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Growth of *Escherichia coli* on glucosamine 6-phosphate: selection of a constitutive hexose phosphate transport system mutant

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Glucosamine 6-phosphate was found to be a substrate but not an inducer for the hexose phosphate transport system of *Escherichia coli*. Wild-type cells grow very poorly on glucosamine 6-phosphate. A mutant was selected that will grow rapidly on glucosamine 6-phosphate because it contains a constitutive hexose phosphate transport system.

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La glucosamine-6-phosphate est un substrat mais non un inducteur du système de transport hexose phosphate chez *Escherichia coli*. La croissance des cellules du type sauvage est très faible avec la glucosamine-6-phosphate. Comme *E. coli* possède un système de transport hexose phosphate constitutif un mutant qui se développe sur la glucosamine-6-phosphate a été sélectionné.

[Traduit par le journal]

Introduction

Wild-type *Escherichia coli* possesses an inducible transport system for hexose phosphates (Winkler 1966; Pogell *et al.* 1966). The compounds so far known to be substrates for this system are glucose 6-phosphate, glucose 1-phosphate, fructose 6-phosphate, mannose 6-phosphate, and 2-deoxyglucose 6-phosphate (Dietz and Heppel 1971*b*). At first it was thought that several of these compounds might also be inducers of this system, for example, fructose 6-phosphate. However, Winkler (1970) has shown that when *E. coli* is presented with fructose 6-phosphate, this compound must first be converted by the cells to extracellular glucose 6-phosphate, probably by a periplasmic glucose 6-phosphate isomerase (Friedberg 1972) which then induces the transport system. Thus it is possible that only glucose 6-phosphate is the true inducer.

Recognizing the broad specificity of the hexose phosphate transport system, it is surprising that we can find no mention of glucosamine 6-phosphate as a potential substrate. There is only the statement that in *B. subtilis* glucosamine 6-phosphate is not taken up from the medium (Bates and Pasternak 1965). It is most likely that this amino sugar phosphate neither induces its own transport system nor is an inducer of the hexose phosphate transport system. However, it might be a substrate for the induced hexose phosphate transport system. If this were the case, a mutant containing a constitutive hexose phosphate transport system should be able to grow on the amino sugar phosphate, since it is well known that *E. coli* will grow on glucosamine via an intermediate glucosamine 6-phosphate (Bates and Pasternak 1965).

In fact, it should be possible for one to select for a constitutive hexose phosphate transport mutant, provided that one other requirement is met: that the glucosamine 6-phosphate is not rapidly hydrolyzed by the periplasmic phosphatases (Heppel 1969). Following a similar approach for this type of selection, but using glucose 1-phosphate as the selective

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substrate, it was necessary to start with a strain already possessing a mutation in glucose uptake, to ensure that free glucose, resulting from the hydrolysis of glucose 1-phosphate, could not serve as an alternate growth substrate (Dietz 1976).

Materials and Methods

Bacteria

Strain E15 has the wild-type, inducible transport system for hexose phosphates, and in addition carries a deletion for alkaline phosphatase (Lin 1976).

Media

The synthetic salts (CRM) and tryptone broth media were as previously described (Dietz 1972). Agar for agar plates contained either 15 g nutrient broth powder, 5 g NaCl, and 12.5 g Bacto agar in 1 ℓ, or substituted CRM and the selective substrate for the nutrient broth powder and NaCl.

Reagents

Glucosamine 6-phosphate and glucose 6-phosphate were purchased from Sigma Chemical Co. [^{14}C]glucose 6-phosphate and [^{14}C]glucosamine 6-phosphate were products of New England Nuclear Co. Nutrient broth and Bacto agar were obtained from Difco Laboratories.

Growth of Bacteria

Bacteria were grown in flasks on a reciprocating shaker at 37°C. Growth was followed by measuring the absorbance of the culture in a Klett colorimeter using the No. 42 filter.

