The phosphoenolpyruvate-sugar phosphotransferase system (PTS) catalyzes the transport and concomitant phosphorylation of a number of carbohydrates in *Escherichia coli* and *Salmonella typhimurium* (for a review, see references 19 and 20). The PTS is composed of two general cytoplasmic proteins, enzyme I and HPr, and various sugar-specific, membrane-bound enzymes II. The glucose PTS contains an additional cytoplasmic phosphoprotein, III^Glc^, which is required for glucose and methyl α-glucoside transport. In addition the phosphoprotein III^Glc^, a product of the crr gene (12, 25), is thought to function in the regulation of the synthesis and activity of a number of non-PTS uptake systems, including those for lactose, maltose, melibiose, and glycerol. Phosphorylated III^Glc^ is supposed to activate adenylate cyclase. When III^Glc^ is dephosphorylated (by a PTS sugar) or absent (due to mutation), the adenylate cyclase activity is low, and the intracellular level of cyclic AMP is not sufficient for the expression of certain catabolic operons (1, 19, 20). Non-phosphorylated III^Glc^ inhibits the accumulation of carbohydrates via the non-PTS uptake systems mentioned above. This phenomenon is called inducer exclusion. A direct interaction between III^Glc^ and the lactose carrier has recently been demonstrated (14, 15). With purified III^Glc^ and purified lactose carrier reconstituted in liposomes, it could be shown that these two proteins are sufficient for the observed interaction; transport of β-galactosides is inhibited by non-phosphorylated III^Glc^ (14). In the case of glycerol uptake we have recently demonstrated that non-phosphorylated III^Glc^ inhibits glycerol kinase, but not the facilitator (18). As a consequence no glycerol phosphate, the inducer of the glp regulon, is produced in the cell. PTS sugars not only decrease the uptake of certain non-PTS compounds, as mentioned above, but can also inhibit the uptake of each other. This is the result of competition of the various enzymes II or factors III for the common phosphotransferase pool (4, 23). Thus glucose and methyl α-glucoside can inhibit the uptake of fructose via III^Glc^ (see below).

A different model for regulation has been advanced by Kornberg and co-workers (6–8, 16). In their view III^Glc^, the product of the crr (tgs) gene, is only involved in the regulation of adenylate cyclase (19). The product of a second gene, iex, regulates the activity of the various non-PTS transport systems. The crr and iex genes are thought to be closely linked, but on opposite sides of the ptsHI genes that code for HPr and enzyme I (2, 7). crr and iex mutants have different phenotypes, a major difference being that methyl α-glucoside, a non-metabolizable analog of glucose, inhibits growth of an iex mutant, but not of a crr mutant, on fructose. According to Kornberg this is due to the fact that iex mutants contain an active III^Glc^.

The introduction of a plasmid containing a wild-type crr^+^ allele into the iex strain restores the iex phenotype to that of the iex^+^ parent. The III^Glc^ produced from the plasmid in the iex strain is heat stable and binds normally to the lactose carrier. These results lead to the conclusion that the iex mutation is most likely allelic with crr and results in an altered, temperature-sensitive III^Glc^ that is still able to function in α-glucose and methyl α-glucoside uptake and phosphorylation and in the activation of adenylate cyclase, but is unable to bind to and inhibit the lactose carrier.

Since these two models are not compatible, we have investigated the iex mutant in more biochemical detail. Our main conclusion is that the iex mutation results in an altered III^Glc^ that is still active in glucose and methyl α-glucoside phosphorylation and adenylate cyclase activation, but is unable to bind to and inhibit the lactose carrier.

### MATERIALS AND METHODS

**Bacterial strains and plasmids.** *E. coli* strains HK761 *pts*Δ(Ts) iex^+^ *umgC metB* *ilv* and HK738 *pts*Δ(Ts) iex^+^ *umgC metB* *ilv* were obtained from H. Kornberg. HK738 was constructed by introducing the iex mutation from HK727...
into ts19. HK761 is the isogenic iex\textsuperscript{−} strain. E. coli strain ts19 pts(TS) ilv metB was obtained from V. Gershanko-vitch. PPA3 crr pts(TS) ilv metB was derived from ts19 as a strain being able to grow on maltose in the presence of 5 mM methyl-a-glucose. The plasmid pBCP20 consists of a 1,000-base-pair S. typhimurium chromosomal fragment inserted in pAT153 (26) and containing the crr\textsuperscript{+} gene coding for III\textsubscript{Glc} (13a).

