The Transport of Carbohydrates by a Bacterial Phosphotransferase System

SAUL ROSEMAN

From the McCollum-Pratt Institute and the Department of Biology, Johns Hopkins University, Baltimore, Maryland 21218

ABSTRACT The components and properties of a phosphoenolpyruvate: glucose phosphotransferase system are reviewed, along with the evidence implicating this system in sugar transport across bacterial membranes. Some possible physiological implications of sugar transport mediated by the phosphotransferase system are also considered.

This paper is concerned with a bacterial phosphotransferase system; its properties, and evidence indicating it to be responsible for sugar transport in bacterial cells will be briefly reviewed, and some speculations will be offered concerning the physiological implications of sugar transport via this system.

The discovery of the phosphotransferase system resulted from our long-standing interest in the biosynthesis of carbohydrate containing macromolecules (1). The 9-carbon sugar acid, sialic acid, is a frequent component of these macromolecules, and enzymatic degradation of one of the sialic acids (*N*-acetylneuraminic acid) was found to give pyruvate and *N*-acetyl-D-mannosamine (2). Studies on the metabolism of the latter sugar led to the discovery of a specific kinase that catalyzes the reaction shown in Fig. 1; this kinase is widely distributed in animal tissues (3). Since certain bacterial cells synthesize polymers of *N*-acetylneuraminic acid, or can metabolize *N*-acetyl-D-mannosamine, extracts of these cells were examined for the kinase. The sugar was not phosphorylated by the reaction shown in Fig. 1, but it was phosphorylated when phosphoenolpyruvate (PEP) was substituted for ATP. The bacterial system, designated PEP:glycose phosphotransferase system, or simply phosphotransferase system, was found to catalyze the reaction shown in Fig. 2 (4, 5).

PROPERTIES OF THE ENZYME SYSTEM

Components of the Phosphotransferase System

The complete phosphotransferase system (designated in some of the Figures as PTS) catalyzes the reaction shown in Fig. 2. Phosphoenolpyruvate is the

phosphate donor, and a variety of sugars can serve as acceptors. While most of the enzymatically synthesized monosaccharide phosphate esters have been shown to be the 6-phosphate esters (4, 5), including the products from galactose (6), and the lactose analogue thiomethyl β-D-galactopyranose (7, 8), recently, the product obtained from fructose has been identified as fructose-1-P (9, 10). The products formed from disaccharides have not yet been characterized with the exception of lactose-P, which is synthesized by extracts obtained from *Staphylococcus aureus*, and here the phosphoryl group is linked

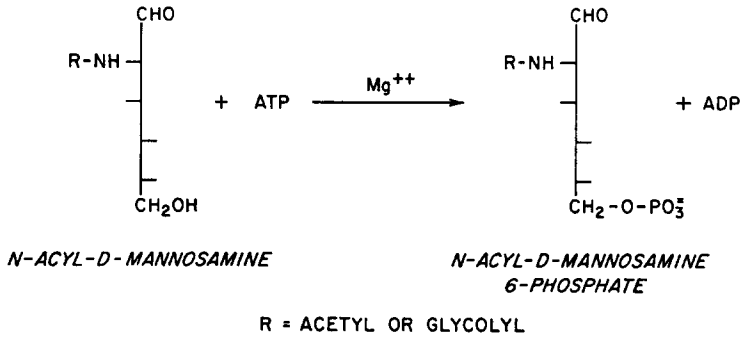


FIGURE 1. *N*-acyl-D-mannosamine kinase (rat liver: 2000-fold). (Ghosh, S., and S. Roseman. 1961. *Proc. Nat. Acad. Sci. U.S.A.*; Warren, L., and H. Felsenfeld. 1961. *Biochem. Biophys. Res. Commun.*; Kundig, W., S. Ghosh, and S. Roseman. 1964. *J. Biol. Chem.*)

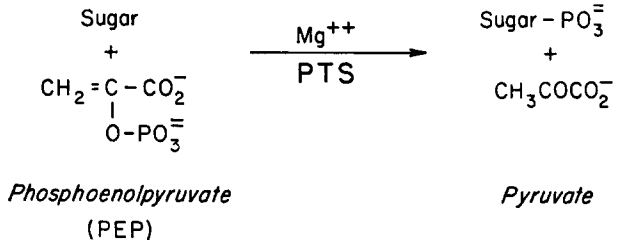


FIGURE 2. Bacterial phosphotransferase reaction.

to C-6 of the galactose moiety (11). The phosphotransferase system requires PEP as the phosphate donor (Fig. 3), which makes the system different from other known sugar kinases. Further evidence that PEP itself is the donor, and not a nucleotide triphosphate generated from PEP, has been published (4).

The complexity of the phosphotransferase system is illustrated in Fig. 4. In the case of Gram-negative organisms (*Escherichia coli*, *Salmonella typhimurium*), the system consists of three protein fractions, Enzyme I, Enzyme II, and a low molecular weight histidine-containing protein, designated HPr. In the Gram-positive organism *Staphylococcus aureus*, an additional protein

component has recently been isolated from cells induced to grow on lactose or mannitol, and the new protein is called Factor III (12). Factor III obtained from mannitol-induced cells, is not the same as the Factor III from lactose-induced cells, each Factor III being required for phosphorylation of the sugar used for growth of the cells from which it was isolated. A protein component distinct from Enzymes I, II, and HPr, has recently been detected in extracts obtained from *Aerobacter aerogenes* (9, 10), and is required for

Active

PEP K_m 4×10^{-4} M

Inactive

Nucleoside Mono -, Di -, and Triphosphates	P – Histidine
Cyclic 3'–5' AMP	P – Serine
Creatine – P	N – Phospho – Glycine
ATP, ADP ± Pyruvate Kinase	Phosphoramidate
P – Glycerate	PPi, Pi
CoA ± Succinate + Pi	DPN, DPNH
Acetylphosphate	TPN, TPNH
	Glucose-6-P

FIGURE 3. Substrate specificity: P-donor.

	Protein Fraction	Occurrence	Sugar Specific	Inducibility
I	Enzyme – I	Cytoplasm	–	–
HPR	Histidine–Protein	Cytoplasm	–	–
II	Fraction or Enzyme II	Membrane	+	+ and –
III	Factor III (*S. aureus*)	Cytoplasm	+	+

FIGURE 4. Components of bacterial PTS.

phosphorylating fructose when the concentration of this ketose is low. The authors therefore consider that the protein functions by increasing the affinity of Enzyme II for fructose.

The requirements for the different protein fractions in the phosphorylating system are illustrated in Figs. 5 and 6. The cellular location of the various proteins is also given in Fig. 4; these conclusions are based on the results of lysis experiments, followed by subcellular fractionation (5). All of the detectable Enzyme II is found in the membrane fraction, while more than 95 % of the other proteins are found in the supernatant fluid. Enzyme II appears to

be an integral component of the membrane, since exhaustive washing of the membrane, or its rupture with either the French Press or by ultrasonic vibration, does not solubilize this component. On electron microscopic examination, the Enzyme II preparations appear to be very similar to the membrane vesicles studied by Kaback and Stadtman (13).

PEP + N-Acetylmannosamine → N-AcMm-6-P + Pyruvate

Incubation	N-Ac-Mm-6-P formed* μmole
I, II, HPr, Mg^{++}	0.38
Minus Mg^{++}	0.03
Minus I	0.00
Minus II	0.00
Minus HPr	0.00

*30 min, 37°C; 20μg HPr; 5μg each, I and II

FIGURE 5. Requirements for *Escherichia coli* PTS.

PEP + TMG → TMG-6-P + Pyruvate

Incubation	^{14}C-TMG-P formed* cpm
I, II, III, HPr, Mg^{++}	15,700
Minus Mg^{++}	150
Minus I	800
Minus II	300
Minus HPr	460
Minus III	900

*Lactose operon induced by growth on galactose

TMG = methyl β - thiogalactoside

FIGURE 6. Requirements for *Staphylococcus aureus* PTS.

The remaining points in Fig. 4 concern sugar specificity, and inducibility of the protein components of the phosphotransferase system. As will be emphasized later, the specific sugar requirements of the system are confined to Enzyme II (and to Factor III where it is required), while Enzyme I and HPr are common to all sugars phosphorylated by the system. The inducibility of some components of the system is shown in Fig. 7 (12). When *S. aureus* is grown under inducing conditions for the lactose operon, and extracts of these

cells are compared with extracts of noninduced cells, there is no detectable increase in Enzyme I and HPr upon induction, while there are dramatic increases in Enzyme II and Factor III. (In these experiments, the lactose analogue thiomethyl β-D-galactopyranoside was used as the substrate; this compound, designated TMG, is often used in both transport and phosphorylation experiments since it is not metabolized, and it is not cleaved by β-galactosidases.) The results therefore show that Enzyme I and HPr are constitutive, while Factor III, and at least one Enzyme II are inducible. Experiments with cells grown on different sugars indicate that most Enzymes II are inducible, except for those which phosphorylate D-glucose and its analogues (such as methyl β-D-glucopyranoside), and D-mannose and its analogues (such as *N*-acetyl-D-mannosamine). In some cases, when cells are grown on a glucose mineral salts medium, the extracts will also phosphorylate galactose

Component	Specific activity in Extracts*	
	Noninduced	Induced
I	52	58
HPr	6.8	6.7
II	0.1	38
III	0.1	2.5

*Spec. act. = μmoles TMG-P formed ($\times 10^2$)/mg protein in crude extracts; 30 min at 37°C. Cells grown in bactopeptone ± galactose or Gal-6-P.

FIGURE 7. Induction of Enzyme II and Factor III (*S. aureus* PTS).

and fructose at low rates, but these rates are substantially increased when the cells are grown on the respective sugars, or the enzymes are derepressed by growth in the absence of glucose.

Attempts are in progress to completely resolve the proteins from each other, and to obtain each in homogeneous form. HPr has been obtained in homogeneous state, and is discussed below. Enzyme I from *E. coli* has been purified about 500-fold (Saier et al., unpublished results). Factor III from *S. aureus* (lactose-induced cells) has been partially purified. The complex called Enzyme II is discussed below.

Partial Reactions of the Phosphotransferase System

Using the purified proteins, it has been possible to demonstrate that the reaction shown in Fig. 2 is the net reaction of the two steps shown in Fig. 8 (4, 12). In the first step, Enzyme I catalyzes the transfer of phosphate from PEP to HPr, giving pyruvate and phospho-HPr. In the second step, Enzyme

II (in the presence of Factor III) (12) catalyzes the transfer of phosphate from phospho-HPr to the sugar, regenerating HPr. The histidine-containing protein therefore serves as a phosphate carrier.

Some of the evidence supporting the formulation of these reactions is shown in Figs. 9 and 10.

$$\text{PEP} + \text{HPr} \underset{\text{Mg}^{++}}{\overset{\text{I}}{\rightleftharpoons}} \text{Phospho-HPr} + \text{Pyruvate}$$

$$\text{Phospho-HPr} + \text{Sugar} \xrightarrow[\text{III}]{\text{II}} \text{Sugar-P} + \text{HPr}$$

$$\text{PEP} + \text{Sugar} \xrightarrow[\text{II, III, HPr}]{\text{I, Mg}^{++}} \text{Sugar-P} + \text{Pyruvate}$$

Factor III detected in induced *S. aureus*

FIGURE 8. Reactions of the phosphotransferase system.

$$\begin{matrix} {}^{32}\text{PEP} \\ + \\ \text{Histidine-Protein} \end{matrix} \quad \underset{\text{Mg}^{++}}{\overset{\text{Enzyme I}}{\rightleftharpoons}} \quad \begin{matrix} {}^{32}\text{P-Histidine-Protein} \\ + \\ \text{Pyruvate} \end{matrix}$$

Enzyme	^{32}P Incorporated into Protein cpm
I + H-P	129,000
I + II + HP	118,000
II + HP	200
I, II or HP	150–200

Incubations (0.5 ml): H-P 0.8 mg, ^{32}PEP 2.5 μm (sp. A. 11 μc/μm) $MgCl_2$ 2.5 μm, Tris-HCl pH 7.4 25.0 μm, Enzymes 0.5 mg; 30 min, 37°C

FIGURE 9. Phosphorylation of HPr by Enzyme I.

Properties of HPr and Phospho-HPr

HPr has recently been obtained in homogeneous form from *E. coli* (7). The purification procedure includes a variety of standard methods for protein purification; the initial step involves heat treatment (100°C, 10 min) of the crude extract, while the last step involves chromatography on DEAE-cellulose. The fractionation on DEAE-cellulose gives two symmetrical protein peaks, exhibiting HPr activity; HPr-1 is eluted from the column before, and is more active than HPr-2. On examination of the two proteins by polyacrylamide disc gel electrophoresis at four pH values, both proteins appear to be homogeneous, but HPr-2 migrates more rapidly than HPr-1 at pH 9.5. Further

examination of the proteins shows that they contain the same amino acids in the same proportions, do not contain detectable quantities of carbohydrate or phosphorus, and are homogeneous and exhibit the same molecular weight in the ultracentrifuge. The only detectable difference between the two proteins is in their amide content; HPr-1 contains 15 amide residues per mole, while HPr-2 contains 10–11. This difference in amide content explains the difference in electrophoretic behavior, and the order of elution from the DEAE-cellulose column. Further investigation suggests that HPr-1 is converted to HPr-2 during the first step in the isolation procedure; i.e., the heat step results in the loss of some amide residues from HPr-1. Thus HPr-2 appears to be an artifact of the isolation procedure. We now believe that HPr-1 is not the native histidine protein. Unheated crude extracts yield a

$$\begin{array}{lcl} {}^{32}\text{P} - \text{Histidine} - \text{Protein} & \text{Enzyme II} & \text{N} - \text{Acetylmannosamine} - 6 - {}^{32}\text{P} \\ + & \longrightarrow & + \\ \text{N} - \text{Acetylmannosamine} & \text{Mg}^{++} & \text{Histidine} - \text{Protein} \end{array}$$

Enzyme	N – Ac – Mm – 6 – ^{32}P cpm
II	17,400
I + II	17,000
I	40
–	30

Incubations (0.5 ml): ^{32}P-H-P 22,500 cpm, N-Ac-Mm 4.0 μm, $MgCl_2$ 2.5 μm, Tris-HCl pH 7.4 25.0 μm, Enzymes 0.5 mg; 30 min, 37°C

FIGURE 10. ^{32}P transfer from ^{32}P-H-P to *N*-acetylmannosamine.

protein with HPr activity that is eluted from the DEAE-cellulose column earlier than HPr-1. Attempts to isolate the native material are in progress.

