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Distribution of the Phosphoenolpyruvate: Glucose Phosphotransferase System in Bacteria

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A survey of the occurrence of the phosphoenolpyruvate-dependent glucose phosphotransferase system was carried out in a number of bacteria, representing both gram-positive and gram-negative facultative anaerobic and strictly aerobic types. The system was found to be present in representatives of genera that are characteristically facultative anaerobes, but the system was absent in members of those genera that are strictly aerobic. Thus, although the phosphoenolpyruvate phosphotransferase system is an important system for the transport of sugars in bacteria carrying out anaerobic glycolysis, it plays no role in sugar transport by those organisms having a strictly oxidative physiology. A fundamentally different system, probably not involving phosphorylation during transport, is indicated in this latter group.

The physiological significance of the phosphoenolpyruvate (PEP): hexose phosphotransferase system first described by Kundig, Ghosh, and Roseman (11) in the transport of sugars has been firmly established in a number of bacteria. Kaback (9), working with isolated membrane vesicles of *Escherichia coli*, showed the absolute dependence of the system on PEP, the necessity of all components of the system for the uptake of sugars, and concomitant phosphorylation of sugars during uptake. Genetic evidence, whereby mutants devoid of a component of the system have been shown to be concomitantly impaired in their capacity to transport a number of sugars, has been obtained with *Aerobacter aerogenes* (19), *Staphylococcus aureus* (5), and *Salmonella typhimurium* (17), as well as with *E. coli* (18). In addition, Kornberg and Smith (10) showed that mutants of *E. coli* devoid of phosphofructokinase (ADP:D-fructose-6-phosphate 1-phosphotransferase, EC 2.7.1.11), although they can metabolize glucose, cannot grow on this substrate because of insufficiency of internal PEP required for glucose transport.

After the demonstration that phosphorylation was not a necessary event during the active transport of hexoses by the fungus *Aspergillus nidulans* (3), the question was raised whether the PEP phosphotransferase system was a widespread characteristic of prokaryotic organisms, or whether it was one of more limited distribution. A cursory examination of the list of organisms in which the system has been studied [in addition to those named above, it has been detected in *Lactobacillus arabinosus*, *Bacillus subtilis* (W. Kundig et al., Fed. Proc. p. 658, 1965), and *Streptococcus* lactis (13)] reveals that all are facultative anaerobes, capable of fermentative metabolism. The key role of PEP in the physiology of such organisms during anaerobic glycolysis is apparent, since PEP is the key product of glycolysis, and hence would represent a readily mobilizable energy source for active transport. The situation in strictly aerobic organisms, which lack a significant fermentative capacity and gain energy chiefly from oxidative phosphorylation, is less clear.

The development of simple methods for the assay of the PEP phosphotransferase system, using frozen and thawed cells and the nonutilizable glucose analogue 2-deoxyglucose by Ghosh and Ghosh (7), or toluene-treated cells by Gachelin (6), made the survey of a number of organisms feasible. This paper reports the results of a survey, including a selected list of representative facultative anaerobic and strictly aerobic organisms.

MATERIALS AND METHODS

Organisms. The following organisms were used: *Achromobacter parvulus* ATCC 4335, *Arthrobacter globiformis* ATCC 8010, *Azotobacter vinelandii* ATCC 12837, *Bacillus cereus* ATCC 6051, *B. megaterium* ATCC 9889, *B. subtilis* ATCC 6051, *Corynebacterium ulcerans* 603/50 (tox$^-$), *E. coli* K-38, *Micrococcus*

luteus ATCC 398, *Mycobacterium smegmatis* (Midwest Culture Service, Terra Haute, Ind.), *Pseudomonas aeruginosa* ATCC 10145, *S. typhimurium* ATCC 1036, and *S. aureus* (Smith).

Assay methods. Organisms were grown on a complex medium containing 0.5% glucose, 0.5% NaCl, and 1% Trypticase (BBL) unless otherwise specified. Cells were harvested during logarithmic phase by centrifugation.