### Growth of cells and preparation of bacterial extracts

Cells were grown in minimal medium A (23) containing the required amino acids and 0.2% of a carbon source and were harvested at the midexponential phase. Cell extracts were prepared by passing the cells through a French pressure cell (24). Membranes were removed by centrifugation for 60 min at 150,000 \( \times \) g.

#### Determination of PTS proteins

The activity of enzyme I, HPr, and III\textsubscript{Glc} was determined in cell-free extracts by phosphorylation in vitro in the presence of an excess of the other purified components (24). Sugar phosphate formation was measured by the ion-exchange method (9). The amount of III\textsubscript{Glc} was determined by rocket immunoelectrophoresis (24).

#### Transport assay

Transport of 0.1 mM methyl-\(\alpha\)-D-\(\text{[U-14C]}\)glucopyranoside (specific activity, 390 cpm/nmol; Amersham Corp.) was measured as described previously (17).

#### Binding of III\textsubscript{Glc} to the lactose carrier

Binding of III\textsubscript{Glc} to the lactose carrier of E. coli was determined as described previously (14). Membranes containing high, elevated levels of the lactose carrier were a gift of J. K. Wright. The membrane preparation contained 17.4 mg of protein per ml and 1.4 mol of lactose carrier per mg of protein.

#### Purified enzymes

Purification of enzyme I, HPr, and III\textsubscript{Glc} has been described previously (24).

#### Transformation

HK738 and HK761 were transformed with pBCP20 by the method of Mandel and Higa (11), selecting for ampicillin resistance.

### RESULTS

#### Nomenclature

In S. typhimurium mutations have been isolated that restore growth of pts mutants on non-PTS compounds such as maltose, glycerol, and melibiose (21, 22). These crr mutants have lowered levels of III\textsubscript{Glc} or lack it altogether. It has been shown that the crr gene is the structural gene for III\textsubscript{Glc} (12, 25). In E. coli two types of suppressor mutations have been reported by Kornberg and co-workers. tgs mutations (17), renamed gsr later (16)]

### TABLE 1. Levels and activities of PTS proteins in E. coli

<table>
<thead>
<tr>
<th>Strain</th>
<th>Enzyme I\textsuperscript{p}</th>
<th>HPr\textsuperscript{p}</th>
<th>III\textsubscript{Glc}\textsuperscript{−}</th>
<th>III\textsubscript{Glc}\textsuperscript{+}</th>
<th>Methyl-a-glucose transport\textsuperscript{d}</th>
</tr>
</thead>
<tbody>
<tr>
<td>HK761</td>
<td>15</td>
<td>12.5</td>
<td>0.8</td>
<td>4.2</td>
<td>7.3</td>
</tr>
<tr>
<td>HK738(pBCP20)</td>
<td>ND \textsuperscript{e}</td>
<td>ND</td>
<td>ND</td>
<td>7.2</td>
<td>ND</td>
</tr>
<tr>
<td>HK738</td>
<td>17</td>
<td>14</td>
<td>0.5</td>
<td>4.0</td>
<td>7.3</td>
</tr>
<tr>
<td>HK738(pBCP20)</td>
<td>ND</td>
<td>ND</td>
<td>1.0</td>
<td>8.1</td>
<td>ND</td>
</tr>
<tr>
<td>PPA3</td>
<td>15</td>
<td>12</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

\[\text{* Enzyme activities were determined by phosphorylation in vitro of methyl-a-glucose and expressed as nanomoles of methyl-a-glucose phosphorylated per minute per milligram of protein.}

\[\text{** III\textsubscript{Glc} was determined by rocket immunoelectrophoresis and expressed as micrograms of III\textsubscript{Glc} per milligram of protein.}

