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The Localization of Glycerol-3-Phosphate Dehydrogenase in *Escherichia Coli**

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Summary. Starved cells of *Escherichia coli* are dependent on an exogenous source of energy. It was of interest to ask whether compounds that are commonly used to supply energy must themselves be transported or whether they can be utilized on the outer portion of the cytoplasmic membrane. The utilization of glycerol-3-phosphate as an energy source is totally dependent on the membrane-bound glycerol-3-phosphate dehydrogenase. In the present report glycerol-3-phosphate was used as the energy source for uptake of amino acids. A mutant was constructed which is unable to transport this ester and the starved mutant could not drive the uptake of glutamine with glycerol-3-phosphate. It is concluded that the enzyme is located on the internal surface of the membrane in intact *E. coli* cells. Further evidence was obtained by showing that no glycerol-3-phosphate dehydrogenase activity could be measured in either intact cells or spheroplasts using ferricyanide as electron acceptor, due to its impermeability. The activity could be measured after destruction of the membrane permeability barrier by toluenization. With membrane vesicles prepared according to Kaback's procedure nearly half of the dehydrogenase activity was accessible to ferricyanide as well as to impermeable competitive inhibitors of the enzyme. Partial inversion during preparation of vesicles is the most probable explanation for the results.

The active transport of amino acids and other substrates in *Escherichia coli* requires metabolic energy (Kaback, 1970; Kennedy, 1970; Harold, 1972) and it is possible to show that starved cells are dependent on an exogenous source of energy (Weiner & Heppel, 1971). Our interest in active transport led us to ask whether compounds that are commonly used to supply energy must themselves be transported or whether they can be utilized on the outer portion of the cell membrane. In the latter case, the "fuel" for active transport processes need not itself be carried across the

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protoplasmic membrane. The utilization of glycerol-3-phosphate as an energy source is totally dependent on the membrane-bound glycerol-3-phosphate dehydrogenase. In this paper we report on studies with glycerol-3-phosphate as the energy source for uptake of amino acids. We show that glycerol-3-phosphate itself must first be transported in order to supply energy for uptake of other compounds. From this fact and other data reported herein, we conclude that glycerol-3-phosphate dehydrogenase is located on the internal surface of the membrane in intact *E. coli* cells.

When membrane vesicles were prepared from these cells according to Kaback's procedure (1971) we were surprised to find that a substantial fraction of the dehydrogenase activity was accessible to an impermeable electron acceptor as well as to impermeable competitive inhibitors of the enzyme. Similar results were obtained for other dehydrogenases. A possible explanation for these results is presented.

Materials and Methods

Bacteria and Growth Conditions

Strain 27 (Berman-Kurtz, Lin & Richey, 1971), a derivative of K12 Hfr Cavalli, was the gift of Dr. E. C. C. Lin and was used for most of the studies reported in this paper. This *E. coli* strain is glycerokinase (−), G-3-P[1] transport (+), G-3-P dehydrogenase (+) and regulatory constitutive for the G-3-P operon. Strain 2712 is derived from Strain 27 and is transport (−). Strain 2712 is a spontaneously arising mutant, selected on the basis of resistance to 25 μg per ml of phosphonomycin in the growth medium. Strain 7 (Hayashi, Koch & Lin, 1964) is constitutive for the glycerol-3-phosphate operon, and was obtained from Strain K10, which in turn was derived from K12 Hfr$^-$ Cavalli-Sforza. *E. coli* ML 308–225 is $i^- z^- y^+$ and has been extensively used for transport studies in whole cells and membrane vesicles. Unless otherwise stated, all cultures were grown in a synthetic minimal medium described by Tanaka, Lerner and Lin (1967), supplemented with 1% sodium succinate (Baker and Adamson, Morristown, New Jersey). Bacterial cultures were maintained on nutrient agar slants that were transferred monthly. Medium lacking inorganic phosphate contained 0.05 M NaCl, 0.05 M KCl, 0.02 M $(NH_4)_2SO_4$, 3×10^{-4} M $MgSO_4$, 10^{-6} M $FeSO_4$ and 10^{-6} M $ZnCl_2$. The pH was adjusted to 7.3.

To reduce endogenous amino acid uptake to a minimum the following procedure was developed. Cells in exponential phase of growth were harvested, washed 3 times with minimal medium, suspended (1 g, wet weight of cells per 200 ml) in minimal medium and aerated for 2 hr at 37 °C. They were then stored for 24 to 48 hr at 4 °C followed by a second 2-hr incubation at 37 °C. This procedure resulted in at least a 10-fold stimulation of amino acid uptake when 10 mM glucose was added.