Transport Assays

Large volumes of cells were harvested by centrifugation and washed twice with CRM medium in the centrifuge. Small quantities of cells were collected on a Millipore filter HAWP 02500, and a small volume of room temperature CRM medium was passed through the filter to wash the cells. The filter was then transferred to a test tube containing CRM medium and the cells were resuspended at approximately 1 mg protein per millilitre. In the assay, the suspension was diluted 10-fold into CRM medium to give a total volume of 0.5 ml, and preincubated for 2 min. At 15 and 45 s after the addition of 5 µl of 5 m*M* radioactive substrate, samples were removed and filtered through a Millipore filter. The filters were washed and counted as previously described (Dietz 1972). Where additions were made they were present during the preincubation. The rate of uptake was calculated from the difference between the 15- and 45-s time points.

Protein Assay

All activities reported are related to the protein content of the cell suspension as determined by the method of Lowry *et al.* (1951).

Assay for Phosphatase Activity

This assay was by the method previously described (Dietz and Heppel 1971*b*).

Data Collection

All experiments were repeated several times and the results reported are from representative trials.

Results

Glucosamine 6-Phosphate and the Hexose Phosphate Transport System

If glucosamine 6-phosphate is a substrate for the hexose phosphate transport system, then it should compete with other substrates, e.g., glucose 6-phosphate, for transport into cells containing this system. As may be seen in Table 1, this is the case, i.e., glucosamine 6-phosphate inhibits the uptake of glucose 6-phosphate. There is often a low level of transport measurable in the uninduced cells, and even here there is some inhibition of glucose 6-phosphate transport by glucosamine 6-phosphate.

Growth of E15 on Glucosamine 6-Phosphate

We found that, as in the case of *B. subtilis*, there is little growth of *E. coli* on glucosamine 6-phosphate, especially when compared to growth on glucose 6-phosphate. In fact after 10 h of incubation cells incubated with glucose 6-phosphate showed a 30-fold increase in optical density, while a separate culture incubated with glucosamine 6-phosphate little more than doubled its optical density. However, there is a slow growth, and after a prolonged incubation the culture will reach a high cell density. This slow growth may be due to a limited hydrolysis of the glucosamine 6-phosphate and growth on glucosamine, or a low level of hexose phosphate transport system activity may provide sufficient substrate for a slow growth.

Selection of a Hexose Phosphate Transport System Constitutive Mutant

Cells which had grown out slowly on glucosamine 6-phosphate were reinocculated into fresh medium and again grown to a high optical density. It took three such cycles before the cells began a rapid growth. After a growth cycle on tryptone broth, the cells were plated out on an agar medium containing 0.05 *M* glucosamine 6-phosphate. After incubation for 2 days at 37°C one of the larger colonies was picked and grown up on tryptone broth. A control incubation of the parent, E15, was carried out in parallel and both cultures were assayed for glucose 6-phosphate uptake. As may be seen in Table 2, the mutant cells are active in glucose 6-phosphate transport, while the control cells are not. Furthermore, when an

TABLE 1. Inhibition of glucose 6-phosphate transport by glucosamine 6-phosphate[a]

	G6P uptake (nmol/mg protein per min)[b]	
Cells	G6P only	G6P + GA6P
Uninduced	0.29	0.20
Induced	12.40	4.10

[a]Strain E15 was grown into early log phase on CRM medium supplemented with 0.4% glycerol. The culture was divided, and one half was made 0.6 m*M* in glucose 6-phosphate and the incubation continued for a further 80 min. Both cultures were then harvested and assayed as described in the text.

[b]The [^{14}C]glucose 6-phosphate concentration was 8×10^{-5} *M*; the glucosamine 6-phosphate where present was at a concentration of 5×10^{-3} *M*.

TABLE 2. Uptake of glucose 6-phosphate: effect of glucose

Cells	G6P uptake (nmol/mg protein per min)
A. E15 (parent)[a]	<0.02
C/1 (mutant)	11.4
B. C/1 (mutant)	5.5
C/1 (mutant) + 0.2 m*M* glucose	6.2

[a]The cells in experiment A were grown on tryptone broth and harvested in late log phase. In experiment B, the cells were grown in CRM medium on glucose at 0.01 *M* and harvested in midlog phase.

excess of unlabeled glucose is present along with the labeled glucose 6-phosphate there is no decrease in the label taken up (Table 2, expt. B). This is to be expected if the glucose 6-phosphate is being taken up without prior hydrolysis.

