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Properties of a Phosphoenolpyruvate:Mannitol Phosphotransferase System in *Spirochaeta aurantia**

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Spirochaeta aurantia **possesses a phosphoenolpyruvate:mannitol phosphotransferase system which catalyzes the transmembrane transport and phosphorylation of mannitol. Assay of cell-free extracts of *S. aurantia* cells revealed that mannitol and sorbitol (and to a lesser extent fructose) were phosphorylated by a phosphoenolpyruvate-dependent mechanism, but that glucose, galactose, fructose, and metabolites of maltose and *N*-acetylglucosamine were preferentially phosphorylated by ATP-dependent kinases. Phosphoenolpyruvate:dependent phosphorylation of mannitol, sorbitol, and fructose required the presence of three protein fractions: Enzyme I, Enzyme II, and the heat-stable phosphoryl carrier protein of the phosphoenolpyruvate:sugar phosphotransferase system (HPr) which were quantitatively separated. Enzyme I ($M_r \approx 80{,}000$) is a soluble protein which is inactivated by both heat and *N*-ethylmaleimide. Enzyme II is an integral membrane enzyme complex which is heat-labile, but partially resistant to *N*-ethylmaleimide inactivation. HPr ($M_r \approx 10{,}000$) is a soluble protein which is partially heat-stable but inactivated by high concentrations of *N*-ethylmaleimide. Synthesis of these three enzymes and of mannitol-1-phosphate dehydrogenase was induced about 100-fold by growth of *S. aurantia* cells in media containing mannitol, sorbitol, or fructose. The phosphotransferase components from *S. aurantia* exhibited poor enzymatic cross-reactivity with those from *Salmonella typhimurium*, but purified HPr from *S. typhimurium* could replace the sphirochete HPr when used in 100-fold excess of that required to saturate the homologous system.**

The phosphoenolpyruvate-dependent phosphorylation of mannitol, sorbitol, and fructose *in vitro* was catalyzed by an enzyme system, the membrane component of which appeared to be a single Enzyme II complex. Nevertheless, of these three sugars, only mannitol was transported into intact cells via the phosphotransferase system-catalyzed group translocation process. The Enzyme II complex (in the absence of the soluble constituents of the Spirochete phosphoenolpyruvate:sugar phosphotransferase system) catalyzed phosphoryl transfer from mannitol 1-phosphate to [^{14}C]mannitol. Sorbitol and fructose did not serve as efficient phosphoryl acceptors when mannitol 1-phosphate was the phosphoryl donor, and sorbitol 6-phosphate and fructose 6-phosphate were not effective phosphoryl donors. Thus, the sugar specificity for transphosphorylation and transport was more restrictive than that for the phosphoenolpyruvate-dependent sugar phosphorylation reaction. The results appear to be inconsistent with a mechanism in which all phosphoryl transfer reactions involve a common phosphorylated Enzyme II intermediate.

In 1964, a novel phosphoenolpyruvate:sugar phosphotransferase system in *Escherichia coli* was described (1). Fractionation and characterization of the protein constituents of the system revealed that phosphoryl transfer required at least three protein fractions and occurred in a sequential fashion as follows (2-9).

$$\text{Phosphoenolpyruvate} + \text{HPr}^{1} \xrightarrow[Mg^{2+}]{\text{Enzyme I}} \text{phospho-HPr} + \text{pyruvate}$$

$$\text{Phospho-HPr} + \text{sugar} \xrightarrow[\text{complex}]{\text{Enzyme II}} \text{sugar phosphate} + \text{HPr}$$

Enzyme I and the low molecular weight heat-stable protein, HPr, were shown to be cytoplasmic proteins, while the Enzyme II complex was localized to the membrane fraction. Subsequent analyses revealed that several distinct and inducible Enzyme II complexes were present in the *E. coli* cell membrane, and that each of these complexes exhibited specificity toward one or a few sugars. The Enzyme II complexes in *E. coli* and *Salmonella typhimurium*, which have been most extensively characterized, catalyzed the phosphorylation of (*a*) glucose and methyl α-glucoside (2-4, 10-13); (*b*) glucose, mannose, glucosamine, and fructose (2-4, 12-14); (*c*) fructose (2, 4, 13, 15-17); (*d*) mannitol (13, 18, 19); and (*e*) sorbitol (13, 18, 19). In addition to its role as a sugar-phosphorylating system, the PTS has been shown to be responsible for the translocation of its sugar substrates across the bacterial mem-

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[1] The abbreviations used are: HPr, heat-stable phosphoryl carrier protein of phosphoenolpyruvate:sugar phosphotransferase system; PTS, phosphoenolpyruvate:sugar phosphotransferase system; Enzyme II^{Fru}, Enzyme II complex which exhibits specificity toward fructose; Enzyme II^{Mtl}, Enzyme II complex which exhibits specificity toward mannitol.

brane (2, 20). The molecular details of the process by which translocation is coupled to phosphorylation have not been elucidated.

Genetic analyses of the phosphotransferase systems in *E. coli* and *S. typhimurium* have revealed that the genes which code for Enzyme I and HPr comprise an operon (the *pts* operon) and that the synthesis of these two proteins is inducible to the extent of about 3-fold (13, 21–23). Any sugar substrate of the PTS can serve as an inducer, but other sugars are not effective (21).[2] In contrast, the genes which code for the sugar-specific Enzymes II map in distinct locations on the bacterial chromosome and, in general, are included in operons coding for the sugar-specific catabolic enzymes. Thus, structural genes in the fructose operon code for the proteins of the Enzyme II^{Fru} complex as well as for fructose-1-phosphate kinase, while structural genes in the mannitol operon code for the Enzyme II^{Mtl} and mannitol-1-phosphate dehydrogenase (17–19, 24). The expression of these operons is specifically induced by the sugar substrate of the catabolic system, by fructose and mannitol, respectively, in the two examples cited above. Examination of a variety of Gram-positive and Gram-negative eubacterial species has suggested a considerable degree of uniformity with respect to this genetic regulatory pattern (25, 26).

