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INTERCALATION OF PERMEASES DURING MEMBRANE GROWTH

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SUMMARY

Cells were grown so that carbohydrate permeases of different specificity are incorporated into the growing membrane. The present results, together with a retraction of a previous result, imply that as the membrane of *Escherichia coli* grows new permeases are intercalated at all regions of the membrane previously possessing transport capability.

INTRODUCTION

If lactose-constitutive cells are grown in the presence of a second sugar such as a glucose, the cells possess an increased capability of transporting the second sugar and still retain the capacity to transport galactosides. In addition, several interactions of the two specific transport processes can be demonstrated. It appears that a portion of the interaction is local, dependent on the proximity on the membrane of the gene product of the Y locus of the lactose operon and the product resulting from growth in the presence of the second sugar.

The original evidence[1] that the interaction results from the propinquity on the membrane of the two "permeases"[2,3] is that a flux inward of the second sugar induces a counter flux of previously accumulated radioactive galactoside. This, in conjunction with the results of physiological experiments of KEPES[4], suggested that the interaction resulted from the envolvement of a common, less specific carrier or transporter element in the transport assembly. It was assumed that this common element can interact with more than one permease element on the outside of the cell.

Recently[5], we exploited this interaction to attempt to study the mechanism of growth of the membrane. First, the organisms were grown so that they produced one permease; then conditions were changed so that they produced only the other permease. We interpreted the results to mean that growth took place in localized regions of the membrane so that there is no interaction of permease elements laid down at different times.

The major purpose of the present paper is to retract that claim and to reinterpret the previous and the new results of the shift experiments to show that indeed the

Abbreviations: IPTG, isopropyl thio-β-D-galactopyranoside; αMG, α-methyl-D-glucopyranoside; ONPG, *o*-nitrophenyl galactoside; TMG, thio-methyl-β-D-galactopyranoside.

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membrane grows by intercalation of groups of new elements of the transport mechanisms in the neighborhood of all old elements.

MATERIALS AND METHODS

Conditions, organisms, techniques, and assays were those employed previously[1,5], except that $2.5 \cdot 10^{-3}$ M azide was used in the first wash solution when the organisms were shifted from one growth media to another. An azide wash was introduced in the shift experiments to eliminate residual inducer, since it is known that the exit rate of previously accumulated galactosides is increased by energy poisons[1]. This wash did eliminate significant increase in the levels of β-galactosidase from cells grown previously in the presence of isopropyl thio-β-D-galactopyranoside (IPTG) as inducer. It caused no lag in the increase in cell mass.

RESULTS

It was hoped to confirm our former working hypothesis by conducting experiments in which a period of induction for one permease was separated from a period of induction of the second permease by an interval in which neither was induced.

When these results (see below) did not accord with the hypothesis of a growth region on the bacterial membrane, we re-examined all our previous results[5] and found that one experiment reported previously was faulty. This was the experiment in which *E. coli* ML 30 cells, induced for many generations with IPTG for galactoside permease, were resuspended in glucose media containing no galactoside. In our previous studies, samples from such culture had not been adequately washed to remove the glucose so that the permease appeared to be more rapidly inactivated than is actually the case. Moreover, in these experiments, never did there appear to be transport sites inhibitable by glucose or α-methyl-D-glucopyranoside (αMG), although there really were.

Thus, Fig. 5 of the previous publication[5] is erroneous. Other results given there are not subject to the same criticism, and we believe they are valid.

In Fig. 1 of the present paper is shown a typical valid experiment of this type in which the cells sampled for assay are adequately washed (twice) to remove the glucose. There is a negligible change in the total amount of galactoside transport capability per unit volume of culture even though the bacteria were followed through 20-fold growth after the shift to glucose media. Thus the transport capability per cell was diluted by the same factor as that by which the concentration of cells was increased. On the other hand, the inhibited rate in the presence of αMG fell from nearly no inhibition at the time of the shift to almost total inhibition at the end of the growth period.