Transport Assays

Transport Activity of Intact Cells. The temperature was maintained at 23 °C throughout the following procedure. For transport measurements a portion of the starved cells

1 Abbreviations used: G-3-P, glycerol-3-phosphate; MTT, (3(4,5-dimethylthiazolyl-2-)-2,5) diphenyl tetrazolium broide; PMS, phenazine methosulfate.

was incubated for 5 min in the presence of either 10 mM glucose or 10 mM DL-glycerol-3-phosphate, as stated. Chloramphenicol was present at 80 μg per ml. To start the reaction, cells were added to 0.5 ml of minimal medium containing 10 mM DL-glycerol-3-phosphate or 10 mM glucose, 80 μg chloramphenicol and 10 μM radioactive transport substrate. For measurement of the uptake of labeled glycerol-3-phosphate, phosphateless medium was used in place of minimal medium. A 0.2-ml portion was removed at 15 and 30 sec, filtered on a 25 mm nitrocellulose filter (type HA, 0.45 μ, Millipore Corp., Bedford, Mass.) and washed with 10 ml of 0.01 M Tris-HCl, pH 7.3 – 0.15 M NaCl – 5×10^{-4} M $MgCl_2$. The filters were dried and counted in a Nuclear Chicago Unilux II liquid scintillation counter, using a solution of 15 g of 2.5 diphenyloxazole and 0.2 g of 1,4-*bis*[2-(4-methyl-5-phenyloxazolyl)benzene] dissolved in 3.81 liters of toluene. The volume of cells was adjusted so that 10% or less of the counts were taken up during the 30-sec assay period.

Transport Activity of Vesicles. These were done essentially as described by Kaback (1971), using 10 μM labeled substrate and 20 mM D(−) lactate or 20 mM DL-glycerol-3-phosphate as energy source, as noted. At various times 0.2 ml was removed and directly filtered on 25 mm nitrocellulose filters and washed with 5 ml of a solution containing 0.05 M potassium phosphate, pH 6.6, 0.01 M $MgSO_4$ and 0.1 M LiCl. The filters were dried and counted as described for intact cells. Tris-malate buffer, 0.05 M, pH 7.0, was used interchangeably with 0.05 M potassium phosphate, pH 6.6, with no significant difference in uptake.

Dehydrogenase Assays

PMS/MTT Assay. This was done as described by Berman-Kurtz *et al.* (1971) except that the 0.2% Triton X-100 was omitted and the concentration of PMS was 60 μg per ml. Substrate-dependent rates were determined in a Gilford Model 240 Spectrophotometer at 570 nm assuming $\varepsilon = 17\ mM^{-1}\ cm^{-1}$. When intact cells were used, they were washed 3 times with 0.01 M Tris HCl, pH 7.3, and suspended in the same buffer (1 g per 80 ml). The substrate was 30 mM DL-G-3-P unless otherwise noted.

Ferricyanide Assay. For this assay 1 ml contained 60 nmoles potassium phosphate, pH 7.0, 30 nmoles KCN and 750 nmoles potassium ferricyanide. The substrate-dependent rate was measured at 420 nm assuming $\varepsilon = 1.0\ mM^{-1}\ cm^{-1}$. The substrate concentration was 30 mM unless otherwise stated.

Other Procedures

Membrane vesicles were prepared according to Kaback (1971) using lysozyme-EDTA spheroplasts derived from cells in early exponential phase of growth. The method was slightly modified in that the lysozyme concentration was 0.1 mg per ml. The vesicles were suspended in 0.1 M potassium phosphate, pH 6.6. Toluenization consisted of adding 0.01 ml of toluene to 1 ml of a 1:80 (w/v) cell suspension and incubating for 10 min at 37 °C. Protein was determined by the method of Lowry, Rosebrough, Farr and Randall (1951). ^{14}C-Glycerol-3-phosphate was synthesized enzymatically from glycerol-^{14}C-(U) (New England Nuclear), using crystalline *Candida mycoderma* glycerokinase (Sigma) and the procedure of Kiyasu, Pieringer, Paulus and Kennedy (1963). Other chemicals were obtained from commercial sources.

