

# Protein Phosphorylation and Allosteric Control of Inducer Exclusion and Catabolite Repression by the Bacterial Phosphoenolpyruvate: Sugar Phosphotransferase System

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*We seek to find nature one, a coherent unity. This gives to scientists their sense of mission, and let us acknowledge it, their aesthetic fulfillment: that every research carries the sense of drawing together the threads of the world into a patterned web.*

Jacob Bronowski

## INDUCER EXCLUSION AND CATABOLITE REPRESSION IN *ESCHERICHIA COLI*

In the early 1940s, inhibition of inducible carbohydrate catabolic enzyme synthesis by exogenous glucose in bacteria was documented (12, 35). By the mid-1960s, several reports had shown that the uptake of various carbohydrate inducers was inhibited by the presence of glucose in the culture medium of *E. coli* (1, 10, 90). At the same time, Makman and Sutherland (27) identified cyclic adenosine 3',5'-phosphate (cyclic AMP) in *E. coli* and showed that exogenous glucose both inhibited its synthesis and stimulated its efflux from the cytoplasmic compartment. Subsequent work, particularly of Pastan and Perlman (50), led to the conclusion that many inducible carbohydrate catabolic enzymes were under the control of cyclic AMP and its receptor protein. Catabolic enzyme synthesis seemed to be regulated by two distinct processes: one was termed catabolite repression and involved inhibition of cyclic AMP synthesis; the other was termed inducer exclusion and involved inhibition of the uptake of the sugar inducer (26, 48). It became clear that several mechanisms responsible for catabolite repression and inducer exclusion were operative in *E. coli* and other bacteria (67, 89).

## GENETIC AND PHYSIOLOGICAL EVIDENCE FOR AN INVOLVEMENT OF THE PTS

Shortly after the discovery of the phosphotransferase system (PTS) (21), mutants of *E. coli*, *Salmonella typhimurium*, and *Klebsiella aerogenes*, pleiotropically negative for the utilization of many carbohydrates, were isolated and characterized. These mutants lacked one of the general, energy-coupling proteins of the PTS, enzyme I or HPr (*ptsI* or *ptsH* mutants, respectively; see, for example, reference

84). Although it was initially suggested that the PTS was responsible for the transport of all of these sugars, it was later found that several such sugars (glycerol, lactose, melibiose, and maltose) were not substrates of the system. Instead, synthesis of the permeases and enzyme systems responsible for their catabolism was not induced in *pts* mutants under normal conditions (13, 49, 77). It appeared that loss of one of the energy-coupling proteins of the PTS hindered the induced synthesis of the requisite catabolic enzymes. An involvement of the PTS in the regulation of non-PTS catabolic enzyme synthesis was suggested. Regulation of catabolic enzyme synthesis appeared to be due to inhibition of inducer uptake (74, 77) and cyclic AMP synthesis (51, 70). The major observations have been reviewed (67) and can be summarized as follows.

(i) Reduced cellular activities of either enzyme I or HPr in leaky *ptsI* or *ptsH* mutants, respectively, coordinately rendered the uptake of radioactive glycerol, lactose, maltose, galactose, and melibiose as well as cyclic AMP synthesis hypersensitive to inhibition by any extracellular PTS sugar, including the nonmetabolizable glucose analog methyl  $\alpha$ -glucoside (70, 71, 76). Thermoinactivation of enzyme I in a temperature-sensitive *ptsI* mutant of *E. coli* in the presence of chloramphenicol resulted in an immediate increase in sensitivity to PTS-mediated regulation, suggesting that enzyme I (and therefore presumably HPr as well) played a direct catalytic role (4).

(ii) Inhibition of sugar uptake or cyclic AMP synthesis by a particular PTS sugar required that the enzyme II which transports and phosphorylates the sugar be catalytically active (16, 53, 71, 76). In wild-type cells and in certain leaky *pts* mutants of *E. coli*, synthesis of the enzymes II of the PTS had to be induced before inhibition of non-PTS sugar uptake was observed upon addition of a PTS sugar (71).

(iii) Mutations in a gene (designated *crr*) which maps adjacent to the *pts* operon rendered all sensitive uptake systems and adenylate cyclase insensitive to PTS-mediated regulation by all sugar substrates of the PTS (70, 74, 76). The *crr* mutants exhibited high-level non-PTS sugar uptake activities comparable to those in the wild-type strain but low

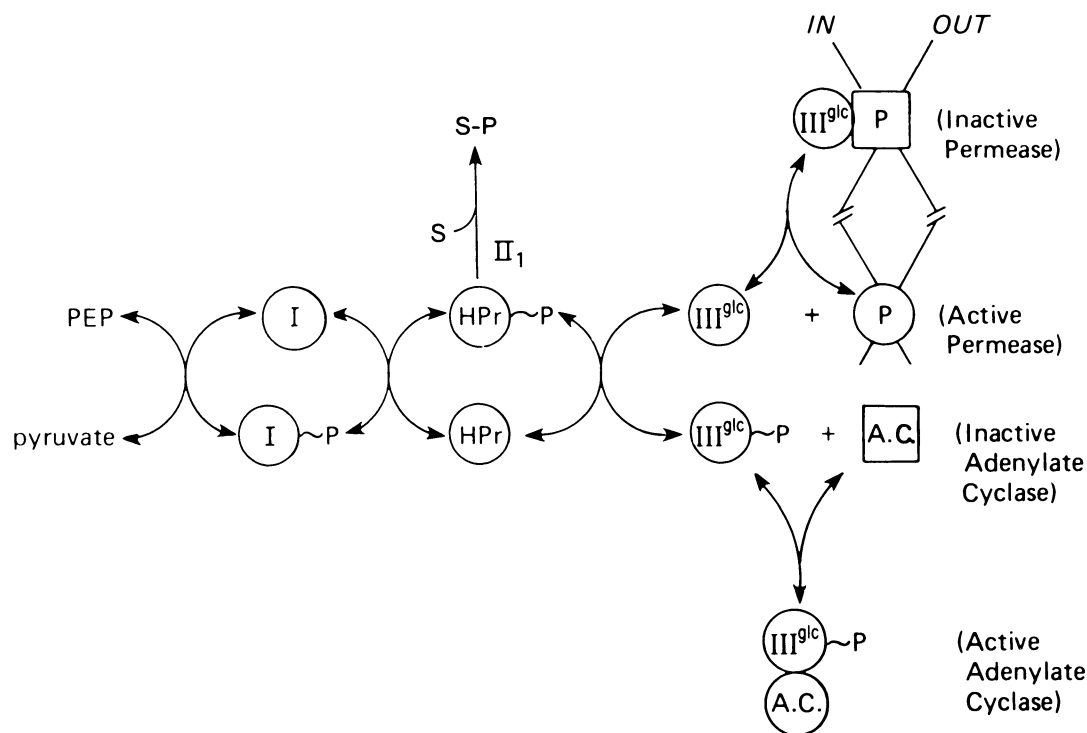


FIG. 1. Proposed mechanism for regulation of non-PTS carbohydrate uptake systems and adenylate cyclase by the PTS in *E. coli* and *S. typhimurium*. Enzyme  $\text{III}^{\text{glc}}$  is the same as RPr (regulatory protein) in the originally proposed model (65, 70, 79). I, Enzyme I; II, an enzyme II specific for a particular sugar (S); P, a non-PTS permease or glycerol kinase; A.C., adenylate cyclase. (Modified from references 65 and 79, with permission.)

adenylate cyclase activity. A few *crr* mutants with regulation-insensitive carbohydrate uptake but regulation-sensitive adenylate cyclase were isolated (11). *crr* mutants exhibited reduced activity of the glucose enzyme III ( $\text{III}^{\text{glc}}$ ) (74). That *crr* is the structural gene for  $\text{III}^{\text{glc}}$  in *E. coli* has been established (6, 30, 31, 64, 83).

(iv) A regulatory mutation within the structural gene for the lactose permease (80), glycerol kinase (44, 80), the *malK* component of the maltose permease (80; M. Schwartz and M. H. Saier, unpublished results, cited in reference 66), or the melibiose permease (80) abolished the PTS-mediated control of that permease without appreciably altering the activity of that permease or the regulatory constraints imposed on other target permeases. Corresponding point mutants of adenylate cyclase have not been described, but cloned truncated *cya* genes which do not encode the C terminus of the protein appear to retain catalytic activity while losing sensitivity to regulation (63; J. Y. J. Wang, personal communication).