The properties of HPr are summarized in Fig. 11. The amino acid composition is unusual in that cysteine, tyrosine, and tryptophane are absent. In fact, the purified protein exhibits an ultraviolet absorption spectrum similar to that obtained with phenylalanine (it contains 4 moles phenylalanine). HPr also contains two histidine residues. Stoichiometry studies with both HPr-1 and HPr-2 show that 0.82–0.95 mole of ^{32}P can be transferred to 1 mole of HPr, using prolonged incubation times, and excessive quantities of ^{32}P-labeled PEP and Enzyme I. Our earlier studies (4) had demonstrated that the phosphoryl residue was linked to histidine, and the stoichiometry experiments therefore suggest the possibility that only one of the two histidine residues accepts phosphate.

Some properties of phospho-HPr are summarized in Fig. 12. The phosphoryl group is linked to N-1 of the histidine imidazole ring (7). This linkage is unlike that found in succinate thiokinase [the phosphohistidine-containing protein first observed by Boyer et al. (14), and DeLuca (15)] in which the phosphate is bound to N-3 of the imidazole ring of a histidine residue. Identification of the attachment site of the phosphoryl group was made possible by the synthesis of the N-1 and N-3 isomers of phosphohistidine by Hultquist

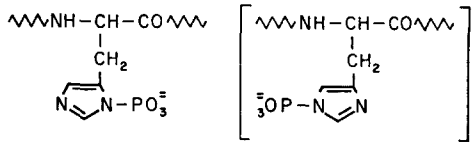
1. Homogeneous
   a. Disc gel electrophoresis; pH 2.3, 4.3, 6.6, 9.5
   b. Ultracentrifuge
2. Molecular Weight
   9340 ± 230 by ultracentrifugation (Yphantis)
   9639 by Σ-amino acid residues
3. Analysis
   a. No carbohydrate or P.
   b. Usual amino acids except Cys, Tyr, Trp
   c. 2 His/mole

FIGURE 11. Some properties of *E. coli* HPr.

$$PEP + HPr \underset{Mg^{++}}{\overset{I}{\rightleftharpoons}} \text{Phospho-HPr} + \text{Pyruvate}$$

1 mole P incorporated / mole HPr

P linked to N-1 of His imidazole ring



1-P-Histidine 3-P-Histidine

FIGURE 12. Phospho-HPr (*E. coli*).

et al. (16, 17). The phosphoryl-N bond in these substances is very labile to acid, and the N-1 isomer is considerably more sensitive than the N-3 isomer.

The Enzyme II Complex

As indicated earlier, many Enzymes II are inducible, while several are found in cells grown on glucose, and are considered constitutive. How many different

Enzymes II can be induced in a single organism? We do not know, but believe that there are many such enzymes, specific for different sugars. For example, the Enzyme II (membrane) fraction from cells grown in a rich medium (broth containing high concentrations of glucose) catalyzes the phosphorylation of glucose and mannose and their analogues, but with little or no activity on a large number of other sugars (4). The sugars that are inactive with this type of preparation are, however, active acceptors when the cells are grown in a mineral salts medium containing the sugars per se. Compounds that can be phosphorylated include pentoses, pentitols, hexoses, hexitols, disaccharides, etc.

Incubation Mixture	μmoles 6-Phosphate formed ($\times 10^2$)	
	α-MeGlu	N-AcMm
II-A, II-B II-Lipids, [P-HPr]*	10.8	12.5
Minus II-A	0.0	0.0
Minus II-B	0.4	2.1
Minus II-Lipids	1.4	0.4

*[P-HPr] generating system = Enzyme I, HPr, PEP, Mg^{++}, phosphate pH 7.4. Incub. 60 min, 37°C, Proteins II-A and II-B, 0.1 mg each; *E. coli* crude lipids or 40 μg phosphatidyl-glycerol. All values corrected for (Minus PEP) control, approximately 1.0.

FIGURE 13. Required components of *E. coli* constitutive Fraction II.

The Enzymes II from *E. coli* grown on glucose have recently been solubilized (18). Fractionation of these extracts show that three components are required for phosphorylating activity, two protein fractions (II-A and II-B), and a lipid fraction (II-lipid). Omission of any of these fractions from incubation mixtures containing a phospho-HPr–generating system gives little or no sugar phosphate (Fig. 13). Further study suggests that II-A contains two specific proteins, one active for glucose and the other for mannose phosphorylation.

The lipid requirements for the reconstituted system have recently been defined. Substitution of a variety of lipids for II-lipid shows that small quantities of phosphatidyl-glycerol (kindly provided by Dr. William Lennarz) are essential for activity.

Purification of II-B has been effected to the extent that it yields a single protein band in sodium dodecyl sulfate, urea polyacrylamide disc gel electrophoresis, and the molecular weight of this protein is approximately 35,000

(19). Homogeneity is not claimed on this basis, since a mixture of proteins containing subunits of about the same size would yield the same results.

Finally, an observation that is potentially of great importance concerns the order of mixing II-A, II-B, II-lipid, and divalent metal. Optimum activity is only observed when a particular sequence of mixing is followed. These results suggest the possibility of studying the requirements for reconstituting intact and functional membrane, and for determining how the proteins interact with membrane lipids and metal ions.

ROLE OF PHOSPHOTRANSFERASE SYSTEM IN TRANSPORT

It is our contention that the PEP:glycose phosphotransferase system is intimately involved in the transport of sugars across bacterial cell membranes.

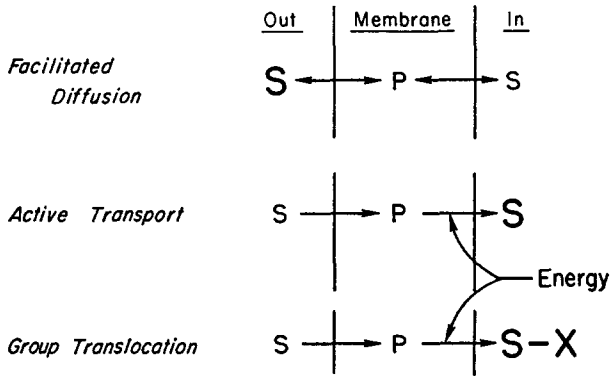


FIGURE 14. Transport systems.

In this section we will present a simple model that suggests how the system may function, and then review some of the evidence in its support.

Several types of transport systems have been defined, and their relationships have been reviewed (20–23). Only the following types will be considered in this discussion:

Simple Diffusion This process involves the diffusion of solutes across membranes, where neither the membrane nor any component thereof acts in a catalytic manner as a "carrier" to expedite the process. When simple diffusion occurs in living cells, it resembles the transport of such solutes across synthetic hydrophobic membranes. Simple diffusion exhibits the following characteristics: (*a*) The process depends upon the existence of an electrochemical gradient across the membrane, and it tends to make the gradient disappear. (*b*) It is not a stereospecific process. For example, L-glucose and D-glucose would penetrate the membrane at the same rate. (*c*) The rate of

penetration is a function of the gradient and of other parameters such as the thickness of the membrane, diffusion coefficient, etc. (*d*) When all other factors are constant, the rate of penetration is directly related to the concentration gradient; i.e., the rate does not approach a limiting value, V_{max} with increasing concentration of substrate. Other features of simple diffusion, such as solvent flow or "drag," have been reviewed (22). Simple diffusion of sugars across bacterial membranes is not thought to be a significant physiological process, either in the entry or exit mechanisms.

Facilitated Diffusion and two other processes of interest in this discussion are illustrated in Fig. 14. As indicated in the figure, facilitated diffusion is similar to simple or passive diffusion in the sense that the solute moves down a concentration gradient, and the process does not require the expenditure of metabolic energy (except for the energy required to maintain an intact membrane). It differs from simple diffusion as follows: (*a*) The membrane contains a component (P in Fig. 14) which catalyzes the process, and the rate of the facilitated diffusion process is much more rapid than predicted for simple diffusion. (*b*) The catalytic component, P, is generally stereospecific. This statement implies the presence of different catalytic systems, or permeases, for different solutes, and this fact was demonstrated in bacterial cells many years ago by Rickenberg, Cohen, Buttin, Monod, Kepes, and their collaborators at the Pasteur Institute (23). (*c*) The rate of penetration generally approaches a limiting value with increasing concentration of the solute on one side of the membrane. This value is called V_{max}^{entry}. The rate of entry as a function of solute concentration resembles simple Michaelis-Menten enzyme kinetics, and transport data are frequently handled in the same manner; i.e., by using Lineweaver-Burke plots to determine the K_m of the permease system for the solute, and to determine the V_{max}^{entry}.

Active Transport is similar to facilitated diffusion, except that the solute is moved across the membrane *against* the concentration gradient. This process requires the expenditure of metabolic energy.

Group Translocation will be used here to mean the process shown in Fig. 14, in which penetration of the membrane by a solute occurs concomitant with a chemical reaction resulting in the formation of a derivative, illustrated in the figure as S-X. Metabolic energy is also required for group translocation, and we believe that this is the most general mechanism for the transport of sugars across bacterial membranes.

The transport systems enumerated above encompass the different bacterial permease systems. There is, thus far, no evidence for coupled transport systems of the type found in animals (22), where sugars and amino acids are cotransported with sodium ion, and the energy for transport may be derived from the sodium pump.

In general, wild-type bacterial cells contain a constitutive glucose permease system, but the transport of the few other sugars that have been studied appears to proceed via inducible systems (23). Where possible, transport is measured with nonmetabolizable analogues of the sugar under study, so that accumulation of metabolites by the cell is not confused with accumulation of the solute under study. For this reason, methyl α-D-glucopyranoside is generally used as a glucose analogue, methyl β-D-galactopyranoside as a galactose analogue, and methyl β-thiogalactopyranoside (TMG) as an analogue of lactose (23).

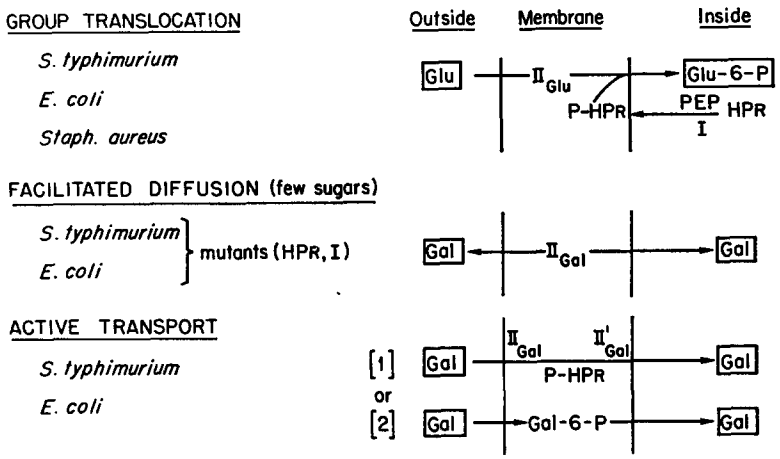


FIGURE 15. Sugar transport via the phosphotransferase system.

Relationship between the Phosphotransferase and Permease Systems

A simple model to explain how the phosphotransferase system can function in the transport processes in Fig. 14, is given in Fig. 15. We believe that most sugars penetrate bacterial membranes by group translocation, mediated by the respective Enzyme II for each sugar. The sugar is phosphorylated as it is transported, and the phosphate is derived from phospho-HPr.

Another mechanism, facilitated diffusion, does occur in certain mutants described below, and with a few sugars. Finally, the active transport process also takes place in a few systems, and again it is proposed that the process is mediated by the specific Enzymes II, and the energy derived from phospho-HPr (i.e. PEP). The sugar may be phosphorylated in the membrane, and the phosphate ester then hydrolyzed by sugar-specific membrane-bound phosphatases of a type that have been described (24), or the Enzyme II–sugar complex changed to a phosphorylated complex (II′ in the figure), which has a low affinity for the sugar, and releases it to the interior of the cell. The latter mechanism would fit the pump mechanism proposed by Kepes (23).

The model offered in Fig. 15 is purely speculative. What, in fact, is the evidence that the phosphotransferase and permease systems are at all related? There are six separate lines of evidence, and these are summarized in Fig. 16. The major emphasis in this brief review will be on the most important correlation, the genetic studies.

While detailed kinetic studies are yet to be performed, there is a good correlation in the action of inhibitors on specific permease and phosphotransferase systems; the inhibitors tested include metal ions, sugars, and sulfhydryl reagents. For example, phosphorylation of galactose and TMG by appropriately induced *E. coli* membrane preparations is inhibited by glucose, while galactose is a particularly potent inhibitor of TMG phosphorylation; these results correspond to the inhibition of transport of the respective sugars in intact *E. coli* cells (25).