PEP: glucose phosphotransferase was assayed by a modification of the method of Ghosh and Ghosh (7) as follows. Cells were washed with 0.15 M of KCl, and resuspended in 0.1 M of phosphate buffer, *p*H 7.0, at a density of approximately 200 mg (wet weight) per ml. The suspension was placed in a freezer at −20 C overnight, then thawed at room temperature, washed, and suspended in phosphate buffer at 5 C. Protein content of a 0.1-ml sample of the cell suspension was determined by the method of Lowry et al. (12) after extraction with 1 ml of 1 N NaOH at 100 C for 15 min.

The reaction mixture contained: 2-deoxy-D-glucose-*UL*-^{14}C (0.05 M, 0.02 μCi/μmole, 0.1 ml; International Chemical and Nuclear Corp., City of Industry, Calif.); PEP (0.01 M, 0.44 ml; Sigma Chemical Co., St. Louis, Mo.); MgCl (0.1 M, 0.09 ml); phosphate buffer (0.1 M, *p*H 7.0, 0.35 ml); frozen-thawed cell suspension (0.2 to 1.0 ml); water (to final volume of 3.0 ml). After incubation at 37 C, the cells were removed from the reaction mixture by centrifugation, and the amount of ^{14}C-2-deoxyglucose phosphorylated was determined in the supernatant by the chromatographic method described by Winkler (23) as follows. An 0.5-ml amount of the supernatant was applied to a column (4 by 0.6 cm) of Bio-Rad AG1-X2 anion exchange resin (100 to 200 mesh) in the formate form; free ^{14}C-2-deoxyglucose was removed from the column with five 0.5-ml portions of distilled water; ^{14}C-2-deoxyglucose phosphate was subsequently eluted with seven 0.5-ml portions of 0.5 M of ammonium formate in 0.2 M of formic acid, directly into vials containing Bray's scintillation fluid (2). Counts were made in a Packard liquid scintillation spectrometer. Results are expressed as the percentage of total counts eluted from the column which appear in the 2-deoxyglucose phosphate peak.

Glucokinase (ATP:D-glucose-6-phosphotransferase, EC 2.7.1.2) was measured by a modification of the spectrophotometric method of Anderson and Kamel (1), using a Coleman-Hitachi double-beam recording spectrophotometer. The reaction mixture contained: tris(hydroxymethyl)aminomethane buffer (0.2 M, *p*H 7.5, 1.0 ml); $MgCl_2$ (0.1 M, 0.2 ml); adenosine triphosphate (ATP) (0.05 M, 0.2 ml); nicotinamide adenine dinucleotide phosphate (0.01 M, 0.2 ml); D-glucose (0.1 M, 0.2 ml); glucose-6-phosphate dehydrogenase (20 units/ml, 0.2 ml); frozen-thawed cell suspension (0.2 ml); water to final volume of 3.0 ml.

Measurement of uptake of ^{14}C-2-deoxy-D-glucose. Cells were grown to logarithmic phase in a synthetic medium containing, per liter of distilled water: glucose, 9 g; $(NH_4)_2SO_4$, 3 g; K_2HPO_4, 3 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; $CaCl_2$, 0.02 g; $FeSO_4 \cdot 7H_2O$, 0.01 g; $MnCl_2 \cdot 2H_2O$, 0.001 g; $NaMoO_4 \cdot 2H_2O$, 0.001 g. After washing with saline, cells were suspended in 10 ml of synthetic medium without glucose at a density not exceeding 0.5 mg of dry cells per ml and incubated at 27 C in a water-bath shaker. Uniformly labeled ^{14}C-2-deoxy-glucose and other additions were added as indicated. Samples of 1 ml were removed at 1-min intervals, filtered through membrane filters (0.45 μm porosity), and washed with 5 ml of cold synthetic medium without carbon source. Filters with cells were transferred to vials containing Bray's scintillation fluid and counted as above.

