*PHOSPHOENOLPYRUVATE-DEPENDENT FORMATION OF D-FRUCTOSE 1-PHOSPHATE BY A FOUR-COMPONENT PHOSPHOTRANSFERASE SYSTEM**

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Fructose[1] is metabolized in *Aerobacter aerogenes* primarily through the pathway: fructose → fructose-1-P → fructose-1,6-P. Previous evidence for this pathway was the detection of an inducible fructose-1-P kinase[2, 3] and the demonstration that a mutant lacking frustose-6-P kinase grew well on fructose but not on glucose, whereas a mutant lacking fructose 1,6-diphosphatase grew on glucose but not on fructose.[3] How widespread the occurrence of this new pathway is has not yet been assessed; however, the pathway has recently been demonstrated in *Escherichia coli*,[4] and fructose-1-P kinase occurs in *Bacteroides symbiosis*.[5]

In the present communication, we report on the initial reaction in the fructose-1-P pathway. We show that in *A. aerogenes* fructose is phosphorylated with PEP[1] specifically at carbon atom 1 by a phosphotransferase system of the type originally described by Kundig *et al.*[6] for other sugars and which has subsequently been implicated in the transport of sugars into bacterial cells as well as in their phosphorylation.[7–15]

$$\text{PEP} + \text{HPr} \xrightarrow{\text{Enzyme I}} \text{Pyruvate} + \text{Phospho-HPr} \qquad (1)$$

$$\text{Sugar} + \text{Phospho-HPr} \xrightarrow{\text{Enzyme II}} \text{Sugar phosphate} + \text{HPr} \qquad (2)$$

We have resolved the phosphorylation system for fructose into four components: enzyme I, HPr, and two components required for enzyme II activity.[1] The enzyme II components are a high-molecular-weight protein and an inducible specifier protein which manifests itself in crude systems as a factor (i.e., "K_m factor") that increases the affinity of enzyme II for fructose. A mutant strain lacking the specifier protein for fructose exhibits defective growth on fructose but grows normally on other substrates.

Materials and Methods.—Preparation of bacterial extracts: *A. aerogenes* PRL-R3 was grown on a mineral medium plus 0.5% carbohydrate or on nutrient broth.[2, 3] Cells in the early stationary phase were harvested by centrifugation at 4°C, washed in cold 0.85% NaCl, and stored frozen. Unless stated otherwise, further operations were carried out at 0–5°C. Frozen cells were suspended in 0.1 *M* Tris-HCl[1] (pH 7.6) containing 0.2% mercaptoethanol (2 ml per gram of wet cells), broken by sonication,[3] and centrifuged at 40,000 × *g* for 20 min to yield a supernatant (the crude extract) which contained 30–40 mg/ml protein.[16]

Assay for PEP-dependent conversion of fructose to fructose-1-P (fructose assay): The incubation mixture for fructose-1-P formation contained (in a volume of 0.2 ml): the components of the PEP-fructose 1-phosphotransferase system, 0.05 mM $MgCl_2$, 6.0 mM PEP, 1.0–200 mM fructose,[17] and 40 mM Tris-HCl (pH 7.6). The mixture was incubated at 25°C for 10 min after which the reaction was stopped by heating at 100°C for 6 min. The precipitate was removed by centrifugation, and the supernatant was assayed for fructose-1-P with an end point assay based on the assay for fructose-1-P kinase.[2] The amount of enzyme II, enzyme I, and HPr used in the incubation mixtures was such that

the rate was linearly proportional to the component being assayed and also such that doubling the concentrations of the other two components did not significantly change the rate.

Assay for PEP-dependent conversion of mannitol to mannitol-1-P (mannitol assay): The assay cuvette contained (in a volume of 0.15 ml): the components of the PEP:mannitol 1-phosphotransferase system, 133 mM glycylglycine (pH 7.5), 0.667 mM NADP, excess glucose 6-phosphate dehydrogenase, excess phosphoglucose isomerase, excess mannitol 1-phosphate dehydrogenase[18] 0.33 mM NAD, 6.67 mM $MgCl_2$, 3.33 mM PEP, and 3.33 mM mannitol. The reaction was monitored at 340 mμ at 25°C. The criteria for establishing the appropriate amount of each component of the phosphotransferase system were the same as used in the fructose assay.