Results

Transport of Glutamine in Whole Cells

Glutamine was selected as an example of an amino acid that was transported by *E. coli* at a relatively fast rate. We investigated the transport of

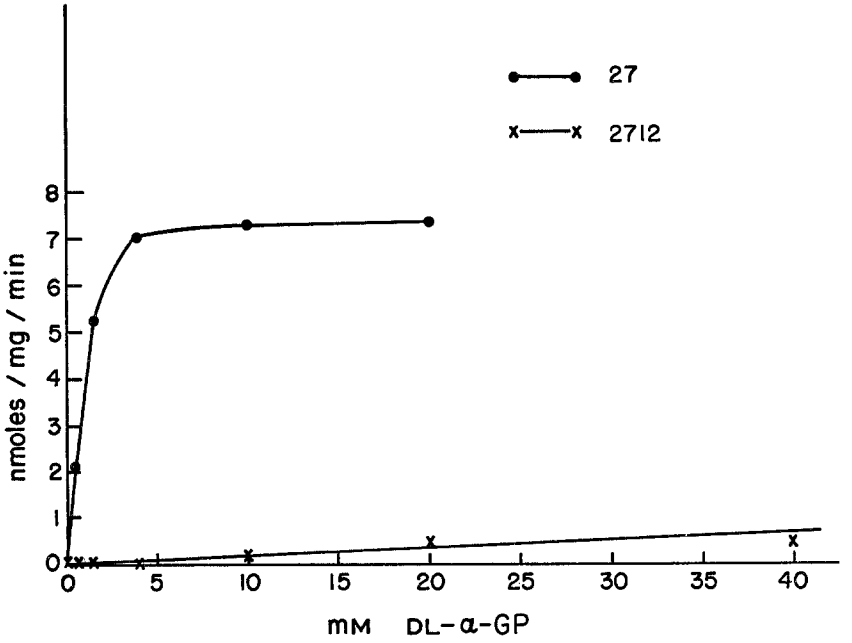

Fig. 1. Glycerol-3-phosphate stimulates the uptake of L-glutamine by starved intact cells of Strain 27 (transport positive for glycerol-3-phosphate) but not by cells of Strain 2712 (transport negative). Cells in exponential phase of growth were starved by two cycles of aeration in minimal medium without a carbon source (*see* Materials and Methods). For transport measurements a portion of the starved cells was incubated for 5 min in the presence of glycerol-3-phosphate (DL-α GP). Glutamine, ^{14}C-labeled, was present at a final concentration of 10 μM. Details of the transport assay in Materials and Methods

glutamine in starved cells when glycerol-3-phosphate was provided as an energy source. Our aim was to find out if this phosphate ester must first be transported across the cell membrane before it can stimulate the uptake of amino acids. Two mutants were used: (1) Strain 27 is glycerokinase (−), G-3-P dehydrogenase (+), G-3-P transport (+) and constitutive for the L-glycerol-3-phosphate system (Berman-Kurtz *et al.*, 1971). The property of being kinase (−) is useful because preparations of glycerol-3-phosphate contain small quantities of glycerol that would interfere with the study. It is important to have a dehydrogenase constitutive strain so that the dehydrogenase is always present and utilization of G-3-P depends only on its transport. (2) Strain 2712 was selected from 27 on the basis of resistance to phosphonomycin, and is transport (−). In Strain 27, the transport of glycerol-3-phosphate exceeds 90 nmoles/mg protein per min; this is the highest rate that we have seen for any uptake system. Strain 2712 takes up only 2 nmoles/mg per min.

Cells were starved (*see* Materials and Methods) so that the endogenous uptake of amino acids was reduced to 10 to 14% of that observed with fresh cells given a source of energy and it could be fully restored by various carbon sources. In Strain 27, glycerol-3-phosphate was able to stimulate the uptake of glutamine by eightfold (Fig. 1). However, Strain 2712, which could not transport glycerol-3-phosphate into the cell, also could not transport glutamine in response to glycerol-3-phosphate in the medium (Fig. 1). There was no significant increase over the low endogenous rate. It is curious that the K_m for stimulation of glutamine uptake by G-3-P is approximately 1 mM. This is much greater than the K_m of G-3-P uptake, which is 12 μM. We cannot explain the requirement for this high concentration. Conceivably a high external concentration alters the exit rate, or some kinetic parameter might have been altered by starvation. A high concentration of G-3-P is required inside the cell because of the weak K_m of the glycerol-3-phosphate dehydrogenase. It is believed that the data of Fig. 1 support the conclusion that glycerol-3-phosphate must first enter the cell in order to be oxidized and thus furnish the energy of active transport.

