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Enzymes II of the Phosphotransferase System Do Not Catalyze Sugar Transport in the Absence of Phosphorylation

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In *Salmonella typhimurium*, glucose, mannose, and fructose are normally transported and phosphorylated by the phosphoenolpyruvate:sugar phosphotransferase system. We have investigated the transport of these sugars and their non-metabolizable analogs in mutant strains lacking the phospho-carrier proteins of the phosphoenolpyruvate:sugar phosphotransferase system, enzyme I and HPr, to determine whether the sugar-specific, membrane-bound components of the phosphoenolpyruvate:sugar phosphotransferase system, the enzymes II, can catalyze the uptake of these sugars in the absence of phosphorylation. This process does not occur. We have also isolated mutant strains which lack enzyme I and HPr, but have regained the ability to grow on mannose or fructose. These mutants contained elevated levels of mannokinase (fructokinase). In addition, growth on mannose required constitutive synthesis of the galactose permease. When strains were constructed which lacked the galactose permease, they were unable to grow even on high concentrations of mannose, although elevated levels of mannokinase (fructokinase) were present. These results substantiate the conclusion that the enzymes II of the phosphoenolpyruvate:sugar phosphotransferase system are unable to carry out facilitated diffusion.

The bacterial phosphoenolpyruvate (PEP): sugar phosphotranferase system (PTS) catalyzes the transport and concomitant phosphorylation of a number of sugars according to the following scheme (for a review, see reference 18):

$$\text{HPr} + \text{PEP} \underset{\text{Mg}^{2+}}{\overset{\text{Enzyme I}}{\rightleftharpoons}} \text{HPr}\sim\text{P} + \text{pyruvate}$$

$$\text{HPr}\sim\text{P} + \text{sugar}_{\text{out}} \xrightarrow[\text{Mg}^{2+}]{\text{Enzyme II}} \text{sugar phosphate}_{\text{in}} + \text{HPr}$$

The phosphoryl group is transferred sequentially from PEP to enzyme I to HPr. The actual translocation step is catalyzed by a family of membrane-bound enzymes II, each of which is specific for one or a few sugars.

Several lines of evidence, including studies with mutants lacking enzyme I or HPr or both, have suggested that phosphorylation and transport are tightly coupled processes. It is not clear, however, whether the enzymes II can catalyze the transport of their sugar substrates in the absence of phosphorylation. We will call this hypothetical process, which would result in the appearance of free sugar inside the cell instead of sugar phosphate, enzyme II-mediated facilitated diffusion.

Mutants of *Escherichia coli* or *Salmonella typhimurium* lacking enzyme I or HPr are unable to grow on glucose, mannose, or fructose, hexoses normally transported by the PTS (18). But from such results one cannot conclude that the enzymes II do not catalyze facilitated diffusion: PTS-mediated sugar phosphorylation is also deficient in *ptsI* or *ptsH* mutants, and kinases are not necessarily available to supply this function. A few transport studies which address this problem have been reported, but the results are inconclusive. Whereas Simoni and Roseman (24) have concluded from studies with *pts* mutants of *Staphylococcus aureus* that the enzyme II specific for lactose is unable to catalyze facilitated diffusion of lactose, most work with *E. coli* and *S. typhimurium pts* mutants suggests that enzymes II can act as sugar carriers in the absence of enzyme I or HPr or both (5, 9, 15, 21, 25). The studies with enteric bacteria are difficult to interpret, however, since the *ptsI* mutants used often contained residual levels of enzyme I, and more important, non-PTS transport systems may have been responsible for the observed uptake. For instance, Saier et al. (19) showed that growth of *ptsHI* deletion mutants on glucose is dependent on constitutive synthesis of the galactose permease.

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We have investigated sugar transport in *ptsHI* deletion mutants of *S. typhimurium*. Substrates of three different enzymes II were examined: the enzyme II specific for glucose, mannose and 2-deoxyglucose; the enzyme II specific for glucose and methyl α-glucoside; and the enzyme II specific for fructose. Our results indicate that these PTS components are unable to catalyze the transport of their sugar substrates in the absence of phosphorylation. A preliminary account of this work has appeared elsewhere. (P. W. Postma, Abstr. 11th FEBS Meet. 1977, B7-1 351).

MATERIALS AND METHODS

Chemicals. D-[*U*-^{14}C]galactose (50 μCi/0.03 mg), D-[1-^{14}C]mannose (50 μCi/0.17 mg), D-[*U*-^{14}C]fructose (250 μCi/9.7 mg) and [^{14}C]methyl β-D-galactopyranoside (50 μCi/2.03 mg) were obtained from New England Nuclear Corp. D-[*U*-^{14}C]glucose (284 mCi/mmol) and 2-deoxy-D-[1-^{3}H]glucose (20 Ci/mmol) were purchased from Amersham. 2-Deoxy-D-[1-^{3}H]galactose (20 Ci/mmol; Amersham) was a gift of J. van Steveninck. 2-Deoxygalactose was obtained from Sigma Chemical Co.; methyl β-D-galactopyranoside was from Koch-Light Lab. Pyruvate kinase, lactate dehydrogenase, glucose-6-phosphate dehydrogenase, phosphoglucose isomerase, and PEP were purchased from Boehringer. All other reagents were obtained from standard commercial sources. All sugars used in this study were of the D-configuration.