Recent studies have established that the phosphotransferase system in enteric bacteria is intimately involved in the regulation of intracellular metabolic rates (27–30), and it has been suggested that the complexity of the system evolved in order to allow greater control over cellular metabolism (6, 28). Unfortunately, few studies have dealt with this problem. Elucidation of the structural and genetic regulatory features of phosphotransferase systems in evolutionarily divergent bacterial genera might provide evidence for a less complex primordial phosphotransferase system which progressively evolved in some bacteria (but not in others) to the degree of complexity found in the enteric bacteria.

With this possibility in mind, we have examined phosphotransferase systems in diverse bacterial species (31, 32).[2] One of the species examined, *Spirochaeta aurantia,* strain J1, is a facultatively anaerobic spirochete which is capable of utilizing numerous sugars for growth (33–35). Of these sugars, only mannitol appears to be metabolized via a phosphoenolpyruvate-dependent pathway. Other sugars are preferentially phosphorylated by ATP-dependent kinases. An examination of the Spirochete phosphotransferase system revealed that although the individual protein components of the system appeared to be structurally similar to those in eubacteria, there were also several differences. First, the Spirochete membrane appeared to be functionally less complex than those of eubacteria, containing only a single Enzyme II complex with specificity toward mannitol. Second, in *S. aurantia,* expression of the genes which code for mannitol phosphate dehydrogenase and the three protein components of the PTS appeared to be subject to coordinate regulation. Third, we have demonstrated that the Enzyme II^{Mtl} complex of *S. aurantia* catalyzes a chemical reaction in the absence of the soluble proteins of the system. This reaction involves the transfer of the phosphoryl moiety of mannitol 1-phosphate to [^{14}C]mannitol. Although this reaction has since been demonstrated in eubacterial extracts (36), the ratio of the rates of sugar phosphate-dependent to phosphoenolpyruvate-dependent phosphoryl transfer is much greater in the Spirochete crude extract. The present communication presents these results. A preliminary account of this work has appeared (32).

[2] Unpublished results.

EXPERIMENTAL PROCEDURES

Growth of Bacteria—The organism used in this study, *Spirochaeta aurantia* strain J1 (ATCC 25083), was obtained from Dr. E. Canale-Parola, Department of Microbiology, University of Massachusetts, Amherst. Morphological, physiological, and metabolic properties of this organism have been described (33–35, 37). The growth medium consisted of 0.4% Bacto-yeast extract, 0.2% Bacto-peptone, and 0.2% sugar in 10 mM potassium phosphate buffer, pH 7.4. Solid medium of the same nutrient composition also contained 1.5% Bacto-agar. *S. aurantia* cells were cloned prior to use, and characteristic orange colonies were selected. By virtue of the distinctive morphological features of this organism (33, 37), phase contrast microscopy could be used to verify the purity of bacterial cultures.

Cells were grown aerobically in liquid medium at 30° in Erlenmeyer flasks filled to less than 50% of capacity. Cultures were rotated at 250 rpm. Employing these conditions, several sugars were found to increase the generation time and cell yield. In agreement with the results of Breznak and Canale-Parola (33), maltose was utilized more rapidly and efficiently than other carbon sources tested (generation time of 2.7 h; cell yield of 0.82 mg of dry cells/ml). Glucose, mannitol, fructose, and galactose were utilized at slightly slower rates (generation times of 2.9 to 3.4 h). Inclusion of sorbitol in the culture medium retarded the growth rate slightly. Of the carbon sources tested, only sorbitol was not fermented.

Sugar Uptake Studies and Enzyme Analyses—The rates of uptake of ^{14}C-labeled sugars were determined employing bacterial cultures which had been harvested after at least 24 h of continuous growth with the cells dividing exponentially. Even when well defined growth conditions were employed, appreciable variation in uptake rates was observed with different batches of cells, and uptake rates were found to decline as cells approached the stationary growth phase. Bacteria were harvested by centrifugation and washed three times with Medium 63 (21) before resuspension in the same salts medium supplemented with 0.04% yeast extract and 0.02% Bacto-peptone as exogenous sources of energy. The cell density was adjusted to 0.16 mg, dry weight, of cells/ml. Prior to all uptake studies, the viability of a culture was verified by showing that more than 80% of the cells were actively motile. Uptake of radioactive sugars was measured at 30° with constant aeration as described previously (21, 29).

In vitro enzyme assays were performed with cell-free extracts derived from bacteria which had been grown in the indicated medium for at least 24 h. Cells were harvested in the late exponential or early stationary growth phase, washed three times with Medium 63, resuspended in dilute phosphate buffer, pH 7.4, containing 1 mM dithiothreitol, and ruptured by passage through a French pressure cell at 10,000 p.s.i. Cell debris was removed by low speed centrifugation, and the particulate and soluble fractions were separated by centrifugation at 200,000 × *g* for 2 h. Control experiments showed that the specific activities of the components of the Spirochete phosphotransferase system were the same in extracts derived from cells harvested during the exponential and early stationary growth phases. Phosphorylation assays for the protein components of the phosphotransferase system were conducted at 30° employing assay conditions similar to those described previously (29). Specific conditions were as indicated in the legends to the tables and figures. Radioactive sugar phosphates were separated from neutral sugar by the Dowex 1-X2 resin column procedure (4). Other enzymes were assayed as described previously (13), except that mannitol-1-phosphate dehydrogenase activity was determined at pH 8.0. Protein was estimated by the Biuret procedure with bovine serum albumin as standard.

Analytical Procedures—High voltage electrophoresis was conducted as described (38) employing 0.05 M sodium borate buffer, pH 10, or 0.05 M pyridinium acetate buffer, pH 6.5 (180 ml of pyridine and 7.2 ml of glacial acetic acid in 1.6 liter of water). Solvent systems employed for descending paper chromatography were 1-butanol:pyridine:water (10:3:3; Solvent A) and ethyl acetate:acetic acid:formic acid:water (18:3:1:4; Solvent B) (39).

