Sugar Phosphate: Sugar Transphosphorylation and Exchange Group Translocation Catalyzed by the Enzyme II Complexes of the Bacterial Phosphoenolpyruvate: Sugar Phosphotransferase System*

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The bacterial phosphoenolpyruvate:sugar phosphotransferase system catalyzes the concomitant transport and phosphorylation of many sugars. Sugar phosphorylation with phosphoenolpyruvate requires several soluble proteins as well as the integral membrane Enzyme II complex. We have demonstrated transfer of phosphate from sugar-P to 14C-sugar in extracts of Salmonella typhimurium, Escherichia coli, and Staphylococcus aureus. Transfer occurred specifically from glucose-6-P to glucose, mannose, or methyl α-glucoside; from mannitol-1-P to mannitol; from sorbitol-6-P to sorbitol; and in S. aureus from galactose-6-P to methyl β-thiogalactoside. The stoichiometry, phosphoryl donor specificities, and phosphoryl acceptor specificities of the transphosphorylation reactions were determined. Mutant analyses, induction studies, and competitive inhibition studies established that each transphosphorylation reaction was catalyzed by the Enzyme II complex specific for the substrate sugar. Extraction of soluble and peripheral proteins from the membrane with butanol and urea did not diminish activity, and full activity was observed in membranes prepared from a strain which lacked the soluble proteins of the phosphotransferase system due to deletion of the pts and err genes.

In vitro kinetic studies showed that the pH optima for sugar phosphotrans: sugar phosphotransphorylation were more acidic than the corresponding phosphoenolpyruvate-dependent sugar phosphorylation reactions. Optimal reaction rates were observed when the concentration of substrate sugar phosphate was high (>10 mM) and the concentration of substrate sugar was low (<100 μM). Substrate inhibition was a characteristic of the transphosphorylation reaction. A dependence on divalent cation was noted, but this dependence was less pronounced than that for phosphoenolpyruvate-dependent sugar phosphorylation.

Studies with intact bacteria showed that under appropriate conditions sodium arsenate, which reduced the intracellular concentration of phosphoenolpyruvate to low values, reduced the maximal extent of phosphotransferase system-mediated [14C]methyl α-glucoside accumulation without affecting the initial rate of sugar uptake. Intracellular sugar, accumulated in the presence of arsenate, was recovered largely as the phosphate ester. Nonradioactive extracellular sugar substrates of the glucose Enzyme II complex stimulated release of [14C]methyl α-glucoside from cells preloaded with this sugar. Under these conditions the intracellular sugar phosphate pool was preferentially reduced. The results suggest that the sugar phosphotrans: sugar phosphotransphorylation reaction can occur in a vectorial fashion with the sugar moiety of intracellular sugar phosphate being expelled from the cell as the phosphoryl moiety is transferred to an incoming sugar molecule.

In the preceding report (1), we demonstrated that a phosphoenolpyruvate:sugar phosphotransferase system (2, 3) in Spirochaeta aurantia catalyzed mannitol 1-phosphate[14C]mannitol transphosphorylation in vitro. The soluble proteins of the Spirochaete PTS were not required, and the available evidence suggested that this activity was attributable to the membrane-associated Enzyme II complex. A rigorous demonstration of this possibility was not attempted because the Enzyme II complex of the Spirochaete PTS is an integral membrane constituent which has not been extensively studied either genetically or biochemically. Instead, we have conducted detailed analyses of transphosphorylation reactions catalyzed by the phosphotransferase systems in Salmonella typhimurium, Escherichia coli, and Staphylococcus aureus, the organisms for which the most detailed genetic (4-6) and biochemical (7-9) analyses of PTS function have been conducted.

The abbreviations used are: PTS, phosphoenolpyruvate:sugar phosphotransferase system; HPr, heat-stable phosphoryl carrier protein of the PTS; Enzyme II, Enzyme II complex which exhibits specificity toward mannitol; Enzyme II, Enzyme II complex of the PTS which exhibits specificity toward glucose and methyl α-glucoside; Enzyme II, Enzyme II complex of the PTS which exhibits specificity toward sorbitol; Enzyme II, Enzyme II complex which exhibits specificity toward lactose.
ducted. The results of these studies are reported here. Preliminary accounts of this work have appeared (10, 11).