Phosphatase Activity of Whole Cells

The above point is pursued further in an experiment comparing the phosphatase activity of whole cells of mutant and parent against a variety of hexose phosphates. It can be seen in the data of Table 3 that the mutant has a very similar pattern of activity compared to the parent and that there has been no major change in the specific activity.

Growth Studies with the Mutant

The mutant and parent were compared for growth on glucosamine 6-phosphate. It is evident from the growth curves in Fig. 1 that the mutant grows much better than the parent on this substrate (the linear plot shows this most clearly). There is, however, a substantial lag when the mutant cells, pregrown on tryptone broth, are transferred into the glucosamine medium: the lag is overcome when the cells are pregrown on glucosamine 6-phosphate (Fig. 2). This most probably reflects a requirement for the synthesis of the enzymes necessary for growth on the glucosamine moiety. Indeed, when the cells are pregrown on glucosamine itself, the lag upon transfer to glucosamine 6-phosphate is removed. Figure 3 illustrates this point, comparing

TABLE 3. Comparing apparent phosphatase activities of whole cells towards various hexose phosphates

	Substrates			
Cells[a]	Glucose 1-P	Glucosamine 6-P	Glucose 6-P	Galactose 6-P
	Activity (μmol Pi/min·mg^{-1} protein)			
E15 (Parent)	0.44	0.39	0.34	0.005
C/1 (Mutant)	0.63	0.42	0.33	0.006

[a]Cells were grown up in CRM medium; in the case of E15 the carbon source was 0.01 *M* glucose 6-phosphate, while the mutant C/1 was grown on 0.01 *M* glucose. The cells were harvested in late log phase and assayed as described in the Material and Methods section.

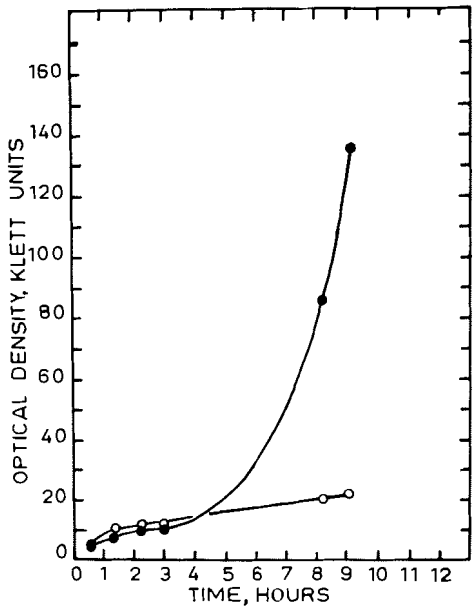

FIG. 1. Growth on glucosamine 6-phosphate. Cells were first grown up into midlog phase on tryptone broth. At zero time a 2% inoculation was made into CRM medium containing 50 m*M* glucosamine 6-phosphate. (○), strain E15; (●), strain C/1.

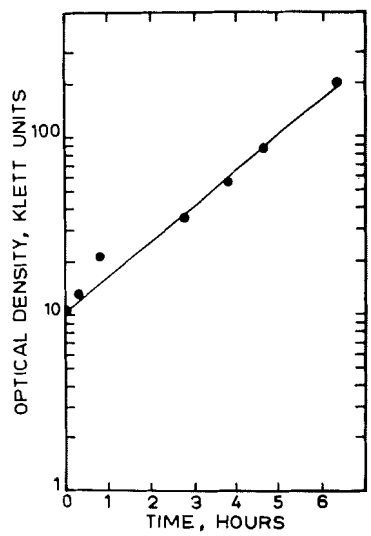

FIG. 2. Growth of cells reinoculated into glucosamine 6-phosphate. C/1 cells, grown up into midlog phase on glucosamine 6-phosphate were reinoculated at 2% into fresh 0.01 *M* glucosamine 6-phosphate.

cells pregrown on glucose with those pregrown on glucosamine.