Bacterial strains. Table 1 lists the derivation of all bacterial strains used in this study.

Media and growth conditions. Cells were grown at 37°C on a rotatory shaker in nutrient broth or medium A [1 g of $(NH_4)_2SO_4$, 10.5 g of K_2HPO_4, 4.5 g of KH_2PO_4, and 0.1 g of $MgSO_4$ per liter of distilled water]. For growth of bacteria, medium A was supplemented with 20 μg of tryptophan per ml and the indicated carbon source. Minimal plates were made from this medium plus 1.5% (wt/vol) Difco agar.

Genetic methods. Mannose$^+$ (Man$^+$) and fructose$^+$ (Fru$^+$) revertants were isolated on minimal plates containing 0.2% mannose or fructose, either spontaneously or by placing a filter disk saturated with diethyl sulfate in the middle of the plate. Cells resistant to 2-deoxygalactose were isolated on minimal plates containing 0.4% DL-lactate and 1.5 mM 2-deoxygalactose (14). Preparation of P22-transducing lysates and transduction with phage P22 were performed as described by Blume and Balbinder (1).

Preparation of cell-free extracts and enzyme assays. Cell-free extracts were prepared as described earlier (16), but cells were disrupted with an Aminco French pressure cell at 1,200 kg/cm^2. Kinase activities were determined in the 200,000 × *g* supernatant fraction in two ways. (i) For labeled sugars: Galactokinase, glucokinase and mannokinase (fructokinase) were determined at 37°C in a reaction mixture (final volume, 0.1 ml) containing 10 mM ATP, 5 mM $MgCl_2$, 2.5 mM dithiothreitol, 12.5 mM KF, 50 mM potassium phosphate buffer (pH 7.5), various amounts of the supernatant fraction, and 10 mM labeled sugar (galactose, glucose, or mannose [fructose], respectively). The sugar phosphates formed were assayed by ion-exchange chromatography on Dowex AG 1-X2 (12), and

TABLE 1. *Strains of S. typhimurium used in this study*[a]

Strain no.	Relevant genotype	Origin	Source/reference
SB3507	*trpB223*		E. Balbinder
SB2309	*trpB223* Δ*(trzA-ptsHI)41*		P. E. Hartman, 3
SB2950	*trpB223* Δ*(trzA-ptsHI crr)49*		P. E. Hartman, 3
PP406	*trpB223* Δ*(trzA-ptsHI crr)49 mak-151 galC286*	Fru$^+$ SB2950, DES	A[b]
PP463	*trpB223 cysA20 mak-151 galC286*	P22(*cysA20*) × PP406	A
PP483	*trpB223* Δ*(trzA-ptsHI)41 mak-151 galC286*	P22(SB2309) × PP463	A
PP442	*trpB223* Δ*(trzA-ptsHI crr) 49 mak-151*	P22(PP406) × SB2950	A
PP453	*trpB223* Δ*(trzA-ptsHI crr)49 mak-151 galC286 galP2902*	2DG res, PP406	A
PP458	*trpB223* Δ*(trzA-ptsHI crr)49 mak-151 galC286 galP287*	2DG res, PP406	A
PP116	*trpB223* Δ*(trzA-ptsHI)41 galC1891*		16
PP635	*trpB223* Δ*(trzA-ptsHI)41 galC1891 mak-152*	Man$^+$ PP116, spont	A
PP638	*trpB223* Δ*(trzA-ptsHI)41 galC1891 mak-153*	Man$^+$ PP116, spont	A
PP639	*trpB223* Δ*(trzA-ptsHI)41 galC1891 mak-154*	Fru$^+$ PP116, DES	A
PP269	*trpB223* Δ*(trzA-ptsHI)41 galP1903*		16
SB2701	*trpB223 mem-1 cysA20*		17
PP339	*trpB223 mem-1* Δ*(trzA-ptsHI)41*	P22(SB2309) × SB2701	A
SB3666	*trpB223 mtlC594 ptsG204*		17
PP490	*trpB223 mtlC594 ptsG204 ptsI413*	Glc$^-$ SB3666, DES	A
PP564	*trpB223 mtlC594 ptsG204 cysA20*	P22(*cysA20*) × PP490	A
PP695	*trpB223 mtlC594 ptsG204* Δ*(trzA-ptsHI)41*	P22(SB2309) × PP564	A
—	*cysA20*		P. E. Hartman, 3

[a] Genetic nomenclature according to Sanderson and Hartman (22). *mak* denotes a mutation resulting in elevated levels of mannokinase (fructokinase). DES, Diethyl sulfate; Fru, fructose; Man, mannose; Glc, glucose; 2DG res, resistant to 2-deoxygalactose; spont, spontaneous.