Measurement of Glycerol-3-Phosphate Dehydrogenase in Whole Cells

Hayashi *et al.* (1964) have shown that L-glycerol-3-phosphate is oxidized to dihydroxyacetone phosphate by a membrane-bound dehydrogenase that is not linked to NAD. This enzyme can be measured in intact cells with the electron acceptor PMS which is permeable and the tetrazolium dye MTT. Such measurements for Strains 27 and 2712 are shown in Table 1. Very low activity is revealed by Strain 2712 because it cannot transport glycerol-3-phosphate into the cell. When toluene is added to remove the permeability barriers, the activity of Strain 27 is unchanged, but that of Strain 2712

Table 1. Glycerol-3-phosphate and succinate dehydrogenase activity in intact cells of Strains 27 and 2712 and spheroplasts of Strains 27 and 2712

Strain	G-3-P, specific activity		Succinate, specific activity	
	− Toluene (units/mg)	+ Toluene (units/mg)	− Toluene (units/mg)	+ Toluene (units/mg)
Strain 27, cells	80	80	14	18
Strain 2712, cells	5	80	18	22
Strain 27, spheroplasts	50	50	—	—
Strain 2712, spheroplasts	3	50	—	—

Activity was measured by the PMS/MTT assay as described in Materials and Methods. One unit is 1 nmole MTT reduced per min; specific activity is units per mg protein.

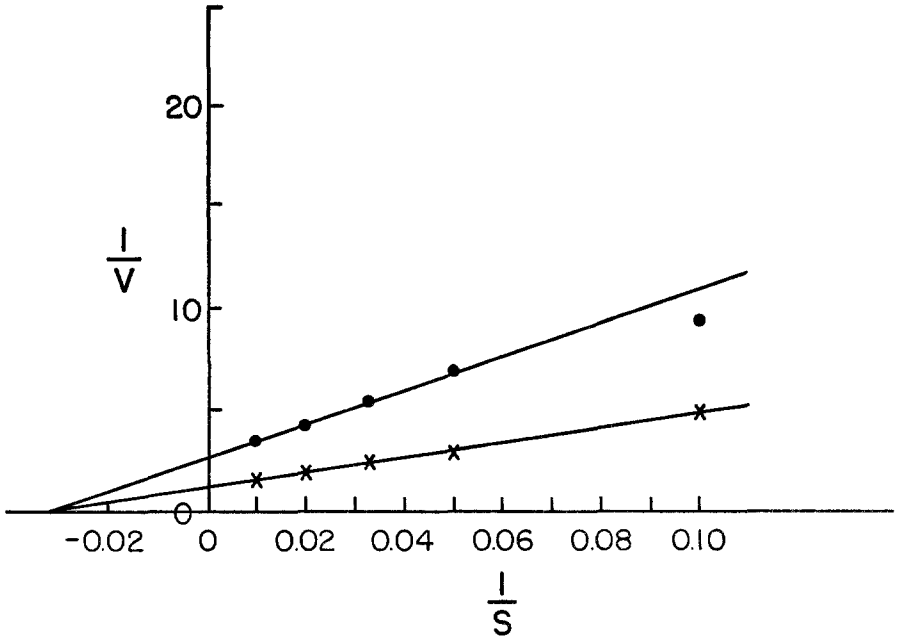

Fig. 2. Plot of $\frac{1}{S}$ *vs.* $\frac{1}{V}$ for the glycerol-3-phosphate dehydrogenase activity of membrane vesicles in the presence and absence of Triton X-100. The PMS/MTT assay (*see* Materials and Methods) was used in this experiment and the concentration of Triton X-100 was 0.2%. (—•—) No Triton X-100. (—×—) Triton X-100 present

increases up to the same level. (This also confirms that the defect in Strain 2712 is specifically a loss of transport function and not a change in regulation from constitutive to inducible, which would have altered both transport activity and the level of the dehydrogenase.) As a control we measured succinic dehydrogenase; its activity was equal in the two strains and was increased only slightly by treatment of the cells with toluene.

The K_m of this dehydrogenase in sonic extracts has been reported to be 2 mM (Hayashi & Lin, 1965) and we obtained a value of 33 mM for DL-αGP in the membrane preparation (Fig. 2). If whole cells are used to measure the K_m, a value of 0.3 mM DL-glycerol-3-phosphate is obtained (data not shown). This difference is probably due to a combination of a tight affinity transport system and a weak affinity enzyme. The KCN present in the assay does not abolish the transport of glycerol-3-phosphate but only reduces it, because the endogenous energy reserves are not affected by cyanide. In fact, if FCCP is added to the cells to inhibit active transport, the K_m measured with whole cells increases to 6 or 7 mM. Under these conditions G-3-P is probably entering by diffusion.