\[\text{*** Methyl-a-glucose transport was measured in glycerol-grown cells as described in the text and expressed as nanomoles of methyl-a-glucose taken up per minute per millgram (dry weight).}

\[\text{\textsuperscript{d} ND, Not determined.}

renders an E. coli strain resistant to methyl-a-glucose or 5-thio-D-glucose on both PTS and non-PTS compounds. Presumably they lack III\textsubscript{Glc} since methyl-a-glucose transport is lowered or absent and they have a low level of adenylate cyclase activity (6, 8). tgs in E. coli has been equated with crr in S. typhimurium. In addition, an E. coli crr mutation has been described ([5, 8], renamed iex later (16)] that renders a strain resistant to methyl-a-glucose on non-PTS compounds, but leaves it sensitive on PTS sugars. It has been shown that the iex gene is different from the crr gene (8, 16). Not to confuse matters more than necessary, we will designate tgs (gsr) in E. coli and crr in Salmonella by crr; iex as described by Kornberg et al. will be used to designate the additional mutation found in E. coli.

#### Properties of iex and crr mutants

The growth characteristics of the iex mutant HK738 and its corresponding iex\superscript{+} parent HK761 are shown in Table 1. They confirm and extend the results reported by Kornberg and co-workers earlier on iex strains (8, 16), i.e., the iex mutant does not grow on fructose, but grows well on maltose in the presence of methyl-a-glucose (Table 1) or thioglucose (data not shown), as is the case with the parent strain. In contrast, crr mutants isolated in the same pts(TS) background, see below, were able to ferment methyl-a-glucose on all carbon sources. A representative strain, PPA3, is shown in Table 1. Growth at 42°C on certain non-PTS compounds is impaired in the parent since it contains a temperature-sensitive enzyme I (3). The iex mutation in strain HK738 was isolated as a suppressor of pts(TS) that allows the strain to grow on maltose at 42°C similar to the iex mutant HK727 described before [8]. Both regain growth on other non-PTS compounds such as glycerol and lactose as well, similar to S. typhimurium crr mutants previously described. However, in S. typhimurium crr mutants, III\textsubscript{Glc} is absent, and methyl-a-glucose transport is impaired. The inhibition of growth on fructose by methyl-a-glucose suggests that III\textsubscript{Glc} is still active in the E. coli iex strain. In addition, growth on non-PTS compounds such as succinate, which is strongly dependent on cyclic AMP, is not impaired. This also suggests that III\textsubscript{Glc} is still functional. Indeed, Kornberg et al. (8) showed that adenylate cyclase activity in an iex strain is close to that in a wild-type strain, in contrast to a crr strain.

#### PTS proteins in iex and crr mutants

To resolve these conflicting results obtained with E. coli iex and crr mutants and S. typhimurium crr mutants, we determined the levels of the various PTS proteins in such strains. Table 2 shows that the iex strain HK738 contained the same amount of III\textsubscript{Glc} (as
measured with an antibody against IIIGlc as the parent HK761. The crr mutant PPA3 lacked IIIGlc completely. Most pts crr double mutants of S. typhimurium, isolated as being able to grow on non-PTS compounds, lack most or all IIIGlc (21, 25). The same type of mutation has been isolated in E. coli strains resistant to methyl α-glucoside on glycerol (10). It should be remembered that the iex mutation in E. coli is isolated in the same way, i.e., suppressing the growth defect of a ptsI(Ts) mutant on maltose at 42°C.

Transport studies with methyl α-glucoside, a substrate of the IIIGlc-IIIGlc transport system, show that the rate of transport is almost the same in wild-type HK761 and iex mutant HK738 (Table 2). The crr strain PPA3 is unable to catalyze methyl α-glucoside transport. It should be pointed out that in intact cells (or, for that matter, in toluenized cells) IIIGlc is most likely not the rate-limiting component for uptake or phosphorylation of methyl α-glucoside (4, 23).