(v) High-level expression of one of the target non-PTS permeases or glycerol kinase rendered it less sensitive, or even fully insensitive, to PTS-mediated regulation (33, 37, 38, 72, 81; D. K. Keeler, B. U. Feucht, and M. H. Saier, Jr., Fed. Proc. 36:685, 1977). These uptake systems were maximally inhibited when expressed in relatively small amounts. These observations suggest that overexpression of a target permease or enzyme allows the inhibitor to be titrated out. This interpretation further implies that the inhibitor is present in limiting amounts.

#### PROPOSED MOLECULAR MECHANISM FOR PTS-MEDIATED REGULATION

Based primarily on the observations reported in the preceding section, a mechanism for PTS-mediated regulation

was proposed, first by Saier and Feucht (70) and Saier and Stiles (79) and subsequently by others (14, 58). This model is illustrated in Fig. 1. The left-hand portion of the figure shows the phosphate transfer chain of the PTS. The phosphoryl group of phosphoenolpyruvate (PEP) can be sequentially transferred to enzyme I, then to HPr, and finally to sugar in the presence of the components of the sugar-specific enzyme II complex.  $\text{III}^{\text{glc}}$  is the central regulatory protein, originally termed RPr, which can be phosphorylated as a result of transfer of the phosphoryl moiety from phospho-HPr. Consequently, this protein is assumed to exist in the cell in two alternative states: as an underivatized protein and in a phosphorylated form. It is further assumed that adenylate cyclase and the carbohydrate uptake systems which are sensitive to PTS-mediated regulation possess allosteric regulatory sites by virtue of which their catalytic activities are sensitive to regulation. The allosteric effector molecules are assumed to be the derivatized and free forms of  $\text{III}^{\text{glc}}$ . Thus, the carbohydrate permeases and glycerol kinase may normally exist in an active configuration, but binding of free  $\text{III}^{\text{glc}}$  to the allosteric site would alter the conformation of the protein such that it would function with reduced efficiency. Uptake activity would therefore be inhibited. In contrast, adenylate cyclase may normally exist in a relatively inactive state which exhibits a low rate of cyclic AMP synthesis. Possibly this enzyme can be activated when the phosphorylated form of  $\text{III}^{\text{glc}}$  binds to its allosteric regulatory site. Thus, according to the model illustrated in Fig. 1, the non-PTS sugar uptake systems are subject to negative control by free  $\text{III}^{\text{glc}}$ , whereas adenylate cyclase is subject to positive control by phospho- $\text{III}^{\text{glc}}$ .

Let us consider the physiological consequences of this model. Suppose the bacterial cell is energy proficient (possesses a sufficient cellular pool of PEP) and is suspended in

a medium that lacks a sugar substrate of the PTS. Under these conditions, the energy-coupling proteins of the PTS, enzyme I and HPr, as well as  $\text{III}^{\text{Glc}}$ , will exist in their phosphorylated states. Consequently, little free  $\text{III}^{\text{Glc}}$  will be present in the cell, the non-PTS permeases should function at maximal capacity, and adenylate cyclase should exist as the activated complex. Inducer uptake and cyclic AMP synthesis will therefore occur at maximal rates. Let us now consider the consequences of the addition of a PTS sugar to the culture medium. The presence of such a sugar should initiate vectorial phosphorylation of the sugar where the immediate source of the phosphoryl group would be expected to be a phosphoenzyme III or phospho-HPr. Since enzyme I~P, HPr~P, and  $\text{III}^{\text{Glc}}\sim\text{P}$  have all been shown to be of high energy and in equilibrium with one another, all of these proteins would be expected to be drained of their phosphoryl moieties provided that the activity of the sugar-specific enzyme II complex were sufficiently great. Under these conditions, the enzyme I-catalyzed reaction is assumed to be rate limiting. Consequently, phospho- $\text{III}^{\text{Glc}}$  would be converted to free  $\text{III}^{\text{Glc}}$ . The regulatory consequences of this dephosphorylation process would be to deactivate adenylate cyclase and inhibit the carbohydrate permeases. The proposed effect of an extracellular PTS sugar on the phosphorylation state of  $\text{III}^{\text{Glc}}$  in intact cells has recently been substantiated (39).

#### DIRECT BIOCHEMICAL EVIDENCE FOR THE ALLOSTERIC REGULATION OF LACTOSE PERMEASE FUNCTION BY $\text{III}^{\text{Glc}}$

In recent years, extensive biochemical evidence for a regulatory involvement of  $\text{III}^{\text{Glc}}$  in lactose (*lac*) permease function has accumulated. Four experimental approaches involved studies of (i) the effect of  $\text{III}^{\text{Glc}}$  on lactose uptake in membrane vesicles, (ii) the direct binding of  $\text{III}^{\text{Glc}}$  to the *lac* permease protein in membrane fragments, (iii) cooperativity of lactose- $\text{III}^{\text{Glc}}$  binding to the *lac* permease in vivo, and (iv) demonstration of  $\text{III}^{\text{Glc}}$  inhibition of lactose permease activity in reconstituted proteoliposomes. These results are described in this and a subsequent section.

Partially purified  $\text{III}^{\text{Glc}}$  was shocked into *E. coli* membrane vesicles that possessed high activity of the lactose permease. Intravesicular (but not extravesicular)  $\text{III}^{\text{Glc}}$  inhibited lactose uptake, and in the presence of enzyme I and HPr, inhibition was abolished by intravesicular PEP (9). These results provided the first biochemical evidence for the involvement of  $\text{III}^{\text{Glc}}$  as RPr in the proposed regulatory process.

The results reported by Dills et al. (9) have recently been confirmed and extended, using vesicles prepared by the method of Beneski et al. (3) and a *Salmonella* strain which carried the *lac* operon on an episome (32). The latter workers showed that in the presence of a PTS sugar such as the glucose analog methyl  $\alpha$ -glucoside, thiomethyl- $\beta$ -D-galactoside uptake into the vesicles via the lactose permease was reduced by an amount which was dependent on the relative concentrations of  $\text{III}^{\text{Glc}}$  and the *lac* permease. Methyl  $\alpha$ -glucoside was not inhibitory when vesicles were prepared from a bacterial strain which was genetically deleted for the soluble enzymes of the PTS, enzyme I, HPr, and  $\text{III}^{\text{Glc}}$ . When native  $\text{III}^{\text{Glc}}$  alone was shocked into the mutant vesicles, thiomethyl- $\beta$ -D-galactoside uptake was reduced, in agreement with the results of Dills et al. (9). Intravesicular  $\text{III}^{\text{Glc}}$  was also found to inhibit the activities of the galactose and melibiose permeases, substantiating the suggestion that

dephosphorylated  $\text{III}^{\text{Glc}}$  inhibits several non-PTS permeases from the cytoplasmic side of the membrane.

Further evidence supporting the involvement of  $\text{III}^{\text{Glc}}$  as the allosteric regulatory protein which binds to the *lac* permease came from direct protein-protein binding studies (46, 47). In these studies, advantage was taken of an *E. coli* strain which contained a multicopy plasmid carrying the *lacY* gene and greatly overproduced the lactose permease (88). Membrane fragments isolated from this strain contained 5 to 15% of their total protein as the lactose permease. Direct binding of  $\text{III}^{\text{Glc}}$  to these membrane fragments was studied under various conditions and in the presence or absence of the other proteins of the PTS by rapidly and quantitatively pelleting the membranes in an Airfuge ultracentrifuge. Soluble enzymes not bound to the membrane remained in the supernatant fraction, which was removed. After the membranes were sedimented, the purified  $\text{III}^{\text{Glc}}$  (and other enzymes of the PTS which became membrane associated) was released from the membranes by extraction with a buffered solution containing 1 M NaCl. The activities of the enzymes were then measured in the salt extract after removal of the membranes.