Activity of the glycerol-3-phosphate dehydrogenase can also be measured by MTT reduction in spheroplasts, but it is reduced more than 40% compared with intact cells. This results from specific inactivation due to the 20% sucrose solution used in preparation, as will be discussed later.

No activity can be measured in either intact cells or spheroplasts using ferricyanide as electron acceptor, due to its impermeability.

It should be noted that all the activity that can be measured is membrane bound. Since G-3-P must be taken up in order to be oxidized and serve as energy source for amino acid uptake, it must be localized on the internal membrane surface in intact cells. The measurements of dehydrogenase activity just cited confirm this conclusion.

Uptake of L-*Glycerol-3-Phosphate and Proline by Vesicles*

The uptake of glycerol-3-phosphate could be measured in vesicles of Strain 27 prepared by Kaback's procedure (1971), and it was stimulated by D (−) lactate (Fig. 3). Similar results have been independently obtained by Dietz (1973). The kinetics of uptake in the absence of added carbon source suggested that glycerol-3-phosphate could enter and thereupon stimulate its own further uptake. Vesicles from Strain 2712 showed an uptake rate of only 6 nmoles/mg per min compared with 18 for Strain 27. The rate for Strain 27, although measured below substrate saturation (at 10 μM) is considerably higher than the rates observed for most amino acids. This may be caused in part by rapid metabolism of glycerol-3-phosphate in vesicles. Since the mutation from 27 to 2712 reduced uptake substantially in both whole cells and vesicles we believe that the same uptake system is being measured in both cases.

The uptake of proline by vesicles was then investigated, using DL-glycerol-3-phosphate or D (−) lactate as energy source. Although Strains 27 and 2712 showed identical uptake rates in the presence of 20 mM D (−) lactate, with 1 mM DL-αGP the rate of proline uptake was much faster in Strain 27 than in Strain 2712 (Fig. 4). When the concentration of G-3-P exceeded 10 mM, Strains 27 and 2712 showed identical uptake rates. This is probably due to leakiness of the vesicles so that at high concentrations enough glycerol-3-phosphate enters by simple diffusion to provide energy for uptake (Fig. 5). These data show that glycerol-3-phosphate must enter the vesicle in order to be oxidized and provide energy for amino acid uptake. In this respect vesicles are similar to whole cells, and presumably the transporting membrane is oriented in the same direction as whole cells.

