylglucoside-inhibited sites are 43% of the total; whereas after 3 h of induction, the α-methylglucoside-sensitive sites are 13% of the total. To restate, the number of sensitive sites per cm^3 of culture is constant after the upswing in the α-methylglucoside sites is observed; *e.g.* the number of sensitive *o*-nitrophenylgalactoside sites after 80 min (next to last point in Fig. 6) of induction is proportional to $6.4 \cdot 10^{-3}$ μmoles/ml·min while at the last point, taken after 3 h of induction, the number of sites sensitive was proportional to $5.7 \cdot 10^{-3}$ μmoles/ml·min.

Differentiation between catabolite repression and competition for transport sites

By growing organisms in a chemostat or with the slow addition of limiting nutrient by a mechanical pump, it is possible to vary greatly the catabolite repression of the lactose operon, as has been shown by CLARK AND MARR[14]. The results of such experiments were included in Table I where the pertinent values for batch culture were also presented. The β-galactosidase is extremely stable, so that these values are a good measure of the degree of repression.

If we take into consideration the known instability of the galactoside permease, then the somewhat increased crypticity of the artificially slowed growing culture is comprehensible. The important conclusion is that while glucose limitation causes a much reduced catabolite repression of the lactose operon, the induction of glucose permease is almost as efficient as with an excess of glucose. With a nitrogen-limited chemostat with glucose as the carbon source, the effects of catabolite repression are evident and extreme in both the β-galactosidase and in the permease.

DISCUSSION

It has been generally felt that the permease is the limiting step in transport. In the steady state, obviously, the rate of all steps involved in the transport process must be equal, as has been well discussed by HEARON[15]. Yet a number of arguments would indicate the transport velocity largely reflects the kinetic properties of the permease itself. Most evident is the fact that cryptic transport is much slower. Possibly the most impelling fact is the finding that on induction the amount of permease as measured by cellular hydrolysis of *o*-nitrophenylgalactoside increases linearly with the rate of growth. This was shown before[5] and is shown again in the present paper.

The observation that K_m for plateau uptake of gratuitous substrates is invariant throughout the induction process leads to the same conclusion. This was found in the original work on galactoside permease[1] and confirmed with more accurate methods[5]. Additional facts pointing to the limitation by permease of the transport are the first-order inactivation of permease during growth or in resting culture[7] and that the K_m for the hydrolysis of *o*-nitrophenylgalactoside is invariant to the partial inactivation of the permease with *N*-ethylmaleimide reported in the present paper. The same conclusion follows from the point that the K_m is the same after proper corrections have been applied for cryptic transport to the transport of *o*-nitrophenylgalactoside in glucose-grown cells and succinate-grown cells, even though the amount of permease for galactosides is markedly different. Finally, we should mention the fact that different substrates for the galactoside permease tend to inhibit each other in a reasonably competitive manner. This once again would suggest that the competition was for the entity that has the specificities and properties attributed to the permease. Recently, we have carried out some detailed theoretical derivations for various models for permease action[16]. An important conclusion of this theory is the conclusion that agents which influence a step which takes place on the external face of the membrane will more greatly inhibit the entry process and will do relatively less to the exit process. On the other hand, the reverse is true for conditions that influence a part of the process that takes place on the internal face of the membrane, *i.e.* efflux will be more markedly affected than will influx by agents acting on a site on the inside of the membrane. From this theorem, then, the action of *N*-ethylmaleimide and formaldehyde should be on the portion of the process which is nearer the external environment since these agents decrease the rate of entry by a much larger factor than they decrease the rate of exit[5]. Together these facts would point to the idea that permease is itself limiting and is on the external membrane.

On the other hand, the argument is weakened by the observation that "counter turnstyling" is an important process—that the flux of one substance out of the cell is greatly augmented by the flux in of a second substrate for the same permease and even of the same transporter system when different permeases interact. This must mean that the transporter element of the cell is in fact limiting as well. Secondly, if the transport assembly consists of many permeases communicating to a single transporter molecule, then the observation that the K_m is invariant to induction and that the v_{max} increases with cell synthesis only shows that completed assemblies are added to the growing membrane, and that it is the kinetic properties of the com-

pleted assemblies that are under study and not the kinetics of one aspect of the transport process. No one is sure what is being inactivated by sulfhydryl reagents, except that it is near the external membrane. What is being inactivated when the cultures are aerated or starved in various ways[7] is unknown, except once again that it is something on the external face of the membrane and does not involve the transporter. Consequently, the most pertinent variable to answer this type of question is to see how the effective K_m varies as the overall permease composition of the bacteria is altered. This puts special stress on our new finding that K_m for *o*-nitrophenyl-galactoside transport is invariant to the admixture of glucose permease with galactoside permease, arising when both are formed together in glucose-grown constitutive cells.