By this assay, small amounts of  $\text{III}^{\text{Glc}}$  were found to bind to the membrane fragments. In some experiments, HPr (but not enzyme I) also became associated with the membranes, but HPr binding was absolutely dependent on the presence of  $\text{III}^{\text{Glc}}$  (47). In retrospect, this result is not surprising since  $\text{III}^{\text{Glc}}$  has been shown to bind HPr with high affinity (19). No binding of the PTS proteins to the membranes was observed when synthesis of the lactose permease had not been induced in the cells from which the membranes had been isolated. The formation of a membrane-bound complex between the lactose permease,  $\text{III}^{\text{Glc}}$ , and HPr suggested that the *lac* permease recognition site on  $\text{III}^{\text{Glc}}$  differs from the HPr recognition site on  $\text{III}^{\text{Glc}}$ . In this connection, it is worth noting that a functional complex of the soluble PTS enzymes involved in methyl  $\alpha$ -glucoside phosphorylation has been shown to be present in association with the membrane-bound enzyme  $\text{II}^{\text{Glc}}$  of *E. coli* vesicles (69). A consideration of these results suggests that the moieties of  $\text{III}^{\text{Glc}}$  which bind to the lactose permease may also be responsible for its binding to the enzyme  $\text{II}^{\text{Glc}}$ .

The presence of a substrate of the lactose permease markedly enhanced the binding of  $\text{III}^{\text{Glc}}$  to the membranes, and this effect was specific (46). All substrates of the *lac* permease tested (including lactose, thio- $\beta$ -D-digalactoside [TDG], thiomethyl- $\beta$ -D-galactoside, and melibiose) stimulated binding more than fourfold over background activity, but sugars which did not bind to the permease were without effect (46). As expected, high-affinity substrates of the *lac* permease promoted  $\text{III}^{\text{Glc}}$  binding at low concentrations (half-maximal stimulation occurred at 15  $\mu\text{M}$  TDG), while low-affinity substrates promoted  $\text{III}^{\text{Glc}}$  binding only at high concentrations (half-maximal enhancement occurred at 15 mM lactose). Binding showed a pH dependency with an optimum at pH 6.0 as had been demonstrated previously for TDG binding to the permease (20). Under optimal conditions, as much as 50% of  $\text{III}^{\text{Glc}}$  added to the membrane suspension became membrane bound, showing that nearly stoichiometric amounts of  $\text{III}^{\text{Glc}}$  bound to the permease and that nonspecific or artifactual adsorption of the protein to the membranes could not account for the results. Isogenic control strains which were *lacY* did not exhibit galactoside-promoted binding of  $\text{III}^{\text{Glc}}$  to the membranes.

Earlier experiments had shown that the sulfhydryl reagent *N*-ethylmaleimide inactivated the lactose permease, and

high-affinity substrates of the system such as TDG protected against inactivation (20). Accordingly, the effect of *N*-ethylmaleimide on III<sup>Glc</sup> binding was studied in the presence and absence of TDG (46). The reagent virtually abolished galactoside-promoted binding of III<sup>Glc</sup>, but when TDG was added prior to treatment with *N*-ethylmaleimide, the binding capacity was largely retained. These results substantiate the conclusion that binding of III<sup>Glc</sup> to the membranes depends on an active configuration of the lactose permease.

The results of Osumi and Saier (46, 47) provided convincing evidence for the regulatory mechanism proposed in 1975. Although the concentrations of TDG and lactose which half-maximally promoted binding of III<sup>Glc</sup> to the permease were significantly lower than the previously reported dissociation constants, this apparent anomaly was explained by the cooperative effects of sugar and III<sup>Glc</sup> binding. Thus, the affinity of the permease for the sugar substrates should be enhanced by the binding of III<sup>Glc</sup> and vice versa.

The results described above have been confirmed and further quantified (36, 40). Moreover, the cooperative effect of III<sup>Glc</sup> binding on galactoside binding, predicted from the cooperative effect of galactoside binding on III<sup>Glc</sup> binding, was demonstrated. Approximately one molecule of III<sup>Glc</sup> was reported to bind per molecule of lactose carrier. The  $K_d$  of the permease for III<sup>Glc</sup> was estimated to be  $10 \pm 5 \mu\text{M}$ , and the binding of III<sup>Glc</sup> was reported to result in a fourfold increase in the apparent affinity of the permease for the galactoside substrates.

Since phosphorylated III<sup>Glc</sup> did not bind to the *lac* permease (40, 46, 47), the results support the conclusion that only free III<sup>Glc</sup> binds to the permease to inhibit its activity. Consequently, the ternary complex, lactose-lactose permease-III<sup>Glc</sup>, exhibits low transport activity relative to the binary complex of the sugar substrate with the permease. When phosphorylated by the sequential reactions of the PTS, III<sup>Glc</sup> dissociates from the lactose permease, and the carrier assumes its active conformation. Since HPr can bind to the permease-III<sup>Glc</sup> complex as discussed above, direct phosphorylation of the membrane-bound III<sup>Glc</sup> by phospho-HPr should be possible.

The estimated  $K_d$  of the lactose permease for III<sup>Glc</sup> ( $10 \mu\text{M}$  as reported by Nelson et al. [40]) is of considerable physiological interest. According to their purification data, III<sup>Glc</sup> represents about 0.5% of the total soluble protein in the *E. coli* cell, a value which corresponds to an intracellular concentration of about 20 to 50  $\mu\text{M}$ . In view of these values, it seems reasonable to assume physiological significance in vivo to the protein-protein interaction between III<sup>Glc</sup> and the lactose permease measured in the in vitro assays. Moreover, maximal inhibition of lactose uptake by the PTS-mediated mechanism should not exceed 70 to 80%. The earlier uptake results showed that maximal inhibitory effects were of about this magnitude and that residual activity could not be further depressed by addition of very high concentrations of sugar substrates of the PTS (4, 76, 80). Since other uptake systems, such as those specific for glycerol and maltose, could be inhibited by >90%, it might be assumed that these systems bind III<sup>Glc</sup> with higher affinity (i.e., with a  $K_d$  of <10  $\mu\text{M}$ ).

In subsequent experiments, the purified lactose permease was reconstituted in an artificial phospholipid bilayer by the procedure of Newman et al. (41, 42). Inhibition of [<sup>14</sup>C] lactose counterflow was demonstrated upon addition of purified III<sup>Glc</sup> (29) to the proteoliposomes (M. J. Newman et al., unpublished results; see reference 67). Increasing concentrations of III<sup>Glc</sup> resulted in increasing degrees of inhibi-

tion, with the maximal inhibitory response equal to about 60%. The permease would be expected to insert randomly into the proteoliposomes, with about 50% of the proteins in one orientation and the other 50% in the opposite orientation. Since all permease proteins would be expected to exhibit comparable counterflow activity regardless of orientation, but only those with their cytoplasmic side facing the extravesicular medium would be exposed to the exogenously added III<sup>Glc</sup>, one would expect about 50% maximal inhibition if III<sup>Glc</sup> binding results in complete loss of activity. The results obtained were thus in good agreement with expectation. Nelson et al. (40) also reported the reconstitution of PTS-mediated inhibition of the lactose permease. While the rate of active [<sup>3</sup>H]TDG transport and the degree of inhibition by III<sup>Glc</sup> were reported to vary from preparation to preparation, 0.6 mg of III<sup>Glc</sup> per ml was reported to inhibit the initial rate of uptake by an average of 18%. While this modest inhibitory response cannot be considered to provide compelling evidence for the proposed regulatory mechanism, it serves to independently confirm, and therefore strengthen, the conclusions resulting from the studies of Newman et al. (see reference 67).

#### REGULATION OF GLYCEROL KINASE BY III<sup>Glc</sup>

The target of III<sup>Glc</sup> action, responsible for the inhibition of glycerol uptake, is glycerol kinase, the first enzyme of glycerol metabolism (56). In vitro experiments were performed which showed that, in crude extracts derived from glycerol-grown wild-type cells of *S. typhimurium*, glycerol kinase was progressively inhibited by the addition of increasing concentrations of III<sup>Glc</sup>. Half-maximal inhibition occurred at about 1 mg of III<sup>Glc</sup> per ml (about 50  $\mu\text{M}$ ). This concentration of III<sup>Glc</sup> was substantially in excess of that required for half-maximal inhibition of the lactose permease (about 200  $\mu\text{g/ml}$ , or 10  $\mu\text{M}$ ) (37, 40, 46). Since the intracellular concentration of III<sup>Glc</sup> in *E. coli* and *S. typhimurium* is normally about 0.5 to 1.0 mg/ml (82), extrapolation of these in vitro results to the in vivo situation would lead to the prediction that glycerol uptake by whole cells could not be inhibited more than 50% by a sugar substrate of the PTS. Since inhibition in excess of 95% is frequently observed in vivo, it must be concluded that the in vitro assay conditions did not mimic those in vivo. The enzyme in the crude extract may not have been in its native state, or a component of the extract may have inhibited binding of III<sup>Glc</sup> to the kinase.