Quantitative Separation of Protein Components of Spirochete Phosphotransferase System—Three protein fractions were required for the transfer of phosphate from phosphoenolpyruvate to [^{14}C]mannitol. These enzymatic components (termed Enzyme I, Enzyme II, and HPr, see below) were quantitatively separated as follows. A cell-

free extract derived from mannitol-grown *S. aurantia* cells and prepared as described above was centrifuged for 2 h at 200,000 × *g*. The particulate fraction was resuspended in 10 mM potassium phosphate buffer containing 1 mM dithiothreitol and was recentrifuged. The washed pellet fraction, containing more than 95% of the Enzyme II^{Mtl} activity of the extract and appreciable contaminating Enzyme I activity, was dialyzed overnight at 4° against 25 mM Tris/HCl buffer, pH 9.0, containing 1 mM dithiothreitol. The membrane fraction was again pelleted by high speed centrifugation and was resuspended in a neutral buffer solution containing 1 mM dithiothreitol. The preparation retained full Enzyme II^{Mtl} activity but was devoid of detectable Enzyme I or HPr activity.

The two soluble protein constituents of the Spirochete PTS were quantitatively separated by gel filtration. A 5-ml aliquot of the membrane-free supernatant, obtained by high speed centrifugation, was applied to a Sephadex G-100 column (2 × 50 cm) which had been pre-equilibrated with 25 mM Tris/HCl buffer, pH 7.4, containing 1 mM dithiothreitol. Enzyme I activity eluted as a single symmetrical peak shortly after the void volume (V_e/V_0 = 1.16). The peak of HPr activity eluted much later (V_e/V_0 = 1.92). No cross-contamination of the two activities was detected.

Fractions containing enzyme activity were pooled, concentrated by lyophilization, and dialyzed to remove salt which was inhibitory in the PTS assay. Enzyme I and HPr were both stable to lyophilization. These enzymes as well as Enzyme II were stable at 0° for up to 2 weeks and could be stored at −60° for up to 1 year without appreciable loss of activity.

Materials—Mannitol 1-phosphate (which is identical with mannitol 6-phosphate since mannitol is a symmetrical molecule) and sorbitol 6-phosphate were synthesized by quantitative reduction of mannose 6-phosphate and glucose 6-phosphate, respectively, with $NaBH_4$; boric acid was removed as described (40). Alternatively, the barium salts of the hexitol phosphates were purchased from Sigma Chemical Co. and converted to the sodium salts with Na_2SO_4. The corresponding [^{3}H]hexitol phosphates were synthesized by reduction with NaB^3H_4 and purified by paper chromatography employing Solvent A. ^{14}C-labeled sugars were purchased from New England Nuclear Corp. and were used at a specific activity of 5 μCi/μmol. The tricyclohexylammonium salt of phosphoenolpyruvate was synthesized by the method of Clark and Kirby (41) and twice recrystallized from aqueous ethanol. Sugars and sugar phosphates (of the D-configuration unless otherwise noted) and other compounds were obtained from commercial sources. They were of the highest purity available.

RESULTS

Uptake of ^{14}C-labeled Sugars by Spirochaeta aurantia—*S. aurantia* strain J1 cells can utilize a large number of sugars as sources of carbon and energy. These sugars include maltose, glucose, mannitol, fructose, galactose, and *N*-acetylglucosamine but not sorbitol (see "Experimental Procedures" and Ref. 32 and 33). In order to gain information about the transport systems responsible for the uptake of these sugars, rates of ^{14}C-sugar uptake were measured after growth of *S. aurantia* in the presence of one of the above mentioned sugars. Representative results are summarized in Experiment 1 of Table I. Although considerable quantitative variability was noted with different cell preparations, it appeared that each of the sugars tested was taken up via a distinct transport system. Glucose, for example, was the only ^{14}C-sugar tested which was taken up from the medium regardless of the carbon source present during growth. Uptake activities for mannitol, maltose, and fructose were each specifically induced when the cells were grown in the presence of the corresponding sugar substrate. Unexpectedly, mannitol uptake activity was observed following growth of *S. aurantia* in the presence of mannitol, fructose, or sorbitol, and nearly maximal uptake rates were observed when cells were grown in medium containing maltose plus one of these three sugars (data not shown). In spite of this broad inducer specificity, only one of the three effective inducers appeared to be a substrate of the system under the experimental condition employed (Table I).

TABLE I

Uptake of ^{14}C-sugars by Spirochaeta aurantia cells grown in presence of different carbon sources

Cells were grown and prepared for the uptake experiments as described under "Experimental Procedures." Uptake rates were linear with time and cell density. In Experiment 2, the nonradioactive sugar was added to the cell suspension 5 min before addition of [^{14}C]mannitol.

Radioactive substrate (20 μM)	Nonradioactive sugar (2 mM)	Uptake rate with cells grown in media containing:			
		Mannitol	Fructose	Glucose	Maltose
		% control			
Experiment 1					
Mannitol		100[a]	90	<2	<2
Sorbitol		<2	<2	<2	<2
Fructose		<2	10	<2	<2
Glucose		42	27	72	65
Maltose		<2	<2	<2	65
Experiment 2					
Mannitol		100[a]			
Mannitol	Mannitol	4			
Mannitol	Sorbitol	39			
Mannitol	Fructose	98			
Mannitol	Glucose	101			
Mannitol	Maltose	104			

[a] 100% corresponds at a rate of uptake of radioactive sugar of 1.3 μmol/g, dry weight, of cells.

Uptake of fructose was only observed when fructose was present during growth, and appreciable sorbitol uptake was not observed regardless of growth conditions.

Although mannitol-grown cells accumulated radioactive metabolites only when incubated with [^{14}C]mannitol, the structurally related nonradioactive inducers of the mannitol transport system inhibited uptake of the radioactive polyol. As shown in Experiment 2 of Table I, sorbitol and fructose were weakly inhibitory although glucose and maltose were not. The results suggest that the mannitol transport system possesses differing degrees of affinity for the three sugars which induced mannitol uptake activity.