Purification of phosphotransferase system components: Sixty ml of crude extract were chromatographed on a column (4.80 cm × 40.0 cm) containing 700 ml of Sephadex G-200 equilibrated with 0.04 *M* Tris-HCl (pH 7.6) in 0.2% mercaptoethanol. The enzyme I fractions from the Sephadex columns were pooled and concentrated tenfold by ultrafiltration with an Amicon ultrafilter (type UM-1 10,000 mol wt cutoff), and the same was done for the HPr fractions. The locations on the elution profiles from which the pooled fractions were taken are indicated in the legend of Figure 1.

Results.—PEP-dependent phosphotransferase systems in crude extracts: The kinetic parameters for PEP-dependent phosphorylation of fructose (Table 1) are

TABLE 1. *Kinetic parameters of PEP-dependent fructose-1-P formation.*

Growth substrate	V_{max} (μmoles/min/mg)	K_m (mM)
Fructose	0.0103	1.0 or less[17]
Mannitol	0.0092	40–60
Glucose	0.0063	20–70
Glycerol	0.0069	25
Nutrient broth	0.0066	80

V_{max} and K_m values were determined with 0.04 ml of crude extract in the fructose-1-P formation assay. In cases of multiple determinations the average V_{max} and the range of K_m values are given. K_m and V_{max} values were obtained from Lineweaver-Burk plots (also for Tables 2 and 3).

a direct reflection of enzyme II properties, since enzyme II was the rate-limiting component in crude extracts under the assay conditions used. Addition of enzyme I (*M*) and HPr (*M*)[1] had no effect on the rates obtained with these assays, whereas addition of enzyme II (*M* or *F*) gave a linear increase in rate up to about three times the unsupplemented rate. Omission of either PEP or fructose resulted in negligible fructose-1-P formation. Distinction of the product from fructose-6-P was made possible by the specificity[2] of the fructose-1-P kinase. The data in Table 1 indicate that there is a constitutive enzyme II activity for fructose with a K_m of 20–80 mM. However, in fructose-induced cells, this constitutive, high K_m enzyme II activity is not present and a new enzyme II activity with a K_m for fructose of 1 mM or less is found.

Sephadex chromatography of crude extracts: To facilitate analysis and to provide a comparison of the constitutive PEP: fructose 1-phosphotransferase system with the inducible one, a crude extract of mannitol-grown cells as well as fructose-grown cells was chromatographed. Chromatography of crude extracts on Sephadex G-200 (Fig. 1) gave a separation of all three previously established components of PEP-dependent phosphotransferase systems. Omission of any one of the three peak fractions from either the mannitol or fructose assays re-

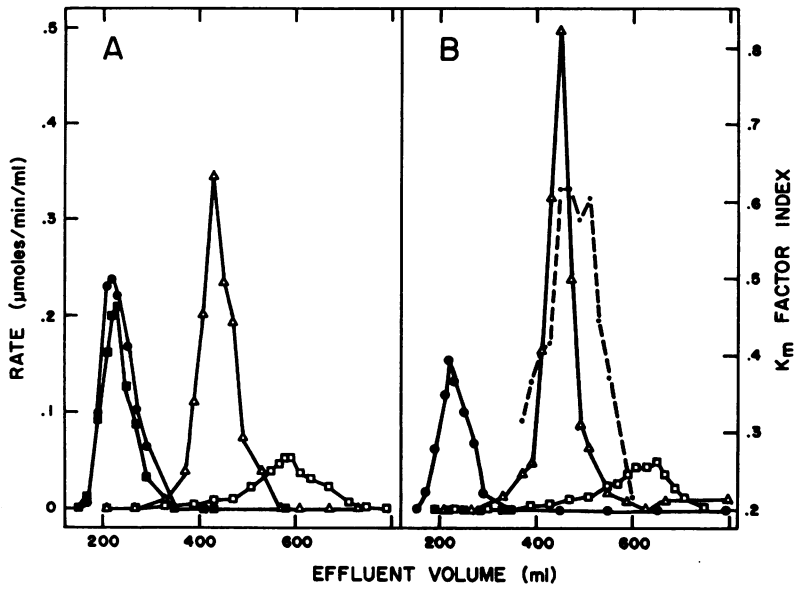

FIG. 1.—Chromatography of crude extracts on Sephadex G-200.

