*TWO CLASSES OF PLEIOTROPIC MUTANTS OF AEROBACTER AEROGENES LACKING COMPONENTS OF A PHOSPHOENOLPYRUVATE-DEPENDENT PHOSPHOTRANSFERASE SYSTEM**

By Shuji Tanaka and E. C. C. Lin†

DEPARTMENT OF BIOLOGICAL CHEMISTRY, HARVARD MEDICAL SCHOOL, BOSTON, MASSACHUSETTS

Communicated by Herman M. Kalckar, February 16, 1967

During a study of the phosphorylation of N-acyl-D-mannosamine by extracts of *Escherichia coli* and other bacteria, Kundig, Ghosh, and Roseman[1] discovered a type of phosphotransferase system consisting of three protein components acting according to the following scheme:

$$\text{PEP} + \text{HPr} \underset{\longleftarrow}{\overset{\text{enzyme I}}{\longrightarrow}} \text{P-HPr} + \text{pyruvate} \qquad (A)$$

$$\text{P-HPr} + \text{sugar} \xrightarrow{\text{enzyme II}} \text{sugar-P} + \text{HPr}, \qquad (B)$$

where PEP denotes phosphoenolpyruvate; HPr, a small heat-stable protein containing histidine; and P-HPr, the phosphorylated protein.

Their data indicated that enzyme I and HPr have broad functions serving a family of enzymes II, each specific for one or a few sugars.[1, 2]

We have recently demonstrated that the dissimilation of D-mannitol by *Aerobacter aerogenes* depends upon a phosphotransferase system similar to the one described above. In the absence of an inducible enzyme II specific for D-mannitol (referred to as "enzyme II" in this work), *A. aerogenes* fails to grow on this compound.[3]

In the present report some of the phenotypic consequences of mutations affecting enzyme I or HPr are described. In contradistinction to the loss of enzyme II, which renders the cell incapable of utilizing a particular compound, the lack of enzyme I or HPr pleiotropically affects the metabolism of a number of polyhydric alcohols and sugars.

Materials and Methods.—Bacteria: Strain 5P14, a double auxotroph of *A. aerogenes* 1033 (ref. 4), provided by B. Magasanik, was used as the starting cell line. Its strict requirement for arginine and guanine permitted distinction of mutant progeny from contaminating organisms. For our convenience this strain has been called strain 2002 (ref. 3) and will be referred to as such in this communication. Strain 2006, a mutant lacking enzyme II, hence unable to grow on D-mannitol, was obtained from strain 2002 (ref. 3). Strain 2050, lacking enzyme I, and strain 2070, lacking HPr, were both isolated as D-mannitol-negative clones from populations of strain 2002 treated with ethyl methanesulfonate.[5] MacConkey agar in which D-mannitol was substituted for lactose[6] was used for the detection of these mutants. Strain 2051, a spontaneous revertant exhibiting normal growth rate on agar with D-mannose as carbon source, was derived from a population of cells of strain 2050 recycled in liquid medium with the same carbon source. Strain 2071 was isolated as a spontaneous revertant by directly plating 10^9 cells of strain 2070 on agar with D-sorbitol as carbon source.

Chemicals: 1-C^{14}-D-mannitol (25 c/mole) was obtained from Nuclear-Chicago Corp.; D-mannitol, L-arabinose, D-mannose, and D-fructose from Eastman Kodak Co.; glycerol and maltose from Fisher Scientific Co.; D-arabitol, ribitol, D-sorbitol, D-ribose, and D-galactose from Pfanstiehl Chemical Co.; i-inositol, lactose, and acid-hydrolyzed casein from Nutritional Biochemicals Corp.; D-glucose from Merck and Co.; and PEP (sodium salt) from Calbiochem.

Growth of cells and preparation of components for enzyme assays: Growth rates were determined at 37° in 50 ml of mineral medium with 0.2% carbon source and 4×10^{-4} *M* each of arginine and guanine. The cultures were incubated in 300-ml nephelometric flasks on a rotary shaker at about 240 cycles per min. Growth was monitored by turbidity readings as before.[3] The composition of the mineral medium has also been described.[3] For assays of enzyme I, enzyme II, and HPr or for the preparation of these components for use as reagents in the assay, cells were grown to stationary phase in 250 ml of media in 2-liter Erlenmeyer flasks.