**EXPERIMENTAL PROCEDURES**

**Materials** — [1-3H]Mannitol-1-P was synthesized by phosphorylation of [14C]mannose with ATP and crystalline yeast hexokinase and subsequent quantitative reduction with NaBH₄. The reduced product was purified by paper chromatography employing ethyl acetate:acetic acid:formic acid:water (18:3:1:4) as solvent (12, 13). Glucose was isolated by passage through a French pressure cell at 10,000 p.s.i. A second procedure, used for preparation of butanol-urea-extracted membrane proteins (8), Escherichia coli and S. Typhimurium cells, involved treatment with lysozyme before passage through a French press. Washed cells (2 to 4 g, wet weight) were suspended in 20 ml of 25 mM Tris/HCl buffer, pH 7.4, containing 0.5 mM dithiothreitol, 10 mM EDTA, and 2 mg of lysozyme. The suspension was stirred at room temperature for 10 min and the following additions were made: MgCl₂ (15 mM, final concentration), DNPase (0.2 mg), and RNase (0.1 mg). After an additional 10 min, EDTA was added to a final concentration of 20 mM and the suspension was centrifuged at 20,000 rpm for 30 min in the SS-34 rotor. The membrane pellet was washed once with 200 ml of 25 mM Tris/HCl, pH 7.4, containing 0.5 mM dithiothreitol for passage through a French press cell at 10,000 p.s.i. and was subsequently sedimented by centrifugation at 200,000 x g for 2 h.

Membranes, prepared by the lysozyme-French press method, were extracted with urea and butanol by a modification of the procedure of Kündig and Rossmann (8). Washed membranes from 2 to 4 g (wet weight) of cells were resuspended in 20 ml of 25 mM Tris/HCl buffer, pH 7.4, in a 50-mI Erlenmeyer flask. Crystalline urea (10 g) was added with slow stirring at room temperature, and the complete dissolution of the urea was usually reached in 3 h. Membranes were then resuspended in 20 ml of 25 mM Tris/HCl buffer, pH 7.4, containing 1 mM EDTA and 0.5 mM dithiothreitol, and the suspension was centrifuged at 37,000 x g for 1 h at 4 °C. Particulate material was separated from the supernatant fraction by centrifugation (3 h at 200,000 x g). The resuspended membranes were dialyzed twice against 2 liters of 25 mM Tris/HCl buffer, pH 7.4, containing 1 mM EDTA and 0.5 mM dithiothreitol. Enzyme II activities for mannitol, methyl-α-glucoside, and mannose were recovered quantitatively with a 2-fold increase in specific activity. These activities were stable for at least a week at 4 °C and indefinitely at -60 °C.

Cell-free extracts were prepared from Staphylococcus aureus strains after disruption of cells by exposure to lysostaphin and subsequent sonication as described by Simoni et al. (20). Cell debris was removed by low speed centrifugation, and the particulate fraction was separated from the soluble proteins by centrifugation at 200,000 x g for 2 h. Membranes were washed once before assay.

**Assay for Sugar Phosphorylation in Vitro** — The standard assay for sugar-P and phosphoenolpyruvate-dependent ¹⁴C-sugar phosphorylation was similar to that described previously (1, 17). Unless otherwise indicated, the reaction mixture contained 50 mM potassium phosphate buffer, pH 7.4, 10 mM MgCl₂, 20 mM KF, 1 mM dithiothreitol, ¹⁴C-sugar at 100 μM for methyl-α-glucoside, or 50 μM for all other sugars, and the phosphorylation donor ATP at 5 mM for phosphoenolpyruvate or 10 mM for sugar phosphates. The final volume was 1 ml. Phosphoenolpyruvate-dependent reactions were measured in the presence of the saturating amount of the soluble enzymes of the PTS with from 0.5 to 10 μg of membrane protein/tube. The transphosphorylation reactions were measured in the absence of the soluble proteins of the PTS with 50 to 1000 μg of membrane/protein/tube. All assays were conducted at 37 °C.

**Isolation of Enzyme II Mutants** — A positive selection procedure described previously (16, 21) was used for the isolation of mutants which were defective for the Enzyme II complex of the PTS. The bacterial strain used in these studies was S. typhimurium strain LJ49 (ppc-201 manA54). This strain lacked phosphoenolpyruvate carboxylase (16, 21) as well as the Enzyme II complex of the PTS which exhibits specificity toward glucose, mannose, glucosamine, and fructose (16, 22, 23). The mutant could not utilize the PTS (due to the ppc-201 mutation) and fermented glucose in complex medium in a process which depended exclusively on the activity of the Enzyme II complex (due to the manA54 mutation) (16). Enzyme II mutants were isolated and characterized as follows.

Strain LJ49 was plated on hands in plates of minimal medium with 10 g of glucose, 10 g of yeast extract, 50 mM potassium phosphate buffer, pH 7.4, 10 mM MgCl₂, 5 mM KF, and 0.4% galactose. Colonies were isolated on plates containing 15% of the original glucose concentration and grown on several media to ensure the stability of the mutants. At least two independent isolates of each mutant were used for the experiments described in the text.

**Preparation of Enzyme II Complexes** — Cells were routinely disrupted by passage through a French pressure cell at 10,000 p.s.i. A second procedure, used for preparation of butanol-urea-extracted membrane proteins (8), was used for preparation of the Enzyme II complexes of Escherichia coli and Staphylococcus aureus. The Enzyme II complexes were purified by the anion exchange method described previously (7).