1. HPR restores transport in osmotically-shocked *E. coli*.
2. Sugar-P are accumulated during transport in whole cells.
3. Genetic correlations
4. Identity between inhibitors of PTS and permease systems.
5. Specificities of Fractions II correspond to permeases.
6. Sugar uptake by membrane vesicles (Kaback) =
   (a) PEP dependent (b) accumulates Sugar-P.

FIGURE 16. Correlations between PTS and sugar permease systems.

As indicated earlier, most Enzymes II are inducible, but some, such as the Enzyme II that phosphorylates glucose, are constitutive. The same pattern holds for the sugar permease systems. In addition to this qualitative correlation, induction of specific permeases is accompanied by a concomitant quantitative increase in the activity of the corresponding Enzyme II (and Factor III; Fig. 7).

Finally, Kaback has utilized membrane vesicles from *E. coli* to study sugar uptake (26). He finds that the membranes are capable of taking up sugars, provided that they are supplemented with PEP; the sugars are accumulated in the vesicles as the phosphate esters. Furthermore, using double-labeling techniques, the sugar-phosphate ester accumulated by the vesicles was shown to be formed during transport, rather than after penetration into a pool of the free sugar inside the membranes.

Restoration of Transport in Osmotically Shocked Cells by HPr

In these experiments (27), we discovered that the osmotic shock procedure of Nossal and Heppel (28), resulted in a marked decrease in the ability of the

cells to transport sugars, despite the fact that the cells remained viable. Futher study showed that the cells also lost most of their HPr. When the shocked cells were first incubated with purified HPr, the ability to transport TMG and methyl α-glucoside was restored. Fig. 17 shows the results obtained with TMG in *E. coli* (i^+ y^+ z^-) induced for the lactose operon. A variety of experiments showed that the effect was very specific for HPr, and the cell membranes had not lost their specificity for TMG.

The model in Fig. 15 shows HPr on the inside of the membrane (or perhaps in the membrane). Incubation of the shocked cells with relatively high concentrations of HPr would therefore require (to fit the model) that a small quantity of HPr either penetrates the membrane, or is absorbed to it (by forming a complex with Enzyme II?) in order to be functional.

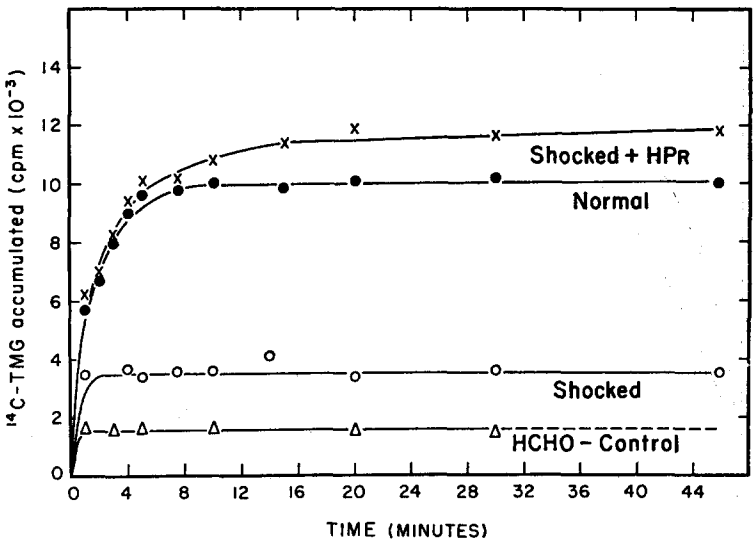


FIGURE 17. Restoration of TMG transport in osmotically shocked *E. coli* by HPr.

Accumulation of Sugar Phosphates during Transport

To our knowledge the first demonstration that sugars were accumulated as their phosphate esters during transport was that of Rogers and Yu (29); the sugars were galactose, which accumulated as the 6-phosphate ester, and α- and β-methylglucosides. Subsequent work by other laboratories showed that essentially all of the methyl α-glucoside taken up during the 1st min by *E. coli* was detected as the phosphate ester. One of these experiments (30) is shown in Fig. 18. In *S. aureus*, it appears that essentially all sugars are converted to the phosphate esters during transport (8, 11, 31).

Genetic Correlations

The most important evidence relating the phosphotransferase and permease systems has been obtained with mutants. As will be seen, the observed physiological behavior of the mutants conforms to the predicted behavior implicating the phosphotransferase system in the transport process.

The model relating the phosphotransferase and transport systems in Fig. 15, has been slightly modified in Fig. 19, primarily to emphasize again that the specificity of each sugar permease system is derived from the specificity of the different Enzymes II. Fig. 19 also shows that most sugars are thought to be transported by group translocation, while some may be transported by

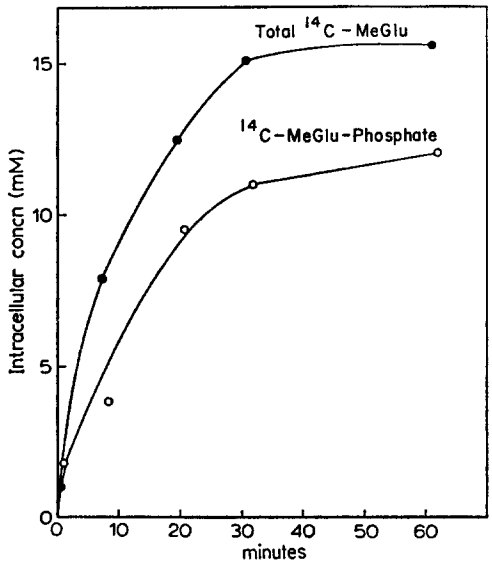


FIGURE 18. α-Me-glucoside uptake by *E. coli* (Winkler, 1966).

active transport. Finally, the figure shows another critical feature of this model, namely that Enzyme I and HPr are required for all sugars transported via the phosphotransferase system when it catalyzes either group translocation or active transport (facilitated diffusion will be considered below).

The model in Fig. 19 leads to certain obvious predictions, which are illustrated in Fig. 20. (*a*) In mutants containing a defective Enzyme II specific for a particular sugar, then only the transport of that sugar should be affected. (*b*) In mutants defective in either Enzyme I or HPr, the group translocation and active transport of all sugars transported by the phosphotransferase system should be affected. As shown in the figure, Enzymes II in mutants defective in Enzyme I or HPr should be present in the membranes, as they are in the wild type. Thus, it is possible that facilitated diffusion could occur

in Enzyme I or HPr mutants. Whether or not facilitated diffusion *will* occur depends upon the exact mechanism required for the transport of each sugar. In the simplest model, we might predict two possibilities, one that the dissociation between the Enzyme II–sugar complex on the inside of the membrane proceeds at a reasonable rate and facilitated diffusion therefore takes place (S_1 in Fig. 20), or, it does not (S_2 in Fig. 20).

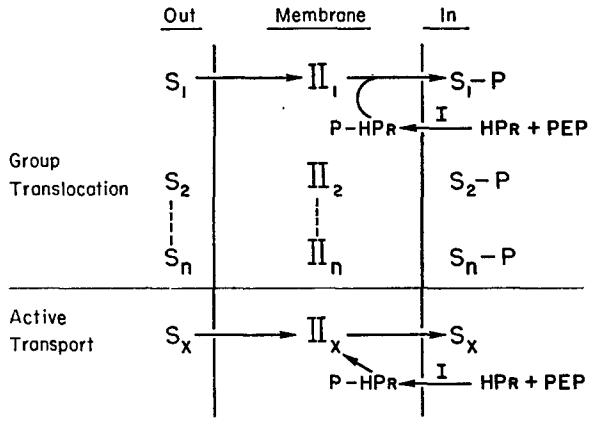


FIGURE 19. Sugar transport in wild-type cells.

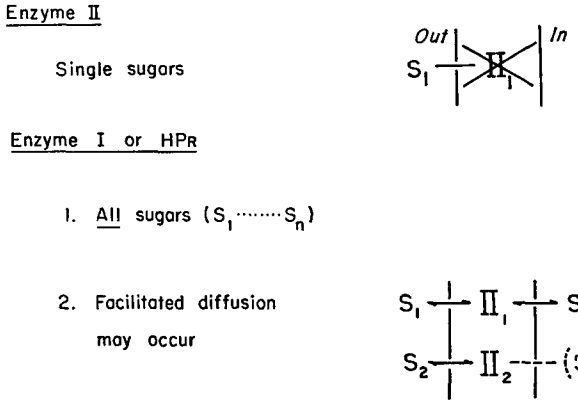


FIGURE 20. Predicted transport defects in PTS mutants.

The properties of mutants defective in specific Enzymes II, listed in Fig. 21, agree with their predicted properties. In addition to the reported results, detailed kinetic studies have recently been performed in this laboratory (Simoni et al., unpublished results) with Enzyme II and Factor III mutants of *S. aureus* (lactose operon). The transport studies showed that such mutants were unable to take up TMG regardless of the external concentration, and therefore that neither the process of passive nor facilitated diffusion occurs at

significant rates in such cells. The mutants did take up methyl α-glucoside at rates comparable with those of the parent strain.

The second predicition in Fig. 20 concerns the properties of Enzyme I and HPr mutants. As shown in Fig. 22, several such mutants have been reported. The original *S. aureus* mutant of Egan and Morse (31), which was unable to utilize 11 carbohydrates for growth and was shown to be defective in its ability to transport the sugars, has recently been shown to be an Enzyme I

Organism	Carbohydrate Affected		
	Growth	Transport	
A. aerogenes	Mannitol$^-$	Mannitol$^-$	Lin (1968)
E. coli	β-Glucosides$^-$	β-Glucosides$^-$	Fox, Wilson (1968)
*S. aureus**	Lac$^-$	Lac$^-$	Morse et al. (1968)

*Factor III$^-$ mutants behave similarly

FIGURE 21. Specific carbohydrate defects in Enzyme II mutants.

Organism		Carbohydrates Affected*		
		Growth	Transport	
E. coli	I$^-$	5	?	Tanaka et al. (1967)
A. aerogenes	I$^-$ or HPR$^-$	5	?	Tanaka, Lin (1967)
S. typhimurium	I$^-$ or HPR$^-$	9	negative	Simoni et al. (1967)
S. aureus	I$^-$	11	negative	Egan, Morse (1965)
E. coli	I$^-$	12	negative	Fox, Wilson (1968)

*Includes glycerol, hexoses (Glc, Gal, Man), glucosides, GlcNAc, ketohexoses, pentoses (?), disaccharides (Lac, Mal, Suc, Mel)

FIGURE 22. Multiple sugar defects in Enzyme I and HPr mutants.

mutant (12). This, and the other mutants listed in the figure, are unable to grow on, or ferment, from 5 to 12 carbohydrates. The defect was shown to be in transport in the last three cases shown in the figure, but was not studied in the first two (31–35). The classes of compounds not utilized include those listed at the bottom of the figure (and the hexitols, sorbitol, and mannitol). The question concerning the pentoses is based on the observation that these compounds are used for growth by the mutants, but generally only after a prolonged lag period that is not observed with the parent strains. We may add that one of the substances not utilized in the *E. coli* mutant isolated by

Fox and Wilson (35) is the disaccharide lactose, suggesting that this disaccharide is also transported in *E. coli* via the phosphotransferase system [1]

Fig. 22 also shows that an Enzyme I mutant of *E. coli* obtained in one laboratory (34) is unable to grow on five sugars, but *can* utilize others, while a similar mutant obtained in another laboratory is unable to utilize 12 sugars (35). These apparently conflicting results are explained by the fact that the first mutant was shown to be "leaky" (35); in our own assays of this mutant, 5–10 % of the Enzyme I levels found in the wild type were detected in extracts of the mutant. Thus, we may conclude that the level of Enzyme I (and presumably of HPr) required for the efficient utilization of a sugar depends on the sugar; some sugars can be utilized at low levels of Enzyme I and HPr, where others cannot. The range of sugars which is affected by mutations in Enzyme I or HPr is therefore not yet established, and may have to be extended

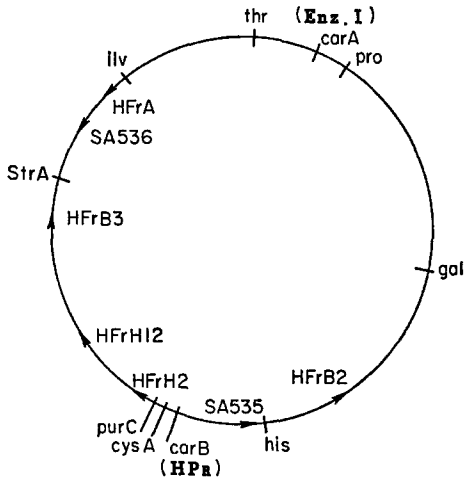


FIGURE 23. Genetic map of *Salmonella typhimurium*.

as more "complete" mutants are obtained. To our knowledge, no deletion mutants have yet been isolated; such mutants would not be "leaky."

Properties of an Enzyme I Mutant of Salmonella typhimurium

Both Enzyme I and HPr mutants have been obtained from *S. typhimurium* (32), and the map location of the genes controlling the synthesis of these proteins has been determined (36), as shown in Fig. 23. The properties of the HPr mutant agree with those of the Enzyme I mutant, described in detail below.

[1] The special problem of lactose and galactose transport in *E. coli*, whether these sugars are or are not transported via the phosphotransferase system, the role of the M protein in lactose transport, and of the galactose-binding protein in galactose transport are discussed elsewhere in this symposium.