RESULTS

PEP: glucose phosphotransferase activity. The results of assays carried out with frozen-thawed cell preparations from the 13 organisms studied are shown in Table 1. It is apparent from the data that those organisms representing genera that are facultative with respect to oxygen requirement (*Achromobacter, Bacillus, Corynebacterium, Escherichia, Salmonella, Staphylococcus*) all show significant activity. On the other hand, representatives of strictly aerobic genera that possess extremely limited or no fermentative capacity (*Arthrobacter, Azotobacter, Micrococcus, Mycobacterium, Pseudomonas*) show very little or no activity by the assay method employed; the highest activity detected in this latter group (*P. aeruginosa*) was only one-fourth that of the

TABLE 1. *PEP phosphotransferase and glucokinase activities of frozen-thawed cell preparations*

Organism	PEP phosphotransferase activity[a]		Glucokinase-specific activity[b]
	Cell protein	2-Deoxyglucose phosphorylated	
	mg	%	
Achromobacter parvulus	12.5	40.9	.016
Arthrobacter globiformis	10.0	0.9	.038
Azotobacter vinelandii	14.3	1.7	.014
Bacillus cereus	10.4	68.8	
B. megaterium	8.5	67.8	.037
B. subtilis	5.0	56.9	.048
Corynebacterium ulcerans	14.3	23.1	.013
Escherichia coli	14.3	69.0	.021
Micrococcus luteus	8.4	0.9	.027
Mycobacterium smegmatis	9.6	2.5	.023
Pseudomonas aeruginosa	8.1	5.9	.032
Salmonella typhimurium	15.0	70.7	.028
Staphyloccus aureus	9.3	40.0	.053

[a] Reaction mixture incubated for 60 min.

[b] One unit = 1 μmole of glucose-6-phosphate formed/min. Results expressed as units per milligram of protein.

lowest in the facultative group (*C. ulcerans*). Moreover, it is probable that this low activity detected in *P. aeruginosa* represents limited phosphorylation of 2-deoxyglucose by a hexokinase occasioned by the presence of ATP in the cell preparation, as well as ATP generated from adenosine diphosphate in the presence of added PEP. A control experiment, in which ATP was substituted for PEP in the reaction mixture at the same concentration, showed 8.5% phosphorylation of 2-deoxyglucose by *P. aeruginosa*.

The conditions used in the assays recorded in Table 1 represent large excesses with respect to incubation time and cell protein, and were designed to detect low amounts of activity. As shown in Fig. 1 and 2, when using *S. typhimurium*, 3 mg of protein and 15 min of incubation are sufficient to give maximal activity.

It was important to rule out the possibilities that failure to detect PEP phosphotransferase activity in the aerobic organisms was due to: (i) failure of the freezing and thawing procedure to decryptify the cells, (ii) presence in the cell preparations of inhibitory agents or strong phosphatases that would hydrolyze sugar phosphates quickly. The first possibility was ruled out by demonstrating that glucokinase, an enzyme expected to be present in all of these organisms, could be assayed in all of the frozen and thawed preparations. Table 1 shows that, although glucokinase activities showed a wide variation, the enzyme was nevertheless detected in organisms that did not show PEP phosphotransferase activity, and, taken as a group, these organisms were not significantly lower in glucokinase than were the phosphotransferase-positive organisms.

The second possibility, that negative results were due to inhibitors or phosphatases, was ruled out by experiments with mixed cell preparations. Table 2 shows that the activity of a preparation from *S. typhimurium* was not diminished when it was mixed with a preparation from *P. aeruginosa*, even when the latter was present at a twofold excess with respect to cell protein.