(*A*) *Extract of mannitol-grown cells.* Preliminary recombining of fractions established the approximate location of the phosphotransferase system components. The final assays shown in the figure were as described in *Materials and Methods.* The types of assays used and the source of coupling components were: (1) fructose assay for enzyme II(●)—0.02 ml enzyme I (*M*) 10×, 0.02 ml HPr (*M*) 10×, 0.2 *M* fructose; (2) mannitol assay for enzyme II (■)—0.005 ml enzyme I (*M*) 10×, 0.01 ml HPr (*M*) 10×; (3) mannitol assay for enzyme I (△)—0.005 ml enzyme II (*M*), 0.01 ml HPr (*M*) 10×; (4) mannitol assay for HPr (□)—0.005 ml enzyme II (*M*), 0.005 ml enzyme I (*M*) 10×.

The effluent volume ranges used for component pools and the notation used for them in the text are: enzyme II (*M*) 210–240 ml, enzyme I (*M*) 380–490 ml, HPr (*M*) 510–670 ml. Pooled fractions concentrated tenfold are designated "10×."

(*B*) *Extract of fructose-grown cells.* The types of assays used and the source of coupling components were: (1) fructose assay for enzyme II (●)—0.02 ml enzyme I (*M*) 10×, 0.02 ml HPr (*M*) 10×, 0.2 *M* fructose; (2) mannitol assay for enzyme II (■)—0.005 ml enzyme I (*M*) 10×, 0.01 ml HPr (*M*) 10×; (3) mannitol assay for enzyme I (△)—0.005 ml enzyme II (*M*), 0.01 ml HPr (*M*) 10×; (4) mannitol assay for HPr (□)—0.005 ml enzyme II (*M*), 0.005 ml enzyme I (*M*) 10×; (5) fructose assay for K_m factor (*dotted line*)—0.04 ml enzyme II (F), 0.02 ml enzyme I (*M*) 10×, 0.02 ml HPr (*M*) 10×, 0.1 ml of K_m factor fractions (final assay volume was 0.22 ml). The assay was run at 2.0 mM and 200 mM fructose and the ratio of 2.0 to 200 mM activity was taken as the K_m factor index value for each fraction assayed.

The effluent volume ranges used for component pools and the notation used for them are: enzyme II (*F*) 210–240 ml, enzyme I (*F*) 390–490 ml, HPr (*F*) 510–690 ml. K_m factor samples for other experiments were taken from unpooled portions of tubes from 450 to 500 ml.

sulted in 5 per cent or less of the activity of the complete systems. The relative molecular weights of the three components common to both the mannitol and fructose crude extracts suggest that the first peak is enzyme II, the second is enzyme I, and the third is HPr. Consistent with the assignment of the first peak as enzyme II, the substrate specificity of this fraction was determined by the inducer; when mannitol was the inducer, both mannitol and fructose were phosphorylated, whereas when fructose was the inducer, fructose was phosphorylated but mannitol was not. The identity of the third peak as HPr was

further substantiated by the observation that these fractions are heat-stable (10 min at 100°C gave no activity loss), which is characteristic of HPr proteins.[7]

Modification of enzyme II affinity for fructose: The affinity of enzyme II (*F*) for fructose (Table 2) was determined by the source of enzyme I and HPr used in the assay. With enzyme I and HPr fractions obtained from the fructose column, enzyme II (*F*) had a K_m for fructose of 1 mM or less, which is the same as that obtained using a crude extract of fructose-grown cells. When enzyme II (*F*) was assayed with enzyme I and HPr fractions from the mannitol column, the K_m for fructose was 67 mM, which is in the constitutive range. When enzyme II (*M*) was used, the K_m values were unchanged from those observed in the crude extract regardless of the source of enzyme I and HPr. The affinity of enzyme II

TABLE 2. *Kinetic parameters of enzyme II for fructose phosphorylation by Sephadex G-200 fractions.*

Phosphotransferase components	V_{max}*	K_m (mM)
Enzyme II (*F*), enzyme I (*F*), HPr (*F*)	0.32	1.0 or less
Enzyme II (*F*), enzyme I (*M*), HPr (*M*)	0.14	67
Enzyme II (*M*), enzyme I (*M*), HPr (*M*)	0.33	65
Enzyme II (*M*), enzyme I (*F*), HPr (*F*)	0.28	65

The assay for fructose-1-P formation is described in *Materials and Methods*. Aliquots of Sephadex-purified fractions were: 0.04 ml enzyme II (*F* or *M*), 0.02 ml tenfold concentrated enzyme I (*F* or *M*), 0.04 ml tenfold concentrated HPr (*F* or *M*)

* V_{max} values are expressed as μmoles per min per ml of enzyme II.