The growth properties of the Enzyme I mutant are illustrated in Fig. 24. In these experiments, the cells are tested in mineral medium containing the indicated carbohydrates as the sole source of carbon, and the rate of growth is determined. The values given in the figure are the time periods required for the cell population to double, and the results show that there is no detectable growth of the mutant on nine carbohydrates (up to 10 hr), ranging from glycerol through the disaccharides maltose and melibiose. In a rich medium (broth), which supports growth of the mutant, it is also unable to ferment the nine carbohydrates. In these experiments, all sugars are used at 0.2 % final concentration in the growth medium. Similar results are obtained with glucose at 0.5 % concentration. However, at a concentration of 1 %, the mutant cells grow slowly on glucose (generation time, 300 min, compared

	MINUTES PER GENERATION TIME	
	Parent	Mutant
Glucose	75	—
Mannose	120	—
Fructose	95	—
Glycerol	130	—
Mannitol	80	—
Sorbitol	75	—
N-Acetylglucosamine	80	—
Maltose	120	—
Melibiose	90	—
Galactose	75	92
Glucose-6-P	70	73

Parent = his-1367 rfb-816; mutant=SB703
Growth in mineral salts, histidine, 37°C shaker.

FIGURE 24. Growth of *Salmonella typhimurium* on carbohydrates.

with 75 min in the case of the wild type). While the mutant grows on three pentoses (D-ribose, D-xylose, and L-arabinose), it requires a prolonged lag period before growth is initiated, and with ribose, at least, the generation time in the exponential phase is about twice that obtained with the parent strain. Finally, the mutant does grow on galactose (although at a somewhat slower rate than the parent), and grows very well on glucose-6-P.

Analysis of crude extracts obtained from the mutant (Fig. 25) shows that it lacks detectable levels of Enzyme I; if present, the enzyme would have to be at less than 0.5 % of the level in extracts of the parent strain. The figure also shows a consistent finding that we cannot yet explain; i.e., the mutant contains about three times more Enzyme II activity for methyl α-glucoside phosphorylation than found in the parent strain.

Genetic analysis (32, 36) of the Enzyme I mutant also establishes a point

that is critical to a comparison between enzyme levels and physiological behavior, namely that the defect results from a single mutation.

The two simplest explanations for the behavior of the mutant are shown in Fig. 26. The mutant cannot utilize glucose (except at high concentrations), but can grow as well as the parent on glucose-6-P. (We must emphasize that glucose-6-P is transported by a specific, inducible permease system, not by the glucose permease, reference 30). Therefore, the block in the mutant must be somewhere between the conversion of external glucose to internal glucose-6-P. The block may be in transport, or it may be in phosphorylating glucose *after* it enters the cell. The first will be called the transport and the second, the phosphorylating hypothesis. In the phosphorylating hypothesis, the func-

	Specific Activity in Crude Extracts*	
	Parent	Mutant
Enzyme I	0.36	0.00
Enzyme II	0.12	0.39
HPr	0.16	0.20

*Specific activity = μmoles MeGlu-6-P formed/mg protein/30 min at 37°C

FIGURE 25. Assay for phosphotransferase system in *S. typhimurium*.

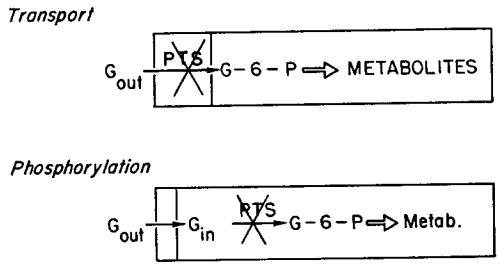


FIGURE 26. Possible defects in *S. typhimurium* mutant (I^-).

tion of the phosphotransferase system is to act as a "trap" after glucose enters the cell through its glucose permease.

The two hypotheses are readily distinguishable, since in the transport mechanism, the mutant should display an inability to bring the sugars it cannot utilize into the cell, while in the phosphorylating mechanism, the rate of entry of the sugars (and the K_m) should not be affected. (In connection with the phosphorylating mechanism, we may note that the mutant contains normal levels of the specific ATP-requiring glucokinase (37) (which suggests, but does not prove, that the defect in the mutant is in transport.) One further test of the two hypotheses is that if the mutant can, somehow, be induced to take up glucose, then according to the transport hypothesis it should possess all of the machinery necessary for its utilization, and should therefore be able

to grow on this sugar, whereas the phosphorylating hypothesis predicts that it should not. The two hypotheses were tested in the following series of experiments.

The uptake of six labeled sugars by parent and mutant cells is illustrated in Fig. 27. In these, and in all subsequent transport experiments, uptake was measured as quickly as possible after the addition of labeled sugar to the cell suspension in the transport medium (7 sec), and was followed for periods up to 10 min. The figure shows that the parent strain rapidly takes up the labeled

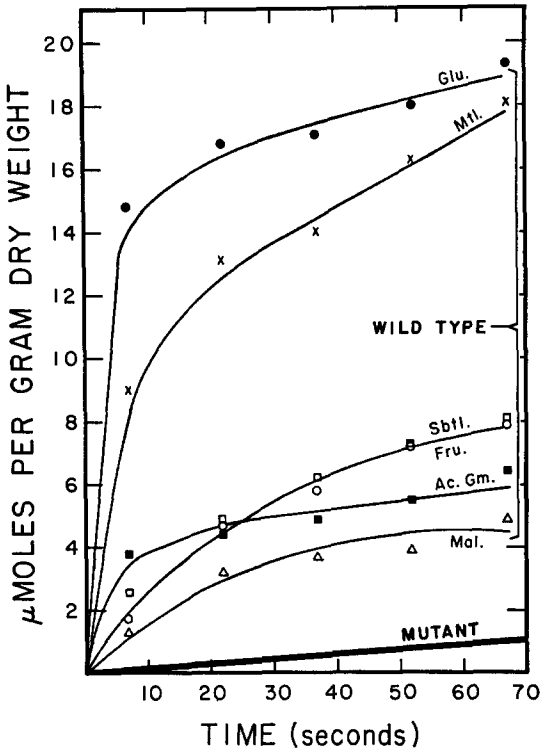


FIGURE 27. ^{14}C sugar uptake.

sugars from the medium, whereas none of the sugars are taken up by the mutant at a significant rate, and in no case is uptake detected at the first measurable time point, 7 sec. These results suggest that the defect in the mutant is in the transport process. However, the sugars employed for the experiment shown in Fig. 27 are metabolizable, and it was therefore important to repeat these studies with a nonmetabolizable analogue. Methyl α-D-glucopyranoside was selected for this purpose, since it is known to be transported via the glucose permease system (23). The results of one experiment are shown in Fig. 28, and clearly indicate a dramatic difference between parent and mutant strains in their ability to take up the glycoside.

A series of experiments of the type shown in Fig. 28 were performed with different concentrations of methyl α-glucoside (over a 35-fold range), and the resulting kinetic data were used to obtain the V_{max}^{entry} for both parent and mutant cells. The calculations were based on the initial time point (7 sec), which was as close as we could get to measuring the initial rate. The results

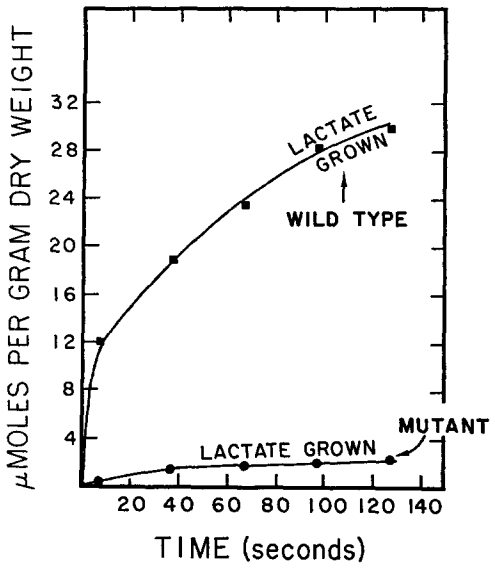


FIGURE 28. Methyl α-glucoside uptake.

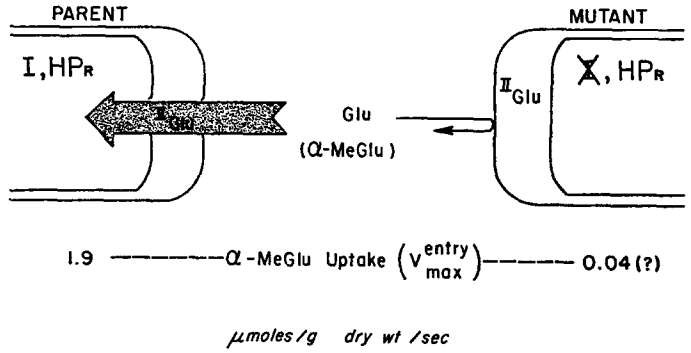


FIGURE 29. Glucose permease defect in *Salmonella* mutant (lactate grown cells).

are illustrated schematically in Fig. 29. There is a *minimum* of 50-fold difference in the rates of entry of methyl α-glucoside between the mutant and parent strains. This ratio is considered minimum for two reasons: (*a*) The values obtained with the mutant at 7 sec were so close to the controls, formaldehyde-treated cells, that their significance must be questioned. In addition, the values obtained with the mutant at 7 sec did not increase with increasing

external concentration of methyl α-glucoside, unlike those obtained with the parent strain. The estimate of 0.04 for the V_{max}^{entry} in the mutant is therefore a guess, and represents the *maximum* rate of entry of the glucoside into the mutant cells. (*b*) The V_{max}^{entry} of the glucoside into the parent cells is a *minimum* value. In order to obtain initial rates of entry, it was necessary to assume that the rate remained constant from zero time to the first measurable point, 7 sec. The shape of the progress curve shown in Fig. 28 indicates that this assumption is probably not correct, and that the initial rate was probably much greater than observed at 7 sec. Thus, despite the linearity of the Lineweaver-Burke plots, we may conclude that the V_{max}^{entry} in the parent cell given in Fig. 28 is probably an underestimate. In any case, the results with the glycoside

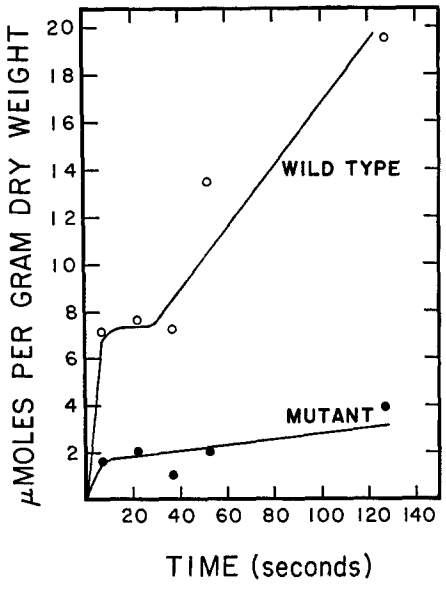


FIGURE 30. Methyl β-galactoside uptake.

amply confirm those obtained with the sugars, and lead to the following important conclusion.

A single mutation in *Salmonella typhimurium* results in an inability of the cell to synthesize Enzyme I of the phosphotransferase system. The physiological consequence of this defect is an inability to grow on, or ferment, nine carbohydrates because these substances are not transported into the cell.

One remaining point must be emphasized, the fact that the *rate* of transport is the critical parameter. In the case of the mutant, methyl α-glucoside uptake was almost undetectable at 7 sec, but after extended periods of time, the internal concentration of the sugar reached that of the external concentration. Glucose is, of course, metabolized, and its internal concentration cannot be determined. Nevertheless, a significant quantity of radioactivity was accumu-

lated by the mutant at 10 min when labeled glucose was used (about 20 % of that observed with the wild type). Thus, it appears that the problem in glucose utilization by the mutant is an inadequate *rate* of transport. The prime importance of the *rate* of transport is considered again later in this discussion.

If the phosphotransferase system is involved in the transport of the nine sugars not utilized by the Enzyme I mutant, then what about the sugars that are utilized? Are the utilizable sugars transported via a different system? To study this question, we examined one of the utilizable sugars, galactose. Galactose is transported by four different permease systems in *E. coli* (38, 39). The properties of the inducible galactose transport system in *Salmonella typhimurium* are identical with one of the four systems described in *E. coli*, called the MG permease. This system transports methyl β-D-galactopyranoside (which is not utilized for growth), D-galactose, and D-fucose (also not

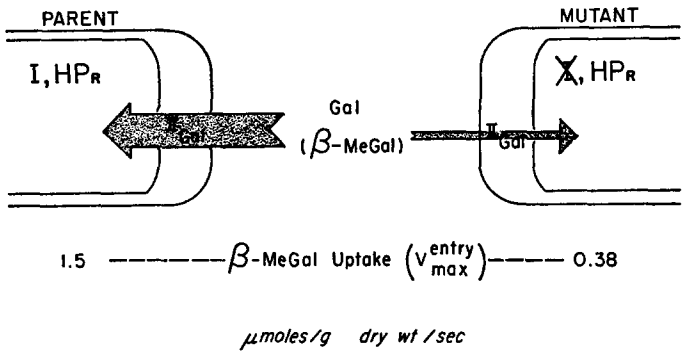


FIGURE 31. Methylgalactoside (MG) permease defect in *Salmonella* mutant. (Grown on galactose or lactate plus fucose.)

used for growth), and is induced by growth of the cells on galactose, or in the presence of D-fucose.

Transport studies were conducted with induced cells, with labeled methyl β-galactoside as substrate, and were performed in the same manner as described for methyl α-glucoside. One such experiment, illustrated in Fig. 30, clearly shows a significant difference between the two types of cells. The peculiar shape of the uptake curves, noted at all concentrations of methyl β-galactoside tested, remain to be explained.