Sugar Phosphorylation in Vitro—In experiments conducted in parallel with those described in Table I, *S. aurantia* cells were grown in the presence of one of several carbon sources, and crude extracts containing both the soluble and particulate protein fractions were prepared. These extracts were tested for the presence of enzymes catalyzing sugar phosphorylation with either ATP or P-enolpyruvate as phosphoryl donor (Table II). Extracts derived from mannitol- and fructose-grown cells phosphorylated mannitol, sorbitol, and, to a lesser extent, fructose when phosphoenolpyruvate served as the phosphoryl donor. Extracts derived from glucose- or maltose-grown cells were greatly depressed for these activities. The γ-phosphoryl moiety of ATP was not readily transferred to the radioactive hexitols. By contrast, fructose and glucose were preferentially phosphorylated by ATP-dependent kinases, while methyl α-glucoside, an effective substrate of the phosphoenolpyruvate:glucose phosphotransferase system in enteric bacteria (1–4), was not phosphorylated. Other experiments showed that galactose and metabolites of maltose and *N*-acetylglucosamine were preferentially phosphorylated by ATP-dependent mechanisms. Fructose-1-phosphate kinase activity (13) was not detected in extracts derived from fructose-grown cells. The results provide evidence for an enzyme system which transfers the phosphoryl moiety of phosphoenolpyruvate to mannitol, sorbitol, or fructose, and indicate that the

TABLE II

Phosphorylation of ^{14}C-sugars with phosphoenolpyruvate or ATP as phosphate donor catalyzed by cell-free extracts of Spirochaeta aurantia

Cells were grown in nutrient medium supplemented with the carbon source indicated below (0.2%). Crude extracts were prepared and assayed for sugar phosphorylation as described under "Experimental Procedures." Rates of sugar phosphorylation were constant with time. ATP-dependent phosphorylation of glucose and fructose was linear with protein concentration. Other reactions were not linear but were sigmoidal with protein concentration. The values recorded below represent the rates of sugar phosphorylation at a protein concentration of 1 mg/ml.

^{14}C-Labeled substrate (50 μM) and phosphate donor (5 mM)	Rate of sugar phosphorylation (μmol of sugar phosphorylated/h/g of protein at 30°) after growth of cells in medium containing:			
	Mannitol	Fructose	Glucose	Maltose
Mannitol				
P-enolpyruvate	7.0	4.7	0.1	0.05
ATP	0.4	—[a]	—[a]	—[a]
Sorbitol				
P-enolpyruvate	6.8	5.1	0.1	0.03
ATP	0.3	—[a]	—[a]	—[a]
Fructose				
P-enolpyruvate	1.3	1.0	<0.05	<0.05
ATP	—[a]	300	320	—[a]
Glucose				
P-enolpyruvate	1.7	1.6	1.9	2.1
ATP	—[a]	2000	2200	—[a]
Methyl α-glucoside				
P-enolpyruvate	<0.05	<0.05	<0.05	<0.05
ATP	<0.05	—[a]	—[a]	—[a]

[a] Not determined.

inducer specificities for the phosphorylation of these three sugars are the same.

In order to characterize the product of the *Spirochaeta*-catalyzed phosphoenolpyruvate:mannitol phosphoryl transfer reaction, the negatively charged radioactive product was subjected to paper electrophoresis in two buffers and to chromatography in two solvent systems (see "Experimental Procedures"). In all cases, the radioactive product migrated with authentic mannitol 1-phosphate. The product was treated with an excess quantity of crystalline *Escherichia coli* alkaline phosphatase (Sigma). More than 95% of the product was cleaved to a neutral ^{14}C-compound with the electrophoretic and chromatographic properties of mannitol.

Properties of Spirochete Phosphotransferase System—Sigmoidal kinetics were observed when the phosphoenolpyruvate-dependent phosphorylation of [^{14}C]mannitol was studied as a function of protein concentration (see legend to Table II). This result suggested that more than a single protein was required for catalysis of this phosphoryl transfer reaction. As described under "Experimental Procedures," one particulate and two soluble protein activities, all of which were required for phosphoryl transfer from phosphoenolpyruvate to [^{14}C]mannitol, were quantitatively separated. When sugar phosphorylation was measured with one component limiting in the presence of excessive quantities of the other two, hyperbolic kinetics were observed (data not shown).[3] Inclusion of EDTA in the reaction mixture strongly inhibited phosphoenolpyruvate-dependent [^{14}C]mannitol phosphorylation, and this inhibition was fully reversed by addition of excess Mg^{2+} (32). In the respects discussed above, the Spirochete PTS resembled that from *E. coli* (1-4).[3]

[3] One interesting difference between Enzyme I from *Escherichia coli* or *Salmonella typhimurium* and that from *Spirochaeta aurantia* was observed. While the latter enzyme gave hyperbolic kinetics when reaction rate was plotted as a function of Enzyme I concentration, sigmoidal kinetics were observed with the former enzyme.

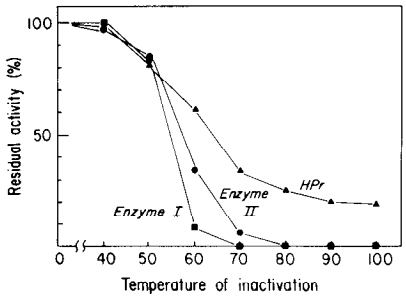

FIG. 1. Heat inactivation of the enzyme components of the *Spirochaeta aurantia* phosphotransferase system. The protein components of the Spirochete phosphotransferase system were quantitatively separated as described under "Experimental Procedures." Subsequently, aliquots of the enzyme preparations were incubated at the temperatures indicated on the *abscissa* for 5 min and then chilled to 0°. Phosphoenolpyruvate:mannitol phosphotransferase activity was measured as described under "Experimental Procedures" at 30° in the presence of limiting quantities of the enzyme being investigated. All values are expressed as percentage of the original activity.

The two soluble proteins of the Spirochete PTS were mixed with Enzyme I and HPr from *Salmonella typhimurium* and were passed through a calibrated Sephadex G-100 column. Elution profiles of the four activities were determined employing the homologous purified components. Enzyme I of *S. aurantia* eluted from the column immediately before and overlapping with Enzyme I of the *Salmonella* PTS, while the Spirochete HPr eluted just ahead of the *Salmonella* HPr. The molecular weights of the two Spirochete proteins were estimated at 80,000 and 10,000.

The heat stabilities of the three components of the Spirochete PTS are reproduced in Fig. 1. Enzymes I and II were quantitatively lost upon exposure to high temperature. In contrast, appreciable HPr activity remained even after the protein solution was boiled (Fig. 1). Likewise, all three enzymes were sensitive to inactivation by *N*-ethylmaleimide. High concentrations of this sulfhydryl reagent were required to destroy Enzyme I and HPr activities. Surprisingly, a much lower concentration of *N*-ethylmaleimide partially inhibited the Enzyme II^{Mtl} activity, but higher concentrations were without further effect (Ref. 32 and see below).