**Bacterial Strains** — Bacteria were grown as described in previous communications (15-17). Most of the strains employed in the present studies are listed in Tables I and II and have been described (15-18). Salmonella typhimurium strain LJ144, which carries the F' episome and exhibits 5-fold elevated activities of Enzyme I, HPr, and the Enzyme III complex (18), was used as a source of the soluble proteins of the PTS. These proteins were partially purified as described previously (7, 19).

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**Analytical Procedures** — Paper electrophoresis of sugar phosphates was conducted with the starch gel-anion exchange method described previously (6). The alternate technique was 12% polyacrylamide gel electrophoresis followed by silver staining. The migration of the proteins was determined with the aid of stained markers. The migration of the carbohydrates was determined from scans of the autoradiograms employing the silver nitrate-sodium hydroxide reagent (25), and radioactive compounds were located by scintillation counting after the chromatographic strips were run into 1-inch segments.

**Transport of W-Sugars** — Uptake of W-sugars by intact bacterial cells, and efflux of nonmetabolizable sugars from the cells was determined as described previously (18) and as specified in the figure and table legends. All assays were conducted in a modified Medium 63 (21) at 37 °C.

**Analytical Procedures** — Paper electrophoresis of sugar phosphates was conducted with two buffer systems, pyridyl acetate, pH 6.5, and tetrazole. Further separation was achieved by high performance liquid chromatography on a TSK gel 410 SW3000 column. The absorbance was monitored at 214 nm. The migration of the sugars was determined from scans of the autoradiograms employing the silver nitrate-sodium hydroxide reagent (25), and radioactive compounds were located by scintillation counting after the chromatographic strips were run into 1-inch segments.

**Relative mobilities in Solvent B of ATP, glucose-6-P, methyl-α-glucoside, and glucose-6-phosphate** were 0.4, 1.0, 2.6, 9.8, 3.8, and 9.0 in that order.
Systems Are Responsible for Transphosphorylation Reactions

The results suggest that a close relationship (and possibly an implicit association) between Enzyme II complexes is responsible for both the P-enolpyruvate or sugar phosphate was the phosphoryl donor. These results are consistent with the conclusion that the three of the activities studied; some of the mutants had previously affected the two reactions for the appropriate substrates. The suggestion that the Enzyme II complexes catalyzed the transphosphorylation reactions was further substantiated by the finding that the glucose-Enzyme II complex, lowered both reactions to 20% of the wild type activity. The manAI2 mutation, which affected the Enzyme II complex for the phosphorylation of glucose, mannose, glucosamine, and fructose (16), drastically reduced the phosphorylation of mannose in both reactions, and the mttI-61 and stl-113 mutations, known to abolish mannitol and sorbitol Enzyme II activities (16), simultaneously affected the two reactions for the appropriate substrates. In other experiments (not shown) the specific Enzyme II mutations did not affect the phosphorylation of sugars which were not substrates of the affected enzyme when either P-enolpyruvate or sugar phosphate was the phosphoryl donor. These results are consistent with the conclusion that the Enzyme II complexes are responsible for both the P-enolpyruvate-dependent and the sugar-P-dependent reactions.

In a more extensive genetic study of Enzyme II′ specific function, 80 mutants which were defective for Enzyme II′ (glu mutants) were isolated (see "Experimental Procedures"). Each of these mutants was assayed for the three known catalytic activities thought to be associated with the Enzyme II′ complex: (a) phosphoenolpyruvate:methyl α-glucoside phosphotransferase activity; (b) glucose 6-phosphate:methyl α-glucoside transphosphorylation; and (c) methyl α-glucoside transport. Most of the mutants assayed had quantitatively lost all three of the activities studied; some of the mutants had coordinately lost these activities to an incomplete degree; and only two mutants were isolated which exhibited marked differential loss of one or two of these activities without a concomitant decrease in the activity of the other reaction(s). The results suggest that a close relationship (and possibly an interdependence) exists between the different Enzyme II′ catalyzed reactions.

The suggestion that the Enzyme II complexes catalyzed the transphosphorylation reactions was further substantiated by examining the sensitivities of the different Enzyme II′ activities to irreversible inhibition by N-ethylmaleimide. Treatment of membranes with 2.5 mM N-ethylmaleimide completely eliminated mannitol-1-P-1′C]mannitol transphosphorylation and phosphoenolpyruvate-dependent mannitol phosphorylation activities without reducing the corresponding activities assayed with [14C]mannose as phosphoryl acceptor. Results obtained with [14C]methyl α-glucoside phosphorylation activities in the same membrane preparations were variable.

In a previous communication, each of the Enzyme II′ complexes of the PTS was shown to be inducible (17). The induction of transphosphorylation activities in S. typhimu-
Induction of transphosphorylation activities in Salmonella typhimurium

S. typhimurium strain LJ62 cells (15) were grown in minimal Medium 63 containing the carbon source indicated below at a final concentration of 0.5%. Washed membranes were prepared and assayed for sugar-P-sugar transphosphorylation as described in the legend to Table I. Phosphoryl donors (10 mM) were as follows: glucose-6-P with methyl α-glucoside and mannose; mannitol-1-P with mannitol; sorbitol-6-P with sorbitol. Corresponding Enzyme II-dependent phosphorylation reactions, have been reported (17). Strain LJ62 carried the opr-401 mutation which resulted in the genetic loss of cyclic AMP phosphodiesterase activity.