Furthermore, and perhaps equally important, at prolonged times under a variety of conditions, the parent strain accumulated methyl β-galactoside, whereas the mutant could only equilibrate internal with external concentrations of the glycoside. (These studies were conducted with cells grown on galactose, or induced by growth on lactate plus fucose, and ± lactate in the transport medium as an energy source.) Thus, we conclude that the mutant, unlike the parent, takes up galactose by facilitated diffusion.

A kinetic analysis of methyl β-galactoside uptake was conducted, and the results are illustrated in Fig. 31. The V_{max}^{entry} of uptake of the glycoside differs by a factor of four for the parent and mutant cells. Despite this difference, the results show that unlike methyl α-glucoside, which is taken up by the mutant at a rate too slow to be measured with accuracy, the mutant does take up methyl β-galactoside at a significant rate, by a process characterized above as facilitated diffusion. While the facilitated diffusion process in the mutant is slower than the active process in the parent strain, it transports galactose at a rate sufficient to support growth of the mutant.

The results obtained with methyl β-galactoside in the Enzyme I mutant may be explained by one of the predictions made earlier in this discussion (Fig.

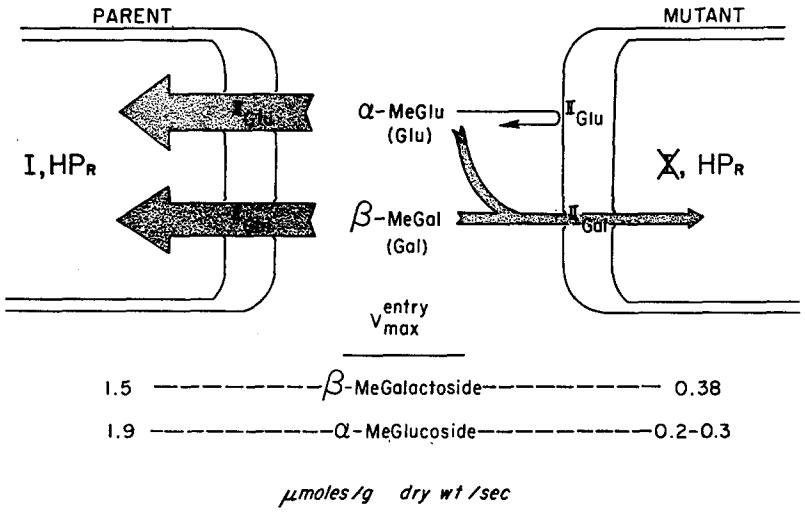


FIGURE 32. α-Methylglucoside (α-MeGlu) transport via the methylgalactoside (MG) permease. (Galactose or lactate/fucose grown cells.)

20). That is, Enzyme I or HPr mutants should be unable to grown on, or transport all carbohydrates utilized by the phosphotransferase system, but it is possible in some cases for a facilitated diffusion process to occur, since the Enzymes II are present in the membranes of such mutants. The rate of facilitated diffusion is dependent on parameters such as the rate of dissociation of the Enzyme II–sugar complex on the inside of the membrane, or more generally, on the rate of translocation in the absence of phosphorylation.

The results with methyl β-galactoside lead to one final, but important experiment, which was performed to answer a question raised earlier. That is, if the prime function of the phosphotransferase system is in sugar transport, then the cytoplasmic and other membrane-bound enzymes required for sugar utilization should be intact in Enzyme I (or HPr) mutants. Therefore, if it

were possible somehow to induce entry of glucose into such mutants, the cells should be able to utilize and to grow on this sugar. Fortunately, the inducible MG permease in *E. coli* is not specific for galactose, methylgalactoside, and fucose, but also transports glucose (40). To determine whether *S. typhimurium* behaves similarly, transport experiments were conducted with methyl α-glucoside in mutant and parent cells grown on lactate, and in a medium containing lactate plus fucose (to induce the MG permease). As reported above, methyl α-glucoside is not significantly transported in *uninduced* mutant cells (Fig. 29). However, there was a significant uptake by the *induced* mutant cells, and the results of the kinetic analysis are shown in Fig. 32. The calculated V_{max}^{entry} for methyl α-glucoside in induced mutant cells was about half

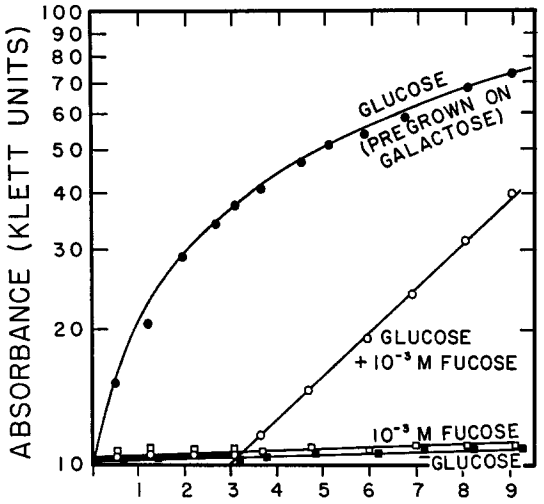


FIGURE 33. Glucose utilization by Enzyme I mutant of *Salmonella typhimurium* mutant induced for the MG permease. The abcissa is time in hours, while the ordinate represents relative cell number ($\log_{10}$ scale).

that obtained with methyl β-galactoside in the same organism. Again, after prolonged incubation, the internal concentration of methyl α-glucoside did not exceed the external concentration, and we therefore conclude that the MG permease–induced mutant is capable of transporting methyl α-glucoside by the process of facilitated diffusion.

Methyl α-glucoside transport in the mutant induced for the MG permease should be a measure of the ability of the mutant to transport the natural substrate, glucose. Since the mutant transported galactose by facilitated diffusion at a rate adequate to support growth, it was possible to test the hypothesis that the mutant contained all of the machinery necessary for growth on glucose, excepting the normal transport mechanism. That is, experiments could be conducted to determine whether or not the mutant could grow on glucose when it entered the cell via the inducible MG permease.

The results of two experiments along these lines are shown in Fig. 33. In one case, the mutant was grown on galactose in a mineral medium, washed, and transferred to a glucose medium. Growth commenced as indicated in in the figure, without a lag, and at a rapid rate, but the rate decreased with time. The reduction in growth rate is explained by dilution of the MG permease, by growth under noninducing conditions.

In the second type of experiment, the mutant was first grown on nutrient broth and therefore did not contain the induced MG permease. After washing, the cells were transferred to mineral medium containing glucose, or fucose, or both sugars. After a lag period of the type normally observed in inducing systems, the mutant grew in the medium containing both sugars, and at a slow, but exponential rate.

Thus, from these experiments it can be seen that the internal machinery for glucose utilization is intact, and that glucose can be utilized for growth provided that it can enter the mutant cell. The only defect in the mutant is in the transport process, and the major function of the phosphotransferase system must be in transport of carbohydrates.

Conclusions

The correlations outlined in Fig. 16 indicate the identity between the phosphotransferase and sugar permease systems. Detailed examination of the properties of the *S. typhimurium* mutant permitted us to show that the phosphotransferase system functions in the transport process according to the predictions of a simple model (Figs. 19 and 20). Thus, we must conclude that only one of the two hypotheses offered in Fig. 26 to explain the behavior of the mutant is correct. The phosphotransferase system does not act as a "sugar trap" (phosphorylating hypothesis), but is required for sugar transport.

To our knowledge, there is no experimental evidence against this conclusion, and as summarized above (Fig. 16), when the properties of the two systems, phosphotransferase and permease, are compared by a variety of methods, they agree in all respects.

DISCUSSION

The PEP:glycose phosphotransferase system is required for the transport of most sugars across bacterial membranes. The following questions remain to be answered: (*a*) Are all sugars transported in all bacteria by the phosphotransferase system or some modification of it? (*b*) Are other solutes transported by this system, or some modification of it? (*c*) Do other organisms contain the phosphotransferase system? (*d*) How is the phosphotransferase system controlled, so that sugars are transported only at rates that permit their utilization, and do not result in the accumulation of excessive quantities of the sugar or

sugar phosphate? (*e*) In molecular terms, what is the precise mechanism by which the phosphotransferase system mediates transport?

Some speculation can be offered in an attempt to answer the last question. First, we must note the possible difference in binding sites of the complex called Enzyme II on the inside and the outside of the membrane. As illustrated in Fig. 34, the outside should only bind sugar, while the inside may bind one

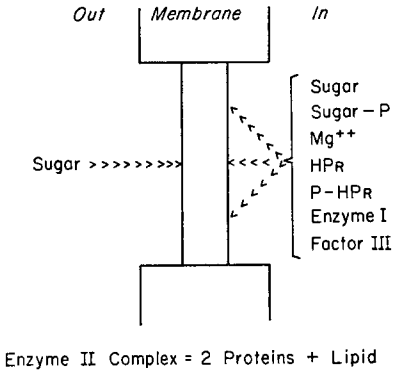


FIGURE 34. Possible binding sites: Enzyme II.

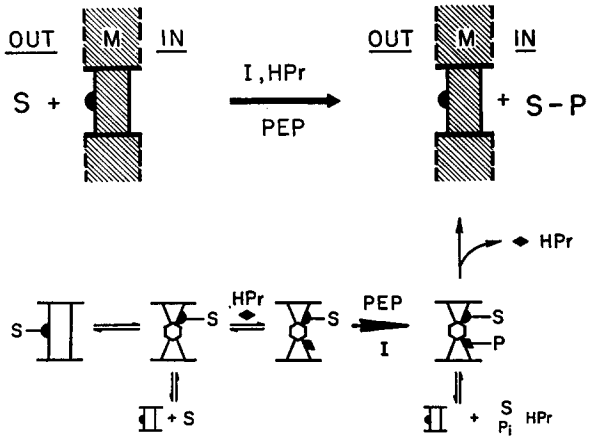


FIGURE 35. Sugar transport via the phosphotransferase system.

or more of many components. These differences could well lead to important conformational changes in Enzyme II, changes that may actually determine whether or not a sugar can be transported by this system. Such conformational changes would lead to transport in a manner analogous to the "translocase" of Mitchell (22), except that they may even be more complex.

A simple scheme for explaining the three processes of interest, facilitated diffusion, active transport, and group translocation of sugars is shown in Fig. 35. While the model shown in the figure may resemble science fiction, it does offer a working hypothesis. The sugar on the outside is bound to its site on

Enzyme II. A conformational change occurs which brings the sugar to the inside, still bound to Enzyme II. If the complex dissociates at a rapid rate, this process would explain facilitated diffusion (lower left of Fig. 35), a process that does occur in a few cases of bacterial mutants lacking Enzyme I or HPr. If the binding is tight, no appreciable dissociation will occur. Enzyme II also has a binding site for HPr, and when this protein is bound, the complex on the inside of the membrane consists of Enzyme II–sugar-HPr. Phosphorylation of the complex by Enzyme I yields the phospho-complex. If the phosphoryl group is transferred to the sugar, then the important process of group translocation is explained (top right of Fig. 35). If the complex dissociates with concomitant release of HPr, Pi, and sugar, then the process of active transport is explained. The latter process resembles in many respects the model for active transport proposed by Kepes (23).

Clearly, we are only at the earliest stages in reaching a full understanding of sugar transport in bacteria. We must eventually comprehend precisely how the phosphotransferase system mediates this process, and finally, how the Enzyme II complex is inserted into the membrane to become fully functional. However, it is equally clear that experiments can now be conducted that may provide answers to these questions.

POSSIBLE PHYSIOLOGICAL IMPLICATIONS

Is the rate of sugar transport independent of, or related to the rates of other important physiological processes such as glycolysis and growth? How is the rate of sugar transport controlled?

Since there is almost no information available to answer these questions, the following discussion can only be speculative, teleological, and will involve many assumptions. Nevertheless, these considerations may be of value since they will lead to hypotheses that can be examined experimentally.

A bacterial cell must adapt to widely divergent, and sometimes rapidly changing environmental conditions. If the rate of sugar transport cannot be varied to meet these changing conditions, the result can be disastrous. For example, if the rate of transport is too low, growth will be limited or negligible. If the rate is too high, energy will be wasted and potentially toxic substances will accumulate; sugar phosphates are apparently toxic at high concentrations. Thus, under ideal conditions, the rate of sugar transport will be regulated so that sugar is transported into the cell only as it can be metabolized.

To meet these requirements, the transport process and its regulatory mechanisms should exhibit the following characteristics:

1. In actively growing cells, sugar transport should be closely linked to and controlled by anaerobic glycolysis, so that the rates of both processes are interdependent. Stationary phase cells, which accumulate storage products such as glycogen, will be considered separately.

2. In actively growing cells, the rate of sugar transport should be inversely related to the concentration of ATP available to the cell, but directly related to the rate at which ATP is utilized. In other words, when the quantity of ATP is high, and greatly exceeds the need for biosynthetic reactions (other than for the synthesis of storage products), then sugar transport should stop; however, if ATP is being used at a rapid rate, then the rate of sugar transport should be maximal to supply the necessary fuel for ATP synthesis.

3. The energy used for sugar transport should be conserved where possible, particularly under anaerobic conditions.

4. When one or more steps are required to convert the transported sugar to an intermediate in the glycolytic pathway, then the rate of transport should not exceed the rate of conversion to the glycolytic intermediate.