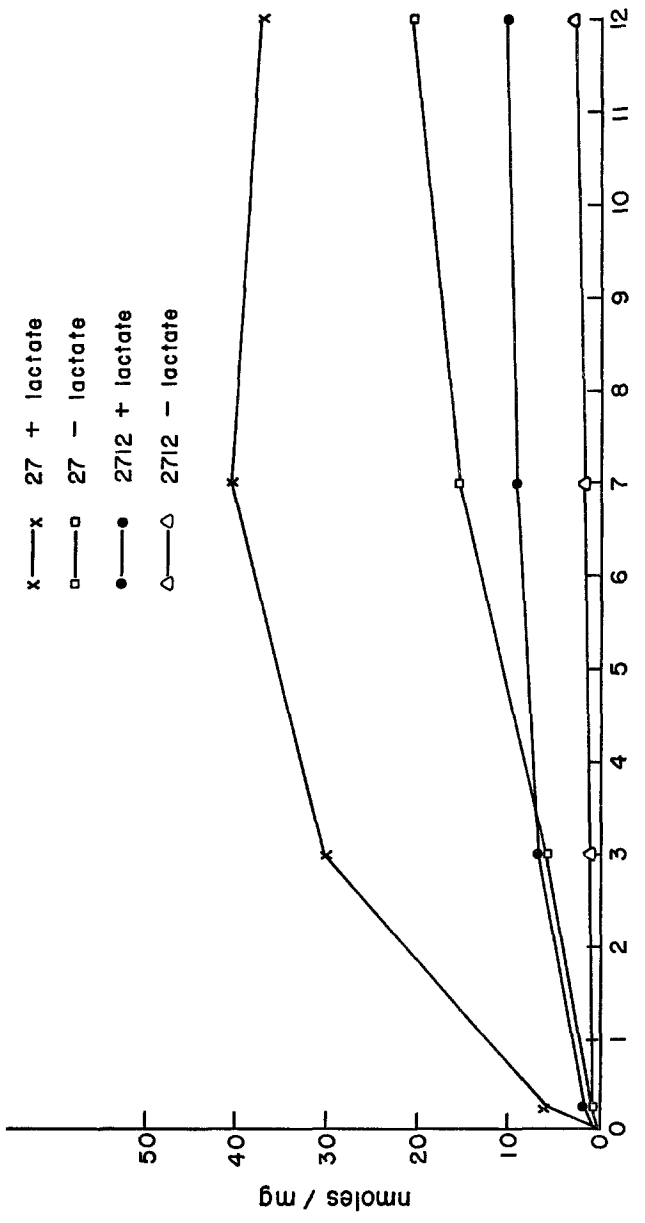

Fig. 3. The uptake of L-glycerol-3-phosphate in vesicles of Strains 27 and 2712 prepared by Kaback's procedure. The concentration of L-glycerol-3-phosphate was 10 μM, and 20 mM D(−) lactate was used as energy source. The transport assay is described in Materials and Methods

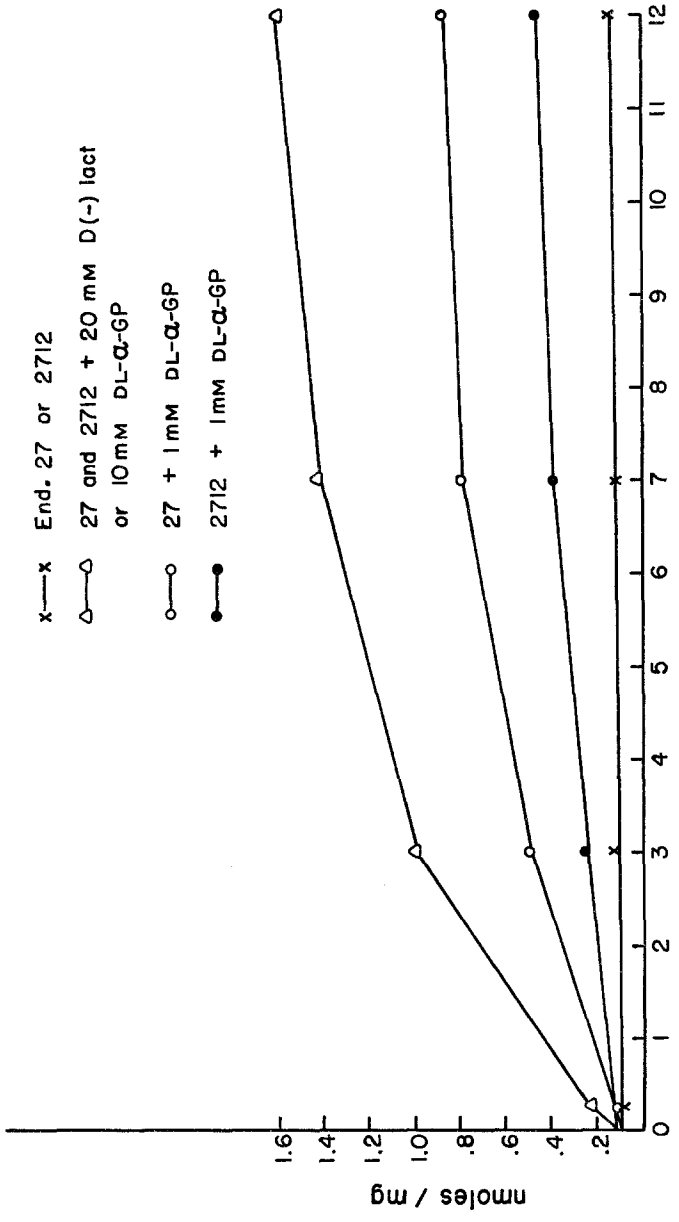

Fig. 4. Uptake of L-proline in membrane vesicles of Strains 27 and 2712 prepared by Kaback's procedure. The concentration of L-proline was 10 μM. Procedure described in Materials and Methods. End.: endogenous uptake, in absence of an energy source. αGP: glycerol-3-phosphate