[b] A, This study.

kinase activities were expressed as nanomoles of sugar phosphate formed per minute per milligram of protein. (ii) Spectrophotometrically: The glucokinase reaction mixture contained 50 mM Tris-hydrochloride, pH 7.2, 10 mM $MgCl_2$, 2 mM ATP, 0.2 mM $NADP^+$, 1.25 mM glucose, and glucose-6-phosphate dehydrogenase (10 μg/ml). Fructokinase was determined in the same medium plus phosphoglucose isomerase (0.15 μg/ml) and using fructose instead of glucose. Kinase activities were expressed as nanomoles of NADPH formed per minute per milligram of protein at 20°C. Mannokinase was determined as follows. The reaction mixture contained 50 mM Tris-hydrochloride (pH 7.2), 10 mM $MgCl_2$, 0.15 mM NADH, 1 mM azide, 2 mM PEP, 2 mM ATP, lactate dehydrogenase (15 μg/ml), pyruvate kinase (15 μg/ml), and 2 mM mannose. Kinase activity was expressed as nanomoles of NADH oxidized per minute per milligram of protein at 20°C. Mannokinase activity was corrected for NADH oxidation in the absence of added mannose. The enzyme II specific for mannose and glucose was assayed as described earlier (14), using the membrane fraction and 10 mM [^{14}C]-mannose as a substrate.

Transport studies. Transport of labeled compounds was performed as described previously (16). In the case of the experiment described in Fig. 1, rapid uptake was measured, as described earlier (26). The rate of transport is expressed as nanomoles of substrate accumulated per minute per milligram (dry weight) at 20°C.

Oxygen consumption. Oxygen consumption was measured with a Clark-type electrode in medium A (final volume, 1.65 ml). Substrates were added as indicated. The oxidation rate was corrected for oxygen consumption in the absence of added substrate and expressed as nano-atoms of oxygen consumed per minute per milligram (dry weight) at 25°C.

Protein. Protein was determined by the method of Lowry et al. (13), using bovine serum albumin as a standard.

Determination of generation times. The rate of growth of mutant strains on a given carbon source was determined as described earlier (17). Growth at 37°C was followed by measuring the increase in optical density at 600 nm.

RESULTS

Hexose transport in mutant strains defective in enzyme I and HPr. Two PTS-deficient strains of *S. typhimurium* were used in this study, SB2309 and SB2950 (3). Both are deleted in the genes coding for enzyme I (*ptsI*) and HPr (*ptsH*). The deletion in SB2309 includes the entire *ptsH* and most of the *ptsI* gene; the deletion in SB2950 includes the entire *ptsHI* operon and extends into *crr*, a gene(s) involved in the regulation of carbohydrate transport and metabolism (18). Neither of these mutant strains grows on glucose, mannose, or fructose (cf. Table 3). But where the PTS is concerned, growth is not necessarily a reflection of transport capacity. Even if enzyme II-mediated facilitated diffusion occurred in the absence of enzyme I and HPr, cells still could not grow unless the first reaction in sugar metabolism, the formation of sugar phosphates, were catalyzed by an alternate pathway.

It has previously been shown (18) that the non-metabolizable sugar analog 2-deoxyglucose is a specific substrate for transport via the enzyme II specific for glucose and mannose, and the analog methyl α-glucoside is a specific substrate for transport via the enzyme II specific for glucose. Uptake of these sugars by SB2309 is shown in Fig. 1. This strain is clearly deficient in methyl α-glucoside transport; the initial rate of uptake in wild-type *pts*$^+$ strains is approximately 20 nmol/min per mg (dry weight), whereas in SB2309 it is less than 0.04 nmol/min per mg (dry weight). In contrast, 2-deoxyglucose is transported by these cells; the sugar reaches equilibration within 2 to 3 min. This residual uptake of 2-deoxyglucose is not mediated by the enzymes II of the PTS, but instead occurs via an alternative, non-PTS transport system. In *S. typhimurium* it has been shown that mannose, glucose and 2-deoxyglucose are also substrates of the galactose permease (16). When a *galP* mutation was introduced into SB2309, the resulting galactose permease-deficient strain, PP269, transported 2-deoxyglucose at a rate less than 0.02 nmol/min per mg (dry weight). This