The sizes, heat stabilities, and *N*-ethylmaleimide sensitivities of Enzyme I and HPr from *S. aurantia* led to the likelihood that these two proteins were evolutionarily related to Enzyme I and HPr of the phosphotransferase systems from enteric bacteria. Although the Spirochete phosphotransferase components did not show appreciable enzymatic cross-reactivity with those of *S. typhimurium* or *Staphylococcus aureus*, the availability of highly purified HPr from *S. typhimurium* (42) allowed a more rigorous test of cross-reactivity. Employing Enzymes I and II from *S. aurantia*, free of detectable Spirochete HPr, high concentrations of *Salmonella* HPr could replace the Spirochete protein (Fig. 2). An amount of *Salmonella* HPr which was about 200-fold in excess of that which saturated the homologous system was required for saturation of the phosphoryl transfer reaction catalyzed by the Spirochete enzymes.

Phosphoryl Donor Specificity of Spirochete Phosphotransferase System—The capacities of various phosphate esters to serve as phosphoryl donors for the phosphorylation of radio-

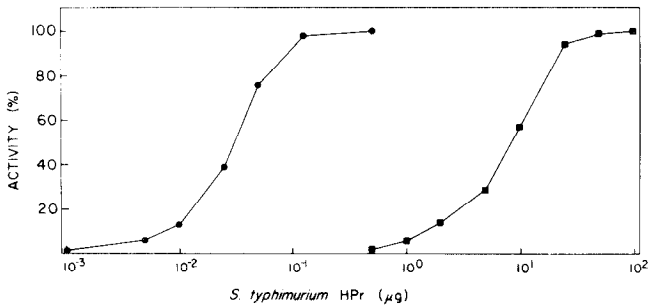

FIG. 2. Relative effectiveness of purified HPr from *Salmonella typhimurium* in catalyzing [^{14}C]mannitol phosphorylation employing Enzyme I and Enzyme II^{Mtl} from *S. typhimurium* (●) or Enzyme I and Enzyme II^{Mtl} from *Spirochaeta aurantia* (■). Phosphorylation was measured as described under "Experimental Procedures." 100% corresponds to the activity observed with a saturating concentration of the homologous HPr protein in the assay tube. The concentration of *Salmonella* HPr (micrograms of protein/0.25 ml, final volume) is given on the *abscissa*. The results show that *S. typhimurium* HPr was able to substitute for the *S. aurantia* HPr provided that the concentration of the former protein was about 200-fold higher than normally employed with the homologous enzymes.

active mannitol, sorbitol, and fructose are tabulated in Table III. ATP, ADP, 2-phosphoglycerate, and 3-phosphoglycerate exhibited low activity when [^{14}C]mannitol served as the phosphoryl acceptor (less than 10% of the phosphoenolpyruvate-dependent activity). It is likely that the crude enzyme preparation used in these studies could convert the latter compounds to phosphoenolpyruvate at slow rates. Several other potential phosphate donors were inactive. Surprisingly, mannitol 1-phosphate was an efficient phosphoryl donor when [^{14}C]mannitol (but not [^{14}C]sorbitol or [^{14}C]fructose) was the phosphoryl acceptor (Table III). Other sugar phosphates were inactive.

The ^{14}C-product of the mannitol 1-phosphate-dependent phosphoryl transfer reaction was characterized as described above for the phosphoenolpyruvate-dependent reaction. The results were fully consistent with the conclusion that this product was [^{14}C]mannitol 1-phosphate.

Induction of Mannitol-1-phosphate Dehydrogenase and Phosphotransferase Enzymes in Spirochaeta aurantia—Eubacterial species which utilize mannitol via a phosphotransferase system possess a mannitol-inducible mannitol-1-phosphate dehydrogenase. Mannitol-1-phosphate dehydrogenase activity was high in extracts derived from mannitol or fructose-grown *S. aurantia* cells, but low after growth of cells in glucose- or maltose-containing medium (Table IV). Assay of the three enzymes of the phosphotransferase system (Enzyme I, Enzyme II, and HPr) in the same cell-free extracts revealed that these activities were induced about 100-fold. These three enzyme activities and the mannitol phosphate dehydrogenase appeared to be induced coordinately within experimental error.

The particulate and soluble fractions were also assayed for phosphoenolpyruvate-dependent [^{14}C]sorbitol and [^{14}C]fructose phosphorylation activities as well as for mannitol 1-phosphate-dependent [^{14}C]mannitol phosphorylation activity. In all cases, coordinate induction was observed (Table IV).

Properties of Phosphoryl Transfer Reactions—Although the phosphoenolpyruvate-dependent phosphorylation of mannitol, sorbitol, and fructose required the three protein fractions of the Spirochete phosphotransferase system, only the particulate fraction (Enzyme II) was required for phosphoryl transfer from mannitol 1-phosphate to [^{14}C]mannitol (32). Moreover, only this last reaction was insensitive to inhibition by EDTA

TABLE III

Phosphate donor specificity of phosphoenolpyruvate:mannitol phosphotransferase system of Spirochaeta aurantia

In Experiment 1, phosphorylation of [^{14}C]mannitol was assayed as described under "Experimental Procedures" with 50 μM [^{14}C]mannitol, the phosphate donor indicated below at 5 mM (with the exception of pyridoxal phosphate (2 mM) and NAD (1 mM)) and crude extract of mannitol grown *S. aurantia* cells at a final protein concentration of 0.85 mg/ml. In Experiment 2, phosphorylation of [^{14}C]sorbitol was conducted essentially as described above. In Experiment 3, phosphorylation of [^{14}C]fructose was conducted essentially as described above except that the concentration of protein was 4.2 mg/ml.