Inhibition studies. In a 1962 study of the specificity of glucose transport in *E. coli*, Rogers and Yu (15) concluded that a free hydroxyl group at C-6 of the sugar was the primary functional group. Subsequent characterization of the PEP phosphotransferase system provided an explanation for this observation; phosphorylation of

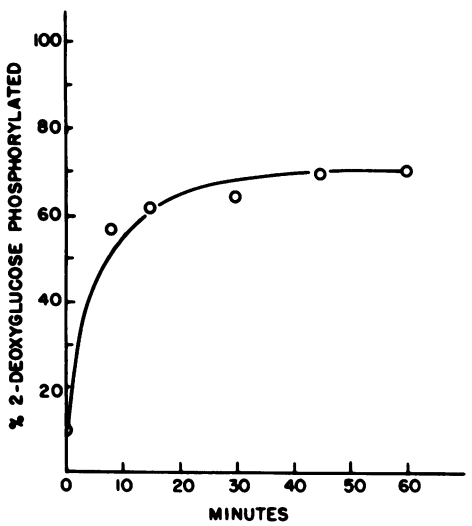

FIG. 1. *Rate of phosphorylation of 2-deoxyglucose by frozen and thawed Salmonella typhimurium. Reaction mixture as specified in Materials and Methods; 6.5 mg of cell protein.*

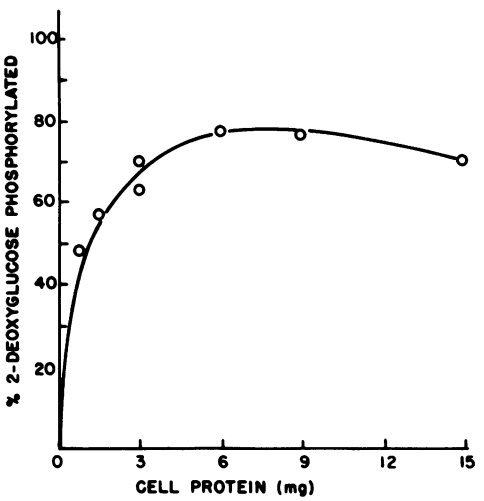

FIG. 2. *Effect of cell density on phosphorylation of 2-deoxyglucose by Salmonella typhimurium. Reaction mixture as specified in Materials and Methods; incubated 30 min.*

TABLE 2. *PEP phosphotransferase activities of mixed preparations*

Cell prepn	Amt of protein	PEP phosphotransferase activity[a]
	mg	
S. typhimurium	3.0	70.5
P. aeruginosa	6.5	4.8
S. typhimurium + *P. aeruginosa*	3.0 + 6.5	70.9

[a] Reaction mixture incubated for 30 min. Results expressed as per cent 2-deoxyglucose phosphorylated.

glucose and its analogues takes place at the C-6 hydroxyl group during transport. Thus, 6-deoxyglucose, which lacks a free hydroxyl group at C-6, would not be expected to be transported by organisms possessing the PEP phosphotransferase system, nor would it be expected to competitively inhibit glucose or 2-deoxyglucose transport in such organisms. One would expect that in systems not involving phosphorylation of C-6 of glucose, 6-deoxyglucose could be transported, and could compete with the transport of glucose or 2-deoxyglucose. This prediction is shown in Fig. 3 and 4; it is clear that in *E. coli* and *S. typhimurium*, which possess the PEP phosphotransferase system, 6-deoxyglucose had no significant inhibitory effect on 2-deoxyglucose transport, even when present at a 100-fold concentration. On the other hand, uptake of 2-deoxyglucose was strongly inhibited by 6-deoxyglucose in *A. globiformis* and *P. aeruginosa*. That this inhibition was of the competitive type is shown in Fig. 5.

It is thus clear that PEP phosphotransferase-positive and -negative organisms can also be differentiated on the basis of susceptibility of their glucose transport to inhibition by 6-deoxyglucose.