(*M*) for mannitol in the mannitol phosphorylation reaction was also unaffected by the source of enzyme I and HPr. The K_m remained at about 0.05 mM, a value similar to that reported by Tanaka *et al.*[19] for a crude extract of mannitol-grown cells.

Further testing of the enzyme I and HPr fractions indicated that the factor responsible for the K_m shift (the K_m factor) was located on the fructose G-200 column somewhat behind enzyme I. Testing the fractions in this region with an assay designed to detect the K_m shift gave the peak indicated by the dotted line in Figure 1*B*. In this assay, enzyme II (*F*) is coupled with enzyme I (*M*) and HPr (*M*) to give a high K_m fructose system. Addition of K_m factor causes a shift toward the low K_m type of system. The K_m factor index plotted in Figure 1*B* is a nonlinear function of the K_m which approaches 1 as the K_m approaches zero. A plot of K_m factor index versus volume of K_m factor fraction in the assay gives a roughly linear response for index values below 0.8. It can be seen in Figure 1*B* that the K_m factor does not coincide exactly with enzyme I. That the K_m factor is not an enzyme I was also shown by the fact that enzyme I is inactivated by air oxidation, whereas the factor is not. Furthermore, purification of enzyme I–K_m factor fractions on DEAE-cellulose results in loss of the K_m factor activity and recovery of enzyme I activity. These observations allow elimination of postulations concerning multiple enzyme I–HPr systems as the basis for the K_m shift.

It was found that mannitol inhibits the high K_m fructose reaction catalyzed by enzyme II (*M*). When 100 mM fructose was used, the rate was reduced to 30 per cent of the noninhibited value by 2.0 mM mannitol regardless of the source of

enzyme I and HPr fractions used. A fivefold increase in mannitol caused no further inhibition, indicating saturation at 2.0 mM mannitol. The *Ki* value for mannitol could not be determined due to limitations of the assay system.[17] Enzyme II (*F*) showed no inhibition by mannitol when enzyme I and HPr from either Sephadex column were used. The inhibition suggests that the fructose activity of enzyme II (*M*) is catalyzed for the most part by the same enzyme II which phosphorylates and is specifically induced by mannitol.

Activation of enzyme II (*F*) was consistently found to accompany the change in K_m when the K_m factor was present, as is shown by the changes in V_{max} (Tables 2 and 3). In the assay used for the K_m factor in Figure 1*B*, addition of the factor caused a twofold increase in V_{max} as well as a shift in K_m from the constitutive range to 1 mM or less. The possibility existed that some enzyme II-activating factor (without a direct effect on enzyme II affinity) could cause an artifactual K_m shift by raising the enzyme II activity to such an extent that the assay was limited by enzyme I or HPr. This possibility was excluded by confirming that the assays were linearly dependent on enzyme II concentration and were unaffected by doubling enzyme I and HPr levels, both with and without K_m factor.

Properties of mutant QQ17: Mutant QQ17 was isolated as a fructose-negative, glucose-positive mutant as previously described.[3] In liquid media it grew at about one-third the rate of PRL-R3 on 0.5 per cent D-fructose but grew normally on D-glucose, D-mannose, mannitol, and glycerol. From the enzyme assays summarized in Table 3, it is evident that QQ17 lacks the K_m factor. Crude extracts of QQ17 cells induced on fructose have the high K_m constitutive-type enzyme II activity for fructose. Addition of K_m factor fractions from the fructose G-200 column almost completely restored both the K_m and V_{max} values to those observed in the wild type. Growth of QQ17 on mannitol (also on glycerol, glucose, and nutrient broth) gave a constitutive-type enzyme II activity, the same as the wild type. The fact that fructose-1-P kinase is present in the fructose-induced QQ17 cells indicates that the induction procedure used was effective in getting whatever compounds are required for induction of this enzyme (and, presumably, the K_m factor) into the cells.

