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Role of Inducer Exclusion in Preferential Utilization of Glucose over Melibiose in Diauxic Growth of *Escherichia coli*

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The role of inducer exclusion in diauxic growth of *Escherichia coli* on glucose and melibiose was investigated. The amounts of glucose and melibiose in the culture medium were determined during the diauxie. Glucose was consumed during the first growth cycle of the diauxie, and melibiose was consumed during the second cycle. The addition of adenosine 3',5'-cyclic monophosphate to the culture medium released both transient and catabolite repressions on the melibiose operon by glucose. Biphasic growth without a transient lag phase was observed in the presence of adenosine 3',5'-cyclic monophosphate. Preferential utilization of glucose over melibiose was observed even under such conditions. Thus, it is clear that inducer exclusion alone is sufficient to ensure the preferential utilization of glucose over melibiose. Similar results were obtained from a glucose-lactose diauxie. Inducer exclusion itself was not affected by adenosine 3',5'-cyclic monophosphate.

Monod (15, 16) found that in the presence of two carbohydrates, such as glucose and lactose, the growth curve exhibited two successive growth cycles, separated by a period of lag. This type of growth behavior has been termed diauxie. It was possible to classify various carbohydrates into two groups according to whether or not diauxic growth occurred when each carbohydrate was paired with glucose (15, 16). One class of carbohydrates did not give rise to diauxic growth when associated with glucose. The other class did. Later it was established that the former class of sugars is incorporated by cells via the phosphoenolpyruvate-sugar phosphotransferase system (PTS), whereas the latter class of carbohydrates is not (22). For instance, when glucose and lactose were added as carbon sources to cells of *Escherichia coli*, glucose was utilized in the first growth cycle, and then lactose was utilized in the second cycle (16). Thus, cells of *E. coli* utilized glucose preferentially to lactose. In other words, lactose cannot be utilized in the presence of glucose in the medium. Three control mechanisms are operating behind this phenomenon, namely, transient repression, catabolite (permanent) repression, and inducer exclusion (12, 13). Transient repression refers to the transient inhibition of gene expression, which occurs when glucose or other PTS sugars (sugars which are transported via the PTS) are added to a culture medium. Catabolite repression is distinguished kinetically. It is the permanent inhibition of gene expression. These two regulatory processes have been extensively studied with the lactose operon as a model system (4, 12, 13, 16, 18, 19, 29). Perlman and Pastan (18, 19), and Ullmann and Monod (29) have shown that adenosine 3',5'-cyclic monophosphate (cyclic AMP) released these repressions. Although expression of the lactose operon required cyclic AMP (4, 18, 19), the intracellular level of cyclic AMP was reduced by the presence of glucose in the culture medium (14). The PTS has been shown to be involved in the reduction of the intracellular level of cyclic AMP (20, 25). Thus, β-galactosidase and the lactose transport carrier, both of which are products of the lactose operon, are not synthesized in the presence of glucose, and lactose cannot be utilized. Inducer exclusion is the regulatory process by which PTS sugars selectively inhibit transport of non-PTS carbohydrates (carbohydrates which are not transported via the PTS), such as lactose and melibiose. Even in cells having the lactose operon expressed, utilization of lactose is still restricted by glucose, because of inhibition of lactose transport by glucose. Both the repressions and the inducer exclusion are caused generally by PTS sugars. Since both transient and catabolite repressions are released by adding a high concentration of cyclic AMP to the culture medium (18, 19, 29), it is possible to test the role of the inducer exclusion in diauxic growth of cells without the involvement of the repressions.

We have been studying the mechanism and regulation of melibiose transport (10, 17, 28). The metabolism of this sugar and its regulation had not been extensively studied. Thus, we investigated inducer (melibiose) exclusion by glucose as a metabolic control of the utilization of this carbohydrate. In this paper we report the effect of cyclic AMP on glucose-melibiose diauxie of *E. coli* and the significance of inducer exclusion in the preferential utilization of glucose over melibiose.