Phosphate donor	Activity (relative to P-enolpyruvate)
	%
Experiment 1 ([^{14}C]mannitol as acceptor)	
Phosphoenolpyruvate	100
ATP	8
ADP	7
2-Phosphoglycerate	6
3-Phosphoglycerate	6
GTP, NAD, AMP, pyridoxal-P, phosphoserine, α-glycerophosphate	<2
Mannitol-1-P	130
Sorbitol-6-P, fructose-1,6-diP, glucose-1-P, glucose-6-P, fructose-1-P, fructose-6-P, mannose-1-P, mannose-6-P	<2
Experiment 2 ([^{14}C]sorbitol as acceptor)	
Phosphoenolpyruvate	100
ATP	6
Mannitol-1-P, sorbitol-6-P, fructose-1-P, fructose-6-P	<2
Experiment 3 ([^{14}C]fructose as acceptor)	
Phosphoenolpyruvate	100
ATP	40,000
Mannitol-1-P, sorbitol-6-P, fructose-1-P, fructose-6-P	<5

(32). The results summarized in Table V show the effects of partial inactivation of Enzyme II with either *N*-ethylmaleimide or heat. The four phosphotransferase activities were depressed to a similar degree by both treatments.

The competitive inhibitory effects of nonradioactive sugars on each of the four phosphoryl transfer reactions are summarized in Table VI. All four reactions were most strongly inhibited by mannitol, less strongly inhibited by sorbitol, and only weakly inhibited by fructose. Glucose was essentially without effect. These results are consistent with the suggestion that a single component of the particulate fraction (the Enzyme II^{Mtl}) mediated catalysis of all four phosphoryl transfer reactions.

Effects of Sugars on Inactivation of Enzyme II^{Mtl} by N-Ethylmaleimide—The inactivation of the Enzyme II^{Mtl} by *N*-ethylmaleimide occurred to differing degrees employing different membrane preparations. Moreover, the extent to which inactivation occurred was influenced by the presence of sugars. As summarized in Table VII, mannitol greatly enhanced the sensitivity of the enzyme to inactivation. Sorbitol exerted a lesser effect, fructose was still less effective, and glucose was almost without effect. The order with which these sugars enhanced sensitivity to inactivation by *N*-ethylmaleimide was the same as their apparent binding affinities to the active site of the Enzyme II complex (Table VI). Moreover, the same effects were observed regardless of the sugar phosphorylation reaction assayed (Table VII). Although the basis

TABLE IV

Induction of mannitol-1-phosphate dehydrogenase and protein components of mannitol phosphotransferase system in Spirochaeta aurantia

Crude extracts, prepared as described in Table II, were centrifuged at 200,000 × *g* for 2 h in order to separate soluble proteins from particulate material. More than 90% of the mannitol-1-phosphate dehydrogenase, Enzyme I, and HPr activities were present in the soluble fraction; more than 90% of Enzyme II activity was in the particulate fraction. These enzymes were assayed in the appropriate fraction as described under "Experimental Procedures." Each of the phosphotransferase system components was assayed in the presence of excess amounts of the other two protein components. All assays were linear with time and with the amount of the rate-limiting enzyme. All phosphotransferase assays were conducted at 30°.

Enzyme assay	Substrates	Activity after growth of cells in media containing:			
		Mannitol	Fructose	Glucose	Maltose
		%			
Mannitol-1-P dehydrogenase	Mannitol-1-P, NAD	100[a]	78	5	2
Enzyme I	Mannitol, P-enolpyruvate	100[b]	75	6	2
HPr	Mannitol, P-enolpyruvate	100[c]	73	2	0.7
Enzyme II	Mannitol, P-enolpyruvate	100[d]	70	1	0.4
Enzyme II	Sorbitol, P-enolpyruvate	100[e]	90	2	0.6
Enzyme II	Fructose, P-enolpyruvate	100[f]	80	<2	<2
Enzyme II	Mannitol, mannitol-1-P	100[g]	85	1	0.5
Uptake	Mannitol	100[h]	90	<2	<2

[a] 100% = an increase in A_{340} of 0.18 unit/min/mg of soluble protein at 23°.

[b] 100% = 1.7 μmol of sugar phosphorylated/min/g of soluble protein.

[c] 100% = 0.37 μmol of sugar phosphorylated/min/g of soluble protein.

[d] 100% = 0.9 μmol of sugar phosphorylated/min/g of membrane protein.

[e] 100% = 1.2 μmol of sugar phosphorylated/min/g of membrane protein.

[f] 100% = 0.1 μmol of sugar phosphorylated/min/g of membrane protein.

[g] 100% = 0.38 μmol of sugar phosphorylated/min/g of membrane protein.

[h] 100% = 1.3 μmol of sugar taken up/min/g, dry weight, of cells.

for this enhancement of *N*-ethylmaleimide sensitivity is not understood, the results provide further evidence for the conclusion that a single Enzyme II complex in the membrane catalyzes all four phosphoryl transfer reactions.

Kinetics of Phosphoryl Transfer Reactions—Although the phosphoenolpyruvate-dependent phosphorylation of [^{14}C]-mannitol exhibited hyperbolic kinetics with an apparent K_m for mannitol of about 2 μM, the kinetics for mannitol-1-P:[^{14}C]mannitol transphosphorylation exhibited the phenomenon of substrate inhibition (Fig. 3*A*). At high concentrations of [^{14}C]mannitol or nonradioactive mannitol-1-P, the rate of transphosphorylation was diminished. Moreover, when reaction rate was plotted *versus* the concentration of mannitol-1-P, sigmoidal kinetics were observed (Fig. 3*B*). This behavior appears to be a consequence of substrate inhibition and does not indicate cooperative behavior. The kinetic behavior of the transphosphorylation reaction will be dealt with in greater detail in a subsequent publication.[4]

[4] A. W. Rephaeli and M. H. Saier, Jr., manuscript in preparation.

TABLE V

Effects of partial inactivation of Enzyme II on rates of sugar phosphorylation

Partial inactivation of Enzyme II with *N*-ethylmaleimide and heat was conducted as described (32). Sugar phosphorylation was assayed as described under "Experimental Procedures" in the presence of excess Enzyme I and HPr.

^{14}C-Substrate	Phosphate donor	Residual activity after treatment with:	
		N-Ethylmaleimide	Heat
		%	
Mannitol	P-enolpyruvate	25	30
Mannitol	Mannitol-1-P	28	38
Sorbitol	P-enolpyruvate	17	22
Fructose	P-enolpyruvate	29	44

TABLE VI

Inhibition of ^{14}C-sugar phosphorylation by nonradioactive sugars

Phosphorylation of ^{14}C-sugar was assayed as described under "Experimental Procedures" with the concentration of radioactive substrate at 50 μM, the phosphate donor concentration at 5 mM, and the concentration of nonradioactive inhibitor at 5 mM.