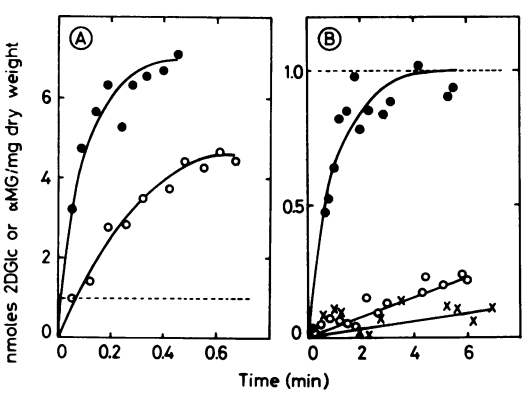

FIG. 1. *Transport of 2-deoxyglucose and methyl α-glucoside in various pts*$^+$ *and ΔptsHI strains. Cells were grown in a medium containing 0.4% DL-lactate. Transport of 0.5 mM [*^{3}*H]2-deoxyglucose (specific activity, 2,800 cpm/nmol) and 0.5 mM [*^{14}*C]methyl α-glucoside (specific activity, 2000 cpm/nmol) was measured, using a rapid uptake apparatus (26). Cell concentration was 10 mg (dry weight) per ml (SB3507), 53 mg (dry weight) per ml (SB2309) or 49 mg (dry weight) per ml (PP269). (A) ●, 2-Deoxyglucose transport in SB3507; ○, methyl α-glucoside transport in SB3507. (B) ●, 2-Deoxyglucose transport in SB2309; ×, 2-deoxyglucose transport in PP269; ○, methyl α-glucoside transport in SB2309. Broken line represents equilibration. 2DGlc, 2-Deoxyglucose; αMG, methyl α-glucoside. Note the differences in both scales.*

should be compared to a rate of approximately 70 nmol/min per mg (dry weight) obtained with wild-type *pts*$^+$ cells grown under similar conditions. The notion that the galactose permease is responsible for 2-deoxyglucose uptake in SB2309 was confirmed by the observation that, whereas galactose had no effect on 2-deoxyglucose uptake in the *pts*$^+$ strain, this uptake was inhibited by galactose in *ptsHI* deletion mutants (for instance, the uptake of 0.25 mM 2-deoxyglucose was over 85% inhibited by 1 mM galactose).

In addition to these studies with non-metabolizable sugar analogs, we also examined the transport of glucose and mannose. The *ptsHI* deletion mutant, SB2950, transported glucose and mannose at low rates (cf. Fig. 2A), but a strain lacking the galactose permease in addition to enzyme I and HPr (PP458) did not transport these sugars (cf. Fig. 2B). The introduction of the *galP* mutation had no effect on the levels of the enzymes II. For instance, the rate of PEP-dependent mannose phosphorylation catalyzed by membranes derived from strains SB2950 and PP458 was essentially identical (cf. Table 2).

PTS-deficient strains capable of growth on glucose, mannose, or fructose. From the studies described above, it is clear that strains lacking enzyme I and HPr are unable to catalyze facilitated diffusion of sugars via the enzymes II. It seems possible, however, that further mutation might permit enzyme II-mediated transport of sugars in the absence of enzyme I and HPr. It has been shown by Saier et al. (21) that mutants which grow on fructose (Fru$^+$), mannose (Man$^+$), and glucose (Glc$^+$) can be isolated from enzyme I-deficient strains. These cells were found to have elevated levels of mannokinase (fructo-kinase) which apparently functioned in the phosphorylation of these hexoses. It was not clear, however, how the sugars entered the cell.

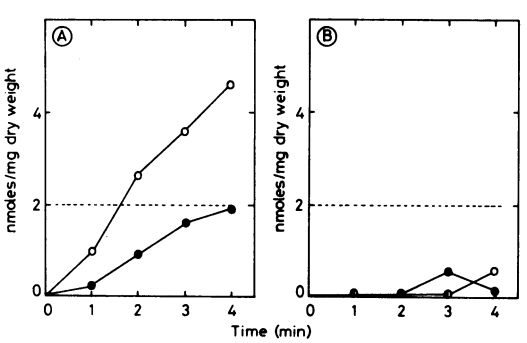

FIG. 2. *Transport of glucose and mannose in SB2950 and PP458. Cells were grown in nutrient broth, and transport was measured with 1 mM [^{14}C]glucose (specific activity, 102 cpm/nmol)* (○) *or 1 mM [^{14}C]mannose (specific activity, 100 cpm/nmol)* (●). *Cell concentration was 7.2 mg (dry weight) per ml (SB2950) or 6.0 mg (dry weight) per ml (PP458). (A) SB2950. (B) PP458. The broken line represents equilibration.*

TABLE 2. *PEP-dependent mannose phosphorylation catalyzed by membranes derived from various mutant strains*

Strain	Relevant genotype	Mannose phosphorylation[a]
SB2950	Δ*pts*	18.0
PP406	Δ*pts galC mak*	14.8
PP442	Δ*pts mak*	17.6
PP458	Δ*pts galC galP mak*	18.6

[a] Cells were grown in nutrient broth. Phosphorylation rate with 10 mM [^{14}C]mannose (specific activity, 54 cpm/nmol) is expressed as nanomoles of mannose phosphorylated per minute per milligram of membrane protein at 37°C.