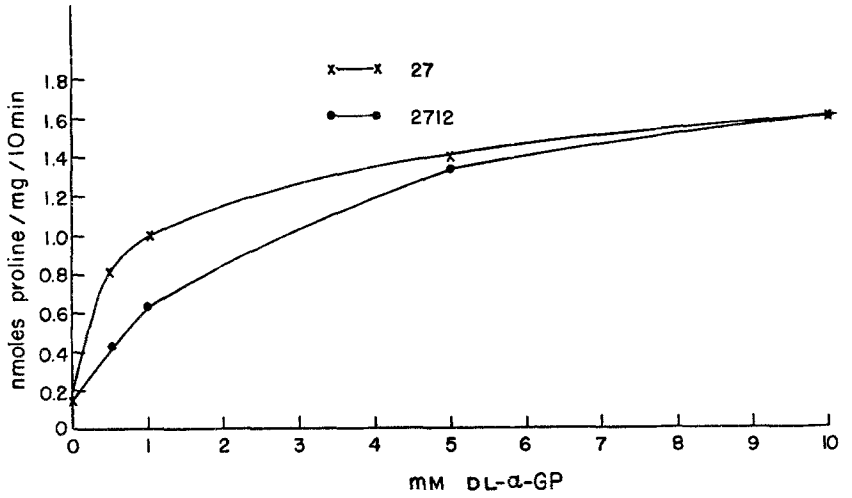

Fig. 5. Rate of uptake of L-proline in Kaback vesicles of Strains 27 and 2712 in the presence of various concentrations of DL-glycerol-3-phosphate as energy source. Procedure as in Fig. 4

Dehydrogenase Activity in Vesicles

Measurement of glycerol-3-phosphate dehydrogenase with the impermeable electron acceptor ferricyanide led to the surprising conclusion that a substantial fraction of the activity was localized on the external surface of the vesicles. Dehydrogenase activity for G-3-P, succinate and D-lactate was assayed in the presence of ferricyanide as electron acceptor (Table 2). Destruction of the membrane permeability barrier by toluenization or by treatment with 0.2% Triton X-100 consistently increased the activity two-

Table 2. Dehydrogenase activity of membrane vesicles of 27 and 2712

Assay	Specific activity − Toluene (units/mg)	Specific activity + Toluene (units/mg)
Ferricyanide Assay		
G-3-P	182	400
Succinate	180	360
Lactate	100	200
PMS/MTT Assay		
G-3-P	140	140
Succinate	60	60
Lactate	20	20

Assays performed as described in Materials and Methods. One unit for the ferricyanide assay is 1 nmole reduced per min per mg protein.

Table 3. Assay of G-3-P dehydrogenase and succinate dehydrogenase in steps of vesicle preparation of Strain 27

Step	G-3-P spec. act. (units/mg)	Total units	Succinate, spec. act. (units/mg)	Total units
Intact cells	80	350	14	60.2
Spheroplasts	51	207	14	60
Spheroplast supernatant	15	11	0	0
Lysed spheroplasts	50	200	23	69
Lysed sphero. supernatant	6	11.9	1	2.4
Wash supernatant	70	19.4	30	8.13
Low speed pellet	60	30	14	7
Vesicles	140	100	100	56

Steps are those described by Kaback (1971). Activity is for the PMS/MTT assay.

fold (Table 2). Fig. 2 shows that treatment with Triton X-100 led only to a change in V_m; K_m for the enzyme was unchanged. The K_m obtained in these experiments was 33 mM of DL-glycerol-3-phosphate in both the PMS/MTT and ferricyanide assays, with vesicles from Strains 27, 2712, 7 and ML 308, and also with vesicles that had been frozen and thawed. Incubation of the vesicles at 37 °C for 20 min to seal any open vesicles did not alter the results (Steck, 1972). As mentioned previously intact spheroplasts are inactive in this assay. Thus, in membrane preparations about one-half of the enzyme is made accessible to ferricyanide.

Dehydrogenase activity was also measured by the PMS/MTT assay; here the electron acceptor is permeable. Strains 27 and 2712 now show identical activities (Table 2) and addition of toluene has no effect. It is presumed that at the high concentration of DL-G-3-P used (30 mM), this compound enters by facilitated or simple diffusion at a rapid rate. Potassium cyanide was present in these assays at a concentration which completely abolishes active transport in vesicles, so that the inability of Strain 2712 to transport glycerol-3-phosphate is not important.

It is of interest to note that the specific activity of α-glycerophosphate dehydrogenase is increased only 1.6- to 1.9-fold in vesicles compared to intact cells while succinic dehydrogenase is increased five- to sixfold. Assays were carried out for both enzymes at each stage of the membrane vesicle preparation to see if α-glycerophosphate dehydrogenase became partially solubilized or inactivated at any point (Table 3). Little if any enzyme appears in the supernatant fraction, but 40% of the enzyme becomes inactivated at the stage of spheroplast formation. A similar irreversible in-

activation is observed after a brief, 1-min exposure of intact cells to 20% sucrose. This is not an osmotic effect because 1 M glycylglycine does not inactivate α-glycerophosphate dehydrogenase. Succinic dehydrogenase was not affected by exposure to sucrose. Membrane vesicles produced by lysis of whole cells with EDTA and lysozyme followed by three cycles of freeze thawing (but no exposure to sucrose) showed no evidence of inactivation during their preparation.