Radioactive substrate	Phosphate donor	Inhibition by:			
		Mannitol	Sorbitol	Fructose	Glucose
		%			
Mannitol	P-enolpyruvate	98	67	5	7
Mannitol	Mannitol-1-P	99	71	0	0
Sorbitol	P-enolpyruvate	>99	98	60	0
Fructose	P-enolpyruvate	>99	>99	92	24

TABLE VII

Inactivation of Enzyme II^{Mtl} by N-ethylmaleimide in presence of various sugars

Washed membranes from mannitol-grown *Spirochaeta aurantia* strain J1 cells (0.25 ml, 10 mg of protein/ml) were incubated at 0° in the presence or absence of the sugar indicated below at a concentration of 10 μM together with 5 mM *N*-ethylmaleimide for 10 min. Subsequently, dithiothreitol was added to a final concentration of 10 mM to inactivate residual *N*-ethylmaleimide. The membranes were then diluted 20-fold and assayed for sugar phosphorylation with the sugar substrate at a concentration of 50 μM as described under "Experimental Procedures." Control experiments showed that the nonradioactive sugar present during incubation with *N*-ethylmaleimide was not inhibitory during the subsequent assay for sugar phosphorylation.

Phosphoryl donor	Phosphoryl acceptor	Residual activity after treatment with *N*-ethylmaleimide in the presence of:				
		No sugar	Mannitol	Sorbitol	Fructose	Glucose
		%				
Phosphoenolpyruvate	Mannitol	11	4	6	9	11
Phosphoenolpyruvate	Sorbitol	10	2	5	8	9
Phosphoenolpyruvate	Fructose	18	4	11	16	19
Mannitol-1-P	Mannitol	16	1	6	14	15

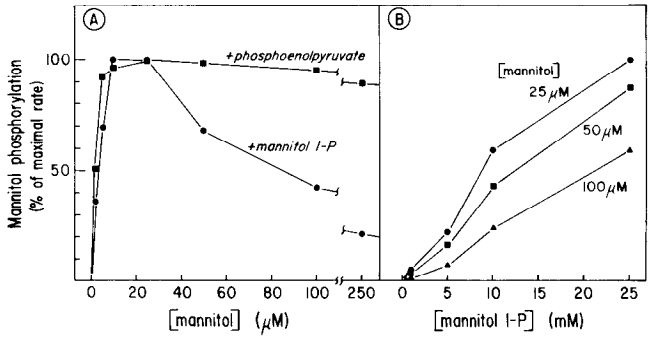

FIG. 3. Dependence of mannitol phosphorylation reactions on substrate concentrations. *A*, reaction rates as a function of [^{14}C]mannitol concentration with 5 mM phosphoenolpyruvate or 10 mM mannitol 1-phosphate as the phosphoryl donor. *B*, rate of the transphosphorylation reaction as a function of the mannitol 1-phosphate concentration employing a concentration of [^{14}C]mannitol equal to 25 μM, 50 μM, or 100 μM. Assay conditions were as described under "Experimental Procedures." Phosphoenolpyruvate-dependent mannitol phosphorylation was studied in the presence of saturating quantities of Enzyme I and HPr. Mannitol 1-phosphate-dependent transphosphorylation was studied employing a membrane preparation which had been washed free of the soluble proteins.

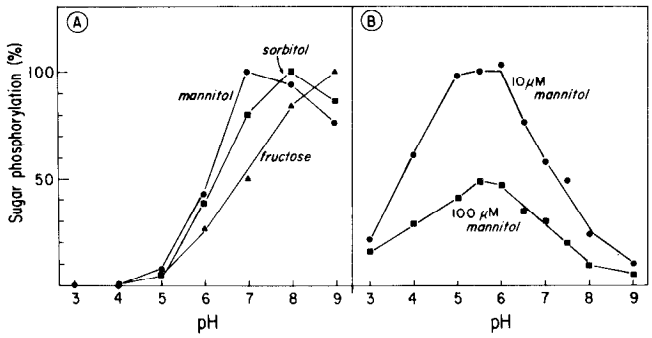

FIG. 4. pH activity curves for sugar phosphorylation catalyzed by the Spirochete phosphotransferase system. *A*, phosphoenolpyruvate-dependent phosphorylation of [^{14}C]mannitol, [^{14}C]sorbitol, and [^{14}C]fructose. *B*, mannitol 1-phosphate-dependent phosphorylation of [^{14}C]mannitol with the radioactive sugar at a concentration of 10 μM or 100 μM. Phosphoryl donor concentrations were 5 mM for phosphoenolpyruvate and 10 mM for mannitol 1-phosphate. Phosphate buffer (50 mM) was employed for all studies. Assay conditions were as described under "Experimental Procedures."

Effects of pH and Buffer on Phosphoryl Transfer Reactions – All of the studies described above were conducted with reaction mixtures containing 50 mM potassium phosphate buffer, pH 7.4. However, the rates of the phosphorylation reactions were strongly dependent on the pH of the solution and also on the buffer employed. Interestingly, the pH optima for the phosphoenolpyruvate-dependent reactions were different for each of the phosphoryl acceptor sugar substrates. Optimal activity with [^{14}C]mannitol as substrate was observed at pH 7.5, while that with [^{14}C]sorbitol was at pH 8, and that with [^{14}C]fructose was near pH 9 (Fig. 4*A*). Thus, lower affinity substrate binding correlated with pH optima at more basic pH. None of the phosphoenolpyruvate-dependent sugar phosphorylation reactions could be demonstrated at a pH lower than 4 (Fig. 4*A*). By contrast, the activity-pH profile for the mannitol-1-P:[^{14}C]mannitol transphosphorylation reaction was shifted to a more acidic pH range, and appreciable activity was observed below pH 3. The optimal pH for this reaction was 5.5 (Fig. 4*B*). Fig. 4*B* also illustrates the pH independence of the substrate inhibition phenomenon discussed above. As will be demonstrated in a subsequent publication (36), the occurrence of the activity-pH curve for transphosphorylation in an acidic pH range, relative to the corresponding phosphoenolpyruvate-dependent sugar phosphorylation reaction, appears to be a characteristic feature of these Enzyme II-catalyzed reactions.