More recent studies with homogeneous glycerol kinase have provided some clarification regarding these uncertainties (44). Regulatory properties of this enzyme are summarized in Table 1. Inhibition of glycerol phosphorylation by both of the two allosteric effectors of glycerol kinase, fructose 1,6-diphosphate (FDP) and III<sup>Glc</sup>, was found to be strongly pH dependent. For example, while fructose 1,6-diphosphate was inhibitory at neutral or slightly acidic pH values, it did not inhibit at basic pH values. III<sup>Glc</sup> showed corresponding behavior, and both its affinity and its maximal inhibitory response were influenced by pH. At pH 7.0 in MES [2-(*N*-morpholino)ethanesulfonic acid] buffer, the  $K_i$  of the kinase for III<sup>Glc</sup> was about 10  $\mu\text{M}$ , while this value decreased to 4  $\mu\text{M}$  when the pH was brought to 6.0. These values reflect much higher affinities than that measured by Postma et al. (56) at pH 7.5 with a crude system. They also reflect higher-affinity binding than reported previously for the interaction between III<sup>Glc</sup> and the lactose permease (40, 47). The in vitro results obtained by Novotny et al. at pH 6 therefore account for the fact that the inhibitory effect of a

TABLE 1. Comparison of the regulatory activities of FDP and III<sup>Glc</sup> of the PTS on glycerol kinase from *E. coli*<sup>a</sup>

Characteristic	Characteristics of inhibition by:	
	FDP	III <sup>Glc</sup>
Nature of inhibition	Allosteric; noncompetitive	Allosteric; noncompetitive
pH optimum	~6.0	~6.0
Positive cooperativity?	Yes	Yes
Complete inhibition?	No	No
Dimer sensitive?	No	Yes
Tetramer sensitive?	Yes	Yes
<i>glpK</i> (R) mutants sensitive?	Yes	No
<i>glpK</i> (I) mutants sensitive?	No	Yes

<sup>a</sup> See text and Novotny et al. (44) for details of the experiments and interpretation of the results.

PTS sugar on glycerol uptake is stronger than that on lactose uptake (4, 76). The *in vitro* conditions at pH 6 presumably resemble the *in vivo* conditions.

In addition to the pH dependency, Table 1 summarizes several characteristics of glycerol kinase regulation which were revealed by detailed kinetic analyses. Both FDP and III<sup>Glc</sup> appeared to inhibit the enzyme noncompetitively by an allosteric mechanism which involves positive cooperativity with respect to binding of the inhibitory ligand. Under appropriate assay conditions, sigmoidicity with respect to either FDP or III<sup>Glc</sup> concentration was observed. That complete inhibition by neither FDP nor III<sup>Glc</sup> could be demonstrated may have been reflective of the presence in the purified enzyme preparation of a non-native form of the enzyme which had retained catalytic activity but had lost sensitivity to allosteric regulation. It should be noted that, while glycerol kinase can clearly be inhibited by III<sup>Glc</sup> to an extent in excess of 95% *in vivo*, no corresponding *in vivo* data provide information about the maximal degree of inhibition possible with FDP as the allosteric effector.

If glycerol kinase rather than the glycerol permease is the target of PTS-mediated inhibition of glycerol uptake, three predictions can be made. First, phosphorylation of III<sup>Glc</sup> with PEP, enzyme I, and HPr should completely reverse the inhibitory effect; second, the activity of the facilitator should be insensitive to inhibition by III<sup>Glc</sup>; and third, mutants which specifically render uptake of glycerol resistant to PTS-mediated regulation (75, 80) should possess an altered glycerol kinase which is altered in the allosteric regulatory site of the enzyme which functions to bind III<sup>Glc</sup>. All three of these predictions have been verified (44, 56). Since the mutations which rendered glycerol uptake insensitive to PTS-mediated regulation had to map within the structural gene for glycerol kinase (*glpK*), these mutants were designated *glpK*(R) by analogy with the *lacY*(R), *melB*(R), and *malK*(R) mutants described previously (see references 67 and 80). It has been shown that some *glpK*(R) mutants of *S. typhimurium* possess a glycerol kinase that retains sensitivity to feedback inhibition by FDP (80). Further, *in vivo* assays indicated that some *E. coli glpK*(I) mutants, which are insensitive to feedback inhibition by FDP, were still sensitive to PTS-mediated inhibition of glycerol uptake (80). It was therefore suggested that the allosteric binding sites for III<sup>Glc</sup> and FDP are distinct.

This conclusion has been verified by *in vitro* assays (Table 1) (44). *glpK*(R) and *glpK*(I) mutants of *S. typhimurium* were isolated and assayed for sensitivity of glycerol kinase to

inhibition by both FDP and III<sup>Glc</sup> *in vitro*. Glycerol kinase isolated from a *glpK*(R) mutant was completely insensitive to regulation by III<sup>Glc</sup> at pH 6.5, the optimal pH for measuring regulatory interactions. However, the enzyme was fully sensitive to inhibition by FDP. By contrast, glycerol kinase in the *glpK*(I) mutant was insensitive to regulation by FDP but retained full sensitivity to regulation by III<sup>Glc</sup>. Glycerol kinase from still a third class of mutants [*glpK*(RI)] was insensitive to both inhibitory agents (44). These studies established that the allosteric binding sites on glycerol kinase for FDP and III<sup>Glc</sup> are distinct, although a single point mutation can evidently give rise to the abolition of sensitivity to both agents. A molecular analysis of these three classes of *glpK* mutants should prove most interesting.

The biochemical properties of glycerol kinase and III<sup>Glc</sup> have been reviewed (67). The recent cloning and sequencing of the *glpK* gene (54) should facilitate genetic analyses, leading to a detailed understanding of the structural and functional basis for PTS-mediated inhibition of glycerol uptake. Because of the ease with which glycerol kinase can be prepared in crystalline form, it is likely that the detailed mechanism of its regulation by III<sup>Glc</sup> and the three-dimensional structure of the glycerol kinase-III<sup>Glc</sup> complex will be elucidated more easily than those of the various permeases or adenylate cyclase.

The genes encoding four PTS-regulated permeases and enzymes, the lactose, melibiose, and maltose permeases and glycerol kinase (*lacY*, *melB*, *malK*, and *glpK*, respectively), have been sequenced. Since all four proteins are believed to bind III<sup>Glc</sup>, a common allosteric binding site of similar sequence might be expected to exist within each of these proteins. Computer analyses of these four proteins have failed to reveal a common sequence which might serve as the III<sup>Glc</sup> binding site (A. Reizer and M. H. Saier, Jr., unpublished results). Such an observation would be consistent with expectation for binding sites which arose by convergent evolution in structurally dissimilar proteins.

PTS-mediated regulation of glycerol kinase, rather than of the permease, is fully consistent with the inducer exclusion mechanism proposed previously (65, 70). This conclusion results from the fact that the inducer of the glycerol regulon is  $\alpha$ -glycerophosphate. Inhibition of either entry or phosphorylation of glycerol should prevent accumulation of the inducer in the cytoplasm. Further, since the glycerol permease is believed to catalyze the fully reversible facilitation of glycerol across the membrane while glycerol kinase catalyzes the first irreversible step of glycerol metabolism, the kinase would be expected to be the target of allosteric regulation. Most allosterically regulated enzymes catalyze reactions which possess a large negative free energy and are therefore essentially irreversible (68).