5. In a medium containing more than one sugar, all except one should be excluded to prevent the synthesis of extraneous, inducible proteins. When the

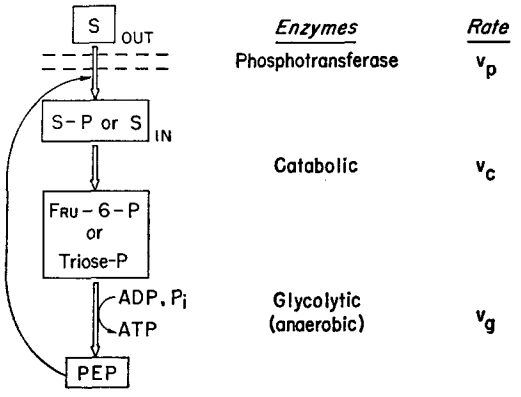


FIGURE 36. Feedback control of transport via glycloysis (group translocation; active transport).

first sugar has been depleted from the medium, then a second should be admitted to the cell (diauxic growth).

6. When the uptake and catabolism of a sugar depends upon the synthesis of inducible proteins, and the sugar is the sole source of energy available to the cell, then transport of the sugar should take place, even at a low rate, to permit induction of the necessary proteins.

The process of sugar transport, mediated by the phosphotransferase system, exhibits many of the characteristics enumerated above. The other properties of the sugar transport process can be explained by postulating certain features of the phosphotransferase system that remain to be proved experimentally. In discussing these properties, frequent reference will be made to Fig. 36, which shows the relationship between sugar transport and anaerobic glycolysis.

The figure refers to the primary events that occur after a sugar is transported; i.e., conversion of the sugar to glycolytic intermediates. Reference is

not made to three other processes, which will be considered separately; exit of the sugar from the cell, conversion to storage products, and utilization for synthesis of polymers such as capsular polysaccharides and cell walls. Under conditions of active growth, the bulk of the transported sugar is used for fuel, a smaller quantity for the synthesis of cell materials, and only traces for the synthesis of storage products (41, 42).

Two types of control over the phosphotransferase systems are postulated, and they are designated primary and secondary. The *primary controls* will include HPr, phospho-HPr, the external sugar, and the product of sugar transport per se (either the internal phosphorylated or free sugar). *Secondary controls* will consist of genetic regulation, the catabolic and glycolytic enzymes, and the products obtained from glycolysis, PEP, and ATP. The more obvious regulatory mechanisms involve the secondary controls, and these will be considered first. Genetic control has been discussed at length; for example, most Enzymes II are inducible, and the concentration of Enzyme II for a particular sugar will control its rate of transport.

Transport in the following discussion means either group translocation or active transport, unless specific reference is made to facilitated diffusion, a process that is only of occasional significance in bacteria.

Conservation of Metabolic Energy

As shown by Kepes (43), a molecule of ATP or its equivalent (i.e., PEP) is required to transport a molecule of sugar against a gradient. In metabolic systems that operate aerobically, where sugars are converted to CO_2, the expenditure of a molecule of ATP to bring the sugar into the cell is not particularly wasteful in view of the large yield of ATP derived from the sugar. However, under anerobic conditions, the situation is quite different; If a molecule of glucose is transported into the cell at the expense of a molecule of ATP, and the transport product is not glucose-6-P, but glucose per se, then the combined processes of transport and anaerobic glycolysis (either to lactate or to ethanol plus CO_2) would yield 1 molecule of ATP. The decided advantage of group translocation by the phosphotransferase system, is that the energy used for transport is not wasted. In the case of glucose, the transport product is glucose-6-P, and the combined processes of transport and glycolysis give rise to 2 moles of ATP per mole of sugar taken up from the medium. Similar considerations apply to a number of other sugars, including mannose, fructose, mannitol, *N*-acetylglucosamine, etc. In each case, the product of transport is the phosphosugar, and the energy used for transport is conserved.

The idea that sugars should be converted to their phosphate derivatives during transport was postulated many years ago, and received considerable attention in early work on sugar transport (20). Unfortunately, the hypothesis

was discarded when the transport process was found to operate independently of hexokinase.

Some recent examples where the phosphosugar is required for the first step in catabolism, and is formed during transport, are particularly interesting. An *E. coli* β-glucosidase requires the corresponding 6-phosphate ester of the glucoside, not the free sugar; the 6-phosphate ester is the product of the transport process (35). Similarly, the β-galactosidase of *S. aureus* hydrolyzes lactose-P, not lactose, and it is lactose-P that is the product of the transport process (11).

In a teleological sense, the phosphorylated sugars should be the products of transport of many, but not of all sugars. For example, *E. coli* β-galactosidase requires lactose, not lactose-P, and lactose is the final product of transport in this organism. Similarly the phosphotransferase system converts galactose to galactose-6-P, but there is no known metabolic pathway for this sugar-P; that is, there is no known mutase that converts galactose-6-P to the desired metabolite, galactose-1-P. (Galactose-1-P is formed from galactose by the widely distributed galactokinase and ATP.) Thus, it appears reasonable that the final product of galactose transport is the free sugar (44, 45), despite the fact that galactose-6-P has been reported in one strain (29).

From these considerations, we conclude that the energy required for transport is conserved where possible, and the process operates by group translocation, but where the desired transport product is the free sugar, then the process of active transport is invoked.

The same point of view may be extended to the facilitated diffusion process. As considered in detail below, the energy requiring transport processes, group translocation and active transport, may be under strict control by various regulatory mechanisms, particularly by coupling to glycolysis. However, facilitated diffusion requires no metabolic energy, and since the uncontrolled entry of sugars into the cell may inhibit metabolic processes, rapid facilitated diffusion could be disadvantageous. The results obtained with the *S. typhimurium* Enzyme I mutant indicate that the rate of facilitated (or possibly passive) diffusion *is* very slow with most sugars. For example, the mutant was unable to grow on 0.2 % and 0.5 % glucose, but did grow slowly at 1 % concentration. Similarly, the rate of entry of metabolizable sugars was extremely low in the mutant (Fig. 27), and the rate of entry of the nonmetabolizable analogue, methyl α-glucoside, was too low to be accurately measured. However, the glucoside *did* enter the cell, since at prolonged incubation times, the internal concentration finally reached the external concentration.

Galactose, however, was an exception. The mutant grew on galactose at almost normal rates, and detailed kinetic studies showed that the facilitated diffusion rate in the mutant (of methyl β-galactoside) was about 25 % of the

rate of transport in the parent strain. This result can be rationalized by the arguments used above.

The desired end product of galactose transport (and lactose transport in *E. coli*) is the free sugar. If 1 mole of PEP is required to transport this monosaccharide, the combined processes of transport and glycolysis under anaerobic conditions would yield 1 mole of ATP. However, when the rate of facilitated diffusion is adequate to supply the sugar to the cell, the net yield is 2 moles of ATP. Thus, it is possible to understand why galactose, unlike the other monosaccharides, can be taken up at a significant rate by facilitated diffusion.

If facilitated diffusion operates at an adequate rate, why then is there another, energy-requiring process (active transport) available to the cell? Perhaps the answer lies in the fact that the cell must be capable of growth at low, as well as at high concentrations of sugar in the medium. A minimum internal concentration of sugar is required to support the minimum rate of glycolysis necessary for growth. At low external sugar concentrations, active transport, unlike facilitated diffusion, can maintain this minimum internal concentration of sugar. These suggestions lead, however, to an additional problem.

For each bacterial species, there are apparently two classes of sugars, one which is transported at a significant rate by facilitated diffusion, and one which is not. Regulation of the transport rate of the latter group can be accomplished by several mechanisms, such as the level of PEP, and these controls are considered in detail below. For the class of sugars where two mechanisms are available, facilitated diffusion and active transport, what are the controls which determine whether one or the other process is invoked?

Exit Process

One control mechanism that could be used to maintain internal concentrations of sugar or sugar-P at utilizable levels, when the external medium contains excessive quantities of the sugar, is the exit process, which would simply move excess carbohydrate out of the cell.

Many studies have been conducted on the exit process in bacteria, and some of these have been reviewed (23). In the case of galactose, for example, entry and exit may be mediated by different systems (44, 45), although a possible complicating factor with galactose is that it is transported by several permeases (39). The lactose system in *E. coli* has been studied extensively (23, 46), and here, and in general (23), it appears likely that exit and entry of each sugar is mediated by one system.

An exit mechanism of particular interest is called counter-flow (or counter-transport), which is not energy-requiring, and is meant to describe the process where a molecule of sugar external to the cell exchanges with a molecule inside the cell. The two sugars can be different, and the internal compound may even

be the sugar phosphate (30, 31). Under special circumstances, this type of exit may be physiologically significant; for example, when the level of an inducible permease is negligible, counterflow may be invoked to bring the inducer into the cell via a different permease.

Despite the demonstrated ability of bacterial cells to transport sugars from inside to outside, we do not believe the exit process to be of importance in growing cultures. Lactose is utilized as rapidly as it is taken up from the medium (23, 43). From our own experiments, we believe this is also true for glucose. Furthermore, nonmetabolizable analogues such as TMG and methyl α-glucoside are lost from cells at very low rates when the cells are placed in sugar-free media (provided that the cells can supply the necessary energy to maintain the gradient). Finally, under anaerobic conditions, the exit process could lead to cessation of growth, since energy would be wasted by transporting a sugar into the cell at the expense of a mole of PEP, and then permitting it to leave.

If the exit process is inconsequential during normal growth, how is the internal sugar concentration maintained at the desired level?

Coupling of Transport to Glycolysis; Pivotal Role of PEP

Phosphoenolpyruvate can be synthesized by several reactions, but we will assume that the major pathway is anaerobic glycolysis in cells actively growing on carbohydrates. On this basis, the end product of sugar transport followed by glycolysis is PEP (Fig. 36), and the end product, PEP, is required for the transport of more sugar. Sugar transport is thus under feedback control by its product. In biochemical systems, this type of feedback regulation is unusual; most feedback processes involve inhibition; i.e., the end-product inhibits the first reaction. Here, the end product is *required* for the first reaction.

PEP, rather than ATP, is a suitable candidate for the driving force behind sugar transport for several reasons. First, PEP participates in relatively few reactions, and the internal pool of PEP may be more easily regulated than that of ATP. Second, the rate of PEP synthesis will depend on the rate of anaerobic glycolysis, which should be relatively independent of fluctuations in the degree of aerobiosis; the rate of ATP synthesis could fluctuate considerably with shifts in aerobiosis. Third, it is important from considerations raised earlier that the rate of sugar transport not be completely independent of the rate of ATP synthesis. Since anaerobic glycolysis is controlled by aerobic oxidation, the necessary balance for PEP synthesis can be achieved as discussed below.

To examine these points, Fig 36 shows the rates of three processes: v_p (sugar transport); v_c (the rate of conversion of the transport product to a glycolytic intermediate by enzymes such as glycosidases, isomerases, etc.); and v_g (the rate of anaerobic glycolysis).

In the growing cell, steady states are achieved for at least brief periods of

time (41, 47). Under these conditions the concentration of metabolites (in Fig. 36) remains relatively constant, and $v_p = v_c = v_g$ the transported sugar will be used at the rate at which it is brought into the cell. Since the rate of synthesis of PEP (v_g) depends upon the rate of sugar transport (v_p), and conversely, the rate of sugar transport depends upon the rate of synthesis of PEP, it is seen that the transport and glycolytic processes are *mutually self-regulating*. Under optimum conditions, we may presume that the rate-controlling factor is sometimes V_{max}^{entry} of the sugar, and that v_c and v_g are capable of handling sugar as fast as it can be transported; for example, the lactose operon in *E. coli* can synthesize 50 times more β-galactosidase than is required to hydrolyze the maximum quantity of lactose that can be transported.

In addition to the limiting situation determined by V_{max}^{entry}, the other limit would result when anaerobic glycolysis stops. The rate of glycolysis is determined by a number of factors; only one will be examined here. When cells are in a medium containing adequate quantities of inorganic phosphate, anaerobic glycolysis is controlled by the availability of ADP. In cells respiring at a rapid rate, but where the demand for ATP is limited, then most, if not all of the available adenosine nucleotide will be in the form of ATP. The lack of ADP brings glycolysis to a halt. Thus, despite maximum levels of ATP, PEP is not being synthesized, and sugar transport stops. In fact, Kepes (43) has called attention to the lack of correlation between sugar transport rates and the levels of intracellular ATP.

These theoretical considerations explain certain experimental observations. At concentrations of azide and dinitrophenol that should uncouple oxidative phosphorylation, the transport rate of methyl α-glucoside is greatly stimulated, rather than inhibited (48). The explanation for this observation is that uncoupling of oxidative phosphorylation is known to increase the rate of formation of PEP (49). That PEP plays a pivotal role in the uptake process is also supported by the fact that under the conditions where azide stimulates methyl α-glucoside transport, the well known anaerobic glycolysis inhibitor, iodoacetate, markedly depresses the rate (48). Similar considerations apply to the results of experiments where the cells are supplied with rapidly metabolizable compounds during methyl α-glucoside uptake (43, 50, 51); these compounds are found to inhibit uptake. However, the addition of azide to the medium, which forces the cell to utilize the rapidly metabolized substances exclusively by anaerobic glycolysis, markedly stimulates methyl α-glucoside transport. The authors state that "accumulation is greater when the cells depend for their energy supply on endogenous respiration or on the anaerobic fermentation of an exogeneous substrate rather than on the active oxidation of such a substrate in the absence of poisons" (50).

Thus we may state that evidence exists to support the contention that sugar transport is linked to anaerobic glycolysis, through the requirement for PEP,

and that regulation of glycolysis by any of the available mechanisms, such as the ratio of ADP to ATP, will in turn control the rate of sugar transport.