DISCUSSION

The phosphoenolpyruvate:sugar phosphotransferase system is an enzyme system which appears to be largely restricted to the prokaryotic world. Within the bacterial kingdom, however, little effort has been devoted to the detection and characterization of the system in phylogenetically distant organisms. The information that is available suggests that while the PTS is found in a number of strict and facultative anaerobes which utilize sugars via the glycolytic pathway, it is absent from most strict aerobes (25, 43).[2] Detailed enzymological studies have been restricted to a few eubacterial species (1–8). However, in those organisms which have been examined, considerable evolutionary conservatism has been noted. Thus, Enzyme I and HPr from different eubacterial species cross-react enzymologically and catalyze the phosphorylation of several sugars. In all organisms which have been examined, these two proteins are either constitutively synthesized or are inducible to an extent of 2- to 3-fold (21).

In *Escherichia coli* and *Salmonella typhimurium*, Enzyme I and HPr are coded for by genes which apparently comprise an operon (21–23, 44). By contrast, the synthesis of each of the Enzyme II complexes (which may consist of a pair of sugar-specific proteins, the Enzymes II and III (26)) is generally induced 5- to 50-fold by growth in the presence of the sugar for which that Enzyme II complex exhibits specificity (29). In these bacteria, the genes which code for the "general" proteins of the PTS and those which code for the sugar-specific proteins clearly do not comprise elements of a single regulon (24).

The genetic and enzymological complexity of the eubacterial PTS leads to the question of its evolutionary origin. To this end, we have examined divergent bacterial genera in order to identify simpler variants of the PTS. The fructose-specific PTS in *Rhodopseudomonas spheroides* and *Rhodospirillum rubrum* represents such a system (31). Two fructose-inducible protein fractions, one a peripheral membrane protein ($M_r \approx$ 200,000) and the other, an integral membrane constituent, were essential for the phosphoenolpyruvate-dependent phosphorylation of fructose. No soluble HPr-like protein was found (31). The characterization of this system led to the possibility that the complex eubacterial phosphotransferase systems, which phosphorylate many sugars, arose from a sugar-specific PTS by duplication of the genes (or operons) coding for an Enzyme II complex, followed by evolutionary divergence leading to altered sugar substrate specificities. The recent identification of distinct but similar operons which code for the enzymes responsible for the catabolism of mannitol, sorbitol, and galactitol in *E. coli* (18, 19) strengthens this possibility.

Further evidence for this view is presented in this communication. We show that in *Spirochaeta aurantia*, only a single sugar, mannitol, appears to be transported and phosphorylated by the PTS-mediated group translocation process. Mannitol phosphorylation *in vitro* depended on two soluble proteins, Enzyme I and HPr, as well as the integral membrane Enzyme II complex. The system is therefore structurally similar to the eubacterial PTS. Surprisingly, the synthesis of

these proteins as well as that of mannitol-1-phosphate dehydrogenase was induced about 100-fold when mannitol was included in the culture medium, and synthesis of these four enzymes appeared to be subject to coordinate regulation (Table IV). It therefore appears that the genes which code for these proteins comprise a single regulon (24). Such a suggestion is teleologically consistent with the conclusion that in *S. aurantia* strain J1 Enzyme I and HPr function exclusively in the transport and phosphorylation of mannitol.

Examination of the phosphoryl donor specificity of the Spirochete PTS led to the discovery of the Enzyme II-catalyzed transphosphorylation reaction (Table III and Ref. 32). The sugar acceptor specificity of this reaction was more restrictive than that of the phosphoenolpyruvate-dependent reaction. Although the phosphoryl group of phosphoenolpyruvate could be transferred to mannitol, sorbitol, and fructose, phosphoryl transfer from mannitol 1-phosphate occurred at an appreciable rate only when mannitol was the phosphoryl acceptor. Moreover, sorbitol 6-phosphate and fructose 6-phosphate were not efficient phosphoryl donors. It is interesting that the phosphoryl acceptor specificity of the transphosphorylation reaction coincided with the transport specificity of the system (Tables I and II and Ref. 32) while the phosphoryl acceptor specificity of the phosphoenolpyruvate-dependent reaction correlated with the inducer specificity (Table II). These properties of the Spirochete PTS are summarized in Table VIII.

An interesting qualitative parallel appears to exist between the mannitol phosphotransferases in *S. aurantia* and *Staphylococcus aureus*. Both enzyme systems catalyze the *in vitro* phosphorylation of sorbitol with phosphoenolpyruvate as phosphoryl donor but they do not catalyze the *in vivo* transport of sorbitol (36). Moreover, the phosphoenolpyruvate-dependent phosphorylation of sorbitol in *S. aureus* extracts occurred with greater facility than did mannitol 1-phosphate- or sorbitol 6-phosphate-dependent sorbitol phosphorylation (36). These acceptor specificities are similar, although quantitatively less restrictive than those for the Spirochete PTS.

Kinetic analyses of the sugar phosphate-dependent Enzyme II-mediated sugar transphosphorylation reactions in *S. typhimurium* argue against a mechanism involving a phosphorylated Enzyme II intermediate.[4] The acceptor and donor specificities of the phosphoenolpyruvate and sugar phosphate-dependent reactions in *S. aurantia* and *S. aureus* support this argument. Specifically, the results appear to be inconsistent with a mechanism in which all phosphoryl transfer reactions involve a common phosphorylated Enzyme II intermediate.

TABLE VIII

Properties of phosphoenolpyruvate:sugar phosphotransferase system in Spirochaeta aurantia

Property	Mannitol	Sorbitol	Fructose
Inducer specificity for PTS protein synthesis	+	+	+
Phosphoryl acceptor specificity for the phosphoenolpyruvate-dependent reaction	+	+	+
Phosphoryl acceptor specificity for the mannitol-1-P-dependent reaction	+	–	–
Transport substrate specificity for the PTS	+	–	–
Phosphoryl donor specificity of sugar phosphates for mannitol phosphorylation	+		

In the accompanying communication, the enzymology of the Enzyme II-catalyzed transphosphorylation reactions in enteric bacteria is examined, and the relationship of this process to vectorial transmembrane sugar transport is considered (36).

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