#### EVIDENCE FOR TRANSMEMBRANE SIGNALING IN PTS-MEDIATED REGULATION

If cooperative binding of  $\beta$ -galactosides and III<sup>Glc</sup> to the *lac* permease (noted previously) is an inherent characteristic of the regulatory process and not an artifact of the *in vitro* assay procedure, this cooperativity should be demonstrable *in vivo*. Assuming that a single regulatory protein, III<sup>Glc</sup>, is responsible for the control of several uptake systems, those specific for glycerol, maltose, lactose, galactose, and melibiose, the binding of III<sup>Glc</sup> to the lactose permease should render it unavailable for interaction with another system (i.e., glycerol kinase or the maltose permease). Further, if addition of thiodigalactoside or another substrate of the *lac*

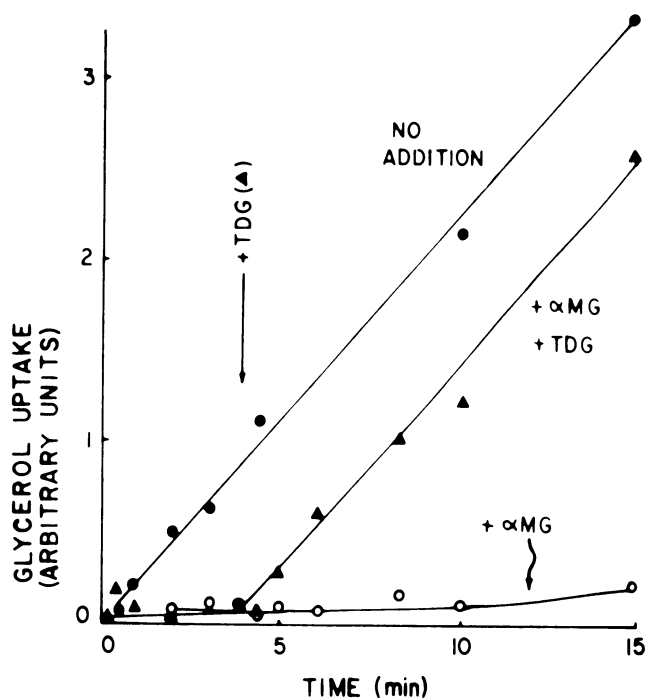


FIG. 2. Time course for relief of PTS-mediated inhibition of glycerol uptake by TDG in *E. coli* T52RT, which overproduces the lactose permease. Shown are glycerol uptake in the absence of PTS-mediated inhibition (●), the inhibitory effect of methyl  $\alpha$ -glucoside ( $\alpha$ MG; ○), and the relief of inhibition caused by addition of a saturating concentration of TDG (▲) to cells in which glycerol uptake is inhibited by the inclusion of methyl  $\alpha$ -glucoside in the medium. The concentrations of methyl  $\alpha$ -glucoside and TDG were 1 and 0.1 mM, respectively. (Reproduced from reference 73, with permission.)

permease to the cell enhances the affinity of the *lac* permease for  $\text{III}^{\text{Glc}}$ ,  $\text{III}^{\text{Glc}}$  should be drained off the other target permeases or catabolic enzymes, thereby relieving inhibition of their activities.

Experiments were therefore designed to test this hypothesis. The *E. coli* strain, T52RT, which was used for the in vitro binding studies (46, 47) was used for the initial in vivo studies (73). Cells were grown in minimal lactate-plus-glycerol medium with a saturating concentration of the *lac* operon inducer isopropyl- $\beta$ -thiogalactoside to induce high-level synthesis of the lactose permease. Glucose was added 2 h before harvesting to induce synthesis of the glucose enzyme II. Washed cells were then assayed for glycerol uptake. Uptake of [ $^{14}\text{C}$ ]glycerol was linear with time, but addition of the nonmetabolizable glucose analog methyl  $\alpha$ -glucoside resulted in immediate and virtually complete inhibition (Fig. 2). This inhibition was attributed to dephosphorylation of phospho- $\text{III}^{\text{Glc}}$  upon addition of the glucoside as subsequently verified by Nelson et al. (39). If saturating amounts of TDG were then added to the cell suspension, the inhibitory effect of the glucoside on glycerol uptake was immediately and quantitatively relieved (Fig. 2). Relief from inhibition presumably resulted from competition between the two uptake systems for  $\text{III}^{\text{Glc}}$ . Similar observations were made with the maltose permease (73).

The effect observed with *E. coli* T52RT was not observed in a strain which lacked or contained normal levels of the lactose permease, but growth of wild-type *E. coli* in the presence of isopropyl- $\beta$ -thiogalactoside plus cyclic AMP

resulted in enhanced synthesis of the lactose permease so that the addition of a galactoside relieved inhibition of glycerol uptake. TDG also relieved the inhibition of glycerol uptake caused by the presence of other PTS substrates such as fructose, mannitol, glucose, 2-deoxyglucose, and 5-thio-glucose. Further, cooperative binding of sugar and  $\text{III}^{\text{Glc}}$  to the melibiose permease in *S. typhimurium* was demonstrated. These results are consistent with a mechanism of PTS-mediated regulation involving a fixed number of allosteric regulatory proteins ( $\text{III}^{\text{Glc}}$ ), which may be titrated by the increased number of substrate-activated permease proteins.

As noted above, the constituent of the maltose permease which interacts with  $\text{III}^{\text{Glc}}$  to effect its allosteric inhibition has been tentatively identified by genetic means as the product of the *malK* gene (Schwartz and Saier, unpublished results; see references 66 and 67). The interaction of  $\text{III}^{\text{Glc}}$  with the maltose permease has recently been demonstrated with a strain of *E. coli* which overproduces the MalK, MalG, and MalF\* proteins (C. P. Broekhuizen, P. W. Postma, and H. Shuman, unpublished results). The *malF\** mutation rendered the system independent of the maltose-binding protein (MalE).  $\text{III}^{\text{Glc}}$  binding to the membrane-bound complex was dependent on the presence of maltose, suggesting that this system exhibits positive cooperativity, as do the lactose and melibiose permeases (73). Interestingly, the MalK protein, which possesses an adenosine triphosphate binding site and can be labeled with 8-azido- $[\gamma\text{-}^{32}\text{P}]$ adenosine triphosphate, showed enhanced azido-adenosine triphosphate binding upon  $\text{III}^{\text{Glc}}$  (but not phospho- $\text{III}^{\text{Glc}}$ ) binding. These properties are consistent with the genetic evidence mentioned above suggesting that  $\text{III}^{\text{Glc}}$  (but not phospho- $\text{III}^{\text{Glc}}$ ) binds to MalK. They are also in agreement with properties demonstrated previously for the lactose permease (46).

The work described above suggested that the cooperativity in the binding of a sugar substrate to the external surface of the permease and of  $\text{III}^{\text{Glc}}$  to the internal surface of the permease represents a form of transmembrane signaling. Only when a substrate of the permease is present in the external medium does  $\text{III}^{\text{Glc}}$  bind with high affinity to its allosteric regulatory site on the cytoplasmic side of the membrane. Cooperativity between external sugar and internal  $\text{III}^{\text{Glc}}$  binding allows the effective allosteric regulation of several permeases and enzymes by a limited amount of  $\text{III}^{\text{Glc}}$ . In the absence of transmembrane signaling, far more cytoplasmic  $\text{III}^{\text{Glc}}$  would be required to effect regulation of the various systems. Although the *crr* gene, encoding  $\text{III}^{\text{Glc}}$ , is part of the *pts* operon, and therefore would be expected to be inducibly expressed in response to the presence of a PTS sugar in the growth medium (6, 61, 77, 78), this gene possesses its own constitutive promoter. Transcription from its own promoter results in about five times as much *crr*-specific messenger ribonucleic acid synthesis as that from the *pts* operon promoter (6). This fact explains the apparent constitutivity of  $\text{III}^{\text{Glc}}$  synthesis as first reported by Saier et al. (77).