If an 80-min growth period in glycerol in the absence of either IPTG or glucose is interposed, similar rapid conversion of non-inhibitable sites to inhibitable sites is observed (Fig. 2). The specific activity of the galactoside permease at the end of the glycerol growth period is less, and a somewhat larger proportion of the transport initially is inhibitable by αMG, presumably because of the formation of a small amount of glucose permease due to the slight glucogenic nature of glycerol. The drop in the number of non-inhibitable sites is as precipitous in this case as when there is no

intervening period, but it does not fall to so low a level. In this case, as in Fig. 1, a factor of 2-fold in growth decreases the number of uninhibitable sites to about one-half. On the assumption that the interaction of αMG with galactoside transport is dependent on a common non-inducible element in the transport[1] which can bind different sugars only if both permeases are affixed locally, these results imply that the new products resulting from growth in glucose are incorporated in all regions of the cell membrane.

When the experimental situation is reversed and glucose grown cells are washed and placed in medium containing IPTG in the absence of glucose, the first formed elements for transporting galactoside interact with the previous capability for glucose transport while later elements do not interact[5]. On repeating this experiment with an intervening period of growth (Fig. 3) where neither transport capability was being induced, virtually identical results were obtained as without this intervening

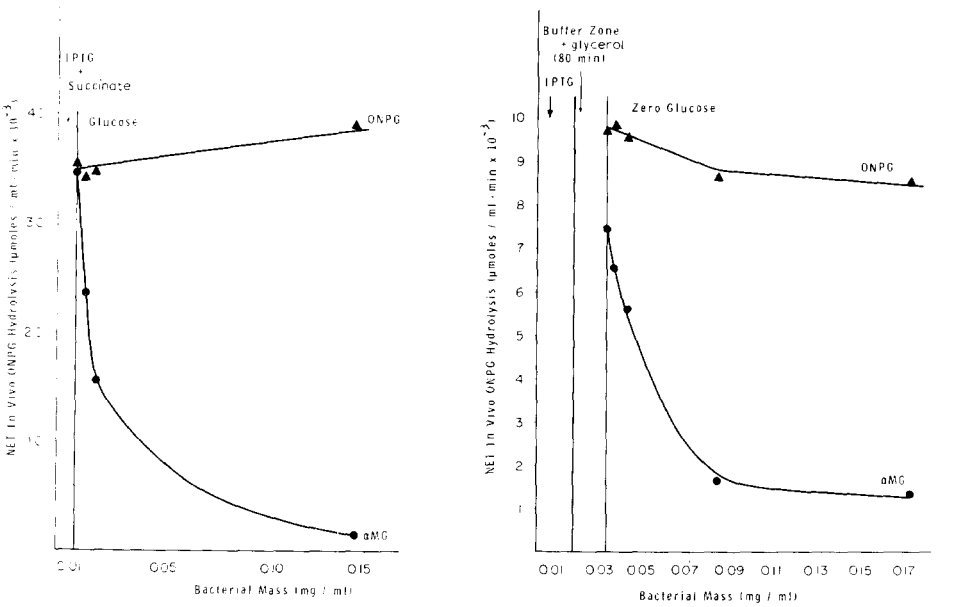

Fig. 1. Differential plot of permease interaction on shift from induction of galactoside permease to glucose permease synthesis. *E. coli* ML30 cells growing with aeration at 37° with a doubling time of 82 min in succinate medium and $5 \cdot 10^{-4}$ M IPTG were washed twice, the first time in azide-containing M-9. The cells were resuspended in glucose-containing (0.5%) medium with no IPTG. Growth was immediate with a doubling time of 53 min. The transport activity of samples taken at indicated times was followed by the hydrolysis at 28° of the chromogenic substrate ONPG. Under these conditions hydrolysis is limited by the transport process and not by internal β-galactosidase: ▲, *in vivo* ONPG hydrolysis without αMG; ●with αMG. All samples are corrected for the formaldehyde control, which eliminates permease transport, but not cryptic transport of hydrolysis due to liberated β-galactosidase. The corrections never amount to more than 10%.