The discussion has thus far centered on two of the three reactions shown in Fig. 36, transport and glycolysis. Is one of these processes always rate limiting, or can the rate-limiting step sometimes be the catabolic pathway leading to glycolysis, which determines v_c? In this connection, it is interesting to note that the parent strain of *S. typhimurium* grows on 16 carbohydrates, but that the growth rate is optimum on glucose, glucose-6-P, galactose, and sorbitol (32). The rate of growth is less, frequently substantially less, on the other 12 carbohydrates. In some of these cases, at least, it appears possible that the rate-limiting step may be v_c, and not v_p.

Obviously, a minimum rate of glycolysis must be achieved to support growth, and according to Fig. 36, this means that a minimum rate of transport and catabolism must likewise be achieved for growth. This concept explains the growth properties of parent and mutant strains of *S. typhimurium* on different sugars. That is, sugars that are transported at a high rate in the parent strain (glucose and galactose) continue to support growth of the mutant even when the transport rate is 10–15 % of that obtained in the parent strain (32). On the other hand, growth of the mutant is not detected on a sugar that is poorly utilized by the parent, such as mannose, although preliminary results indicate that mannose is taken up by the mutant at 25 % of the rate obtained with the parent. Presumably, reduction in the transport rate of mannose cannot be tolerated because this decrease in the transport rate depresses the rate of glycolysis below the required minimum value necessary for growth.

The emphasis in this discussion has been that the rate of sugar transport is regulated to correspond to the rate at which it is used, but are the transport and glycolytic processes always coupled?

Other Metabolic Pathways; Uncoupling of Transport and Glycolysis

Transported sugar has thus far been assumed to travel the pathway shown in Fig. 36, where transport and glycolysis are coupled. However, if the bulk of the sugar that is transported into the cell escapes metabolism via this pathway, then the simplification shown in Fig. 36 is invalid. One such possible fate of transported sugar, the exit reaction, has already been considered, and concluded to be of little physiological significance under the usual conditions of growth. Other possible metabolic fates that await transported sugar are catabolism by pathways other than anaerobic glycolysis, the synthesis of storage products such as glycogen, and the synthesis of polysaccharides such as those found in the cell wall, capsule, and lipopolysaccharides.

The major catabolic pathway for sugar utilization in most strains of bacteria appears to be glycolysis through the Embden-Meyerhof-Parnas pathway

(anaerobic glycolysis). However, two other degradative systems are known, the pentose phosphate pathway of Warburg-Dickens-Horecker-Racker, and the 2-keto-3-deoxy-hexonic pathway of Entner-Doudoroff-Wood; in some bacteria, one or the other of these two pathways is the major catabolic system for degrading sugars. The products of the latter two pathways, however, are further catabolized through the glycolytic pathway, and these degradative pathways are therefore considered to be the second step in Fig. 36, which determines rate v_c. On this basis, they need be of no further concern to this discussion.

When a carbohydrate is the sole source of carbon available to a growing cell, almost all constituents of the cell are synthesized from cleavage products of the sugar obtained by anaerobic glycolysis, aerobic respiration, the degradative pathways discussed above, etc. In these cases, therefore, the transported carbohydrate follows the path shown in Fig. 36, and transport and glycolysis are coupled. However, cell polysaccharides are not synthesized by this route. Numerous isotope experiments have demonstrated that sugars are incorporated directly (i.e., without fragmentation) into polysaccharides, and here, the transport process must provide sugar above that required for glycolysis. In quantitative terms, what fraction of the transported sugar is used for the biosynthesis of polysaccharides?

When *E. coli* grows on glucose as the sole source of carbon, about 75 % of the sugar undergoes glycolysis, and about 25 % is converted to cellular material (41). About 10 % of the latter consists of polysaccharides (cell wall, lipopolysaccharides, etc.) (52). Thus, about 2.5 % of the total sugar used by the cell for growth is incorporated into these structural polysaccharides. It is therefore reasonable to conclude that in the growing cell only negligible quantities of sugar escape the pathway shown in Fig. 36.

Stationary phase cells, and cells that normally produce capsules, can be made to synthesize much larger quantities of polysaccharide. When some strains of capsule-producing bacteria are grown under conditions where nitrogen is limiting, increased quantities of extracellular polysaccharide are formed. Stationary phase cells, maintained in a medium rich in sugar, can accumulate storage products; glycogen, for example can be accumulated up to 20 % of the cell mass. The formation of large quantities of polysaccharides at first suggests that a substantial quantity of transported sugar escapes the pathway shown in Fig. 36, and at least this portion is not coupled to the rate of glycolysis. This conclusion may prove correct, but further consideration suggests that it may not. For example, in the case cited above, if 20 % of the cell mass of *E. coli* is glycogen, and 10 % consists of the structural polysaccharides, the total polysaccharide synthesized from glucose transported by the cell amounts to 7.5 %. Therefore, even in cells that grow to the

stationary phase, the bulk of the transported sugar traverses the route shown in Fig. 36.

Is there any connection between the regulatory mechanisms for glycogen synthesis, sugar transport, and glycolysis in the resting cell, or are these processes uncoupled? PEP has been implicated as a prime factor in sugar transport, and it is therefore of particular interest that the *E. coli* enzyme which catalyzes the synthesis of the glycogen precursor, ADP-glucose, is activated 20- to 40-fold by three products of anaerobic glycolysis, fructose diphosphate, glyceraldehyde-3-P, and PEP (53). In medium containing sugar, stationary phase cells synthesize lipid, protein, nucleic acid, etc. at negligible rates, but can transport sugar, and degrade it by glycolysis. The two processes can continue until glycolysis stops because of lack of ADP. However, if the ADP-glucose pyrophosphorylase is activated by the resulting high levels of hexose-P and PEP, the three processes, transport, glycolysis, and glycogen synthesis can be coupled. This follows from the fact that the net reaction for glucose transport (by group translocation) and glycogen synthesis is: glucose + [glycogen] + ATP + PEP → glucosyl-[glycogen] + ADP + pyruvate + PPi. The incorporation of 1 mole of glucose into glycogen therefore requires 2 moles of energy-rich compounds (PEP and ATP), and these 2 moles can be provided by the transport of an additional mole of glucose, followed by glycolysis. Under optimum anaerobic conditions therefore, 2 moles of glucose would be transported into the cell, one would be incorporated into glycogen, and the other undergo glycolysis. The key point is not the energy balance, however, but rather that glycogen synthesis requires the conversion of ATP to ADP. As discussed earlier, it is precisely this step that may be required to permit the transport of another mole of sugar (by stimulating glycolysis) which results in the formation of the required compound, PEP. Thus, we can see the possibility of three interlocking systems, transport, glycolysis, and glycogen synthesis, where the rate of one process regulates the rates of the other two. Similar considerations would apply to the synthesis of other bacterial polysaccharides, such as the capsular materials.

Some Possible Primary Controls over Sugar Transport

As indicated earlier, primary controls are those involved directly in the sugar transport process, rather than those affected by subsequent metabolic reactions.

The need for such controls may be illustrated by two examples: (*a*) Diauxic growth means utilization of one of two (or more) sugars until the medium is depleted of this compound, followed by induction of the required enzymes and the permease for utilization of a second sugar. Diauxie is a complex phenomenon, involving regulation of induction, catabolite repression, etc. Nevertheless,

in at least one case, where the medium contains glucose and galactose, diauxie is explained by inhibition of sugar transport; glucose inhibits entry of galactose, and the enzymes required for galactose utilization cannot be induced (25). (*b*) When nonmetabolizable substances are used in transport experiments (Fig. 28), the rate of transport decreases substantially after the first few seconds, despite the fact that energy is available to the cell. One explanation for this phenomenon is that the internal sugar leaves the cell as its concentration rises, and the rate of net accumulation therefore declines. However, this explanation appears improbable since the internal concentration of the sugar is very low when the transport rate decreases, far below the concentration of sugar in the medium.

Primary controls over sugar transport are easily visualized (by the biochemist who is willing to substitute imagination for unavailable fact), particularly because of the unique topographical location of Enzyme II. Unlike soluble enzymes which are exposed on all sides to the same solutes, Enzyme II is exposed on one side to the constituents of the growth or transport medium, and on the other to the constituents of the cytoplasm. An attempt to depict the asymmetry of Enzyme II is offered in Fig. 34, and a mechanism for transport based on the asymmetry is shown in Fig. 35. Obviously, since we have no precise information, variations of the mechanism shown in Fig. 35 can be offered to explain all of the phenomena involved in sugar transport. Two such possible regulatory mechanisms will be considered.

COMPETITION FROM WITHOUT AND INHIBITION FROM WITHIN That sugars can compete with each other for a transport site is a well-documented phenomenon. The example of glucose and galactose was cited above. (It is interesting to note that glucose is a potent inhibitor of galactose phosphorylation in the phosphotransferase enzyme system, while galactose inhibits TMG phosphorylation.) In this connection, we may again call attention to the fact that Enzyme II consists of two proteins, A and B, and that fraction II-A appears to contain different, sugar-specific proteins. The conformational change shown in Fig. 35 may be envisioned as a translocation of protein, A_1, from outer to inner side of the membrane, while it remains complexed to protein B, and carries with it a molecule of sugar, S_1. This type of mechanism is completely analogous to the conventional sugar "carrier" hypothesis, where the carrier shuttles from one side of the membrane to the other. Two possible modes of competition from without are: (*a*) Two sugars, S_1 and S_2, compete for the specific carrier proteins, A_1. (*b*) Protein B also has the capacity of binding sugars, but it is nonspecific (and may also have a low affinity relative to A). Following the translocation step, where S_1–A_1 moves from the outer to the inner edge of the membrane, B is exposed to the external medium. If B binds S_2 rather than S_1, the return of A_1 from inside to outside may be prevented, and thus prevent further transport of sugar S_1. Alternatively,

specific protein A_1 may transport competing sugar S_2, if this sugar first binds to B. The sequence would be: $S_2 + B-A_1 \rightleftarrows S_2-B-A_1 \rightleftarrows S_2-A_1-B \rightleftarrows B-A_1-S_2$.

This model also explains counter-flow. A sugar on the inside of the cell, S_1, binds to its specific site, A_1, while the external sugar, S_2, binds to the non-specific site, B. Exchange of one sugar for the other results in counter-flow. When the internal sugar is the phosphate ester, the protein complex A-B would also have to act as a transphosphorylase during the reaction.

Finally, the same model can be used to explain inhibition from within, a process that could prevent the accumulation of potentially toxic sugars and sugar phosphates, and that could result in a very rapid decline in the rate of transport of substances such as methyl α-glucoside (cited above). Inhibition from within is visualized to occur as indicated above, except that the same sugar is on each side of the membrane (it can be the sugar phosphate on the inside). The Enzyme II complex would then be: $S_1-A_1-B-S_1$. Either translocation is prevented, or counter-flow occurs. In either case, the process would be inhibitory to the *net* flow of sugar from outside to inside.

REGULATION BY HPR, PHOSPHO-HPR, AND ORDER OF BINDING The critical event in the mechanisms for sugar transport depicted in Fig. 35 is the conformational change, or translocation, that brings the sugar from the outside to the inside of the cell. In the sequence shown in the figure, which may be designated *HPr-independent translocation*, HPr binds to the complex after the translocation step, and is *then* phosphorylated. Another important possibility is where phospho-HPr binds to Enzyme II, and the translocation step occurs as a *result* of this binding; this mechanism may be designated *phospho-HPr–dependent translocation*. In the latter case, HPr could conceivably prevent transport by competing with phospho-HPr for the binding site on Enzyme II.

Reference was made earlier to the fact that there are two classes of sugars for each strain of bacteria, a class that can be taken up from the medium by the process of facilitated diffusion at a significant rate, and a class that cannot. The mechanism shown in Fig. 35 explains the properties of the first class, while the phospho-HPr–dependent mechanism explains the properties of the second class. Furthermore, the potential inhibitory activity of HPr in the phospho-HPr–dependent mechanism explains why mutants which have a limited ability to phosphorylate HPr (Enzyme I "leaky" mutants) can utilize some, but not all sugars; the degree of inhibiton would depend on the relative affinities of HPr and phospho-HPr for the Enzyme II complex.

Finally, the two mechanisms considered above explain why a single inhibitor, such as the oxidative phosphorylation uncoupler, sodium azide, can have opposite effects on the transport rates of two different sugars. Azide stimulates methyl α-glucoside uptake (as described earlier), but inhibits the active transport of TMG. If methyl α-glucoside is taken up by the phospho-

HPr–dependent mechanism, and TMG by the mechanism shown in Fig. 35, then precisely these results are expected. The inhibitor greatly stimulates the production of PEP, which may convert most of the cellular HPr to phospho-HPr. The increased availability of phospho-HPr would increase methyl α-glucoside uptake. However, the decreased quantity of HPr would inhibit active transport of TMG, as can be deduced from Fig. 35, although it would not affect facilitated diffusion of TMG. In fact, azide does not inhibit facilitated diffusion of TMG but inhibits its retention by the cell (46).

We have speculated that there may be two (or more) mechanisms for sugar transport, HPr-independent and phospho-HPr–dependent translocation; possibly neither one of these mechanisms is correct. However, the important question is whether a single system, such as the phosphotransferase system, possess sufficient inherent flexibility to explain the observed variations in sugar transport systems; a single sugar may be transported differently by different organisms (lactose by *E. coli* and *S. aureus*), while a single organism, such as *E. coli*, transports different sugars by more than one process (facilitated diffusion, group translocation, active transport). The two mechanisms offered above explain these phenomena. For example, what at first appears to be a trivial change, i.e., the order of interaction of the constituents of the phosphotransferase system, results in a qualitative change in the transport process (facilitated diffusion can or cannot occur). We therefore conclude that the phosphotransferase system is flexible enough to act as a general mediator of sugar transport in bacterial cells.