We have isolated similar mutants, starting with *ptsHI* deletion strains, to determine whether the transport of hexoses in these strains involves the PTS enzymes II. As described previously (21), cells were selected for growth on fructose. A few Fru$^+$ colonies were obtained from SB2950 (frequency approximately 3×10^{-9} in the absence of diethyl sulfate and 10^{-8} in the presence of diethyl sulfate). These mutants also grew on mannose and glucose. The growth properties of one such mutant strain, PP406, are shown in Table 3. Since we were unable to obtain the corresponding mutants in SB2309, it seemed possible that a mutation in or deletion of the *crr* locus allowed expression of the Fru$^+$ phenotype (it should be noted that the mutants isolated by Saier et al. [21] were also in a *crr* background). By two successive transductions the extended *ptsHI crr* deletion in PP406 was replaced by smaller *ptsHI* deletion. The resulting strain, PP483, showed the same growth properties on hexoses as PP406 (cf. Table 3).

Attempts to isolate Man$^+$ colonies from either SB2950 or SB2309 on minimal agar plates containing mannose were unsuccessful. The possibility was considered that more than one mutational event is required for growth of *pts* deletion strains on mannose, in contrast to growth on glucose (19) or fructose (see above). The observation that all Fru$^+$ mutants were also Glc$^+$ and Man$^+$ suggested that possibly a second mutation conferred the Glc$^+$ (and Man$^+$) phenotype. We therefore attempted to derive Man$^+$ mutants from the Glc$^+$ *ptsHI* deletion strain, PP116, which is able to transport mannose (16), but does not grow on this carbon source. Starting with this strain, a large number of Man$^+$ mutants was isolated (frequency ca. 10^{-6}). These fell into two classes: Man$^+$ Fru$^-$ (PP635 [cf. Table 3]) and Man$^+$ Fru$^+$ (PP638 [cf. Table 3]). In a typi-

TABLE 3. *Growth rates of mak mutant strains on various carbon sources*

Strain	Relevant genotype	Generation time[a] (min)			
		Glc	Man	Fru	Gal
SB2950	Δ*ptsHI crr*	>600	∞	∞	60
PP406	Δ*ptsHI crr mak-151 galC*	83	98	200	66
PP453	Δ*ptsHI crr mak-151 galC galP*	∞	∞	240	>600
PP483	Δ*ptsHI mak-151 galC*	80	84	260	57
SB2309	Δ*ptsHI*	>600	∞	∞	70
PP116	Δ*ptsHI galC*	70	∞	∞	60
PP635	Δ*ptsHI galC mak-152*	NT	270	∞	60
PP638	Δ*ptsHI galC mak-153*	NT	99	152	55
PP639	Δ*ptsHI galC mak-154*	NT	108	138	60

[a] Generation times were determined with carbon sources at final concentrations of 0.2%. Glc, Glucose; Man, mannose; Fru, fructose; Gal, galactose; NT, not tested; ∞, no detectable growth after 12 h.

cal experiment, 40 of 41 Man^+ colonies displayed the former phenotype. When PP116 was used to isolate Fru^+ colonies directly (PP639 [cf. Table 3]), the results were similar to those obtained by using SB2950.

These data suggest that the Glc^+ and Fru^+ phenotypes are due to two different mutations, which when present together yield a Man^+ phenotype. This was confirmed by transduction studies. When phage P22 was grown on PP406 (Glc^+ Man^+ Fru^+) and crossed with SB2950 on minimal agar plates containing fructose, Fru^+ transductants were obtained. All of these had the same growth properties: Fru^+ Man^- Glc^-. On the other hand, transduction of PP116 (Glc^+ Man^- Fru^-) to Fru^+ by using phage P22 grown on PP406 yielded only Fru^+ Man^+ Glc^+ colonies.

Sugar phosphorylation in PTS-deficient strains. Metabolism of glucose, mannose, and fructose requires the formation of the corresponding sugar phosphates. In wild-type cells this reaction is catalyzed by the PTS. Mutants lacking enzyme I and HPr cannot carry out this function and therefore require alternate phosphorylation mechanism(s). *ptsHI* deletion mutants contain high constitutive levels of glucokinase, but the corresponding kinase for mannose and fructose, mannokinase (fructokinase) (23), is normally present only at low levels (cf. Table 4).

Fru^+ mutants obtained from SB2950, for instance PP406, have over 20-fold elevated levels of mannokinase (fructokinase) (cf. Table 4). Similar results were obtained by Saier et al. (21) with a Fru^+ *ptsI* strain. When phage P22 grown on PP406 was crossed with SB2950 on minimal fructose plates, the resulting Fru^+ strains, for instance, PP442 had elevated levels of mannokinase (fructokinase) (Table 4). It should be recalled that PP406 grew on fructose, mannose, and glucose, whereas PP442 grows only on fructose. Thus, elevated levels of mannokinase (fructokinase) appear to be sufficient for growth on fructose but not on mannose.