Wild type and mutant Staphylococcus aureus cells (6, 37) were grown in staphylococcal broth, harvested in the stationary growth phase, washed three times with Medium 63, and resuspended for cell rupture by the lyostaphin sonication method described under "Experimental Procedures." Buffer-washed membranes in the absence of the soluble protein fraction were used to assay the phosphorylation reaction with galactose-6-P (30 mM) as phosphoryl donor while the phosphoenolpyruvate-dependent reaction was measured in the presence of rate-limiting quantities of the membrane-associated Enzyme II′ with excess quantities of the soluble enzymes of the staphylococcal PTS from strain C22 cells. Incubations were for 1 h at 37”. Values are expressed as micrograms of [14C]methyl β-thiogalactoside-6-P formed/min/mg of membrane protein. All mutant strains were shown to exhibit negative lactose fermentation properties and to be deficient for methyl β-thiogalactoside uptake. In the absence of added soluble enzymes all mutant extracts catalyzed phosphoryl transfer from phosphoenolpyruvate to methyl β-thiogalactoside at rates which were less than 1% of the wild type rate. The rate at which a crude extract from F9 cells catalyzed phosphoenolpyruvate-dependent [14C]methyl β-thiogalactoside phosphorylation was less than 20% of the rate at which the same extract catalyzed methyl β-thiogalactoside phosphorylation with galactose-6-P as phosphoryl donor. Toxylgalactosides (14) were dissolved to a concentration of 0.1 μM in dimethyl sulfoxide and added to the assay mixtures before addition of enzyme. Dimethyl sulfoxide was not inhibitory at the concentration employed.

Under the conditions employed. Mannose-6-P was an effective phosphoryl donor for [14C]mannose phosphorylation, but not for methyl α-glucoside phosphorylation. The only effective phosphoryl donors for [14C]mannitol and [14C]sorbitol phosphorylation were mannitol-1-P and sorbitol-6-P, respectively. These results are consistent with the known sugar substrate specificities of the Enzyme II complexes in Salmonella and Escherichia coli (16, 17, 22, 23).

The strict donor specificity noted above was not observed in all preparations examined. In crude extracts of some E. coli and S. typhimurium strains, several sugar phosphates served as effective phosphoryl donors when [14C]mannitol or methyl α-glucoside was the phosphate acceptor. This broad donor specificity was always lost after preparation of washed or butanol/urea-extracted membranes. Thus, enzyme components in cell-free extracts in addition to the Enzyme II complexes may allow phosphoryl donation from sugar phosphates other than those listed in Table III.

Transphosphorylation Catalyzed by Enzyme II Complexes of Phosphotransferase System in Staphylococcus aureus—Transphosphorylation of methyl β-thiogalactoside was observed in the precocne of galactose-6-P and washed membranes from S. aureus strain C22 (Table IV) (6, 9, 20). Genetic loss of Enzyme I or Enzyme II′ did not reduce the rates of galactose-6-P-dependent transphosphorylation although the phosphoenolpyruvate reactions were reduced to less than 0.2% of the wild type rates (data not shown). By contrast, three independently isolated Enzyme II′ mutants exhibited greatly reduced rates of the phosphoenolpyruvate and sugar-phosphoenolpyruvate reactions in the presence of rate-limiting quantities of the membrane-associated Enzyme II′ with excess quantities of the soluble enzymes of the staphylococcal PTS from strain C22 cells. Incubations were for 1 h at 37”. Values are expressed as micrograms of [14C]methyl β-thiogalactoside-6-P formed/min/mg of membrane protein. All mutant strains were shown to exhibit negative lactose fermentation properties and to be deficient for methyl β-thiogalactoside uptake. In the absence of added soluble enzymes all mutant extracts catalyzed phosphoryl transfer from phosphoenolpyruvate to methyl β-thiogalactoside at rates which were less than 1% of the wild type rate. The rate at which a crude extract from F9 cells catalyzed phosphoenolpyruvate-dependent [14C]methyl β-thiogalactoside phosphorylation was less than 20% of the rate at which the same extract catalyzed methyl β-thiogalactoside phosphorylation with galactose-6-P as phosphoryl donor. Toxylgalactosides (14) were dissolved to a concentration of 0.1 μM in dimethyl sulfoxide and added to the assay mixtures before addition of enzyme. Dimethyl sulfoxide was not inhibitory at the concentration employed.

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The suggestion that Enzyme II\textsuperscript{ac} catalyzed the transphosphorylation reaction was confirmed by inhibitor studies. Hays et al. have shown that 6-O-tosylgalactose and 6-O-tosylmethyl β-galactoside are potent and highly specific competitive inhibitors of Enzyme II\textsuperscript{ac} function (14). As shown in Table IV, Experiment 2, these analogues exerted similar inhibitory effects on the phosphoenolpyruvate and galactose-6-P-dependent phosphor-y1 donor specificity of the sugar-P:methyl β-thiogalactoside transphosphorylation reaction is recorded in Table III. Of the sugar phosphates tested, only galactose-6-P was an effective phosphoryl donor.