A first indication that IIIGlc might be altered in an iex mutant derives from phosphorylation studies in vitro. The specific activity of IIIGlc was determined in the presence of an excess of enzyme I, HPr, and enzyme IIIGlc. Table 2 shows that IIIGlc from the iex strain has only about 60% of the activity of the parental strain. The specific activity of enzyme I and HPr is the same in the iex' and iex strains. A crr mutant is unable to phosphorylate methyl α-glucoside due to the absence of IIIGlc. Since the iex strain contains the same amount of IIIGlc protein as the iex' strain (Table 2), this result suggests that this IIIGlc has altered properties. In yet another way one can show that IIIGlc in HK738 is altered. Heating IIIGlc of the parent and the iex strains for 6 or 30 min at various temperatures results in a negligible decrease of the activity in the parent and an almost complete loss at all but the lowest temperature in the iex strain (Table 3). Clearly, the stability of IIIGlc has been altered by the iex mutation.

No difference could be detected in the apparent molecular weight of IIIGlc in iex' and iex strains as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, electroblotting on nitrocellulose filters, and detection with antibody against IIIGlc (data not shown). A slight difference is observed after crossed immunoelectrophoresis. IIIGlc from the iex strain shows a somewhat broader peak compared with the iex' IIIGlc, but lacks the small shoulder present in the iex' strain. Mixing both types of IIIGlc shows that wild-type and mutant IIIGlc are immunologically identical (Fig. 1).

<table>
<thead>
<tr>
<th>Source</th>
<th>Temp (°C)</th>
<th>Activity of IIIGlc</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>No heat treatment</td>
</tr>
<tr>
<td>HK761</td>
<td>4</td>
<td>0.78</td>
</tr>
<tr>
<td>HK761</td>
<td>70</td>
<td>0.82</td>
</tr>
<tr>
<td>HK738</td>
<td>4</td>
<td>0.37</td>
</tr>
<tr>
<td>HK738</td>
<td>37</td>
<td>0.37</td>
</tr>
<tr>
<td>HK738</td>
<td>45</td>
<td>0.37</td>
</tr>
<tr>
<td>HK738</td>
<td>54</td>
<td>0.37</td>
</tr>
<tr>
<td>HK738</td>
<td>63</td>
<td>0.37</td>
</tr>
<tr>
<td>HK738</td>
<td>70</td>
<td>0.37</td>
</tr>
<tr>
<td>HK738(pBCP20)</td>
<td>70</td>
<td>1.05</td>
</tr>
</tbody>
</table>

*High-speed supernatant samples of the various strains were heated for the indicated times, cooled at 0°C for 1 min, and assayed for remaining activity of methyl α-glucoside phosphorylation at 30°C. Activity is expressed as nanomoles of methyl α-glucoside phosphorylated per minute per milligram of protein. Control samples, not heated, were kept on ice. ND, Not determined.

Binding of IIIGlc to the lactose carrier. We have shown recently that non-phosphorylated IIIGlc of S. typhimurium binds to and inactivates the lactose carrier of E. coli in the presence of β-galactosides (14). The properties of the iex mutant suggested to us that it might contain an altered IIIGlc that is still active in phosphorylation, but unable to bind to the lactose carrier. Table 4 shows that the E. coli IIIGlc from the parental strain (HK761) also binds to the lactose carrier and that this binding requires the presence of a β-galactoside. In contrast, IIIGlc from the iex strain is unable to bind to the lactose carrier. To exclude the possibility that an inhibitory factor is present in the iex mutant, purified IIIGlc was added to an iex supernatant, and the binding of this IIIGlc to the lactose carrier was determined. The binding of purified IIIGlc was equivalent to that in control under these conditions (Table 4).

**Complementation by a crr+ plasmid.** We tested the possibility that the iex gene product is involved in modification of IIIGlc. Conceivably, post-translational modification is required to allow the inhibitory action of IIIGlc, although it does not influence its phosphorylating capacity. A plasmid containing the crr+ gene was introduced into the E. coli strains. This plasmid produces IIIGlc at about the same levels as the chromosomal gene (Table 2) (13). Table 1 shows that the iex strain HK738 acquires the parental phenotype upon transformation with the crr+ plasmid pBCP20, i.e., it becomes sensitive again toward methyl α-glucoside on non-PTS compounds such as maltose, lactose, and glycerol. Thus the crr+ gene is dominant.