The assay of each component of the PEP-dependent phosphotransferase system was based upon the phosphorylation of labeled D-mannitol, as described below, in a reaction mixture containing the remaining two components in excess.

To obtain "enzyme II + HPr," cells from a 250-ml culture of strain 2002 fully grown on 0.2% D-mannitol were suspended in 2.5 ml of 0.1 *M* Tris-HCl buffer, pH 7.6, and disrupted by ultrasonic treatment for 2 min in a model 60 W MSE apparatus (Measuring and Scientific Equipment Ltd., London, England) while being chilled in a −10° bath. After centrifugation at 10,000 × *g* for 20 min at 0° to remove unbroken cells and fragments, the supernatant fraction was centrifuged at 100,000 × *g* for 2 hr at 0°. The pellet, containing about 70% of the enzyme II activity in the original extract, was resuspended in 2.5 ml of Tris-HCl buffer. The 100,000 × *g* supernatant fraction was heated to 100° for 10 min and clarified by centrifugation at 35,000 × *g* for 10 min. This solution retained almost full activity of HPr and was combined with the suspension of enzyme II. The mixture contained no detectable enzyme I activity.

The procedure for obtaining "enzyme I + HPr" has already been described.[3] Cells of strain 2006 grown on 2% antibiotic medium 3 (Difco) supplemented with 1.5% D-glucose were used as the starting material. The extract contained no enzyme II activity.

To prepare "enzyme I + enzyme II," cells of strain 2002 fully grown on 0.2% D-mannitol were suspended in 2.5 ml of Tris-HCl buffer, and disrupted by sonication as above. The extract was centrifuged at 10,000 × *g* for 20 min at 0°. The supernatant fraction was treated with 250 mg of Norit A for 30 min at 0°. The supernatant fraction after centrifugation contained less than 0.5% of the HPr activity originally present in the crude extract.

All three preparations could be stored for several weeks at −20° without significant loss of activity.

Enzyme assays: All enzyme activities are expressed as mμmoles of D-mannitol phosphorylated per min per mg protein at 25°. All the reactions were carried out in the presence of 5×10^{-5} *M* C^{14}-D-mannitol (2.5 c/mole), 5×10^{-3} *M* PEP, 5×10^{-5} *M* $MgCl_2$, and 0.04 *M* Tris-HCl (pH 7.6). For measuring enzyme I activity, 0.1 ml of "enzyme II + HPr" was added; for measuring enzyme II activity, 0.1 ml of "enzyme I + HPr" was added; and for measuring HPr activity, 0.1 ml of "enzyme I + enzyme II" was added. An appropriate amount of the crude extract to be assayed was added to initiate the reaction. In each case the final volume of the mixture was 0.4 ml. The reaction was allowed to proceed for 10 min after which further phosphorylation of the labeled substrate was arrested by the addition of 0.6 ml of 0.2 *M* unlabeled D-mannitol. The labeled D-mannitol phosphate was separated from the free hexitol by passing the mixture through a Dowex-1 formate column. D-mannitol phosphate was eluted and counted in a planchet as previously described.[3]

Protein concentrations were measured with the biuret reaction.[7]

Assay of C^{14}-D-mannitol uptake: Cells grown overnight in 50 ml of medium with 1% casein hydrolysate as carbon source were diluted 100-fold with fresh medium containing 1% casein hydrolysate in the presence or absence of 2% D-mannitol. After reaching 100 Klett units, the cells were washed twice and resuspended at the same density in mineral medium at 0°. Two-tenths ml of this suspension was added to a test tube, equilibrated at 25°, containing 0.1 ml of 5×10^{-5} *M* C^{14}-D-mannitol (25 c/mole), 0.1 ml of chloramphenicol (400 μg/ml), and 0.6 ml of mineral medium. After 30 seconds the cells were quantitatively collected on a Millipore filter, washed, and counted.[8] Under the conditions used, the rate of D-mannitol uptake was linear for at least 1 min. Cells metabolically poisoned with 0.03 *M* azide and 0.001 *M* iodoacetate[9] for 1 hr at 25° immediately prior to the assay served as controls.