In S. aureus, a single pair of sugar-specific proteins (the Enzyme II\textsuperscript{m} and the Enzyme III\textsuperscript{m}) catalyzes the phosphorylation and transport of the hexitols, mannitol and sorbitol (30). In this organism, mannitol is readily taken up from the medium and utilized as a source of carbon, although sorbitol is transported and utilized very slowly. In vitro phosphoenolpyruvate-dependent phosphorylation of \textsuperscript{14}C\textsuperscript{}mannitol occurred at about 5-fold the rate of \textsuperscript{14}C\textsuperscript{}sorbitol phosphorylation under conditions where both sugar substrates were present at saturating concentrations (30). An examination of transphosphorylation activities associated with the Enzyme II\textsuperscript{m} revealed that with mannitol-1-P (10 mM) as phosphoryl donor, and with the \textsuperscript{14}C-sugar substrate at a concentration of 50 μM, sorbitol was phosphorylated at 4% of the rate at which mannitol was phosphorylated. The relative rates of \textsuperscript{14}C\textsuperscript{}mannitol and \textsuperscript{14}C\textsuperscript{}sorbitol phosphorylation with sorbitol-6-P as the phosphoryl donor were 2% and 0.3%, respectively.

### Product Characterization and Stoichiometry of Sugar Phosphate: Sugar Transphosphorylation Reactions

The anionic radioactive products of the phosphoenolpyruvate and sugar-P-dependent reactions obtained from (a) \textsuperscript{14}C\textsuperscript{}mannitol in the presence of the Enzyme II\textsuperscript{m} of S. typhimurium, (b) \textsuperscript{14}C\textsuperscript{}methyl α-glucoside in the presence of the Enzyme II\textsuperscript{m} of S. typhimurium, and (c) \textsuperscript{14}C\textsuperscript{}methyl β-thiogalactoside in the presence of the Enzyme II\textsuperscript{ac} of S. aureus were characterized as the phosphate esters of the corresponding radioactive sugars as follows. All three radioactive products electrophoresed in two buffer systems with sugar phosphates. Their chromatographic mobilities in Solvent A were nearly zero as observed for the standard sugar phosphates, and they co-chromatographed with the standard compounds in Solvent B (see "Experimental Procedures"). Each of the three anionic radioactive products was cleaved by crystalline E. coli alkaline phosphatase (Sigma) to give a single product which co-chromatographed in two solvent systems with the starting free sugar (mannitol, methyl α-glucoside, or methyl β-thiogalactoside, respectively).

The stoichiometry of transphosphorylation could not be determined with washed membrane preparations because the sugar phosphatase activities were in excess of the transphosphorylation activities. Butanol/urea extraction of the membranes yielded preparations which were largely free of phosphatase activity. Employing butanol/urea-extracted membranes, the stoichiometry of transphosphorylation catalyzed by the Enzyme II\textsuperscript{ac} (Experiment 1) and the Enzyme II\textsuperscript{m} (Experiment 2) was determined (Table V). With glucose-6-P as the phosphoryl donor and either glucose or methyl α-glucoside as phosphoryl acceptor approximately 1 mol of glucose was released from the starting sugar phosphate for every mole of sugar phosphorylated. The rate of sugar phosphate hydrolysis was less than 20% of the rate of transphosphorylation. Mannitol, which is not a substrate of the Enzyme II\textsuperscript{ac}, did not stimulate the release of free sugar from \textsuperscript{14}C\textsuperscript{}glucose-6-P. It can also be seen that high sugar concentrations strongly inhibited the transphosphorylation reaction (Table V). Corresponding stoichiometric data for the Enzyme II\textsuperscript{m}-catalyzed transphosphorylation reaction are recorded under Experiment 2 of Table V.

### Table V

Stoichiometry of sugar phosphate:sugar transphosphorylation reactions catalyzed by Enzyme II\textsuperscript{m} and Enzyme II\textsuperscript{ac} complexes of phosphotransferase system of Salmonella typhimurium