MATERIALS AND METHODS

Bacterial strains and growth. *E. coli* W3133 (10), W3133-2 (10), and 7 (6), all derivatives of strain K-12, were used. Strains W3133 and W3133-2 lack the lactose transport system. Thus, melibiose transport is mediated solely by the melibiose transport system in these strains. Strain W3133 possesses a temperature-sensitive melibiose transport system which is inactive at 37°C or above. Strain 7 possesses the lactose transport system. Cells were grown at 37°C with shaking in a basal salt medium (26) supplemented with 2 mM glucose and 5 mM melibiose, unless otherwise stated. Cyclic AMP, when necessary, was added to the culture medium at a final concentration of 5 mM. For transport experiments, cells were grown in the basal salts medium supplemented with 1% tryptone (Difco Laboratories) and 10 mM melibiose and harvested at exponential phase of growth. Growth was monitored in terms of optical density at 650 nm (OD_{650}).

Determination of glucose and melibiose. Samples (2.0 ml of culture medium) were taken at intervals and filtered through membrane filter (Toyo TM 2, pore size 0.45 μm) to remove cells. Glucose in the filtrate was determined with glucose oxidase (Glucose-Test, Wako Pure Chemicals). To determine melibiose in the presence of glucose, the procedure outlined by Asensio et al. (1) for the measurement of lactose was modified. The sample (0.5 ml of the filtrate) was taken and diluted with 3.5 ml of water. To 0.8 ml of this solution was added 0.6 ml of water and 0.1 ml of 5% solution of $NaBH_4$. The solution was kept at room temperature (20 to 22°C) for 2 h to reduce glucose and melibiose, and the remaining D-galactopyranosyl-α-D-glucitol was measured by the anthrone method (21). The same procedure was employed for the determination of lactose in the presence of glucose.

Assay of α-galactosidase. The activity of α-galactosidase was assayed by measuring the hydrolysis of *p*-nitrophenyl-α-D-galactopyranoside as described previously (27).

Transport assay. Transport of [*methyl*-^{14}C]β-D-thiogalactopyranoside (TMG) was measured by the filtration method as described previously (10).

Protein determination. Protein contents were determined by the method of Lowry et al. (11).

Chemicals. Melibiose was purchased from Sigma Chemical Co. Cyclic AMP was from Kohjin Co. [^{14}C]TMG was from New England Nuclear Corp. All other chemicals were of reagent grade and obtained from commercial sources.

RESULTS

Diauxic growth of cells on glucose and melibiose. Both glucose and melibiose can be utilized as carbon sources by *E. coli*. When the two sugars were added to a culture medium, *E. coli* strain W3133-2 showed diauxic growth (Fig. 1). Generation time of the first growth cycle was 65 min and that of the second cycle was 95 min. Generation times of this strain on glucose and on melibiose under similar conditions were 60 to 65 min and 80 to 95 min, respectively. The time of the cessation of growth between the two cycles was about 40 min. When 1 mM glucose and 5 mM melibiose were added, the first growth cycle stopped at an OD_{650} of 0.23. On the other hand, when 2 mM glucose and 5 mM melibiose were added, the first cycle continued until an OD_{650} of 0.4. Thus, the growth yields of the first cycles in the two cases were proportional to the initial concentrations of glucose. Therefore, it seems that glucose was utilized in the first growth cycle and melibiose in the second cycle. The final growth yields of both cases were close.

Strain W3133 possesses a temperature-sensitive melibiose transport system (10), which is active at 32°C and inactive at 42°C. Although melibiose is also transported via the lactose transport system, this strain lacks the lactose system. Thus, melibiose cannot be utilized by this strain at 42°C. When 2 mM glucose and 5 mM melibiose were added as carbon sources, this strain showed diauxic growth at 32°C (Fig. 2). The OD_{650} of the transition point between the two growth cycles was about 0.4. When cells were grown in the same medium at 42°C, growth stopped at an OD_{650} of 0.4 (Fig. 2). Cells did not show the second growth cycle in this case because of the lack of melibiose transport. Since the same amount of glucose should give the same growth yield, it is believed that glucose was utilized in the first growth cycle and melibiose was utilized in the second cycle at 42°C.