TABLE 4. *Mannokinase (fructokinase) in strains of S. typhimurium*

Strain	Relevant genotype	Mannokinase (fructokinase)		Glucokinase
		Mannose	Fructose	
Expt 1[a]				
SB2950	*mak*$^+$	3	1	100
PP406	*mak-151*	60	40	103
PP442	*mak-151*	70	38	93
PP458	*mak-151*	65	45	89
Expt 2[b]				
PP116	*mak*$^+$	14	2.5	100
PP635	*mak-152*	36	4.1	89
PP638	*mak-153*	120	32	120
PP639	*mak-154*	112	27	112

[a] In experiment 1, cells were grown on nutrient broth. Kinase activities were assayed by measuring the formation of sugar phosphate from labeled sugars at final concentration of 10 mM. [^{14}C]Mannose, specific activity 45 cpm/nmol; [^{14}C]-fructose, specific activity, 88 cpm/nmol; [^{14}C]glucose, specific activity, 174 cpm/nmol. All values are expressed relative to glucokinase. A value of 100 corresponds to 860 nmol of sugar phosphorylated per minute per mg of protein at 37°C.

[b] In experiment 2, cells were grown on 0.4% DL-lactate. Kinase activities were assayed spectrophotometrically as described in the text, using 6 mM hexose. All values are expressed relative to glucokinase. A value of 100 corresponds to 56 nmol of sugar phosphorylated per min per mg of protein at 20°C.

If, however, elevated levels of mannokinase (fructokinase) are sufficient for growth of *ptsHI* deletion mutants on fructose, and if such activities are required for growth of these strains on mannose, one would not expect mutants able to grow on mannose but not fructose. But this was the major class (for instance, PP635) when Man^+ mutants were selected on mannose minimal plates starting from a Glc^+ *ptsHI* deletion strain (PP116). In contrast to the Man^+ Fru^+ mutants, PP638 and PP639, isolated from the same background, the Man^+ Fru^- strain PP635, had relatively low levels of mannokinase (fructokinase)

(cf. Table 4). Similar results were obtained when two other Man$^+$ Fru$^-$ strains were tested. Apparently, higher levels of this enzyme are required to support growth on fructose than on mannose. This seems reasonable since the specific activity of mannokinase (fructokinase) is higher with mannose than with fructose as a substrate (Table 4) (21). It should be noted that PP635 grows more slowly on mannose than PP638 and PP639.

Since mutations which result in elevated levels of mannokinase (fructokinase), *mak*, have not been genetically characterized, it is not clear whether different *mak* alleles are mutations in the same regulatory gene resulting in variable levels of mannokinase (fructokinase) or whether these mutations alter the enzyme to give increased activities or both. Preliminary data indicate that kinase activities in extracts derived from PP635 (*mak-152*), PP638 (*mak-153*), PP639 (mak-154), and PP116 (*mak*$^+$) have approximately the same K_m for mannose (0.14 to 0.25 mM).

Sugar transport in PTS-deficient strains able to grow on hexoses. From the genetic studies described above we reasoned that two mutational events were required to allow growth of *ptsHI* deletion mutants on mannose. One of these is responsible for growth on fructose and leads to elevated levels of mannokinase (fructokinase). The other allows growth of *ptsHI* deletion mutants on glucose, and as has been shown previously, such mutations lead to constitutive synthesis of the galactose permease (16, 19).

Uptake studies (Fig. 3A and Table 5) showed that PP406 has high levels of glucose, mannose, 2-deoxyglucose and galactose transport activity as compared with the parent strain, SB2950.

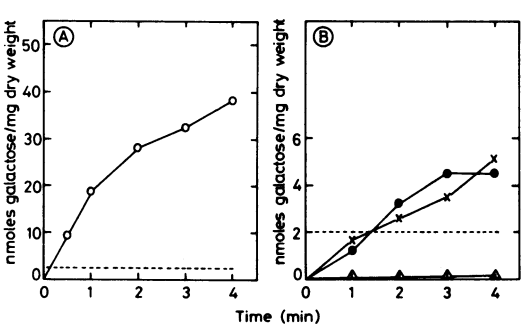

FIG. 3. *Galactose transport in various ΔptsHI crr strains. Cells were grown in nutrient broth. Transport of 1 mM [^{14}C]galactose (specific activity, 60 cpm/nmol) was measured as described in the text, using cells at a concentration of 5.2 to 7.6 mg (dry weight) per ml. (A) ○, PP406. (B) ×, SB2950; ●, PP442; Δ, PP458. Broken line represents equilibration.*

Fructose transport, however, was not increased. Previously we have shown that glucose, mannose and 2-deoxyglucose are substrates for the galactose permease (16), although these hexoses do not function to induce this system. PP406 had apparently acquired the ability to synthesize the galactose permease constitutively. This conclusion is supported by the finding that PP406 transports 2-deoxygalactose, a specific substrate of the galactose permease (14), at high constitutive rates. Two genes are known to be involved in the regulation of galactose permease synthesis, *galR* (19) and *galC* (16). Mutations in *galR* also lead to the constitutive synthesis of the galactose-metabolizing enzymes. Since galactokinase is still inducible in PP406 (cf. Table 6), we tentatively conclude that this strain contains a *galC* mutation. The *galC* mutation was not necessary for growth on fructose; the Fru$^+$ transductant, PP442, did not have elevated galactose permease activity (cf. Table 5).