Comparison of Effects of Glucosamine 6-Phosphate and 2-Deoxyglucose 6-Phosphate on the Adaptation of Cells to Growth on Glycerol

It is well known that the intracellular accumulation of hexose phosphates may be toxic for cells

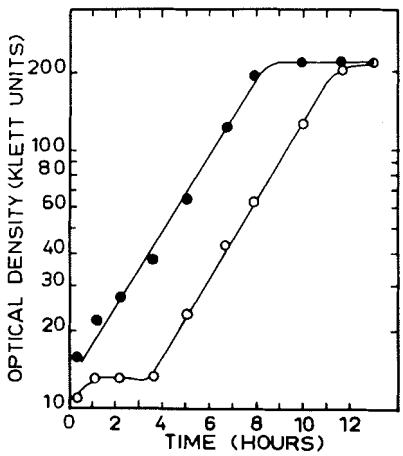

FIG. 3. Transfer of cells from glucose or glucosamine to glucosamine 6-phosphate. C/1 cells were pregrown on 0.01 *M* glucose (○) or 0.01 *M* glucosamine (●), into late log phase and then inoculated at 2% into 0.01 *M* glucosamine 6-phosphate. The small spurt of growth in the case of the glucose-grown cells most probably results from a small amount of glucose carried over in the inoculum.

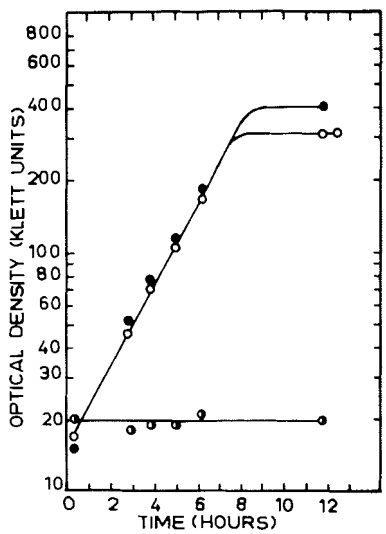

FIG. 4. Adaptation of glucose-grown cells to glycerol. C/1 cells were grown in 0.01 *M* glucose into late log phase and then inoculated into 0.02 *M* glycerol with the additions as noted: (○) control, no additions; (●) plus 0.001 *M* glucosamine 6-phosphate; (◑) plus 0.001 *M* 2-deoxyglucose 6-phosphate.

(Fraenkel 1968). This is especially true for cells forced to adapt to a new carbon source; an example of this is the inhibition by 2-deoxyglucose 6-phosphate, accumulated through the uptake of either the free sugar (Curtis and Epstein 1975; Dietz and Heppel 1971*a*), or the intact hexose phosphate (Dietz, unpublished data), of the adaptation of glucose-grown cells to glycerol. Since the mutant possesses a transport system for glucosamine 6-phosphate, yet requires a long lag period before it can grow on this compound, perhaps an accumulation of toxic levels during this period is in part responsible for the lag phase. This accumulation should be manifested in an inhibition of the glycerol adaptation of glucose-grown cells. However, as may be seen in Fig. 4, this is not the case: the mutant has no problem in growing out on glycerol in the presence of glucosamine 6-phosphate, whereas 2-deoxyglucose 6-phosphate inhibits as expected. Furthermore, the same growth rate was achieved in cultures grown over a 10-fold range of concentrations of glucosamine 6-phosphate (0.2–2.0 m*M*), with glycerol as the primary growth substrate.