Preferential utilization of glucose over melibiose in the diauxie. As shown in Fig. 1 and 2, preferential utilization of glucose over melibiose was suggested. We have confirmed this notion by direct determination of both glucose and melibiose in the culture medium when these two sugars were added as carbon sources, namely, during the diauxic growth. As shown in Fig. 3, glucose was consumed in the first growth cycle, as expected. The first cycle continued until all of the glucose was exhausted. Then the cells stopped growing. After a lag time of about 40 min, the cells started to consume melibiose and grow exponentially again. After the exhaustion of melibiose, growth of the cells ceased. Thus, preferential utilization of glucose over

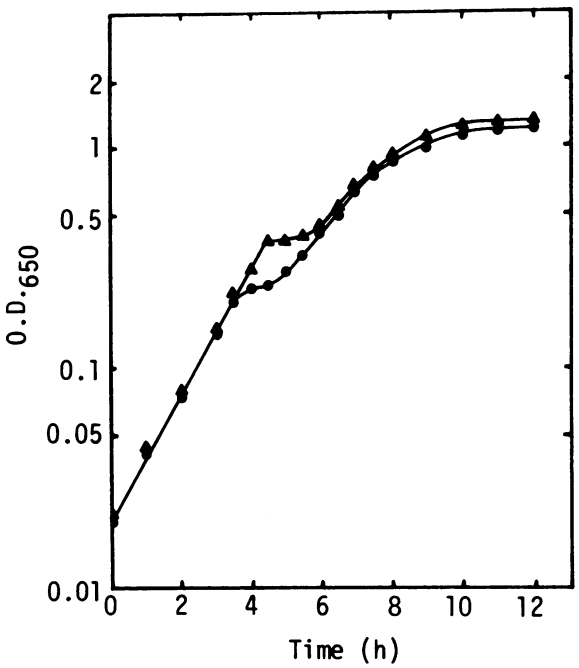

FIG. 1. *Diauxic growth of strain W3133-2 on glucose and melibiose. Cells were grown in two media, one containing 1 mM glucose and 5 mM melibiose (●), the other containing 2 mM glucose and 5 mM melibiose (▲).*

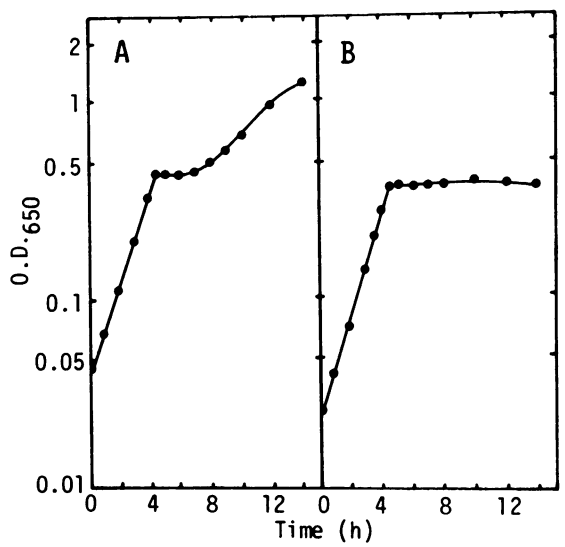

FIG. 2. *Growth patterns of strain W3133 in a medium containing glucose and melibiose. Cells which possess a temperature-sensitive melibiose transport system were grown at 32°C (A) or 42°C (B). Glucose and melibiose were added to 2 mM and 5 mM, respectively.*

melibiose was directly shown and correlated to the growth of cells.

Effect of cyclic AMP on the expression of the melibiose operon. It is well known that the expression of the lactose operon is under the control of both transient and catabolite repressions (4, 13, 18, 19, 29). Similarly, it has been shown that the melibiose operon of *Salmonella typhimurium* is repressed by the addition of glucose to the medium, and the repressions were released by the addition of cyclic AMP (9). We tested the effect of glucose and cyclic AMP on

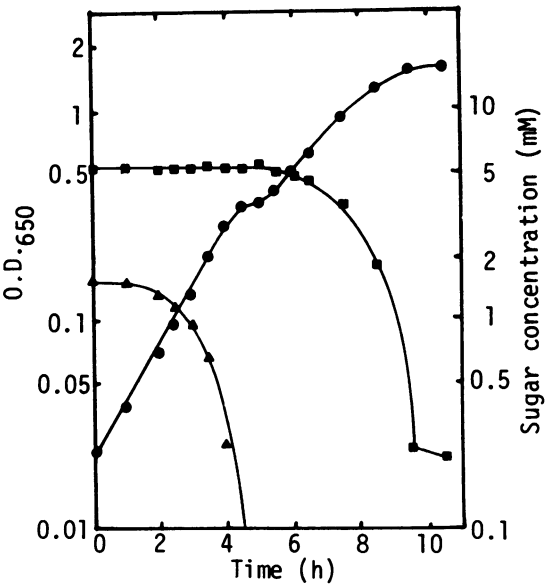

FIG. 3. *Utilization of glucose and melibiose during diauxic growth of strain W3133-2. Cells were grown on 2 mM glucose and 5 mM melibiose. Samples were taken at intervals, and the amounts of glucose* (▲) *and melibiose* (■) *in the medium were determined as described in the text. Optical density of the culture medium* (●) *was measured at the same time.*