Mutant QQ17 allows a simple and readily applied assay for the K_m factor (Fig. 2). The first peak of the 200-mM plot corresponds with the enzyme II (*F*)

TABLE 3. *Enzymatic defect in mutant QQ17.*

Assay type	Assay supplement*	Growth substrate or inducer	PRL-R3 (Parent) V_{max} (μmoles/min/mg)	PRL-R3 (Parent) K_m for fructose (mM)	QQ17 (Mutant) V_{max} (μmoles/min/mg)	QQ17 (Mutant) K_m for fructose (mM)
Enzyme II	—	Fructose	0.013	1.0 or less	0.006	70
Enzyme II	K_m factor	Fructose	0.015	1.0 or less	0.011	1.2–2.0
Enzyme II	—	Mannitol	0.009	60	0.012	67
Fructose 1-P kinase	—	Fructose	0.120	—	0.070	—

Phosphotransferase system activities were determined with 0.04 ml of crude extract in the fructose assay. Supplementation with enzyme I (*M*) or HPr (*M*) had no effect on the assays. Both fructose- and mannitol-induced cells were grown on glucose to the late log phase and then incubated in fructose or mannitol mineral media for 4 hr before harvesting. Fructose 1-P kinase was assayed as previously described.[2]

* K_m factor-containing fraction (0.10 ml) from Sephadex G-200 chromatography (Fig. 1*B*).

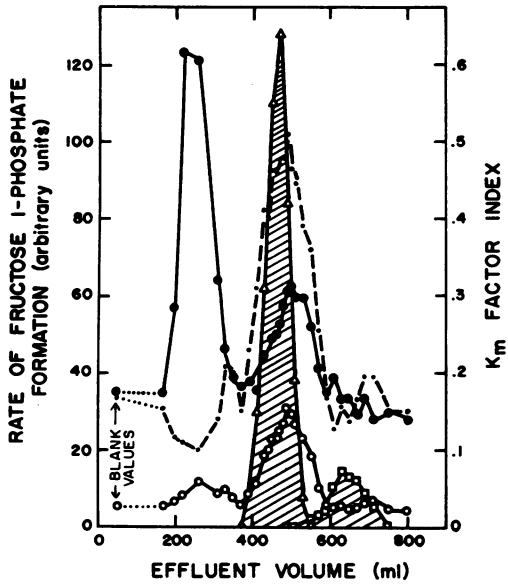

FIG. 2.—Assay for the K_m factor using a crude extract of mutant QQ17. A crude extract of fructose-grown PRL-R3 (wild-type) cells was chromatographed on Sephadex G-200 and enzyme I (△) and HPr (□) (*both shaded*) were assayed as described in Fig. 1.

The K_m factor was determined with the fructose assay using 0.04 ml of a crude extract of QQ17 to which 0.1-ml aliquots of the column fractions were added. Assays were made at 2.0 mM fructose (○) and 200 mM fructose (●) and the K_m factor index (*dashed line*) was taken as in Fig. 1*B*. The QQ17 cells used for the extract were the same fructose-induced cells described in Table 3.

fraction shown in Figure 1*B* which, in this case, stimulates the enzyme II-limited activity of the QQ17 extract. Note that this fraction produces little stimulation at 2 mM fructose, indicative of the high K_m for fructose of this enzyme II activity. The second peak of the 200-mM plot corresponds to activation by the K_m factor of the high K_m enzyme II for fructose present in the QQ17 extract. In this case, a peak also occurs in the 2 mM activity, giving a net change in K_m for fructose as indicated by the K_m factor index values.

Discussion.—In addition to the PEP:fructose 1-phosphotransferase system reported here, we have found in extracts of *A. aerogenes* two other enzymes capable of forming fructose-1-P: an acyl phosphate (hexose phosphate):hexose phosphotransferase[20, 21] and a sugar phosphate phosphohydrolase with phosphotransferase activity (unpublished). Both enzymes are constitutive and require high fructose concentrations (50–200 mM) for maximal rates of fructose-1-P formation, so it is unlikely that they participate in the inducible fructose-1-P pathway. However, the concurrent loss of the low K_m enzyme II activity for fructose and impairment of fructose utilization in mutant QQ17 demonstrates that the PEP:fructose 1-phosphotransferase system is required for normal growth of *A. aerogenes* on fructose. The fact that this system exhibits a specific induction response to fructose (as does fructose-1-P kinase[2, 3]) is also consistent with its participation in fructose metabolism. Furthermore, its existence is compatible with the isolation, by Tanaka and Lin,[9] of pleiotropic mutants of *A. aerogenes* which lack either enzyme I or HPr and which show impaired growth on fructose as well as mannitol and several other carbohydrates.