Results.—Strains 2050 and 2070, originally isolated as D-mannitol nonfermenting mutants, exhibited multiple impairments of growth when tested on agar plates

TABLE 1

GROWTH PATTERNS OF PARENTAL, MUTANT, AND REVERTANT STRAINS ON VARIOUS CARBOHYDRATES

Carbon Source	Strains				
	2002 (Parental)	2050 (Mutant)	2051 (Revertant)	2070 (Mutant)	2071 (Revertant)
Glycerol	++	+	++	++	++
D-arabitol	++	++	++	++	++
Ribitol	++	++	++	++	++
D-mannitol	++	0	++	0	++
D-sorbitol	++	0	++	0	++
i-Inositol	++	++	++	++	++
L-arabinose	++	++	++	++	++
D-ribose	++	++	++	++	++
D-glucose	++	+	++	+	++
D-mannose	++	+	++	+	++
D-galactose	++	++	++	++	++
D-fructose	++	0	++	+	++
Lactose	++	++	++	++	++
Maltose	++	+	++	++	++

Growth was tested on agar plates. Normal growth rate of colonies is denoted by ++, slow growth by +, and no growth by 0.

(Table 1). Strain 2050 not only failed to grow on D-mannitol, but also on D-sorbitol and D-fructose. Its rates of growth on glycerol, D-glucose, D-mannose, and maltose were considerably slower than those of the parental strain 2002. A similar pattern was exhibited by strain 2070, except that it grew normally on glycerol and slightly on D-fructose.

Table 2 gives the doubling times of the parental and the two mutant strains with D-mannitol, D-glucose, and D-arabitol as sole carbon source in liquid medium.

To test whether the multiple impairments in the two mutants were phenotypic expressions of single mutations, a *spontaneous* revertant was obtained from each mutant by selection on a single carbon source. As shown in Tables 1 and 2, strain 2051, selected from strain 2050 on D-mannose, concomitantly regained all the other growth abilities. Similarly, full restoration of the growth properties was observed in strain 2071, selected from strain 2070 for growth on D-sorbitol.

The wild-type, mutant, and revertant strains were grown on casein hydrolysate in the presence or absence of D-mannitol and their cell-free extracts were analyzed for enzyme I, enzyme II, and HPr under conditions in which their activities were proportional to concentration. (See Fig. 1*A* for assay of enzyme I, Fig. 1*B* for assay of HPr, and ref. 3 for assay of enzyme II.) (The slight deviation of enzyme I activity from linearity at very low concentrations suggests the requirement of another factor in the phosphotransferase system or dissociation of enzyme I at high dilution. Table 3 shows that the activity of enzyme I is absent in strain 2050 and the activity of HPr is absent in strain 2070. The missing activity in each case is restored in the revertant.

TABLE 2

DOUBLING TIMES OF PARENTAL, MUTANT, AND REVERTANT STRAINS ON THREE DIFFERENT CARBOHYDRATES

Carbon source	Strains				
	2002 (Parental)	2050 (Mutant)	2051 (Revertant)	2070 (Mutant)	2071 (Revertant)
D-mannitol	52	n.g*	54	n.g.*	49
D-glucose	45	132†	54	160†	54
D-arabitol	47	54	50	65	55

All cells were pregrown on casein hydrolysate and transferred to fresh medium containing the new carbon source at a level of 0.2%. Doubling time is expressed in minutes.

* No growth.

† Slow growth commenced after a lag period of 3–4 hr instead of the usual lag time of about 20 min.