DISCUSSION

The survey of bacterial types carried out here, though limited, clearly points out that the PEP: glucose phosphotransferase system is not universally distributed in organisms capable of actively transporting glucose, but rather is a property of those organisms capable of anaerobic metabolism. Thus, it was not found in representatives of genera that have a strictly oxidative physiology (*Arthrobacter*, *Azotobacter*, *Mycobacterium*, *Micrococcus*, *Pseudomonas*). Though no strict anaerobe was tested, the system presumably is present; Groves and Gronlund (8) supplied evidence that glucose was phosphorylated during transport by *Clostridium perfringens*, thus implicating the PEP phosphotransferase system, although this system was not specifically measured.

In an independent study published after this work was completed, Phibbs and Eagon (14), using different assay methods, could not detect the PEP: hexose phosphotransferase system in cell-free extracts of *P. aeruginosa*, in agreement with this study.

Even genera that are as closely related as *Staphylococcus* and *Micrococcus*, that are differentiated principally on the basis of their fermentative capacity, differ with respect to the presence of the PEP phosphotransferase system in a manner that correlates with their fermentative capacity.

Most strains of *B. subtilis* and *A. parvulus* are strict aerobes and thus appear to represent exceptions from the correlation between fermentative ability and PEP phosphotransferase activity. However, these organisms belong to genera that

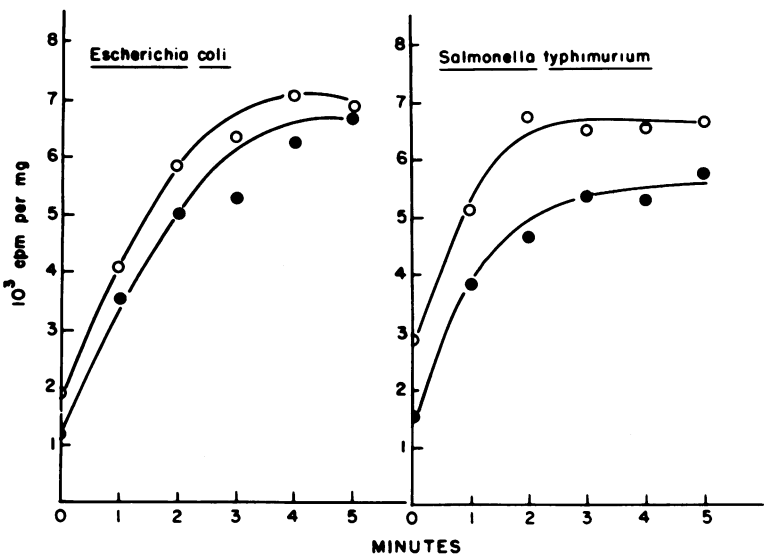

FIG. 3. *Effect of 6-deoxyglucose on uptake of ^{14}C-2-deoxyglucose by Escherichia coli and Salmonella typhimurium. Cells incubated with 10^{-4} M ^{14}C-2-deoxyglucose (0.2 μCi/μmole) in the absence (○) and presence (●) of 10^{-2} M 6-deoxyglucose.*