Table 4 shows that under these conditions plasmid-produced IIIGlc synthesized in the iex strain binds to the lactose carrier. The amount of IIIGlc bound is lower, however, in the
TABLE 4. Binding of III°Glc to the lactose carrier

<table>
<thead>
<tr>
<th>Source of III°Glc</th>
<th>TDG</th>
<th>III°Glc conc</th>
<th>III°Glc bound</th>
</tr>
</thead>
<tbody>
<tr>
<td>III°Glc</td>
<td>-</td>
<td>0.24</td>
<td>2.9</td>
</tr>
<tr>
<td>HK761</td>
<td>-</td>
<td>0.21</td>
<td>9.6</td>
</tr>
<tr>
<td>HK761(pBCP20)</td>
<td>-</td>
<td>0.18</td>
<td>3.3</td>
</tr>
<tr>
<td>HK738</td>
<td>-</td>
<td>0.16</td>
<td>11.5</td>
</tr>
<tr>
<td>HK738(pBCP20)</td>
<td>-</td>
<td>0.17</td>
<td>2.9</td>
</tr>
<tr>
<td>HK738 + III°Glc</td>
<td>-</td>
<td>0.16</td>
<td>8.2</td>
</tr>
</tbody>
</table>

*The binding of III°Glc from various sources to E. coli membrane vesicles containing 1.4 nmol of lactose carrier per mg of protein was determined as described in the text.

Approximately 20 µg of III°Glc from each source was added to each binding assay. Pure III°Glc was purified from S. typhimurium.

When present, the concentration of thiogalactoside (TDG) was 4 mM.

The free concentration of III°Glc was determined by rocket immunoelectrophoresis after centrifugation and is expressed as milligrams of III°Glc per milliliter.

The bound III°Glc was determined by rocket immunoelectrophoresis and is expressed as micrograms of III°Glc bound per milligram of vesicle protein.

case of the HK738(pBCP20) strain compared with the HK761 strain lacking the plasmid, for the following reason. The final concentration of III°Glc was kept the same in both extracts. Since only about half of the III°Glc in the cell-free extract of HK738(pBCP20) originates from the plasmid crr+ gene (Table 2) and the apparent Kₐ for III°Glc is 10 to 15 µM (14), one expects lowered binding at these III°Glc concentrations (approximately 5 µM each of mutant and wild-type III°Glc).

The heat inactivation of III°Glc produced by wild-type and mutant strains containing the plasmid also confirms the prediction. The III°Glc activity present in HK738(pBCP20) is only inactivated partly, whereas in HK738 the activity decreased to zero upon heating at 70°C (Table 3).

**DISCUSSION**

Two conflicting models have been advanced to explain the regulation of uptake and utilization of carbohydrates such as maltose, melibiose, glycerol, and lactose by the phosphoenolpyruvate-glucose PTS. According to one hypothesis, the protein III°Glc (product of crr gene) is the regulatory molecule (19–22), whereas according to Kornberg and co-workers (6–8, 16) an unidentified protein (product of the iex gene) is involved in the inhibition of non-PTS transport systems by PTS carbohydrates (inducer exclusion). Their main argument derives from the fact that two different mutations, tgs and iex, could be isolated in E. coli and that these mutations map at opposite sides of the pts operon. In their view III°Glc, the product of the tgs (gsr) gene, is only involved in glucose uptake and in the regulation of adenylate cyclase, but not in inducer exclusion of non-PTS carbohydrates. The data presented in this paper suggest, however, an alternative explanation for the tgs and iex mutations and the different phenotypes they cause, namely, that both are variable alleles of the crr gene.