Direct Measurement of Glucosamine 6-Phosphate Uptake

Labeled glucosamine 6-phosphate was taken up by cells containing the hexose phosphate transport system as demonstrated in Table 4, and an excess of either unlabeled glucose 6-phosphate or glucosamine 6-phosphate drastically reduced this amount. Figure 5 presents a time course for the uptake of glucosamine 6-phosphate. The rate is linear for about 3 min, then begins to decrease, but even after 10 min the uptake has not plateaued. The reason for this is apparent from the results of the second part of the experiment. At the point indicated by the arrow a sample of the suspension was removed, a 50-fold excess of unlabeled glucosamine 6-phosphate was added, and the time course of retention of the label in this sample was followed as well. Only 25% of the counts incorporated in the first 2½ min of incubation could be chased by the unlabeled substrate. This is to be expected of a metabolically active compound, and is similar to results found with, for example, glucose 6-phosphate.

TABLE 4. Specificity of the uptake of glucosamine 6-phosphate

Substrates	Uptake of [^{14}C]GA6P (nmol/mg protein per min)
Expt. 1[a]	
[^{14}C]GA6P[b]	22.8
[^{14}C]GA6P plus excess [^{12}C]G6P[c]	0.2
Expt. 2	
[^{14}C]GA6P	8.0
[^{14}C]GA6P plus excess [^{12}C]GA6P	0.5

[a]Cells in both experiments 1 and 2 were the mutant C/1 grown up in CRM medium in 0.01 *M* glucose.
[b]GA6P equals glucosamine 6-phosphate.
[c]G6P equals glucose 6-phosphate.

Discussion

We have confirmed that glucosamine 6-phosphate is a very poor growth substrate for bacteria,

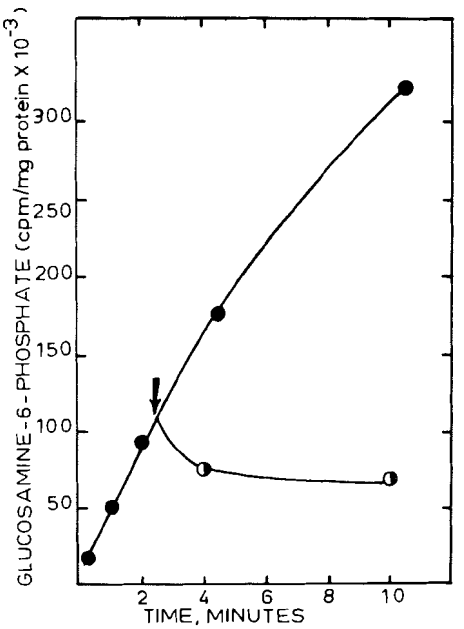

FIG. 5. Time course and chase of glucosamine 6-phosphate uptake. Cells were the parent E15 grown up on glucose 6-phosphate. An amount of 1.5 ml of suspension was supplemented with 15 µl of 0.005 *M* [^{14}C]glucosamine 6-phosphate at the start of the experiment. At the 2.5-min point 0.45 ml of suspension was removed, made 2.5 m*M* in unlabeled glucosamine 6-phosphate, and sampled at the indicated times. All samples were 200 µl.

in this case *E. coli*; although in our conditions it does support a slow growth. This may be the result of a limited hydrolysis of glucosamine 6-phosphate, which we have shown the cells to be capable of, or the low level of hexose phosphate transport may provide sufficient substrate for a slow growth. The former possibility has been minimized by growth under conditions which repress the so-called acid phosphatases of *E. coli* (Dvorak *et al.* 1967) and the alkaline phosphatase-negative character of the parent should further reduce the hydrolysis of the selective agent. Even after two growth cycles on glucosamine 6-phosphate, more than adequate time for the cells to adapt fully, the growth rate remained slow and when the cells were assayed for hexose phosphate transport activity it was present but at a low level. Eventually, the growth rate of the culture increased, and a spontaneous mutant was isolated possessing a constitutive hexose phosphate transport system, and capable of rapid growth on glucosamine 6-phosphate.