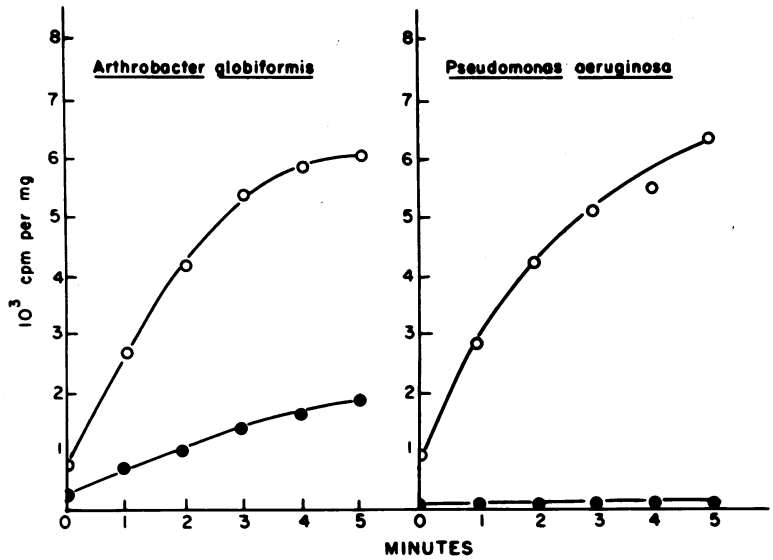

FIG. 4. *Effect of 6-deoxyglucose on uptake of ^{14}C-2-deoxyglucose by Arthrobacter globiformis; and Pseudomonas aeruginosa. Cells incubated with 10^{-4} M ^{14}C-2-deoxyglucose (0.2 μCi/μmole) in the absence* (○) *and presence* (●) *of 2 × 10^{-3} M 6-deoxyglucose.*

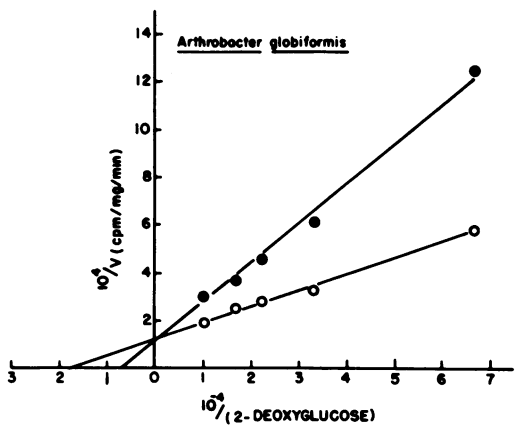

FIG. 5. *Lineweaver-Burk plot of the inhibition of 2-deoxyglucose uptake by 6-deoxyglucose. Arthrobacter globiformis was incubated with 2-deoxyglucose (0.2 μCi/μmole) with no further addition* (○), *and in the presence of 5 × 10^{-4} M 6-deoxyglucose* (●).

are predominantly facultative anaerobes. Thus, their inability to grow anaerobically is probably due to other physiological factors unrelated to the means of sugar transport.

The PEP phosphotransferase system offers extremely important physiological advantages to organisms carrying out anaerobic glycolysis, as pointed out by Roseman (16). First, the system provides a tight linkage between the transport of a sugar and its subsequent metabolism; secondly, under conditions where energy supply is limited, the system allows for conservation of ATP, in that the product of the transport event is a phosphorylated sugar, which can enter catabolic and anabolic pathways directly. These factors are less critical during aerobic metabolism, since PEP is not the principal energy product, and plays a less pivotal role under these conditions. Also, the relative abundance of ATP generated by oxidative phosphorylation may make the ATP conservation aspect less crucial. Thus, it is not unreasonable to expect that strictly oxidative organisms should lack such a system.

The fact that 6-deoxyglucose competitively inhibits the uptake of 2-deoxyglucose and presumably is itself transported by *A. globiformis* and *P. aeruginosa* shows that a free hydroxyl group at C-6 of glucose is not essential for the transport, and indicates that phosphorylation is not necessary for transport in these organisms, either by PEP or any other phosphate donor. This line of reasoning was used by Crane and Krane (4) and by Brown and Romano (3) in demonstrating that phosphorylation was not essential for hexose transport in hamster intestine and *Aspergillus nidulans*, respectively. The hypothesis that phosphorylation accompanies sugar transport was prominent in early studies on sugar transport (22), was then discarded for lack of convincing evidence, and finally was revived by the clear-cut demonstration of the PEP phosphotransferase system in bacteria. Also, Van Steveninck (20, 21) has presented evidence implicating phosphorylation during sugar transport in

yeast. However, Kuo and Cirillo (Bacteriol. Proc., p. 145, 1970) reported that galactose enters yeast as the free sugar. Thus, at present it must be concluded that the PEP: hexose phosphotransferase system represents the only unequivocal demonstration of necessary phosphorylation of sugar during transport, and that this system is not of universal distribution, even among the bacteria.

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