Conclusions

An attempt has been made to explain the behavior of cells under widely divergent conditions on the basis of some known properties of the phosphotransferase system, and some that are speculative. As indicated earlier, however, many of these speculations can be tested. For instance, the isolation of components of the phosphotransferase system in homogeneous form will permit direct determination of the various interactions that have been postulated. With these quantitative values in hand, definite conclusions on the validity of these hypotheses may be obtained.

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Discussion from the Floor

Question from the Floor: Is there any relationship between "y^-" mutants (so-called M Protein mutants) and one of your HPr or Enzyme I or Enzyme II mutants?

Dr. Roseman: I would prefer that Dr. Kennedy answer this question, but apparently he is not here at this time.

What you are asking, if I may rephrase the question, is whether there is or is not a relationship between the permease or permease system in the lac operon of *Escherichia coli* and the phosphotransferase system, or are lactose and its analogues transported by some other mechanism?

I am unable to answer in a definitive manner. However, I would like to repeat some points that I made during the talk, and some others which I could not cover because of lack of time, all of which support the conclusion that lactose is transported in *E. coli* via the phosphotransferase system: (*a*) I showed a slide which represented the first evidence correlating the phosphotransferase and permease systems, and which also was the first time that it was recognized that osmotic shock of bacterial cells results in damage to the transport systems. In our experiment, when cells were induced for the lac operon, and subjected to osmotic shock, they lost much of their HPr, and most of their ability to accumulate TMG and α-methylglucoside. Preincubation of the shocked cells with purified HPr resulted in a complete restoration of the ability to transport these sugars. (*b*) TMG, a lactose analogue, is phosphorylated by extracts of y^+, but not of y^- cells. While this correlation appears to hold for the strains examined, I must add that unlike the kinetics of phosphorylation with many sugars, the kinetics with TMG are not good. In fact, it is necessary to use detergents to obtain significant phosphorylation of TMG. I therefore question the quantitation of the enzymatic activity, and it is perhaps this problem which has thus far not permitted us to obtain a satisfactory correlation in different strains between induction of the "y" gene, and the degree of phosphorylation by extracts of the cells. I should also note in passing that there is no doubt whatsoever about the phosphotransterase system acting as the permease for lactose in *Staphylococcus aureus*. Here, the kinetics are easy to determine with accuracy, and phosphorylation of

lactose or its analogues has been demonstrated beyond question. In addition, induction of the lac operon results in an increase in Enzyme II activity of more than 300-fold. Also, as I discussed during the talk, an additional protein is induced; i.e., Factor III. Factor III is sugar-specific, and soluble, and although we don't know how it functions, it appears to be involved in the second reaction catalyzed by the phosphotransferase system. While Factor III has not yet been detected in *E. coli*, the constitutive *E. coli* Enzymes II have been resolved into two proteins and a lipid component for each of the sugars that is phosphorylated. Thus, it is at least conceivable that the problem in correlating phosphorylation with the induction of the "y" gene in *E. coli* may be complicated by the possibility that more than one protein is induced, one being soluble, and that our mistake was in assaying only the particulate fraction in these cells. Certainly, more work must be done along these lines. (*c*) While "leaky" Enzyme I or HPr mutants in *E. coli* will utilize lactose, this is not true of nonleaky mutants of the type reported by Fox. (*d*) When induced and noninduced whole cells of *E. coli* are treated with *N*-ethylmaleimide according to the procedure of Fox and Kennedy, Enzyme II for phosphorylation of TMG is completely inhibited unless it is protected from the reaction with *N*-ethylmaleimide by TDG. Thus, the properties of Enzyme II for TMG in this type of experiment, at least, corresponds to the properties of the M protein.

Francis C. G. Hoskin (Columbia University): I am somewhat more familiar with the penetration of compounds into giant axons of squids, but having recently spent a year with Professor Hans L. Kornberg at the University of Leicester, England, and for those who are as impressed with Dr. Roseman's presentation as I am, I would like to show two slides.

Fig. 1 shows the relative uptake of β-methylglucoside by *E. coli*; equivalent distribution is shown by the horizontal bars near the abscissae. The mutants K1-1 and K2-1-4 are both phosphoenolpyruvate synthaseless (pps^-) and in addition K2-1-4 is citrate synthaseless (cs^-). In the left-hand frame of Fig. 1 it can be seen that pyruvate (10^{-4} M) has reduced somewhat the rate of uptake of α-methylglucoside (10^{-5} M) by K1-1, but has reduced the rate of uptake virtually to zero for K2-1-4. I emphasize *rate* of uptake, because the initial absolute uptake of about 5–10 times equivalent distribution in the first minute or two seems to be present under a great variety of conditions and may represent something other than active transport—for example, adsorption or interstitial entrappment.

In the right-hand frame of Fig. 1 are seen the results obtained with *E. coli* K1-1/A10 and UH-Ac-2, two pyruvate dehydrogenaseless (pdh^-) mutants having their lesions at two different steps in the complex set of reactions, pyruvate→ acetyl-coenzyme A. It can be seen that, here, pyruvate is without effect on the uptake of α-methylglucoside. These results seemed to indicate that the effect of pyruvate on sugar uptake (for which α-methylglucoside was the model) was only seen when pyruvate was metabolizable to acetyl CoA, and was seen most markedly when one of the major degradative routes of acetyl CoA (citrate synthase) was blocked. However, the possibility of testing directly the effects of acetyl CoA is limited by the poor passage of this compound across the permeability barriers of cells.

Therefore, we partially purified the Roseman system, as described this afternoon,

from *E. coli* K1-1. This is now a noncellular system although, as Dr. Roseman described, still particulate, especially as far as Enzyme II is concerned. We can no longer measure uptake but, as indicated by the heading of Table I, phosphorylation was determined by well-known ion-exchange column separation procedures. The complete system, phosphorylation by which is arbitrarily set at 100, behaves identically as described by Dr. Roseman. As we would have expected from the whole-cell experi-

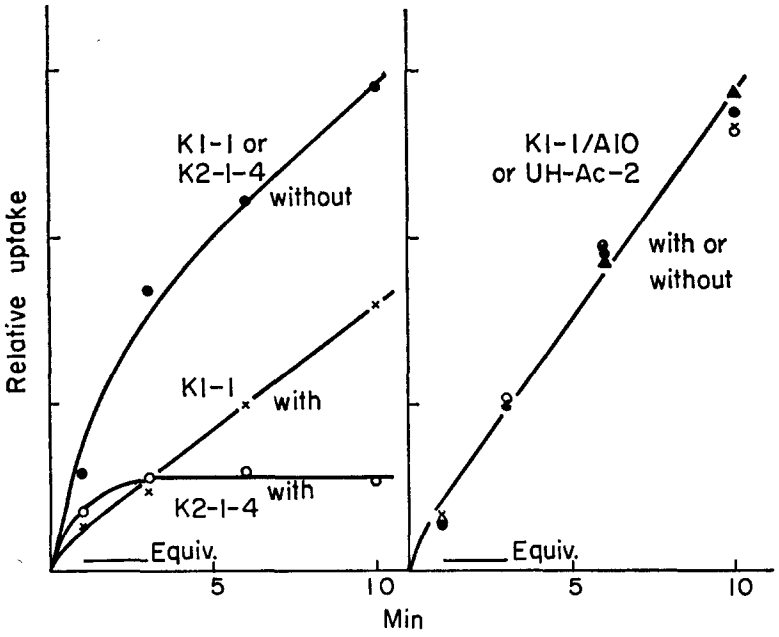

FIGURE 1. Effect of pyruvate on α-methylglucoside uptake.

TABLE I

PHOSPHORYLATION OF α-Me-G BY PEP AND KUNDIG-ROSEMAN SYSTEM FROM *E. COLI* K1-1

Complete system (α-Me-G, PEP,Mg^{++}, I + II, HPr)	100
HPr omitted	6
PEP omitted	26
Pyruvate added	93
Acetyl CoA added	218

ments, pyruvate was without observable effect. As we did *not* expect, however, when acetyl CoA was added a marked stimulation was observed, as can be seen in the last line of Table I. This is a stimulation of phosphorylation and may or may not be directly relatable to stimulation or inhibition of active transport or uptake in its broadest sense. Several explanations may be possible for the seemingly contradictory results in Fig. 1 and Table I. One could be that, in the intact cells, the internal milieu causes acetyl CoA to have different effects from those it has in a more

solubilized system. For example, acetyl CoA might stimulate phosphorylation, but the phosphorylated form of α-methylglucoside might then remain bound to the carrier, thus inhibiting the continuing uptake. Another might be that, as a result of partial purification or dissociation from the cell membranes the enzymes (I and II) may have undergone conformational changes thus subtly altering their properties. I am sure Dr. Roseman may be able to suggest other possibilities.

Regardless of the explanation, these results may serve to illustrate a central theme of this meeting, namely, the importance of structure and organization with respect to our understanding of biological function.

Dr. Roseman: The point that we discussed previously may be involved here. That is, is it possible that acetyl Coenzyme A can act as a detergent? If so, then perhaps the increased rate of the reaction merely reflects a detergent-like action of acetyl CoA. I should emphasize that our Enzyme II preparations closely resemble the vesicles studied by Dr. Kaback in his work on uptake of sugars and amino acids by membrane vesicles. Thus, anything that will make these vesicles more permeable to Enzyme I, HPr, and all the other ingredients used for assaying phosphorylation, could well give an increased rate. In this connection, I do not believe that you have established which of the protein components is really the rate-limiting component in the phosphorylation reaction, and if not, then it would be quite difficult to interpret these experiments.

Dr. Tedeschi: It seems to me that there may be a problem in postulating a general role of a phosphotransferase reaction in transport. The proposed mechanism might not explain the counterflow phenomenon which occurs in bacterial systems as well as in other systems.

Dr. Roseman: The question you ask relates to the exit process, and to counter-flow. We have not really studied these phenomena, and do not know whether or not such processes are catalyzed by the phosphotransferase system alone, or whether they involve other systems. For example, in the studies of Egan and Morse with *Staphylococcus aureus*, it was shown that essentially all sugars that were studied were accumulated principally or primarily as derivatives. Recently, these derivatives have been identified as the sugar phosphate esters. Now, in the original experiments, when cells which had accumulated one sugar as its phosphate ester were incubated with a different sugar, the sugar phosphate was lost from the cell, in exchange for the new external sugar which entered the cell. Thus, in this type of counter-flow experiment, an external sugar enters the cell at the expense of a different internal sugar phosphate, and presumably the external sugar is also accumulated by the cell as a phosphate ester. Similar results were, I believe, obtained by Winkler using methyl α-glucoside in *E. coli*. Is the phosphotransferase system involved in this process? We do not know. It would be entirely feasible, according to the simple model that I have presented, if an Enzyme II could act as a phosphotransferase, where the internal sugar phosphate was the phosphate donor, while the external sugar was the phosphate acceptor. During the transferase reaction, the external sugar would enter the cell concomitant with phosphorylation, while the internal sugar phosphate would leave the cell, concomitant with dephosphorylation. It is of course equally possible that other proteins could catalyze this type of process, and it is of interest to note that sugar phosphatases have been reported in bacteria that are specific for sugar phos-

phates, and that act as phosphotransferases in the presence of other sugars. Also, there have been reports on membrane-bound sugar phosphatases in *E. coli*.

Dr. B. L. Horecker (Albert Einstein College of Medicine, Bronx, N. Y.): I wonder if you can reconcile Heppel's galactose- and glucose-binding protein, and the fact that he loses an essential component of glucose transport in his shocked cells, which in your model implies HPr?

Dr. Roseman: Before I attempt to answer this question, I should first like to ask whether the binding proteins that have thus far been described have clearly been shown to play a role in transport? For this purpose, it seems to me that studies with mutants would provide the best answer. That is, for example, are mutants lacking the binding proteins always defective in transport of the corresponding solute? I wonder if Dr. Boos would like to make some comments along these lines?

Dr. Winfried Boos (Massachusetts General Hospital, Boston, Mass.): I can say two things. First: The binding specificity of the galactose-binding protein is identical with the specificity of uptake of the so-called β-methylgalactoside permease. Sugars which inhibit the galactose uptake by the β-methylgalactoside permease also inhibit the galactose-binding activity of the binding protein. Second: Permease positive and negative strains of *E. coli* contain the binding protein with the same binding activity. Therefore if the galactose-binding protein is involved in galactose transport by the β-methylgalactoside permease it cannot be the only component necessary for the overall process of transport.

Dr. Roseman: In connection with the binding proteins, I would like to add one comment. It is perfectly possible that the sugar-binding protein is a component of the phosphotransferase system. As you will recall, Enzyme II has been resolved into two protein components A and B. Further fractionation of A gave three separate protein fractions, each being specific for a particular sugar. Protein B was not sugar-specific, but was required by all sugars phosphorylated by the system. Thus it is at least conceivable that the galactose-binding protein is one of the A proteins, but this question remains to be solved.

Dr. Werner Kundig (Johns Hopkins University, Baltimore, Md.): I would like to comment on one of the questions raised by Dr. Horecker concerning the shock experiments reported by Dr. Heppel. As you will recall, when the cells were shocked, transport was reduced from 2.90 to 1.2. When the purified binding protein was added to the cells, the ability to transport was only stimulated slightly; i.e., back to 1.7. However, full transport ability was restored by adding back the 55–65% ammonium sulfate fraction of the shock fluid. Now this fraction does not contain the binding protein, but we believe that it does contain HPr. If it is HPr that is restoring the ability of the cells to transport, then I would add that these experiments confirm the observation that we reported several years ago.