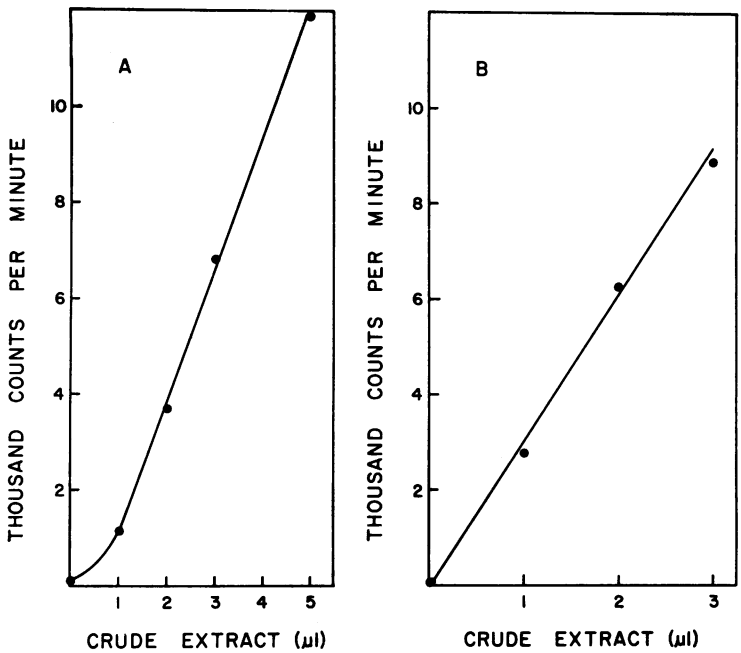

FIG. 1.—(*A*) Activity versus concentration of enzyme I. (*B*) Activity versus concentration of HPr (in this case a significant blank value of 1500 cpm has been subtracted).

Table 3 indicates that the presence of D-mannitol stimulated the production of enzymes I and II. The effect on enzyme I is not specific; addition of glycerol, D-arabitol, D-sorbitol, D-glucose, D-fructose, or D-mannose to the casein hydrolysate medium also resulted in similar elevations (data not shown). The induction of enzyme II by D-mannitol is highly specific. Glycerol, D-arabitol, D-sorbitol, D-glucose, D-fructose, or D-mannose in the growth medium caused no appreciable increase in the specific activity of this enzyme (data not shown). There was also apparently a slight stimulation of HPr formation by D-mannitol. Slight stimulation of HPr formation by other polyhydric alcohols and sugars has likewise been observed.

Table 3 also shows that D-mannitol was able to induce enzyme II in strain 2050 (with no measurable enzyme I activity) and in strain 2070 (with no measurable HPr activity). Moreover, the lack of HPr activity did not prevent the hexitol from

TABLE 3

SPECIFIC ACTIVITIES OF ENZYME I, ENZYME II, AND HPr IN PARENTAL, MUTANT, AND REVERTANT STRAINS

Strain	Growth on Casein Hydrolysate			Grown on Casein Hydrolysate Plus D-Mannitol		
	Enzyme I	Enzyme II	HPr	Enzyme I	Enzyme II	HPr
2002 (Parental)	3.4	0.2	8.2	14	7.4	13
2050 (Mutant)	0.0	0.3	6.6	0.0	4.8	8.8
2051 (Revertant)	2.5	0.1	7.5	12	4.7	9.7
2070 (Mutant)	4.8	0.2	0.0	14	13	0.0
2071 (Revertant)	2.7	0.2	8.2	12	6.5	14

Casein hydrolysate was added at a level of 1% and D-mannitol, when used, at 2%. All enzyme activities are expressed as mμmoles of D-mannitol phosphorylated per min per mg protein at 25°.

TABLE 4

ACCUMULATION OF RADIOACTIVE MATERIAL BY CELLS INCUBATED WITH C^{14}-D-MANNITOL

Strain	Grown on casein hydrolysate (cpm)	Grown on casein hydrolysate plus D-mannitol (cpm)
2002 (Parental)	822	5534
2050 (Mutant)	183	391
2051 (Revertant)	930	5304
2070 (Mutant)	14	80
2071 (Revertant)	889	6214

The incubation conditions were as in *Materials and Methods*. The counts retained by poisoned cells (21 cpm) have been subtracted from each value.

influencing the level of enzyme I in strain 2070. Hence it must be concluded that the mutant cells were sufficiently permeable to D-mannitol for induction to occur.