This mutation is confined to the hexose phosphate transport system, and is not a general derepression of the enzymes of glucosamine catabolism. The mutant, pregrown on glucose, when switched to glucosamine, undergoes a period of quiescence before the onset of exponential growth, and this lag phase may be circumvented by prior growth on glucosamine. These results suggest that internal glucosamine 6-phosphate arising either indirectly from the transport-mediated phosphorylation of the amino sugar via the phosphotransferase system, or directly from the uptake of the sugar phosphate, is an intermediate in the process of induction of the enzymes of glucosamine catabolism. This warrants further investigation.

It is interesting that while 2-deoxyglucose 6-phosphate inhibits growth, glucosamine 6-phosphate does not. We would have expected both compounds to be inhibitory, since transport data indicate that newly transferred mutant cells can accumulate the amino hexose phosphate, while growth studies suggest that the cells cannot catabolize this compound. The absence of toxicity could be the result of either a lower accumulation of glucosamine 6-phosphate relative to the deoxyhexose phosphate, or to differences in the toxicity of the two compounds. These results merit further investigation, and differential sensitivity studies might pinpoint the metabolic step responsible for the growth inhibition.

The results of our growth experiments are in accord with the idea that glucosamine 6-phosphate is a substrate for the hexose phosphate transport system, and evidence from two types of transport studies substantiates this view. Indirectly, this was shown in the competition between unlabeled glucosamine 6-phosphate and labeled glucose 6-phosphate for uptake. A more direct demonstration is to be found in the measurement of uptake using labeled glucosamine 6-phosphate itself. Any question as to the possibility that the glucosamine 6-phosphate is first split to glucosamine and phosphate is laid to rest by the demonstration that unlabeled glucose does not inhibit the uptake of label from the glucosamine 6-phosphate. These results likewise make it unlikely that an appreciable quantity of glucosamine accumulates within the cell since in the presence of glucose it would be expected to exchange out of the cell via the constitutive mannose enzyme II of the phosphotransferase system; and it does not (Table 2, expt. B).

The general significance of the experiments reported herein lies in two areas. In the first place, the use of glucosamine 6-phosphate allows for the selection of mutants in any cell line. Previous protocols required a prior mutation in some step of carbohydrate metabolism. This was necessary since even under conditions of minimal expression of phosphatases, sufficient activity remained to release enough free sugar to allow the cells to grow. A

mutant lacking phosphoglucose isomerase was used in a selection with fructose 6-phosphate (Kadner 1973), while another mutant unable to grow on fructose was employed in a selection with fructose 1-phosphate (Ferenci *et al.* 1971). In the case of selection with glucose 1-phosphate it was necessary to employ a mutant impaired in the uptake of glucose (Dietz and Heppel 1971*b*). A constitutive mutant was also selected as a revertant from a hexose phosphate transport negative mutant (Kadner and Winkler 1973). The strain used in the present experiments E15 does contain a deletion for alkaline phosphatase; of possible benefit in the study of the uptake of phosphorylated compounds under certain conditions. However, since alkaline phosphatase is only derepressed in low phosphate media (Lin 1976), under the conditions of the selection procedure used here i.e., high phosphate, it would be repressed, and should not have any effect on the selection of mutants even in alkaline phosphatase positive cells.

In the second place, glucosamine 6-phosphate should prove an interesting compound with which to prove the mechanism of the hexose phosphate transport system. For example, the role of a pH gradient and the coupling of the hydrogen ion uptake and solute transport continues to intrigue speculators and experimentalists alike. Glucosamine 6-phosphate carries one less negative charge than the other substrates of the hexose phosphate transport system. There is a report that the uptake of at least one proton is coupled to the uptake of one glucose 6-phosphate (Essenberg and Kornberg 1975). It was suggested that this is comparable to the proton linked to lactose uptake (Winkler 1970) which has been implicated in the energy-coupling step of this and many other transport systems. On the other hand, it could also be involved more directly in the neutralization of the charge on the glucose 6-phosphophosphate molecule. It will be interesting to compare the proton fluxes coupled to glucose 6-phosphate and glucosamine 6-phosphate transport. Indeed, we are currently pursuing this problem.

Acknowledgments

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