Fig. 2. Differential plot of permease interaction on shift of previously induced cells into glucose after an 80-min buffer period in the presence of glycerol. *E. coli* ML30 cells growing in M-9 with 0.4% glycerol and $5 \cdot 10^{-4}$ M IPTG were washed twice and resuspended in warm M-9 containing 0.2% glycerol and were allowed to grow for 80 min. The bacteria were again washed once and resuspended into warm 0.2% glucose M-9; aliquots were taken as before. Doubling times were 66 min in glycerol and 49 min in glucose with no observable lag in turbidity increase during shifts. Activity of samples as Fig. 1: ▲, without αMG; ●, with αMG. All samples are corrected for formaldehyde treated controls.

period. The first transport systems for galactosides incorporated into the growing membrane interact strongly, but non-inhibitable capability develops with only an extrapolated delay of a factor of 1.72 in dry weight.

These experiments imply, under the same assumption stated above, that the gene products produced as a result of inducing transcription of the lactose operon are also introduced into the growing cell at random. We had previously interpreted this result to mean there was a pool of the products of the inductive response to glucose that were incorporated into the membrane subsequent to the removal of the inducer.

It also implies that after 2-fold growth in the IPTG inducing medium, regions in the membrane exist that are not inhibited by αMG, even at a concentration of

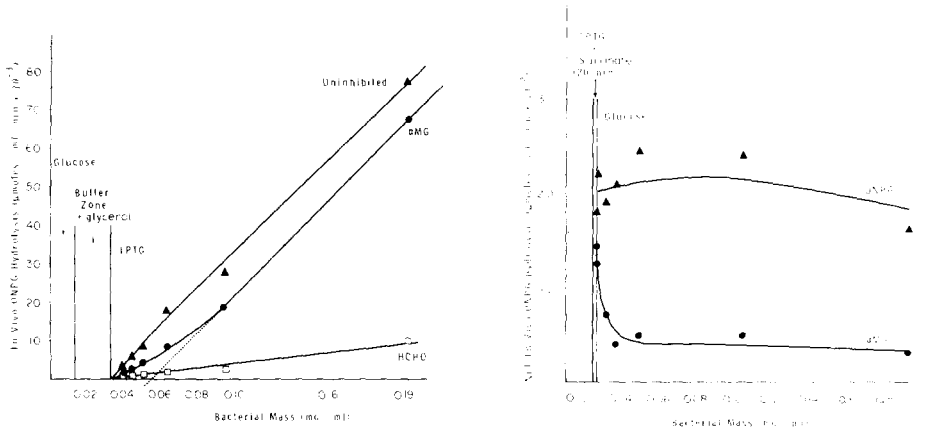

Fig. 3. Differential plot of glucose-grown organisms of galactoside permease production after shift to induction for an 80-min buffer period in the presence of glycerol. *E. coli* ML30, having grown in the presence of 0.2% glucose for many generations, were washed twice and resuspended into warm M-9 containing 0.2% glycerol. The bacteria were allowed to grow for 80 min following a 20-min lag. $1 \cdot 10^{-3}$ M IPTG was then added to the growing suspensions. Doubling times were 54 min in glucose medium and 60 min in the presence of glycerol. The differential rate of ONPG hydrolysis post-shift was 410 μmoles/g per min: ▲, *in vivo* ONPG hydrolysis without αMG; ●, with αMG; □, with formaldehyde.

Fig. 4. Differential plot of permease interaction on shift of bacteria from a 20-min pulse of induction of galactoside permease to glucose-containing media. Doubling times were: pre-shift, 70 min; and post-shift, 58 min. *E. Coli* ML30 cells grown on succinate were induced for the galactoside permease with $1 \cdot 10^{-3}$ M IPTG for 20 min. The cells were washed 2 times, first with azide wash medium, and were then placed in 0.5% glucose-containing M-9 and allowed to grow. Samples were taken as specified: ▲, without αMG; ●, with αMG. All values are corrected for formaldehyde-treated controls.

1.8 mM. As this concentration is 200 times the concentration that gives half-maximal inhibition of the transport process, this appears to be a change in the proportion of qualitatively different sites. Moreover, the sensitivity of transport to αMG concentration of that portion of the activity which is inhibitable by αMG remains the same independent of the degree of induction of the glucose permease (Table II). The half-inhibiting concentration remains about $1 \cdot 10^{-5}$ M, even for cells harvested after a shift of glucose cells to IPTG just as fully resistant sites are appearing.