E. coli tgs mutants as described by Kornberg are very similar to the crr mutants of S. typhimurium and of E. coli K12 (10), whether selected as clones resistant to methyl α-glucoside or as pts(T) crr double mutants able to grow at 42°C on non-PTS carbohydrates. All are impaired in methyl α-glucoside transport and unable to grow on succinate. Growth of a pts(T) tgs double mutant on maltose at 42°C is partly restored compared with the pts(T) parent (8), similar again to pts(T) crr mutants (Table 1). The remaining difference in growth rate in the double mutant might be due to the fact that, as a result of the defective III°Glc, the cyclic AMP level is lowered. Expression of the maltose regulon is dependent on cyclic AMP. According to the iex-hypothesis, one would predict that pts crr double mutants of E. coli would not grow on non-PTS compounds like maltose, since the inhibitory iex-coded protein is still present. This, clearly, is not the case.

E. coli iex mutants and S. typhimurium and E. coli crr mutants apparently differ in one important phenotypic property. Whereas the latter lack III°Glc, the former are thought to possess normal, functional III°Glc as deduced from methyl α-glucoside transport and inhibition of growth on fructose by methyl α-glucoside. Thus, according to Kornberg, growth on non-PTS compounds is resistant to methyl α-glucoside in crr and iex mutants for different reasons. In the case of the crr mutation, the analog cannot enter the cell. In the case of the iex mutants, the unidentified iex-coded protein is mutated or absent.

The data presented in this paper show that in the iex mutant HK738 an altered III°Glc is found, active in phosphorylation in vitro, but unable to bind to the lactose carrier. Although strain HK738 seems different from the S. typhimurium or E. coli crr mutants, this is due to the fact that most crr mutants lack III°Glc altogether, whereas in iex mutants the various functions of III°Glc have been affected to different extents. The normal transport of methyl α-glucoside and the growth on succinate (which requires cyclic AMP) indicate that III°Glc can still be phosphorylated and can donate its phosphoryl group to II°Glc. It is also able to function in the activation of adenylate cyclase. Non-phosphorylated III°Glc is unable, however, to bind to the lactose carrier. A further strong indication for the presence of an altered III°Glc in the iex strain HK738 is the heat lability of the protein.

The allele specificity of crr (tgs) and iex mutations is sufficient to explain their phenotypic differences, assuming that only the inducer exclusion function with respect to non-PTS carbohydrates of III°Glc is altered in iex mutants. In such mutants, growth on non-PTS substrates is no longer inhibited by the altered III°Glc, whereas glucose and methyl α-glucoside can still inhibit the uptake of, for instance, fructose by competition for the common P–HPr pool (4, 23). Since a similar suppression by iex of PTS-mediated inhibition of growth on glycerol, maltose, or melibiose is obtained, it is likely that the interaction of III°Glc with those systems is also impaired.

The genetic evidence that iex and crr mutations lie at opposite sides of the pts operon seems an argument against our hypothesis that both are alleles of the same gene crr. Unfortunately, the published evidence is not sufficient to distinguish unequivocally between the two possibilities. The localization of iex has varied considerably, ranging from 25% cotransduction with ptsF (17) to possibly allelic with ptsH (3), whereas the apparent molecular weight of its gene product is given as 33,000 or 21,000. Furthermore, several of the iex-transducing lambda phages used had to be disregarded in the construction of the genetic map since either the gene products for which they code or the complementation pattern did not agree with the restriction enzyme site mapping. As a result the mapping was done with one lambda phage (e.g., lambda 206) lacking the iex proteins and having
an iex phenotype. Interestingly, this phage also produces a strongly decreased amount of \(3^{*}\). In addition, the possibility has to be considered that different iex alleles produce \(3^{*}\) proteins having a residual activity at lower temperature (iex phenotype), but none at higher temperature (crr phenotype). This could explain several problems related to the complementation studies.

We think our studies have resolved the discrepancies between crr (ijs or gsr) mutations in \(S.\ typhimurium\) or \(E.\ coli\) and iex mutations with respect to their role in glucose-generated catabolite repression or inducer exclusion of non-PTS carbohydrates. Different mutations in the crr gene, coding for \(3^{*}\), can affect the phenotype of pts mutants in different ways, iex and ijs (crr) being most likely alleles of the crr gene affecting different domains of \(3^{*}\) to variable extents.

It would be most interesting to sequence the mutated iex genes to determine what changes have occurred. This could possibly tell us something about the interaction between \(3^{*}\) and its many different target proteins.

**LITERATURE CITED**