The role of the galactose permease in growth of *ptsHI* deletion mutants on hexoses was investigated by introducing mutations in the *galP* gene(s). Since we have shown earlier that *galP* mutations abolish growth of *ptsHI* deletion mutants on glucose (14), we concentrate here on mannose and fructose. Mutant strains defective in the galactose permease were obtained as described previously by selecting for resistance to 2-deoxygalactose (14). When such mutations were introduced into PP406, the resulting strains lost the ability to grow on mannose and glucose even at concentrations as high as 50 mM (1%) sugar. These strains still have elevated levels of mannokinase (fructokinase) (for instance, PP458 [Table 4]), so any mannose entering the cell would be metabolized at a high rate. Under these conditions, no transport or oxidation of galactose, glucose, or mannose could be detected (cf. Fig. 3B and Tables 5 and 7). Since oxidation studies can be performed at higher substrate concentrations than can be used in transport studies with labeled compounds, this result tends to rule out the possibility that the enzymes II catalyze facilitated diffusion even at high sugar concentrations.

As shown above, the galactose permease is not responsible for fructose transport. Although we do not know which system catalyzes uptake of this hexose, the following observations tend to rule out the possibility that PTS enzymes II are involved. (i) The enzyme II for fructose is inducible, but the V_{max} and apparent K_m of fructose oxidation in PP406 are the same in lactate- and fructose-grown cells. (ii) Uptake of labeled fructose is not increased by growth on fructose. (iii) We have previously described a pleiotropic mu-

TABLE 5. *Transport in various mutant strains*

Strain	Relevant genotype	Transport[a]					
		Glucose	Mannose	Fructose	2-Deoxyglucose	Galactose	2-Deoxygalactose
SB2950	Δ*pts*	1.2	0.2	0.6	1.0	1.3	0.3
PP442	Δ*pts mak*	1.2	0.6	1.0	<0.1	1.5	0.2
PP406	Δ*pts mak galC*	16.0	14.0	0.6	11.0	19.0	5.0
PP458	Δ*pts mak galC galP287*	<0.1	<0.1	0.8	<0.1	<0.1	<0.1

[a] Cells were grown in nutrient broth. Transport rate of 1 mM [^{14}C]glucose (specific activity, 102 cpm/nmol), 1 mM [^{14}C]-mannose (specific activity, 100 cpm/nmol), 1 mM [^{14}C]fructose (specific activity, 163 cpm/nmol), 1 mM [^{3}H]2-deoxyglucose (specific activity, 277 cpm/nmol), 1 mM [^{14}C]galactose (specific activity, 60 cpm/nmol), and 1 mM [^{3}H]2-deoxygalactose (specific activity, 403 cpm/nmol) is expressed as nanomoles of substrate taken up per minute per milligram (dry weight) at 20°C.

TABLE 6. *Galactokinase induction in mutant strains*

Strain	Relevant genotype	Galactokinase[a]	
		Lactate-grown cells	Galactose-grown cells
SB2950	*galC*$^+$	22.8	228
PP406	*galC286*	26.2	250
PP442	*galC*$^+$	38.8	314

[a] Cells were grown in the presence of either 0.4% DL-lactate or 0.2% galactose as carbon source. Galactokinase activity is expressed as nanomoles of galactose phosphorylated per minute per milligram of protein at 37°C, using 10 mM [^{14}C]galactose (specific activity, 32 cpm/nmol).

tation, designated *mem*, which results in the loss of almost all sugar transport systems (17). The PTS enzymes II specific for glucose and fructose are not, or are only slightly, affected by this mutation, however. Using a *mem ptsHI* strain, PP339, no Fru$^+$ mutants could be isolated spontaneously, by mutagenesis with diethyl sulfate, or by transduction with phage P22 grown on PP406, excluding these transport systems as the ones responsible for fructose transport. (iv) Lengeler (Abstr. FEBS Symp. Biochem. Membrane Transport, Zurich, 1976, p. 34) has suggested that the enzymes II specific for mannitol and glucitol in *E. coli* are capable of facilitated diffusion, although with low affinity. Furthermore, Kornberg (7) has reported that fructose is a substrate for the glucitol-specific enzyme II. We have found, however, that fructose oxidation in PP406 was not inhibited by these hexitols.