the expression of the melibiose operon of *E. coli*. The addition of glucose to fully induced culture inhibited further increase of α-galactosidase activity (data not shown), suggesting an inhibition of the expression of the melibiose operon by glucose. After a severe transient repression, the enzyme activity began to increase again, but to a lesser extent compared with the control. Thus, the characteristic pattern of the catabolite repression was observed. The addition of cyclic AMP to the culture medium together with glucose prevented the inhibition of the enzyme synthesis by glucose (data not shown).

Effect of cyclic AMP on the diauxie. Since cyclic AMP released the inhibition of the expression of the melibiose operon by glucose, cyclic AMP should have some effect on the glucose-melibiose diauxie. In the absence of cyclic AMP, the melibiose operon was expressed after complete consumption of glucose, judging from the appearance of α-galactosidase activity (Fig. 4). A typical diauxic growth was observed in this case. On the other hand, a biphasic growth curve without a transient cessation of growth was observed in the presence of cyclic AMP (Fig. 4), as reported by Ullmann and Monod (29). The OD_{650} of the transition point of the biphasic growth curve was almost the same as that of the typical diauxic growth. The activity of α-galactosidase, which represents the expression of the melibiose operon, appeared at a very early stage of the biphasic growth in the presence of cyclic AMP. The biphasic growth without a transient cessation in the presence of cyclic AMP suggests that glucose was utilized before melibiose. There are two reasons for this. First, generation times of the first and second phases corresponded to those of growth on glucose and melibiose, respectively. Second, the transient point of the biphasic growth corresponded well to the point of transient cessation of growth in the typical diauxie where glucose was utilized first.

The order of consumption of glucose and melibiose in the presence of cyclic AMP was tested. As shown in Fig. 5, glucose was utilized first, as expected. After complete consumption of glucose, cells started to consume melibiose. The transition point of the biphasic growth corresponded well with the starting point of melibiose utilization. Thus, it became clear that glucose was preferentially utilized over melibiose even in the absence of both transient and catabolite repressions.

Similarly, preferential utilization of glucose over lactose in the presence of cyclic AMP was observed with a lactose-positive strain of *E. coli* (data not shown). Biphasic growth instead of a typical diauxic growth was also observed in this case. Again, the transition point of the biphasic growth corresponded well with the point of the beginning of lactose utilization. Thus, biphasic growth without a transient lag and preferential

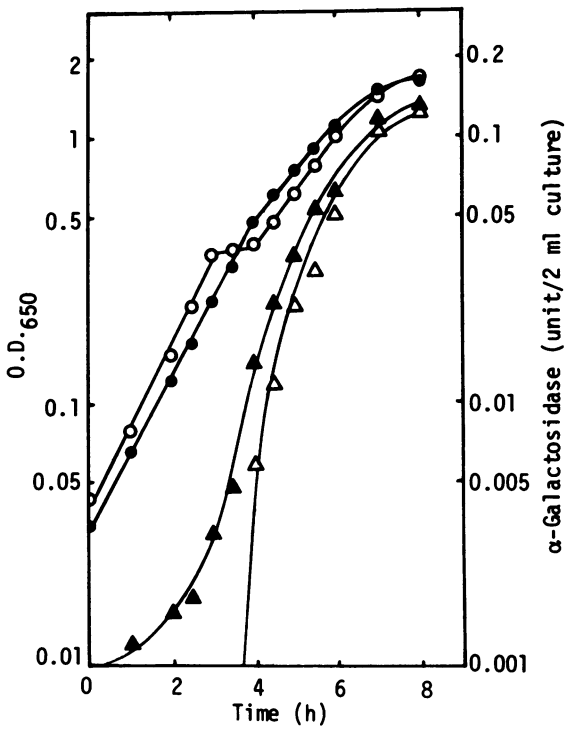

FIG. 4. *Effect of cyclic AMP on the diauxic growth of strain W3133-2. Cells were grown on 2 mM glucose plus 5 mM melibiose either in the absence (○, △) or in the presence (●, ▲) of 5 mM cyclic AMP. Samples were taken at various times from each culture to measure optical density (circle) and α-galactosidase activity (triangle). One unit of α-galactosidase activity is defined as the amount causing release of 1 μmol of p-nitrophenol per min.*