Further evidence on enzyme accessibility was provided by a study of the effects of sorbitol-6-phosphate, a competitive inhibitor of glycerol-3-phosphate dehydrogenase (Rose & Rose, 1969).

Using the PMS/MTT assay, K_I for purified glycerol-3-phosphate dehydrogenase was found to be 1.5 mM by David Tu and 1.4 mM by Christa Mollay, in studies carried out in this laboratory. Friedberg (1972) has shown that this compound cannot permeate the *E. coli* cytoplasmic membrane. When the PMS/MTT assay (*see* Materials and Methods) was applied to Kaback vesicles using DL-glycerol-3-phosphate in the presence of increasing concentrations of sorbitol-6-phosphate, the per cent inhibition reached 50% as a plateau value. This provides further evidence that half of the activity is accessible from the outside.

Discussion

Our results show that in intact *E. coli* the membrane-bound dehydrogenases are localized on the internal surface of the protoplasmic membrane. Experiments like those described above for the stimulation of amino acid uptake by glycerol-3-phosphate in intact cells have been performed with a mutant unable to transport succinate (mutant kindly supplied by Dr. W. Kay). Similar results were obtained; succinate must be transported inside the cell to be oxidized and supply energy for transport. The two dehydrogenases are not available to a substrate which cannot permeate. This would appear to eliminate the possibility that the substrate could be oxidized on the external membrane surface and thus not be transported. The situation is different in mitochondria. Here, the glycerol-3-phosphate dehydrogenase is localized on the outer surface of the inner membrane and G-3-P is oxidized without being transported. In fact, mitochondria are unable to take up G-3-P.

Membrane vesicles present a slightly different picture. At low concentrations of glycerol-3-phosphate (about 1 mM) it is necessary for the substrate to be transported into the vesicle to provide energy for amino acid uptake. At high concentrations the results are complicated by an apparent

high rate of diffusion of substrate into the vesicles. It seems likely that vesicles which are concentrating amino acids are correctly oriented.

While it is not possible to measure any dehydrogenase activity in intact cells or spheroplasts with ferricyanide as the electron acceptor, vesicles show significant activity, with 50% of the total activity being available to this impermeable acceptor. Destruction of the membrane permeability barriers makes all of the enzyme available to ferricyanide. These data, as well as the partial inhibition observed with the sorbitol-6-phosphate suggest that some change has occurred during preparation of membrane vesicles such that one-half of the enzyme is correctly oriented, i.e., accessible only from the inside. There are several possibilities: (a) two populations of vesicles, half correct and half inverted; (b) one population, with each vesicle being a mosaic of differently oriented patches of membrane; (c) all the vesicles are correctly oriented, but the membrane has been altered to allow some accessibility to substrate.

Antiserum against the purified glycerol-3-phosphate dehydrogenase was prepared, to help localize the enzyme. Antiserum completely inactivates the purified enzyme. Experiments with vesicles were complicated by the fact that control sera caused a 50% stimulation of enzyme activity. Specific antisera mixed with vesicles caused no change in activity. This may be due to the summation of 50% stimulation and 50% inhibition. This work must be repeated with more potent sera from which the activating factor has been removed.

Experimental results with other membrane systems would suggest that inversion during preparation of vesicles is the most probable explanation for the present results. Hampton and Freese (1973) present evidence that membrane vesicles prepared from *B. subtilis* are also partially inverted. Asano, Cohen and Brodie (1971) have found that vesicles prepared by sonication of ghosts of *Mycobacterium phlei* can also result in the formation of a mixed population of correctly oriented and inverted vesicles. These populations can be separated by treatment with antibody against an ATPase which is normally internal. Kaback (1972) has summarized evidence in favor of the view that a significant number of vesicles do not become inverted during lysis. The most striking evidence is the inability to detect inside-out vesicles by freeze-etch microscopy. Further experimentation will be required to interpret these different types of experimental results.

These experiments were performed in the laboratory of Dr. L. A. Heppel, who was a constant source of encouragement and advice. Drs. E. C. C. Lin and D. P. Richey were of invaluable help in providing the bacterial strains, in suggesting a way to select for

Mutant 2712 and in providing helpful advice on a number of occasions. Advice from Dr. E. Racker is also gratefully acknowledged.

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