| Experiment 1 | Sugar P (1 mm) and sugar | \textsuperscript{14}C-Sugar formed | \textsuperscript{14}C-Sugar-P formed | μmol/min/mg protein |
|--------------|--------------------------|-------------------------------|-------------------------------------|---------------------|
| None         | (\textsuperscript{14}C\textsuperscript{}Glucose-6-P) | 5                             | 23                                  | 12                  |
| None         | (\textsuperscript{14}C\textsuperscript{}Glucose (0.2 mm)) | 23                            | 2                                   | 5                   |
| None         | (\textsuperscript{14}C\textsuperscript{}Methyl α-glucoside (0.2 nm)) | 7                             | 2                                   | 2                   |
| None         | (\textsuperscript{14}C\textsuperscript{}Methyl α-glucoside (20 mm)) | 7                             | 7                                   | 2                   |
| Mannitol-1-P | None                     | 0.2                           | 0.4                                 | 0.4                 |
| None         | (\textsuperscript{14}C\textsuperscript{}Glucose-6-P) | 21                            | 22                                  | 22                  |
| None         | (\textsuperscript{14}C\textsuperscript{}Glucose (0.2 mm)) | 22                            | 22                                  | 22                  |
| None         | (\textsuperscript{14}C\textsuperscript{}Methyl α-glucoside (0.2 mm)) | 22                            | 22                                  | 22                  |
| None         | (\textsuperscript{14}C\textsuperscript{}Methyl α-glucoside (20 mm)) | 22                            | 22                                  | 22                  |
| Mannitol-1-P | None                     | 0.2                           | 0.2                                 | 0.2                 |
| None         | (\textsuperscript{14}C\textsuperscript{}Glucose-6-P) | 22                            | 22                                  | 22                  |
| None         | (\textsuperscript{14}C\textsuperscript{}Glucose (0.2 mm)) | 22                            | 22                                  | 22                  |
| None         | (\textsuperscript{14}C\textsuperscript{}Methyl α-glucoside (0.2 mm)) | 22                            | 22                                  | 22                  |
| None         | (\textsuperscript{14}C\textsuperscript{}Methyl α-glucoside (20 mm)) | 22                            | 22                                  | 22                  |
Kinetic Characteristics of Transphosphorylation Reactions—The effects of various reagents on the rates of transphosphorylation were studied with glucose-6-P as phosphoryl donor and [14C]methyl α-glucoside or [14C]mannose as acceptor, or with mannitol-1-P as phosphoryl donor and [14C]mannitol as acceptor. Employing our standard reaction conditions, substitution of phosphate with arsenate did not appreciably depress the rates of phosphorylation. Replacement of Mg2+ with 5 mM EDTA depressed the rates of the transphosphorylation reactions from 0 to 50% while inhibiting the phosphoenolpyruvate-dependent reactions completely. Omission of fluoride depressed the amount of product formed, possibly because the residual sugar-P phosphatase activity was strongly inhibited by fluoride under the conditions of the assay.

The effects of pH on the relative rates of several phosphorylation reactions are shown in Fig. 1. Data are included for both the phosphoenolpyruvate-dependent and the sugar-P-dependent reactions. The pH optima for sugar-P:sugar transphosphorylation were lower than those for sugar phosphorylation with phosphoenolpyruvate. Although some variability was noted with different membrane preparations, each of the Enzyme II-catalyzed reactions showed distinctive pH profiles.

Another characteristic feature of the transphosphorylation reactions was the phenomenon of substrate inhibition. Under appropriate conditions, each of the transphosphorylation reactions was inhibited by both the sugar and the sugar phosphate substrates. Representative data are included in Fig. 2 for the Enzyme II"mil"- and Enzyme II"mii"-catalyzed reactions. In both cases, high concentrations of sugar were inhibitory, and increasing the concentration of sugar-P partially reversed this effect. The data illustrate the fact that optimal conditions were highly dependent on the concentrations of both substrates. For most systems, a low sugar concentration (<100 μM) and a high sugar phosphate concentration (between 10 and 30 mM) gave optimal rates of transphosphorylation.

Fig. 3 illustrates the phenomenon of substrate inhibition of transphosphorylation as catalyzed by the Enzyme II"lac" complex of S. aureus. Results obtained with lactose (a substrate which binds to the Enzyme II"lac" with high affinity (6)) are compared with those obtained with methyl β thiogalactoside (a substrate which binds to the Enzyme II"lac" complex with low affinity (6)). The data are plotted both as a function of sugar concentration as catalyzed under "Experimental Procedures" and in the legend to Table I with substrates as follows: A, [14C]mannitol with 5 mM mannitol-1-P (○); [14C]mannitol with 25 mM mannitol-1-P (●); [14C]sorbitol with 25 mM mannitol-1-P (▲); B, [14C]sorbitol with 5 mM sorbitol-6-P (○); [14C]sorbitol with 25 mM sorbitol-6-P (●); [14C]mannitol with 25 mM sorbitol-6-P (▲).
Sugar Phosphate: Sugar Transphosphorylation Catalyzed by PTS 8905

FIG. 3. Substrate inhibition of transphosphorylation reactions catalyzed by the Enzyme IILac of Staphylococcus aureus. Washed membranes from S. aureus strain C22 were prepared and assayed.