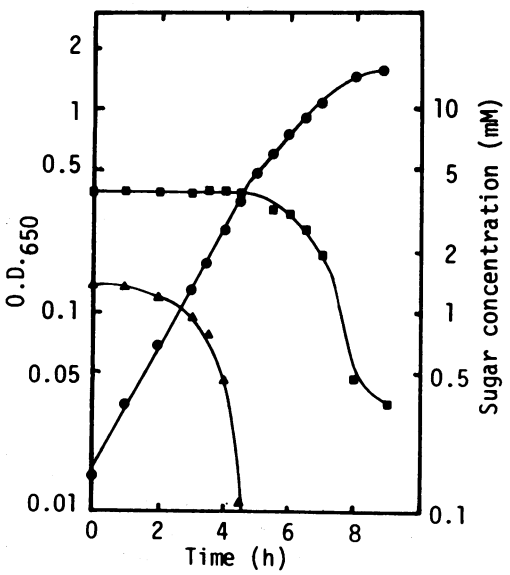

FIG. 5. *Effect of cyclic AMP on the utilization of glucose and melibiose in strain W3133-2. Cells were grown in the presence of 2 mM glucose, 5 mM melibiose, and 5 mM cyclic AMP. At various times samples were taken to measure optical density (●) and the content of glucose (▲) and melibiose (■) in the medium.*

utilization of glucose in the presence of cyclic AMP are not exceptional for the case of melibiose.

Effect of glucose and cyclic AMP on the melibiose transport system. Why was melibiose utilization inhibited by glucose in the absence of both transient and catabolite repressions? The participation of inducer exclusion should be considered to answer this question. As shown in Fig. 6, glucose inhibited uptake of TMG via the melibiose transport system. Addition of cyclic AMP to the assay mixture did not reverse this inhibition. Cyclic AMP alone did not have a significant effect on TMG transport. Thus, inducer exclusion, in this case, an inhibition of melibiose transport by glucose, functions in the presence of cyclic AMP. The effect of glucose and its derivatives on the melibiose transport system was tested in membrane vesicles where inducer exclusion does not work be-

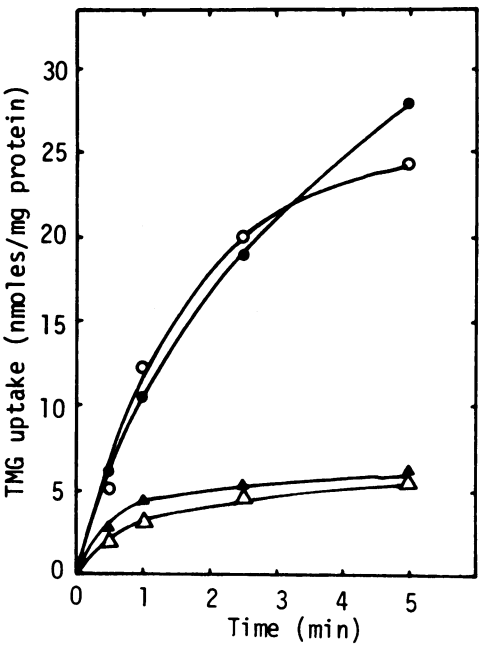

FIG. 6. *Effect of glucose and cyclic AMP on TMG uptake via the melibiose transport system in strain W3133-2. Uptake of TMG was followed at 20°C with the extracellular concentration of [^{14}C]TMG at 0.1 mM. TMG uptake was determined in the absence (●) or in the presence (▲) of 1 mM glucose, in the presence of 1 mM glucose plus 5 mM cyclic AMP (△) or 5 mM cyclic AMP (○). Glucose and cyclic AMP were preincubated with cells for 10 min before initiation of the reaction. Transport was initiated by the addition of [^{14}C]TMG.*

cause of lack of soluble components (enzyme I and histidine-containing phosphate carrier protein of the PTS) involved in this regulation. Glucose, 2-deoxyglucose, and *methyl-α*-glucoside did not significantly inhibit TMG transport via the melibiose transport sytem in membrane vesicles (data not shown), although all of them inhibited TMG transport in whole cells. This means that glucose does not have a significant affinity for the melibiose transport carrier. Thus, the inhibition of TMG transport observed in cells was actually due to inducer exclusion.