Equations for the transport of a permease transporter model system have been presented[16]. The expression for K_m is:

$$\left(\frac{k_{-1}\,k_{-2}}{k_1\,k_2}\right)\left(\frac{\frac{1}{k_{-2}}+\frac{k_2}{k_{-1}k_2}+\frac{1}{k_3}+\frac{1}{k_5+k_6[A]}}{\frac{1}{k_2}+\frac{1}{2k_3}+\frac{1}{2k_4}+\frac{1}{k_5+k_6[A]}}\right)$$

Rate constants with subscripts 1 or 2 have to do with the permease-catalyzed step, 3 and 4 with the diffusion process, and 5 and 6 with the (active) dissociation on the inner membrane. The introduction of a larger number of permease molecules to a common transporter can be taken into account by multiplying each rate constant that has a subscript 1 or 2 by n. These n's will cancel in the first factor. Clearly, the apparent K_m will not change if either the permease-catalyzed steps are dominant in both the numerator and denominator, or if they are negligible in both the numerator and denominator. Thus, it will be exceptional to find an alteration in K_m even if a multiplicity of permease molecules can interact with a common transporter.

Let us now turn to a discussion of the degree of multiplicity of permease molecules to transporter molecules. The interaction between different kinds of permeases shows that the transporter must be capable of interacting with at least two different permeases—galactoside and glucose, or galactoside or maltose, *etc.* Yet there are other reasons to imagine that the multiplicity might be very much higher than just two. For example, the apparent number of transport assemblies is presumably of the order of 200–300 (see refs. 17, 18). These results are obtained by measuring the rate of the loss of "maintainability" of cultures grown under conditions where the permeases are no longer induced. Recently, FOX, CARTER AND KENNEDY[13] have found, through the use of radioactive *N*-ethylmaleimide, that there are of the order of 8000 sites which presumably have a specificity for galactoside transport. There are two alternative possibilities to explain the discrepancy between these two kinds of results. First, we might interpret, as we have done before[7], that the permeases in excess of the actual transport capability of the cell are lost to transport measurements in a way that does not inactivate them but merely uncouples them from their proper transporter. In this interpretation, there might be 8000 permease molecules on a cell but only 200 or 300 of them would actually be capable of interacting with transporters and this number would decrease further when the cells were starved under various conditions. Alternatively, one might picture that all 8000 communicate with transporters but that there is something of the order of 30–40 permeases communicating with each transporter in the system.

In this case then, the loss of activity during starvation conditions would be difficult to explain because one should expect multi-hit type of kinetics if movement of the transporter across the membrane was limiting, but would follow naturally if the permease-catalyzed reaction was limiting.

On the other hand, such a high degree of multiplicity would permit the easy explanation of the fact that very low concentrations of α-methylglucoside inhibit transport of galactosides in cells constitutive for galactoside permease when grown in the presence of glucose. α-Methylglucoside inhibits at a half-maximal concentration of 8–9 μM. Yet the Michaelis–Menten constant for α-methylglucoside uptake is reported in the literature as 30 μM in ML strains, but is 0.1 mM in Salmonella strains[19]. The evidence is quite clear that α-methylglucoside and glucose enter the the cell through the same transport mechanism[20]. This finding of an inhibitory constant smaller than the constant for uptake suggests either a completely different action in inhibition than in its own transport or an allosteric interaction. It certainly eliminates any of the simple types of inhibition so well studied by biochemists and pharmacologists. These findings would be consistent with the idea that in glucose-grown cells there are many glucose permeases per transporter and that the chance of having at least one of these filled would take place at a concentration of glucoside very much lower than that concentration which is required to half-saturate the rate of the entry process.

Now, we turn our attention to the finding that interaction between permeases does not seem to take place unless two kinds of permease are grown into the membrane at the same time. The only exception where such an interaction could be demonstrated was when a few galactoside permeases were introduced into a bacterial cell that already contained a large number of glucose permeases. Then, and only then, would those permeases which were introduced in the next half a generation show such interaction with glucose. This could mean that either there can be an interaction over the distance in space that corresponds to the amount of membrane synthesized in this time or, alternatively, it could mean that there was a pool of residual glucose permease molecules that had been synthesized under the genetic control but had not yet been incorporated into the growing membrane and may have existed in a soluble pool of the kind that KOLBER AND STEIN[21] may have isolated in their recently reported experiments. As yet, we have no idea whether new membrane is synthesized by intercalation into the previous existing membrane or if there is a single region in the cell where new membrane is synthesized or if there are many discrete places on the membrane where growth takes place. In any case, the interaction is not extensive and can not be attributed to competition for soluble substances of the cell.

There are several possible reasons why no interaction ever was observed when the shift was from galactoside permease synthesis to glucose permease synthesis, even for the first few permeases laid down after the shift. One reason may simply be that the new glucose permeases may only interact at the margin between galactoside and glucose permease-containing areas, while most of the older galactoside permease sites do not so interact. Obviously, the proportion of the total galactoside-transporting capability that could be inhibited could only be a very small portion of the total galactoside transport capability of the cell and therefore may have been missed. The second alternative explanation is that in constitutive cells the glucose permease

is the major component, and it is possible that the interaction can only be shown on the process carried out by the minor component on adding the substrate for the major component.

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