As noted above, transmembrane signaling allows the effective regulation of several target permeases while minimizing the requisite cytoplasmic concentration of  $\text{III}^{\text{Glc}}$ . Mitchell et al. (34) have recently examined the effects of  $\text{III}^{\text{Glc}}$  overproduction on lactose permease function. In these studies, transformed cells which overproduced  $\text{III}^{\text{Glc}}$  2- or 10-fold were constructed from a *pts*<sup>+</sup> strain of *E. coli* and plasmids specifically carrying the *crr* gene. Uptake of non-PTS carbohydrates (glycerol, lactose, maltose, and melibiose) in the twofold overproducer was normal, but it was

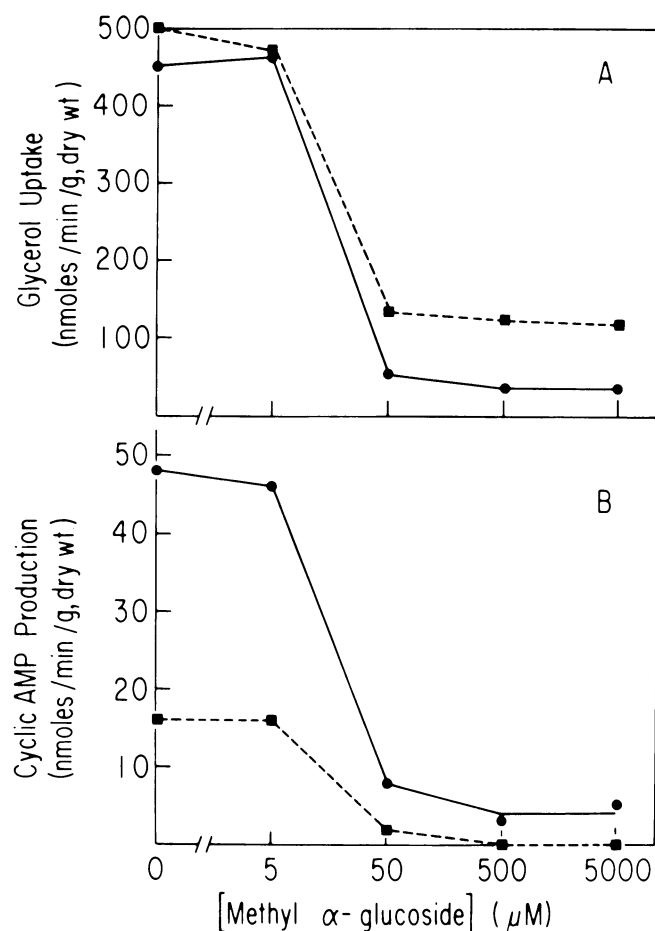


FIG. 3. Effects of *pts* and *crr* gene dosage on regulation of glycerol uptake (A) and cyclic AMP production (B) in *S. typhimurium*. The strains used were LJ98 (*cpd-401 cysA1150*, - - -) and LJ144 [*cpd-401 cysA1150/F'198 (pts<sup>+</sup> crr<sup>+</sup>)*, —]. Cells were grown in medium 63 without iron in the presence of 0.5% glycerol for 4 h before addition of 0.5% glucose, 2 h before harvesting. Logarithmically growing cells were harvested, washed three times with medium 63 at 0°C, and suspended for [<sup>14</sup>C]glycerol uptake measurements and determination of the rates of cyclic AMP production as a function of the methyl  $\alpha$ -glucoside concentration in the medium. Experimental procedures were as described previously (11, 70).

more sensitive to inhibition by PTS sugars than in the control strain. Increased sensitivity to PTS-mediated regulation was most pronounced when the target non-PTS catabolic system was induced to high levels. The results were similar to those illustrated in Fig. 3 for glycerol uptake (see below), where enzyme I, HPr, and III<sup>Glc</sup> were all overproduced about fourfold. By contrast, the 10-fold III<sup>Glc</sup> overproducer (36) fermented the above-mentioned non-PTS carbohydrates poorly, and lactose permease activity was 50% of that in control cells containing the same level of  $\beta$ -galactosidase. It is clear from these results that the mere overproduction of III<sup>Glc</sup> by an enteric bacterium is not an acceptable alternative to transmembrane signaling for increasing the sensitivity of non-PTS permeases to regulation by the PTS. They provide a teleological explanation for the fact that the *crr* gene, encoding III<sup>Glc</sup>, which is a part of the *pts* operon, possesses its own constitutive promoter (6). The presence of this promoter renders III<sup>Glc</sup> synthesis insensitive to induction by PTS sugars. These observations lead to the unavoid-

able conclusion that cooperativity between sugar and III<sup>Glc</sup> binding to the two surfaces of a permease protein is physiologically important *in vivo*. Mechanistically similar transmembrane cooperativity and signaling are likely to occur in bacterial and eucaryotic chemoreception (86, 87) as well as in hormone reception in animal cells (23).

#### PTS-MEDIATED REGULATION OF ADENYLATE CYCLASE ACTIVITY

As discussed briefly in previous sections, substantial genetic evidence coupled with physiological and biochemical analyses have led to the postulate that III<sup>Glc</sup> regulates adenylate cyclase by a mechanism which parallels the process by which it regulates the lactose permease and glycerol kinase. Saier and Feucht (70) demonstrated that mutations in the genes encoding enzyme I, HPr, and the various enzymes II of the PTS in *S. typhimurium* had parallel effects on the *in vivo* regulation of glycerol kinase and adenylate cyclase when studied as a function of sugar concentration. By contrast, mutations in the *crr* gene had opposing effects, giving rise to high-level uptake activities but low-level adenylate cyclase activity. This observation resulted in the prediction that free III<sup>Glc</sup> inhibited carbohydrate uptake while phospho-III<sup>Glc</sup> activated cyclic AMP synthesis (Fig. 1).

In recent experiments this conclusion has been confirmed (M. H. Saier and B. U. Feucht, unpublished results). In these experiments, glycerol uptake and cyclic AMP synthesis were compared in a *Salmonella* strain which could not degrade cyclic AMP because it lacked the enzyme cyclic AMP phosphodiesterase and the same strain bearing an *E. coli* episome (F'198) which carried the entire *pts* operon (*ptsOPHIcrr*). The latter strain possessed four- to fivefold-elevated activities of enzyme I, HPr, and III<sup>Glc</sup>. After growth of these two cell types in a minimal glycerol-plus-glucose medium, glycerol uptake and cyclic AMP synthesis were measured as a function of the concentration of methyl  $\alpha$ -glucoside (Fig. 3). The presence of the episome did not alter the concentration of methyl  $\alpha$ -glucoside to which the two target systems were sensitive, but it increased the sensitivity of the glycerol uptake system to inhibition while activating adenylate cyclase. These results are fully consistent with expectation based on the model presented in Fig. 1.

The literature through 1984, dealing with the PTS-mediated regulation of adenylate cyclase, has been reviewed (57, 67). At that time, attempts to reconstitute adenylate cyclase regulation *in vitro* had been totally without success; consequently, the mechanism proposed on the basis of *in vivo* results could be considered to be only hypothetical. In this section, more recent biochemical experimental results will be discussed which, although insufficient to establish the mechanism of PTS-mediated adenylate cyclase regulation, at least demonstrate an interaction between adenylate cyclase and the enzymes of the PTS.

Harwood and Peterkofsky (17) had demonstrated the regulation of adenylate cyclase by glucose in toluene-permeabilized cells. With this experimental system, it was found that, in addition to magnesium, the regulated form of the enzyme required potassium and phosphate. Stimulation of adenylate cyclase activity by potassium phosphate required the presence of the proteins of the PTS, and since the activity of the PTS was also stimulated, it was proposed that the effect of potassium phosphate on adenylate cyclase was mediated through an effect on the PTS. Cell disruption abolished this effect.



In subsequent communications (24, 59), purified PTS proteins were added to a crude extract of an *E. coli* strain which overproduced adenylate cyclase. It was found that, while the PTS proteins together with PEP had a noticeable inhibitory effect by themselves, the stimulatory effect of potassium phosphate was substantially (about 50%) enhanced by their presence. Moreover, only in the presence of all requisite constituents (enzyme I, HPr, III<sup>Glc</sup>, PEP, and potassium phosphate) was an inhibitory effect of methyl  $\alpha$ -glucoside observed. Inhibition occurred to the extent of about 50%. In these experiments, both PEP and methyl  $\alpha$ -glucoside were used at a concentration of 1 mM, leading to the possibility that the true inhibitor was one of the products of the PTS-catalyzed reaction, either pyruvate or methyl  $\alpha$ -glucoside-6-phosphate.