The question arises as to whether there is a specific transport mechanism which permits the cell to bring D-mannitol into its cytoplasm against a concentration gradient. If so, one would expect cells of strain 2050 and 2070 to retain their potential for accumulating free D-mannitol, although they can no longer phosphorylate it. Cells of parental, mutant, and revertant strains, grown in the presence and absence of the inducer, were tested for their ability to take up radioactive material when incubated with C^{14}-D-mannitol. The results show that substantial retention of labeled material (presumably as D-mannitol 6-phosphate and its metabolites) occurred only in the parental and revertant cells, and was most marked when they were induced (Table 4). The accumulation process was severely curtailed, although not entirely abolished, in the mutant cells. The fact that the uptake in the mutant cells could also be increased several-fold by induction suggests that the mutations affecting enzyme I and HPr were actually slightly leaky and that the residual function could be more readily detected by the highly sensitive *in vivo* experiment. In any event, the large disparities between the wild-type and mutant cells in the ability to accumulate radioactive substances, *with or without induction*, strongly imply that phosphorylation of D-mannitol is indispensable for the retention and that there is no mechanism for concentrating the *free hexitol* against a gradient.

Discussion.—The pleiotropic growth defects associated with the loss of enzyme I and HPr activities establish the importance of this phosphotransferase system for the utilization of not only D-mannitol, but also of D-sorbitol, D-glucose, D-mannose, and D-fructose. The residual capacity of these mutants for slow growth on D-glucose and D-mannose is probably due to the function of minor alternative pathways in this organism.[10-13] Inasmuch as the enzyme I and HPr mutants reported here were both isolated on the basis of D-mannitol fermentation, the possibility cannot be excluded that there exists more than one kind of enzyme I-HPr system in the same organism and that each system governs the metabolism of a different family of carbohydrates. Pleiotropic mutations affecting the utilization of carbohydrates in other species of bacteria, e.g., *E. coli*[14-16] and *Staphylococcus aureus*[17-19] have been reported before. It would be interesting to examine whether any of them lack a component of an enzyme I-HPr system. (In collaboration with D. G. Fraenkel we have just shown that the *E. coli* mutant MM6 (ref. 16) lacks enzyme I. A more detailed account of this finding will be given elsewhere.)

Kundig and co-workers deduced, from the retention of sugar analogues in osmotically shocked cells, that the PEP-dependent phosphotransferase system plays

a direct role in carbohydrate transport.[2] The present study provides genetic evidence of the operation of the phosphotransferase system in the accumulation of certain sugars. The transport of D-mannitol was amenable to analysis because its first phosphorylated product is directly utilized by a specific dehydrogenase.[20, 3] Hence retention of molecules, as distinguished from their passage into the cells,[21-25] is associated with the first step of dissimilation. In such a situation a separate active transport system for the free hexitol would not be required and is in fact not found.

The entry of the free hexitol may be facilitated by a protein, possibly by enzyme II itself. The process of D-mannitol uptake is thus analogous to the capture of glycerol by *E. coli*, except that glycerol is believed to diffuse freely[26] and that glycerol kinase utilizes ATP as the phosphoryl donor.[27] The presence of a specific protein mediating the equilibration of D-mannitol could be tested by the counterflow method successfully used in the study of the lactose permeation system of *E. coli* by Winkler and Wilson.[9] If such a protein exists, its relationship to enzyme II could be examined by the use of temperature-sensitive mutants.

The basic mechanism for D-mannitol uptake has the virtue that a single high-energy phosphate bond is expended for both capture and activation of a substrate molecule. The model proposed by Kundig and collaborators as a process of transport requires phosphorylation and immediate dephosphorylation.[2] Such a model would therefore demand the demonstration of a highly specific phosphatase inside the cell.

Finally it should be pointed out that some sugars may be accumulated in cells by mechanisms different from those discussed here, i.e., without phosphorylation. For example, the transport system for D-galactose also acts on D-fucose,[28] which cannot be phosphorylated at carbon six.

Summary.—Two classes of pleiotropic mutants of *Aerobacter aerogenes* with multiple defects in sugar and hexitol utilization have been isolated. One mutant lacks the enzyme I and another lacks the histidine protein (HPr) of the phosphoenolpyruvate-dependent phosphotransferase system. Both mutants fail to grow on D-mannitol and D-sorbitol and grow slowly or very poorly on D-glucose, D-mannose, D-fructose, or maltose. Neither mutant is able to accumulate significant amounts of radioactive material when incubated with C^{14}-D-mannitol, even after growth in the presence of 2 per cent D-mannitol. A spontaneous revertant selected on a single carbon source was isolated from each mutant. All the other pleiotropic defects were corrected in the revertant. A model for the role of this phosphotransferase in active transport is proposed.