The nature of the defect in enzyme II function in the mutant QQ17, as well as the *in vitro* behavior of the K_m factor, indicates that the PEP:fructose 1-phosphotransferase system in *A. aerogenes* requires for physiologically significant enzyme II activity both an inducible component which manifests itself as a K_m

factor and a higher-molecular-weight component. For the purpose of suggesting a model for enzyme II structure and specificity, we will call the K_m factor the "fructose specifier protein" and the high-molecular-weight component [enzyme II (*F*) fraction] the "heavy protein." Accordingly, the induced enzyme II for fructose phosphorylation could be represented by the following equation: heavy protein + fructose specifier protein (inducible)$\rightleftarrows$enzyme II complex for fructose. The specifier protein would associate with the heavy protein to form a complex which is active, with a low K_m, in the phosphorylation of fructose. The heavy protein is probably membrane-bound, since Kundig *et al.*[7, 22] have found that enzyme II activities in *E. coli* and *B. subtilis* are associated with the membrane. Our findings that the constitutive-type enzyme II for fructose in mannitol-grown cells is inhibited by mannitol and has properties in common (high K_m for fructose, high molecular weight) with the heavy protein are suggestive of the possibility that the heavy protein is constitutive and that it may interact with inducible specifier proteins for substrates other than fructose.[23] Since the specifier protein is induced by fructose and is a determinant of the affinity of the enzyme II system for fructose, it is probable that one of the primary functions of the specifier protein is the determination of enzyme II specificity.

Several investigators[7-9] have provided evidence for a transport role for the PEP-dependent phosphotransferase system in several bacteria. The PEP: fructose 1-phosphotransferase model described above, although not dependent on assumptions concerning the transport function of the phosphotransferase system, is easily compatible with such a function. The discovery of "carrier" or "binding" proteins in several nonphosphorylating transport systems has led to some expectation[7] that enzyme II activities might also involve participation of such proteins. The specifier protein of our model is similar to a binding (or carrier) protein in that both determine the specificity of a complete functional unit. We have used the term "specifier protein" in the same sense as Brew *et al.*[24] because both carrier protein and binding protein imply functions which are not necessarily (but may be) a property of the specifier protein of our model. It is possible, for instance, that the specifier protein alone does not bind the substrate (and, thus, would not manifest itself as a binding protein) and that this function is observed only with the complete enzyme II complex.

Summary.—A phosphoenolpyruvate-dependent phosphorylation of D-fructose has been identified as the initial step in the metabolism of D-fructose via D-fructose 1-phosphate in *Aerobacter aerogenes*. The phosphotransferase system has been resolved into four components which, when recombined, catalyze the formation of D-fructose 1-phosphate but not D-fructose 6-phosphate. The components of this phosphoenolpyruvate: D-fructose 1-phosphotransferase system are enzyme I, HPr, and a two-component enzyme II system. The two enzyme II components are a high-molecular-weight protein and a smaller inducible protein which increases the affinity of the system for D-fructose. A mutant lacking the inducible component shows impaired growth on D-fructose but not on other sugars.

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Station, journal article no. 4422. These data were presented at the 52nd Annual Meeting of the American Society of Biological Chemists.[25]

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[1] Abbreviations: fructose, D-fructose; glucose, D-glucose; P, phosphate; PEP, phosphoenolpyruvate; HPr[6], heat-stable protein; enzyme I[6], PEP:HPr phosphotransferase; enzyme II[6], phospho-HPr:sugar phosphotransferase; tris, tris(hydroxymethyl)aminomethane; DEAE, diethylaminoethyl. The symbol "(*M*)" after a phosphotransferase component indicates that the component used was one of the pooled fractions described in Fig. 1*A*; likewise, the symbol "(*F*)" indicates that the component was one of the pooled fractions described in Fig. 1*B*.

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[23] Tanaka *et al.*[9, 19] have isolated a mutant of *A. aerogenes* which shows impaired growth only on mannitol and which lacks enzyme II activity for mannitol. Their procedure for assaying enzyme II activity is such that even if a specifier protein for mannitol were dissociable under the conditions used (which is unlikely since our conditions are very similar to theirs), loss of a mannitol specifier protein would not be evident as the cause of enzyme II activity loss. Simoni and Roseman have recently discovered a fourth component of the phosphotransferase system of *Staphylococcus* (private communication).

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