FIG. 4. Effect of arsenate on the uptake of [$^{14}$C]methyl α-glucoside by Salmonella typhimurium cells. Cells were grown in Medium 63 containing glycerol (0.5%) for 12 h. Two hours before harvesting with the cells in exponential growth, glucose was added to a final concentration of 0.5%. Cells were washed three times by centrifugation and resuspension in 20 mM Tris/HCl buffer, pH 7.5, and were finally resuspended to a cell density of 0.25 mg dry weight/ml in 20 mM Tris/HCl buffer, pH 7.5, containing 0.5% (NH₄)₂SO₄ and 0.02% MgSO₄. Sodium phosphate (10 mM) (O) or sodium arsenate (10 mM) (A) was added to aliquots of the cell suspensions, and the suspensions were shaken at 37°C for 3 min before initiation of the uptake experiment by addition of [$^{14}$C]methyl α-glucoside (40 μM, final concentration). Aliquots of 1 ml were periodically removed for filtration and determination of intracellular radioactivity as described under "Experimental Procedures." A, strain LJ62 (cpd-401); B, strain LJ101 (cpd-401 ptsI17) (18).

FIG. 5. Effect of extracellular methyl α-glucoside (aMG) on the pools of intracellular [$^{14}$C]methyl α-glucoside and [$^{15}$C]methyl α-glucoside-6-P (αMG-P). Cells were grown, washed, and loaded with radioactive substrate as described in Fig. 4, except that the cell density was 1.0 mg of dry cells/ml. Uptake of [$^{15}$C]methyl α-glucoside (80 μM) was allowed to proceed at 37°C for 2 min in the presence of 5 mM sodium arsenate. Cells were filtered, washed twice, and resuspended at 0°C in the same buffer to a cell density of 0.4 mg of dry cells/ml for the efflux experiments. Efflux was initiated by placing tubes containing aliquots of the cell suspensions, plus or minus 100 μM nonradioactive methyl α-glucoside, in a shaking water bath at 37°C. Aliquots of the cell suspensions were rapidly filtered, washed, and transferred to tubes containing ice-cold water. Radioactivity was extracted from the cells by incubation at 100°C for 5 min and was analyzed for [$^{14}$C]methyl α-glucoside and [$^{15}$C]methyl α-glucoside-6-P as described under "Experimental Procedures." At zero time, 73% of the total intracellular [$^{14}$C]methyl α-glucoside in strain LJ62 (cpd-401, A) was recovered as the phosphate ester. In strain LJ101 (cpd-401 ptsI17, B), 64% of the intracellular [$^{15}$C]methyl α-glucoside was phosphorylated. Experimental points were as follows: O--O, intracellular [$^{14}$C]methyl α-glucoside-6-P, no extracellular sugar added; O--O, intracellular [$^{15}$C]methyl α-glucoside, no extracellular sugar added; Δ-Δ, intracellular [$^{15}$C]methyl α-glucoside, 100 μM extracellular nonradioactive methyl α-glucoside added; X--X, intracellular [$^{15}$C]methyl α-glucoside-6-P, 100 μM extracellular nonradioactive methyl α-glucoside added.
When activity was plotted as a function of galactose-6-P concentration (Fig. 3B), hyperbolic kinetics were observed when the lactose concentration was low, but sigmoidal kinetics resulted when the sugar concentration was high. The sigmoidal nature of these curves resembles those noted previously with the mannitol phosphotransferase system of Spirochaeta aurantia (1) and probably resulted from the inhibitory effect of lactose which was overcome when the galactose-6-P concentration was increased.

Corresponding data, obtained when methyl β-thiogalactoside served as the phosphoryl acceptor, are reproduced in Fig. 3, C and D. It is apparent that the inhibitory effects of the sugar are much less pronounced than when lactose served as the phosphoryl acceptor. This result is expected for a low affinity substrate. An examination of the curves nevertheless reveals that the phenomenon of substrate inhibition is of importance with both substrates in the concentration ranges studied.

A detailed kinetic analysis of the Enzyme II-catalyzed transphosphorylation reactions will be presented elsewhere. Evidence for Vectorial Transphosphorylation in Intact Bacteria—Klein and Boyer demonstrated that E. coli cells which had been poisoned with arsenate contained negligible amounts of phosphoenolpyruvate, but nevertheless accumulated methyl α-glucoside at appreciable rates (31). The results depicted in Fig. 4 confirm this conclusion and compare data for a wild type and an Enzyme I-deficient strain of S. typhimurium. In both strains, arsenate did not reduce the initial rates of [14C]methyl α-glucoside uptake, although the maximal extent of accumulation was depressed.

Employing conditions similar to those used for the uptake experiment depicted in Fig. 4, bacterial cells were loaded with [14C]methyl α-glucoside, washed free of the extracellular sugar by filtration, and resuspended for efflux studies. Fig. 5 shows a time course for the loss of intracellular [14C]methyl α-glucoside and [14C]methyl α-glucoside 6 P in the presence and absence of nonradioactive extracellular methyl α-glucoside. In both the parental strain and the leaky Enzyme I mutant, extracellular methyl α-glucoside stimulated release of the radioactive sugar. Of particular significance was the observation that loss of radioactivity from the intracellular sugar phosphate pool was stimulated to a far greater extent than was that from the free sugar pool.