To test this possibility, each compound was separately added to appropriate enzyme preparations. When the crude extract containing the PTS proteins, PEP, and potassium phosphate was supplemented with methyl  $\alpha$ -glucoside-6-phosphate or pyruvate, inhibition was observed. Methyl  $\alpha$ -glucoside-6-phosphate was less effective than methyl  $\alpha$ -glucoside, and pyruvate was most inhibitory. Further, in the absence of PEP, methyl  $\alpha$ -glucoside was only as inhibitory as was methyl  $\alpha$ -glucoside-6-phosphate, but the effectiveness of pyruvate as an inhibitor was not diminished by PEP removal. These results, while provocative, are difficult to reconcile with the *in vivo* effects of PTS sugars on adenylate cyclase and are not as expected from the proposed mechanism involving III<sup>Glc</sup>-P as an activator of adenylate cyclase. In fact, the results do not establish any mechanism in which a phosphorylated PTS enzyme activator is responsible for the regulatory effects (51, 52). Instead, they are consistent with a mechanism involving an inhibitory effect of pyruvate on adenylate cyclase which is somehow dependent on the presence of the PTS proteins. As discussed below, such a mechanism cannot account for the *in vivo* regulatory interactions which we term PTS-mediated regulation.

In appropriate very slightly leaky *ptsI* mutants of *S. typhimurium*, nanomolar concentrations of methyl  $\alpha$ -glucoside have been shown to be sufficient to inhibit adenylate cyclase *in vivo* via the PTS-mediated regulatory mechanism (11). Nanomolar concentrations of sugar in a slightly leaky *ptsI* mutant would not be expected to cause appreciable cytoplasmic accumulation of pyruvate. Consequently, the accumulation of cytoplasmic pyruvate cannot be responsible for the PTS-mediated inhibition of adenylate cyclase by methyl  $\alpha$ -glucoside *in vivo*.

While not responsible for PTS-mediated regulation, the possibility should be considered that the *in vitro* inhibitory effect of pyruvate noted by Reddy et al. (59) is in some way related to the PTS-mediated inhibition of adenylate cyclase *in vivo*. Pyruvate would be expected to cause PTS protein dephosphorylation via the reaction: enzyme I-P + pyruvate  $\rightleftharpoons$  enzyme I + PEP. Since HPr-P and III<sup>Glc</sup>-P are in equilibrium with enzyme I-P, this reaction would be expected to cause dephosphorylation of both of these phosphoproteins. Methyl  $\alpha$ -glucoside would also be expected to dephosphorylate the PTS proteins if the glucose enzyme II-III pair were sufficiently active. In the report by Reddy et al. (59), only pyruvate, not methyl  $\alpha$ -glucoside, was found to inhibit adenylate cyclase in the absence of PEP. Thus, if the inhibitory effect of pyruvate on adenylate cyclase *in vitro* observed by Reddy et al. was a consequence of PTS protein dephosphorylation and a reflection of PTS-mediated regulation, it would be necessary to propose, first, that the PTS proteins were phosphorylated in the *in vitro* experiments of

Reddy et al. in the absence of added PEP, and, second, that only pyruvate, not methyl  $\alpha$ -glucoside, was capable of dephosphorylating the PTS proteins. This second condition requires that enzyme II<sup>Glc</sup> not be active in the *in vitro* preparation. However, if the inhibitory effect of methyl  $\alpha$ -glucoside on adenylate cyclase, which was shown to be dependent on the presence in the *in vitro* reaction mixture of PEP, is to be explained in terms of either pyruvate generation or phospho-PTS protein dephosphorylation, then the enzyme II<sup>Glc</sup> must have been active. Since neither the phosphorylation state of the PTS proteins in the absence of added PEP nor the activity state of enzyme II<sup>Glc</sup> was investigated, this question remains unresolved. One must conclude that the experimental results of Reddy et al. probably reflect a regulatory interaction which is unrelated to PTS-mediated regulation. Whether or not the *in vitro* observations of Reddy et al. have physiological significance *in vivo* has yet to be determined.

In a more recent report, Liberman et al. found that inorganic phosphate inhibited adenylate cyclase in the presence but not the absence of III<sup>Glc</sup>, even when enzyme I and HPr were totally absent (25). The authors interpreted this result to suggest that III<sup>Glc</sup> can interact directly with adenylate cyclase in the absence of enzyme I, HPr, and PTS-mediated phosphorylation. The possible mediation of this inhibitory effect by other proteins was not ruled out. Nevertheless, an interaction, direct or indirect, between adenylate cyclase and III<sup>Glc</sup> is suggested. The results are in agreement with the model shown in Fig. 1. It should be noted that the possibility of dual control, activation of adenylate cyclase by III<sup>Glc</sup>-P as well as inhibition by free III<sup>Glc</sup>, had been suggested on the basis of genetic and physiological studies conducted with certain *E. coli* strains (11).

The results evaluated in this section are, unfortunately, insufficient to establish a physiologically relevant mechanism by which the PTS controls adenylate cyclase activity. Since tremendous effort has been expended over the past 15 years to establish this or an alternative but related mechanism, the critical reader might justifiably ask whether the earlier evidence ought to be reevaluated in terms of a totally different mechanism. I think that the *in vivo* evidence still supports the essential components of the model outlined in Fig. 1. However, in light of the transmembrane signaling concept, it is possible that adenylate cyclase regulation may involve additional unrecognized components, conditions, or events such as its binding to and release from the membrane in response to PTS activity. Perhaps the failure to reconcile the *in vivo* and *in vitro* data can be attributed to the dissociation of topographical interactions between the enzyme and the membrane which, for unexplained reasons, are lost when cells are disrupted to yield the various subcellular fractions. Further studies will be required for a detailed understanding of this process.

#### EVIDENCE FOR PTS-MEDIATED REGULATION IN GRAM-POSITIVE BACTERIA

Like *ptsI* mutants of *E. coli* and *S. typhimurium*, mutants of *Bacillus subtilis* which lack enzyme I of the PTS are incapable of growth on glycerol (22, 43). The availability of a temperature-sensitive *ptsI* mutant of *B. subtilis* (43) rendered feasible a rigorous physiological study of PTS-mediated regulation of glycerol uptake in this organism (60), comparable to that which had been conducted previously in *E. coli* (4). Thermoinactivation of enzyme I in the presence or absence of chloramphenicol resulted in the loss of methyl



$\alpha$ -glucoside uptake activity and enhanced sensitivity of glycerol uptake to inhibition by all tested sugar substrates of the PTS. The concentration of the inhibiting sugar which half-maximally blocked glycerol uptake was directly related to residual enzyme I activity. Maximal inhibition required that synthesis of the enzyme II specific for the inhibitory sugar be induced prior to the uptake measurement. The results obtained were fully consistent with the conclusion that a PTS-mediated regulatory mechanism analogous to that which had been characterized in enteric bacteria was operative.

In an examination of the phosphorylated proteins of *Streptococcus faecalis*, a protein with a molecular weight of about 55,000 was identified which could be phosphorylated in vitro in the presence of enzyme I and HPr at the expense of [ $^{32}$ P]PEP (7). No enzyme III activity was found to be associated with this protein. Attempts to demonstrate a regulatory function for the protein, analogous to that of III<sup>Glc</sup> in enteric bacteria, led to its identification as glycerol or dihydroxyacetone kinase (8). The PTS-catalyzed reversible phosphorylation of this protein was shown to occur at the N-3 position of a histidyl residue and led to a 10-fold increase in activity.