DISCUSSION

Transport of sugars by the phosphotransferase system involves the concomitant phosphorylation of sugars at the expense of PEP. The complete system is comprised of a number of cytoplasmic phospho-carrier proteins which are sequentially phosphorylated and a set of sugar-specific, membrane-bound proteins designated enzymes II. Except as described in one report (10), no phospho-enzyme II has been demonstrated. Since the discovery of the PTS (11), there has been controversy as to the relationship between the translocation and phosphorylation events which this system catalyzes. Whereas the results of Simoni and Roseman and Saier et al. with *S. aureus* (24) and *S. typhimurium* (19), respectively, have indicated that there exists an obligatory coupling between these two processes, other investigators (5, 9, 15, 25) in studies mainly with *E. coli* have concluded that these two processes are not necessarily linked, that is, that the enzymes II can catalyze sugar translocation in the absence of phosphorylation. The latter studies are not definitive, however, because (i) the *pts* mutants used contained residual levels of the PTS enzymes, (ii) the possibility that sugars were transported by alternate transport systems was not completely excluded, or (iii) both i and ii.

The results reported in this communication strongly support the notion that, in the absence of the cytoplasmic phospho-carrier proteins, the glucose and mannose enzymes II are unable to catalyze facilitated diffusion of their natural substrates. We have used *pts* deletion mutations which completely eliminate enzyme I and HPr and introduced a *galP* mutation which abolishes the galactose permease. Our data show that the rate of 2-deoxyglucose or mannose uptake via the enzyme II specific for mannose and glucose is less than 0.03% that found in the wild-type *pts*$^+$ strain. Similarly, the rate of methyl α-glucoside or glucose uptake via the enzyme II specific for glucose is less than 0.2% of the wild-type rate. These data are based on transport studies performed with radiolabeled sugars at relatively low concentrations. It is possible, however, that the affinity of the enzymes II for their sugar substrates is increased when phosphorylation is abolished. We have therefore isolated strains with elevated levels of mannokinase (fructokinase) and a defective galactose permease to show that, even at concentrations of sugars as high as 50 mM, PTS-mediated facilitated diffusion does

TABLE 7. *Oxidation of substrates by S. typhimurium mutant strains*

Strain	Relevant genotype	Rate of oxidation[a]			
		Glucose	Mannose	Fructose	Galactose
Expt 1					
PP406	Δ*pts mak galC*	125	151	70	17
PP458	Δ*pts mak galC galP287*	<1	<1	60	<1
Expt 2					
PP116	Δ*pts galC*	108	10	<1	ND
PP635	Δ*pts galC mak-152*	ND[b]	137	<1	ND
PP639[c]	Δ*pts galC mak-154*	ND	110	22	ND

[a] Cells were grown with 0.4% DL-lactate as carbon source. The rate of oxidation is expressed as nano-atoms of oxygen consumed per minute per milligram (dry weight) at 25°C. Substrate concentrations were as follows. Experiment 1: 3 mM glucose, 6 mM mannose, 24 mM fructose, and 6 mM galactose. Experiment 2: 6 mM glucose, mannose, and fructose.

[b] ND, Not determined.

[c] When PP639 was grown on fructose, the rates of oxidation for mannose and fructose were 143 and 24, respectively.

not occur at rates sufficient to support growth. Moreover, by using glucose or mannose oxidation as a measure of uptake at high sugar concentrations, we were able to show that in *ptsHI* deletion mutants the enzymes II transported hexoses at less than 0.5% the rate obtained with the wild-type *pts*$^+$ strains.

Although we have established that in the absence of enzyme I and HPr the enzymes II are unable to catalyze facilitated diffusion of their sugar substrates, these results do not unequivocally show that phosphorylation is necessary for translocation. It is possible that the enzymes II and the phospho-carrier proteins form a complex which is required for sugar translocation in the absence of phosphorylation. The recent discovery, however, by Saier et al. (20) that the enzymes II are able to catalyze transphosphorylation (the transfer of the phosphoryl moiety from sugar phosphate to sugar) in the absence of enzyme I and HPr indicates that the enzymes II can function in the absence of the soluble PTS proteins.

In this study we have only addressed the question of sugar translocation in the absence of phosphorylation. Our conclusion that translocation and phosphorylation are tightly coupled processes does not exclude the possibility that sugar phosphorylation can occur in the absence of transport. Although the available evidence is not conclusive (for a review, see references 6 and 18), it appears that this process does not occur at significant rates in vivo.

Mutational events could possibly lead to altered enzymes II with dissociated phosphorylation and transport activities. For instance, Bourd et al. and Erlagaeva et al. (2, 4) and Kornberg and Jones-Mortimer (8) have described mutations in *E. coli*, designated *tgl*, which impair methyl α-glucoside transport but allow normal phosphorylation of this sugar in sonic extracts or in cells made permeable by toluene treatment. On the other hand, we have described mutants of *S. typhimurium* in which the enzyme II specific for mannose and glucose may catalyze the facilitated diffusion of galactose in the absence of phosphorylation (15). Care should be taken in the interpretation of results obtained with mutant strains, however. One must be wary of the possibility that non-PTS systems with altered activities can be responsible for putative PTS-mediated processes. This is especially true in *E. coli* and *S. typhimurium*, in which the PTS is only one of a large number of systems which can transport carbohydrates.

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