Experiments were also performed in which the permease was induced for a short period of time in order to further test the interaction of permeases. Fig. 4 shows the result of inducing succinate-growing *E. coli* ML 30 for the galactoside

transport systems for 20 min, then washing the bacteria, and resuspending them in medium which will induce for the glucose permease. The *o*-nitrophenyl galactoside (ONPG) transport capability very rapidly becomes sensitive to the glucose transport analogue, αMG. Note the significant, but slight, initial increase of ONPG sites after time zero and then the slow decline in the rate of *in vivo* hydrolysis. The rise is the largest we have ever observed and might indicate that the product of the permease gene may have cytoplasmic pools which, even after removal of inducer, are incorporated into the growing membrane. However, these pools constitute no more than one-fourth of the amount of permease incorporated under inducing conditions into the membrane in less than a third of a generation.

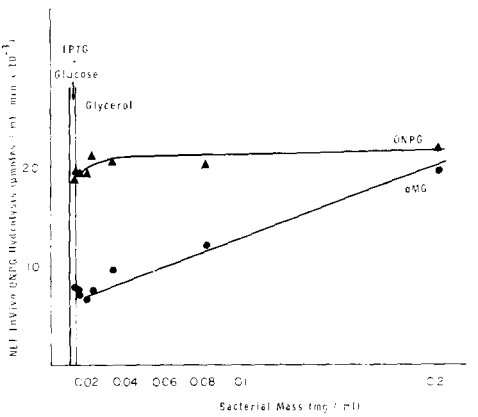

Fig. 5. Differential plot of permease interaction after the simultaneous pulse induction of both the galactoside and glucose permease to non-inducing glycerol containing growth medium. *E. coli* ML30 cells growing in 0.2% glycerol were washed once and resuspended into warm M-9 containing $5 \cdot 10^{-4}$ M glucose and $1 \cdot 10^{-4}$ M IPTG. Induction of both permeases was allowed to take place for 30 min. The bacteria were then washed twice and resuspended in warm 0.2% glycerol-containing M-9. Doubling times were 58 min in the glucose medium and 56 min post-shift in glycerol. Samples were measured as before: ▲, without αMG; ●, with αMG. All values are corrected for formaldehyde-treated controls.

When both the glucose and galactoside permeases were induced at the same time for only 30 min and the cell suspension was washed and resuspended in M-9 and glycerol, the results shown in Fig. 5 were obtained. At time zero, 63% of the galactoside sites were inhibited by αMG.

There is a slight initial increase in the transport capability and a slight initial increase in the proportion of αMG sensitive sites. These probably represent delayed incorporation of the gene products of the two permease genes. There is a gradual loss of the proportion of transport sites for ONPG inhibitable by αMG. At the end of the sampling period, α-methylglucose inhibited only 11% of the galactoside permease activity. One possibility is that the glucose permease is being inactivated by growth of the bacteria with a half-life of approx. 1 h. This inactivation of the glucose permease would therefore release the galactoside sites from inhibition by αMG. However, we have shown that the inactivation, at least under resting conditions, could not account for this[6,7,11] since the inhibitability of glucose on galactoside transport does not change very much as the galactoside transport is inactivated in starving culture.

Previously[6], the rate of inactivation of the galactoside permease was followed

in growing and non-growing cultures. It is now found, in confirmation of these studies, as shown in Table I, that the galactoside permease is inactivated with a half-life of approx. 6 h in growing bacteria and from 3 to 8 h in non-growing cultures, depending on carbon source present. The rate of inactivation of preeexisting galactoside per-

TABLE I

HALF-LIFE FOR THE INACTIVATION OF PERMEASE UNDER VARIOUS CONDITIONS

A growing culture of ML30 in 0.5% succinate and $5 \cdot 10^{-4}$ M IPTG was centrifuged and washed twice in M-9, the first time with azide in the media. The culture was divided and placed in the different media. Carbon sources were present at a concentration of 0.3%, except for growth in glucose in which the concentration was 0.1%. To prevent growth, chloramphenicol was added at a concentration of 50 μg/ml; no further turbidity increase was observed. Doubling times during growth in succinate and glucose were 78 and 60 min, respectively, and are typical. Samples were assayed under standard conditions and were corrected for lysed cells and cryptic transport by formaldehyde controls. Logarithmic plots give straight lines for all but the growth in glucose experiments. All experiments were carried out for a 480-min period at 37 °.