We are grateful to T. H. Wilson, H. M. Kalckar, and J. B. Alpers for helpful comments during the preparation of this manuscript.

* This work was aided by the National Science Foundation (GB-5854) and the U.S. Public Health Service (GM-11983).

† Supported by a Research Career Development Award from the U.S. Public Health Service.

[1] Kundig, W., S. Ghosh, and S. Roseman, these PROCEEDINGS, **52**, 1067 (1964).

[2] Kundig, W., F. D. Kundig, B. Anderson, and S. Roseman, *J. Biol. Chem.*, **241**, 3243 (1966).

[3] Tanaka, S., S. A. Lerner, and E. C. C. Lin, *J. Bacteriol.*, **93**, 642 (1967).

[4] Magasanik, B., M. S. Brooke, and D. Karibian, *J. Bacteriol.*, **66**, 611 (1953).

[5] Lin, E. C. C., S. A. Lerner, and S. E. Jorgensen, *Biochim. Biophys. Acta*, **60**, 422 (1962).

[6] Cozzarelli, N. R., and E. C. C. Lin, *J. Bacteriol.*, **91**, 1763 (1966).
[7] Gornall, A. G., C. J. Bardawill, and M. M. David, *J. Biol. Chem.*, **171**, 751 (1949).
[8] Hayashi, S., J. P. Koch, and E. C. C. Lin, *J. Biol. Chem.*, **239**, 3098 (1964).
[9] Winkler, H. H., and T. H. Wilson, *J. Biol. Chem.*, **241**, 2200 (1966).
[10] Magasanik, A. K., and A. Bojarska, *Biochem. Biophys. Res. Commun.*, **2**, 77 (1960).
[11] Kamel, M. Y., and R. L. Anderson, *J. Biol. Chem.*, **239**, PC3607 (1964).
[12] Kamel, M. Y., and R. L. Anderson, *J. Bacteriol.*, **92**, 1689 (1966).
[13] Kamel, M. Y., D. P. Allison, and R. L. Anderson, *J. Biol. Chem.*, **241**, 690 (1966).
[14] Doudoroff, M., W. Z. Hassid, E. W. Putman, A. L. Potter, and J. Lederberg, *J. Biol. Chem.* **179**, 921 (1949).
[15] Asensio, C., G. Avigad, and B. L. Horecker, *Arch. Biochem. Biophys.*, **103**, 299 (1963).
[16] Fraenkel, D. G., F. Falcoz-Kelly, and B. L. Horecker, these PROCEEDINGS, **52**, 1207 (1964).
[17] Egan, J. B., and M. L. Morse, *Biochim. Biophys. Acta*, **97**, 310 (1965).
[18] *Ibid.*, **109**, 172 (1965).
[19] *Ibid.*, **112**, 63 (1966).
[20] Liss, M., S. B. Horwitz, and N. O. Kaplan, *J. Biol. Chem.*, **237**, 1342 (1962).
[21] Cohen, G. N., and J. Monod, *Bacteriol. Rev.*, **21**, 169 (1957).
[22] Davis, B. D., *Arch. Biochem. Biophys.*, **78**, 497 (1958).
[23] Horecker, B. L., M. J. Osborn, W. L. McLellan, G. Avigad, and C. Asensio, in *Membrane Transport and Metabolism*, ed. A. Kleinzeller and A. Kotyk (New York: Academic Press, Inc., 1960), p. 378.
[24] Koch, A. L., *Biochim. Biophys. Acta*, **79**, 177 (1964).
[25] Fox, C. F., and E. P. Kennedy, these PROCEEDINGS, **54**, 891 (1965).
[26] Hayashi, S., and E. C. C. Lin, *Biochim. Biophys. Acta*, **94**, 479 (1965).
[27] Hayashi, S., and E. C. C. Lin, *J. Biol. Chem.*, **242**, 1030 (1967).
[28] Ganesan, A. K., and B. Rotman, *J. Mol. Biol.*, **16**, 42 (1966).