DISCUSSION

The metabolism of melibiose by *E. coli* has not been extensively studied, whereas that of lactose has been. The first step of the melibiose utilization is the expression of the melibiose operon which codes for α-galactosidase and the melibiose transport carrier. This operon is an inducible system and is under the control of transient and catabolite repressions. The second step is the transport of melibiose, which is under the control of inducer exclusion. The third step is the cleavage of melibiose by α-galactosidase. α-Galactosidase splits melibiose into galactose and glucose, and then these two sugars are metabolized. The first and second steps are regulated by glucose and other PTS sugars added to the culture medium. The third step is not affected by glucose (3). Thus, the utilization of melibiose is severely inhibited by glucose, and diauxic growth is observed when glucose and melibiose are added to the culture medium. Although transient and catabolite repressions are well understood phenomena, the significance and mechanism of inducer exclusion are not clear. To avoid the involvement of the two repressions in the diauxie, we added cyclic AMP to the culture medium. Thus, the significance of inducer exclusion in the diauxie could be evaluated. Addition of cyclic AMP to a culture medium containing glucose and melibiose caused the transient lag phase to disappear (Fig. 4 and 5). This indicates that the short lag phase observed in the absence of cyclic AMP was due to the absence of products of the melibiose operon, which were necessary for the metabolism of melibiose. Time was required to induce the operon after the release of transient and catabolite repressions and inducer exclusion. This release was due to the disappearance of glucose from the medium. In the presence of cyclic AMP, on the other hand, the melibiose operon was expressed at all stages of the growth. Although inducer exclusion of melibiose uptake by glucose occurs, a small amount of melibiose enters the cell in the presence of glucose (Fig. 6). Therefore, induction of the melibiose operon can take place in the presence of both glucose and cyclic AMP (Fig. 4). Thus, the transient cessation of growth did not occur. Utilization of melibiose and lactose did not occur in the presence of glucose in the medium under conditions where transient and catabolite repressions were released. This means that inducer exclusion alone is sufficient to cause preferential utilization of glucose over melibiose (and lactose). On the other hand, transient or catabolite repression alone would not be sufficient for the preferential utilization of glucose over melibiose (and other non-PTS carbohydrates). Although transient repression is a very severe inhibition of gene expression, it continued for only one or two generations, after which it is released (9, 19). Catabolite repression, on the other hand, is a permanent although weak inhibition. Thus, the melibiose operon is expressed to some extent after one or two generations in the presence of glucose (data not shown). Therefore, melibiose can be utilized if

some other inhibitory mechanism is not present.

To explain inducer exclusion it was postulated that PTS sugars might dissipate the membrane potential which was necessary to energize transport of non-PTS sugars (5). This seems unlikely for the following reasons: (i) even the entrance of TMG into metabolically poisoned cells was inhibited by PTS sugars (30); (ii) glycerol transport, which does not require membrane potential for its transport (7), was strongly inhibited by PTS sugars (24); (iii) transport of some amino acids, which requires membrane potential for energization, was energized by glucose (2); (iv) addition of glucose to cells did not dissipate membrane potential. On the contrary, an increase in the membrane potential by the addition of glucose was observed (unpublished data). Thus, the carrier of non-PTS carbohydrates themselves seems to be inhibited more directly by the PTS without involvement of an energization mechanism.

A model for the mechanism of inducer exclusion was proposed by Saier (23). According to the model, a hypothetical regulatory protein, perhaps factor III for glucose, is a key component for the inhibition of carriers of non-PTS carbohydrates. He postulated that binding of free regulatory protein to the carrier of non-PTS carbohydrate would alter the conformation of the protein such that it would function with reduced efficiency. This model has been supported rather convincingly by genetic evidence (23). It would be necessary to evaluate this model from a biochemical point of view.

With respect to transient repression, it is interesting that glucose-6-phosphate, which is a non-PTS compound, induced reduction of intracellular concentration of cyclic AMP and the repression of the lactose operon (8). It was suggested that the translocation of glucose-6-phosphate across the membrane caused the decrease in the intracellular concentration of cyclic AMP (8). It will be important to elucidate the mechanism(s) by which the intracellular level of cyclic AMP is reduced by PTS sugars and non-PTS compounds (8, 20, 23, 25).

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