Conditions	*Half-life (min)*
No carbon source + chloramphenicol	380
Succinate + chloramphenicol	480
Glucose + chloramphenicol	260
Succinate	360
Glucose	360–600

mease by growth in glucose is certainly not greater than on growth on succinate; and therefore, the glucose permeases are not supplanting the galactoside permeases. Together these facts would seem to rule out inactivation of the glucose permease as a cause of the loss of interaction shown in Fig. 5.

A more probable explanation states that there is physical separation of the permeases. Since a 10-fold growth is required to halve the number of inhibitable sites, probably intercalation by new membrane growth, without the necessity of the assumption of mobility or diffusion of the permease molecules in the membrane, will be sufficient to account for this phenomenon.

CONCLUSIONS

The specificity of transport mechanisms has been obscured by the finding that transport processes *via* quite stereospecific permeases can interact with transport processes *via* other highly stereospecific permeases. This interaction must occur through other less specific parts of the transport mechanism. For example, galactose interacts with galactoside transport under all circumstances known to us, while glucose or αMG interact only in cells grown in glucose or a substrate that is rapidly converted intracellularly to glucose, which are also the conditions favoring the synthesis of glucose permease. Thus, WINKLER AND WILSON[8] found that the v_{max} for the initial rate of αMG uptake is 5–6 times higher and the degree of inhibitions of ONPG *in vivo* hydrolysis is considerably elevated in ML308 grown on glucose compared with cells grown on casein hydrolysate, glucose 6-phosphate, gluconate, and fructose. Similarly, we have found that maltose in the growth medium[9] yield cells with high

capability for glucose uptake if amylomaltase is present and lower amounts in cells incapable of converting maltose to glucose, and that the effect of αMG on ONPG transport is larger in the former case than the latter one. The only circumstance known to us where growth conditions resulting in high glucose transport capability and not high degree of inhibitability of the ONPG transport is to grow ML308 in glucose-limited chemostat culture[7].

We can assume broad specificity of the common factor, no matter if the interaction is at the level of a common carrier, or at the level of Enzyme I or the HPr of the phosphotransferase system[10], or some as yet unspecified entity necessary for the transport process without the unique specificity of the permeases to explain these findings. The present communication is concerned with the interaction of galactoside transport with that of αMG. What is the evidence that the interaction is a very local one depending on juxtaposition of permeases on the cell membrane?

In cells possessing both transport capabilities, immediate inhibition of ONPG transport results from addition of glucose[7] and high levels of αMG[5]. The former induces counterflow of thio-methyl-β-D-galactopyranoside (TMG) (ref. 1 and A. KEPES, personal communication) although the latter does inhibit the exit of pre-accumulated TMG. It is almost impossible to believe that these effects are indirect and result from interactions mediated through some diffusible chemical species in the cytoplasm.

On the other hand, it is also quite clear that both glucose and αMG also cause inhibitions of *in vivo* ONPG hydrolysis in an indirect way[7]. This is evidenced by a delayed time to achieve full inhibitions by low levels of αMG or by high levels of glucose, *i.e.* both these agents at the appropriate concentrations have an immediate inhibitory action on permease action, but later develop a higher degree of inhibition. Under the conditions of αMG concentration and time scale of the measurements, the results presented here are the sum of the immediate and delayed action of αMG. The first is no doubt local; the second must surely result from some more general depletions of energy reserves or control signal. Most of the experiments reported in this paper were performed before we could distinguish between the two processes. However, Table II shows that the ratio of the immediate to total degree of inhibition is the

TABLE II

PROPERTIES OF CELLS INDUCED FOR THE GALACTOSIDE PERMEASE AND SUBSEQUENTLY INDUCED FOR GLUCOSE PERMEASE

	Inducible ML30 succinate $5 \cdot 10^{-4}$ M, IPTG	*Inducible ML30 induced cells grown one generation in glucose with no IPTG*	*Constitutive ML308 cells glucose-grown (typical values)*
		(μmoles/g per min)	
β-Galactosidase	12972	8972	3000
Net *in vivo* hydrolysis			
Control	701	521	200
10000 μM αMG	550	288	38
5000 μM glucose	570	354	72
Half-inhibitory concentration		*(μM)*	
For αMG	—	10	10
For glucose	—	4*	10

* Measurement made in 5-cm cells so that effects of glucose consumption could be minimized.

same as a culture undergoing a shift as it is in a glucose-grown culture of ML308 which has been grown indefinitely in glucose medium and has high levels of both galactoside and glucose permeases.