A biochemical search for a protein in *B. subtilis* with catalytic properties analogous to those of III<sup>Glc</sup> in *E. coli* provided evidence for such a protein. In the presence of partially purified *B. subtilis* enzyme I, HPr, and enzyme II<sup>Glc</sup>, a preparation of soluble proteins stimulated glucose phosphorylation 20-fold (M. J. Novotny and M. H. Saier, unpublished results). More recently, the gene for a presumptive III<sup>Glc</sup> protein (*crr*) has been cloned and sequenced (15; G. Gonzy-Tréboul, M. Zagorec, M.-C. Rain-Guion, and M. Steinmetz, Mol. Microbiol., in press). Like the *crr* gene of enteric bacteria, it maps adjacent to the *pts* operon in *B. subtilis*. However, the gene order in these two organisms is different. In *E. coli*, the gene order of the *pts* operon is *ptsOP-ptsH-ptsI-crr*, where the *crr* gene can be read either from the inducible operator-promoter of the *pts* operon (*ptsOP*; 20% relative strength) or from its own constitutive promoter (*crrP*; 80% relative strength) (6). The *crr*-specific promoter is located within the downstream portion of the *ptsI* structural gene (6). In *B. subtilis* the gene order appears to be *ptsG-crr-ptsOP-ptsH-ptsI*. Thus, the *crr* gene is upstream from the *ptsHI* genes instead of downstream, and it is transcribed in the same direction as the presumed *ptsG* gene, encoding the enzyme II<sup>Glc</sup>. In *E. coli* the *ptsG* gene maps far from the *pts* operon. It seems likely that in *B. subtilis* the *ptsG* and *crr* genes comprise an operon and that the operator-promoter region of this operon precedes the *ptsG* gene. However, because the sequence of only the C-terminal part of the enzyme II<sup>Glc</sup> gene was determined (Gonzy-Tréboul et al., in press), this suggestion must be considered to be speculation. Nothing is yet known concerning the regulatory region preceding the *ptsG* region in *B. subtilis*.

Recently, the genes encoding the lactose-proton symport permease from *Streptococcus thermophilus* and *Lactobacillus bulgaricus* were cloned and sequenced (55). The structural gene from the former bacterium was 1,902 base pairs long, encoding a protein of 634 amino acids with a molecular weight of 69,454. The protein possesses an amino-terminal hydrophobic 49,000-dalton region and a carboxy-terminal hydrophilic 20,000-dalton region. The N-terminal hydrophobic region is homologous to the melibiose permease of *E. coli* throughout its entire length (23% overall identity), while the C-terminal hydrophilic portion shows homology to the enzyme III<sup>Glc</sup> of enteric bacteria (about 40% overall sequence

identity throughout its entire length). Among the amino acids which were conserved between the C-terminal domain of the *Streptococcus thermophilus* lactose permease and III<sup>Glc</sup> were found two histidyl residues, one of which in III<sup>Glc</sup> is known to be phosphorylated by phospho-HPr. Since the sugar substrates of the *Streptococcus thermophilus* lactose-proton symporter are not phosphorylated during translocation, it was suggested that the III<sup>Glc</sup>-like region of the protein might serve a regulatory role analogous to III<sup>Glc</sup> in enteric bacteria which interacts directly with several non-PTS permeases and catabolic enzymes to regulate the catabolism of various non-PTS carbohydrates (see above). Preliminary evidence has been presented which suggests a role for the *crr* gene product in PTS-mediated regulation in *B. subtilis* (15, 60). It is interesting to note that the *Bacillus* protein, which shows significant sequence identity with the *E. coli* protein, can substitute for it in an in vivo assay (15).

The results described in this section are consistent with two distinct (but possibly related) mechanisms of PTS-mediated regulation of glycerol kinase or the lactose permease in gram-positive bacteria: one involving direct phosphorylation of a histidyl residue within an enzyme III-like moiety of the target permease or enzyme and the other involving III<sup>Glc</sup>-mediated inhibition of the target permease or enzyme which can be relieved by III<sup>Glc</sup> phosphorylation, as has been demonstrated for gram-negative bacteria. The published physiological and genetic data for *B. subtilis* (60) are consistent with both mechanisms. No physiological or genetic data are yet available supporting the involvement of direct phosphorylation of glycerol kinase in *Streptococcus faecalis* in the regulation of its activity in vivo. It is possible that like the lactose permease of *Streptococcus thermophilus*, the dihydroxyacetone or glycerol kinase of *Streptococcus faecalis* will prove to possess a fused III<sup>Glc</sup> moiety.

Careful scrutiny of the experimental observations discussed in the preceding paragraphs reveals that the necessary physiological experiments (but not the biochemical or molecular genetic experiments) have been conducted only with the glycerol kinase in *B. subtilis* (60), that the requisite biochemical studies (but not the physiological or molecular genetic studies) have been carried out with the dihydroxyacetone or glycerol kinase of *Streptococcus faecalis* (7, 8), and that only the molecular genetic characterization (but not the relevant physiological or biochemical investigations) have been performed with the *Streptococcus thermophilus* lactose symport permease. Interpretation of the results obtained with these three different biochemical systems in terms of a unified mechanism of regulation should therefore be considered speculation. Nevertheless, demonstration of the occurrence of a non-PTS lactose-proton symporter with a fused III<sup>Glc</sup> moiety showing a high degree of sequence identity with the III<sup>Glc</sup> of *E. coli* argues for not only the occurrence of gene fusion during evolution, but also the horizontal transmission of genetic material between distantly related bacteria. It is clear that further studies will be required to establish the molecular details and physiological significance of PTS-mediated regulatory mechanisms operative in gram-positive bacteria.

## CONCLUSIONS AND FUTURE PERSPECTIVES

PTS-catalyzed regulation of inducer uptake in enteric bacteria, mediated by III<sup>Glc</sup> as an allosteric effector, provided the first documented example of a protein phosphorylation-dependent mechanism in which a metabolic or transcriptional process in bacteria was the regulatory target.

This process remains the best characterized of all such processes. In recent years, a variety of protein phosphorylation events have been found to control gene expression, transport function, and metabolic processes in prokaryotes (see reference 7a for an up-to-date review). The recent discovery that bacterial chemotaxis is apparently activated by a protein phosphorylation event, and that methylation-dependent adaptation may be similarly regulated by protein phosphorylation, further extends our recognition of the significance of this derivatization reaction (18, 45, 85, 91). That the PTS mediates chemotaxis in response to gradients of PTS sugars in a process dependent on enzyme I and HPr as well as the *cheA* and *cheY* gene products suggests that HPr(his)P may phosphorylate a histidyl residue of the CheA protein and that dephosphorylation of CheA~P upon addition of a PTS sugar to the culture medium may serve as the chemotactic signal for PTS sugars. Moreover, it has recently been found that phosphorylation of a histidyl residue in isocitrate lyase, the first enzyme in the glyoxylate shunt in *E. coli*, activated this enzyme (62) and that the enzyme possesses a histidyl residue (*his-266*) around which significant identity with the active histidyl residue (*his-91*) in III<sup>Glc</sup> has been demonstrated (28). These observations lead to the possibility that phosphorylation of isocitrate lyase by phospho-HPr serves as one regulatory signal which influences the relative flux of carbon through the Krebs cycle and the glyoxylate shunt. Finally, existing evidence supports the conclusion that transcriptional regulation of the *bgl* operon in *E. coli* and the *sac* operon in *B. subtilis* is controlled by PTS-mediated phosphorylation (2, 80a; M. Steinmetz, personal communication; K. Schnetz and B. Rak, personal communication). The PTS protein phosphotransfer cascade has therefore been implicated in chemotaxis, metabolic regulation, and transcriptional regulation in both gram-negative and gram-positive bacteria. Further studies will be required to establish the importance and extent of PTS-catalyzed protein phosphorylation to the regulation of prokaryotic physiological processes.

While the PTS-mediated mechanism of inducer exclusion represents one of the most thoroughly established mechanisms of its type, the sister process of PTS-mediated catabolite repression is still poorly defined. Moreover, in many bacteria, such as *B. subtilis* or *Rhodobacter capsulatus* in which a PTS is present but cyclic AMP is apparently absent, catabolite repression mechanisms unrelated to adenylate cyclase regulation must be operative (5, 89). It is clear that we are only beginning to understand the mechanisms by which bacteria regulate the metabolism of carbon, nitrogen, phosphorus, and sulfur. The interactions of these regulatory systems which must ultimately result in their coordination have yet to be studied.

#### ACKNOWLEDGMENTS

I thank Mary Beth Hiller for expert word processing assistance. G. Gonzy-Tréboul, B. Poolman, and B. Postma provided unpublished mentioned in this review.

Work in my laboratory was supported by Public Health Service grants 5RO1 AI 21702 and 2RO1 AI 14176 from the National Institute of Allergy and Infectious Diseases.

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