The release of intracellular radioactive sugar is plotted as a function of the extracellular methyl α-glucoside concentration in Fig. 6. It can be seen that 10 to 100 μM methyl α-glucoside maximally stimulated the release of intracellular sugar both in the parental and mutant strains. This concentration corresponds approximately to the Kₘ values reported for methyl α-glucoside uptake by E. coli and S. typhimurium cells (7, 24, 32-35). The stimulation of cellular [14C]methyl α-glucoside release was observed only when sugars of the gluco- and manno-configurations were present in the extracellular medium (Table VI). Other sugars were completely without effect.

Release of radioactive sugar from S. typhimurium cells in the presence of extracellular sugar appeared to be representative of the different transport systems studied. Thus, the release of intracellular [14C]isopropyl β-thiogalactoside in the presence of nonradioactive extracellular sugar was observed although this process was relatively slow (data not shown). A number of the substrates of the lactose phosphotransferase system in S. aureus stimulated release of [14C]isopropyl β-thiogalactoside, and several metabolizable carbon sources, including glucose and glycerol, were equally effective. Essentially all of the intracellular [14C]isopropyl β-thiogalactoside was present as the phosphate ester (36, 37).

**DISCUSSION**

The present report serves to characterize the sugar phosphate-sugar exchange transphosphorylation reactions catalyzed by enzymes present in bacterial extracts. Several lines of evidence supported the conclusion that these reactions were catalyzed by the different Enzyme II complexes of the bacterial phosphotransferase system. First, loss of the catalytic activity of a specific Enzyme II, either as a result of a mutational event or following treatment with a protein reagent, resulted in loss of the corresponding transphosphorylation reaction. Second, the phosphoryl donor and acceptor specificities corresponded to those expected for known Enzyme II complexes of the PTS. Third, induction studies showed that growth conditions which resulted in enhanced activity of a specific Enzyme II complex (as measured with phosphoenolpyruvate as the phosphoryl donor (17, 28, 29)) also enhanced transphosphorylation activity to a corresponding degree. Fourth, ptsI mutations enhanced Enzyme II事迹 activity mea-
sured either with phosphoenolpyruvate or with glucose-6-P as the phosphoryl donor. Finally, the results of competitive inhibition studies using nonphosphorylatable substrate analogues of the Enzyme II complex of *Staphylococcus aureus* were fully consistent with the conclusion that this enzyme complex catalyzed transfer of the phosphoryl moiety of galactose-6-P to methyl β-thiogalactoside or lactose. The genetic and biochemical analyses described led to the additional conclusion that the Enzyme II complexes of the PTS were not only necessary, but also sufficient for catalysis of transphosphorylation. None of the known soluble constituents of the phosphotransferase system (Enzyme I, HPr, and Enzyme III) exerted either positive or negative effects on the rates of transphosphorylation (Table I).

Preliminary kinetic studies revealed several characteristic features of the transphosphorylation reactions catalyzed by the various Enzyme II complexes. First, high concentrations of the sugar phosphate substrate (>10 mM) and low concentrations of the sugar substrate (<100 μM) were required for maximal rates of phosphoryl transfer. This observation presumably reflects the relative affinities of each Enzyme II complex for its sugar and sugar phosphate substrates. The sugar appears to bind to the enzyme surface with much higher affinity than does the sugar phosphate. Second, substrate inhibition was observed with respect to both the phosphoryl acceptor and donor, although inhibition by sugar was more pronounced than that by sugar phosphate. The results suggested that inhibition resulted, at least in part, from competition with the second substrate for a binding site on the enzyme surface and showed that inhibition of the phosphoenolpyruvate-dependent phosphorylation reactions was much less pronounced. Finally, in all cases so far examined, the pH-activity curves for transphosphorylation were shifted to the acidic side relative to the phosphoenolpyruvate-dependent reactions.

Studies with whole cells led to the suggestion that transphosphorylation can occur in a vectorial fashion. In the presence of arsenate, which reduced cellular ATP and phosphoenolpyruvate concentrations to very low values (31), rates of methyl α-glucoside uptake were appreciable. This observation confirmed an early report of Klein and Boyer (31) in which the suggestion was advanced that stable (low energy) phosphate esters might serve as energy sources for methyl N-glucoside uptake. Additionally, we confirmed earlier reports (32, 38, 39) showing that extracellular nonradioactive sugar markedly stimulated the release of intracellular radioactive sugar. In contrast to the conclusions of others (38, 39), our analyses suggest that the immediate source of the released 14C-sugar is the intracellular pool of 14C-sugar phosphate, rather than the intracellular pool of free sugar. While this conclusion remains tentative, the results lead to the suggestion that the Enzyme II complexes of the PTS catalyze the process of "exchange group translocation" in which the phosphoryl group of intracellular sugar phosphate is transferred to an incoming sugar molecule while the sugar moiety of the sugar phosphate is expelled. This suggestion is further substantiated by studies with bacterial membrane vesicles described in the accompanying report (40).

Unpublished results.

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