Another reason for assuming that the interactions reported here are proportional to the local effect is the peculiar dependence on inhibitor concentrations[5]. Inhibition by αMG is half-maximal at $1.0 \cdot 10^{-5}$ M, but at much higher concentrations, the degree of inhibition is never complete and, in fact, depends on the growth history of the organism, not on the conditions of assay. For example, if we computed the specific activities of the *in vivo* ONPG hydrolysis in the presence of αMG for the different kinds of experiments shown in Figs. 1, 2, and 4 at the interpolated time when the cells have grown one doubling in glucose by simply dividing the ordinate by the abscissa, there is a 6-fold variation. The rates are approximately: Fig. 1, 180; Fig. 2, 50; Fig. 4, 30 μmoles/g per min. Since there is presumably the same amount of glucose permease in each case, if the inhibition were due to non-local interactions involving ATP depletion on some non-membrane bound component of the phosphotransferase system, then αMG transport would deplete reserves of those materials to the same degree, and would limit ONPG flux to the same level in all cases.

Yet another reason for assuming that the interactions reflect local membrane geometry is the observation presented here that when both permeases are laid down during one-half a generation, the interaction is initially maximal and then falls until the flux of galactoside is almost completely independent of the presence of αMG (Fig. 5). This fall is not the result of inactivation of either permease. Both activities apparently decay, but much more slowly than does the loss of interaction (present results and refs. 5 and 6).

Together with the conclusion that the interaction is local, the results of the shift experiments imply that the membrane grows by intercalation at all regions so the bacterial membrane containing permease so that newly introduced permeases can interact with permeases previously laid down through common elements of the transport system, *i.e.* the carriers, both old and new. But these observations also suggest several other conclusions:

(1) That the permease is the rate-limiting step of the transport under the usual conditions of assay. This has tacitly been assumed[2,3], but, as pointed out previously[1,12] the experimental observation that the apparent K_m for hydrolysis of ONPG by cells is constant and independent of the degree of induction could be interpreted to mean that new complete and independent assemblies were being introduced into the membrane. In a fully induced cell, more than one galactoside permease interacts with the common element, and consequently, if this element were rate limiting, an increase in transport rate on further growth under non-inducing conditions would be expected. But any increase in the control curves in Figs. 1 and 2 is very small, even when a correction for permease decay is included. Consequently, it can be concluded that, even for fully induced cells, the permease level is rate determining.

(2) Groups of more than one permease molecule are introduced specially and temporally together into the growing membrane. In the case where both IPTG and glucose were added for one-half a generation, the interaction was initially higher than expected from random and independent introduction of a permease molecule into the growing membrane. For either type of permease, approximately a doubling of cell mass is required for 50% interaction when there is a large amount of the others loose

and one-half a generation would give very approximately 25% interaction so that by chance association, we should expect about 1/16 of the cell permease system to show when both are present in small amounts. In fact, this is the level of interaction if cells are induced for one-half generation for one and then for the second one-half generation induced for the other carbohydrate permease. Yet 63–67% of the *in vivo* activity was inhibitable when both permeases were laid down simultaneously. This means that as the membrane grows there are local regions laid down with several permease assemblies.

(3) The permease molecules are rather rigidly bound to the membrane. If the structure of the membrane were fluid, then the initial interaction would be lost. Yet it takes a mass increase of 10-fold to decrease the interaction by one-half.

It appears quite likely that the conclusion that permease molecules can be added at all points of the cell membrane is a reflection that membrane and cell wall growth in *E. coli* also takes place in this manner. Recently E. GREEN AND M. SCHAECHTER (personal communication) have reported with radioautography that membrane-bound tritium does not segregate for many generations, and certainly not in the manner predicted by the original version of the replicon model.

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