Sigler, P. B., Blow, D. M., Matthews, B. W., and Henderson, R. (1968), *J. Mol. Biol. 35*, 143.
Somkuti, G. A., Babel, F. J., and Somkuti, A. C. (1969), *Appl. Microbiol. 17*, 606.
Strauss, J. S., and Kligman, A. M. (1960), *Arch. Dermatol. 82*, 779.
Strauss, J. S., and Pochi, P. E. (1965), *Arch. Dermatol. 92*, 443.
Voss, J. G. (1970), *J. Bacteriol. 101*, 392.
Weber, K., Freedman, R., and Eudy, W. W. (1971), *Appl. Microbiol. 21*, 639.
Weber, K., and Osborn, M. (1969), *J. Biol. Chem. 224*, 4406.
Zierdt, C. H., Webster, C., and Rude, A. W. (1968), *Nat. J. Syst. Bact. 18*, 33.

Reconstitution of Transport Dependent on D-Lactate or Glycerol 3-Phosphate in Membrane Vesicles of *Escherichia coli* Deficient in the Corresponding Dehydrogenases†

Masamitsu Futai

ABSTRACT: By addition of purified D-lactate dehydrogenase to membrane vesicles made from an *Escherichia coli* mutant lacking this enzyme, it was possible to restore D-lactate dependent respiration and transport. This confirms the observations of J. P. Reeves, J.-S. Hong, and H. R. Kaback (1973, *Proc. Nat. Acad. Sci. U. S. 70*, 1917) made with a chaotropic extract of membranes from wild type cells as a source of enzyme. We have extended this procedure and obtained similar results on binding partially purified glycerol-3-phosphate dehydrogenase to membrane vesicles which were uninduced for this enzyme. In both systems, the greatest extent of reconstitution of respiration and stimulation of transport resulted from preincubation of deficient vesicles with dehydrogenase in 0.1 M potassium phosphate (pH 6.6) at 37° for 10 min. The nonpermeable electron acceptor, ferricyanide, completely inhibited the transport of amino acid in reconstituted vesicles driven by glycerol 3-phosphate. This would suggest that, as expected, the enzyme added back to vesicles remains on the outside. Unexpectedly, when vesicles made from cells in which the dehydrogenase had been induced in the normal fashion were examined, the uptake of amino acids driven by glycerol 3-phosphate was partially inhibited by ferricyanide. Transport by spheroplasts was not inhibited by this compound. We favor the interpretation that this dehydrogenase has moved outside during preparation of membranes. D-Lactate-driven transport by reconstituted vesicles was only slightly inhibited by ferricyanide, which was partly explained by the evidence that ferricyanide is a poor electron acceptor for purified D-lactate dehydrogenase.

The oxidation of D-lactate by the membrane bound D-lactate dehydrogenase greatly stimulates the active transport of various amino acids into membrane vesicles (see review by Kaback, 1972). An inducible dehydrogenase, glycerol-3-phosphate dehydrogenase, plays a similar role in membrane vesicles from cells induced for this enzyme (Dietz, 1971). Both enzymes have been solubilized from membranes and purified to apparent homogeneity (Kohn and Kaback, 1973; Futai, 1973; Weiner and Heppel, 1972).

Recently Reeves *et al.* (1973) made the striking observation that D-lactate dehydrogenase in guanidine-HCl extracts of membranes from wild type cells could restore D-lactate-dependent respiration and transport in membrane vesicles from mutants lacking this enzyme. This intriguing discovery encouraged us to attempt to determine if reconstitution was possible with highly purified enzyme and to see if other dehydrogenases are also active in restoration of respiration and transport. In the present investigation purified D-lactate dehydrogenase (Futai, 1973) or partially purified glycerol-3-phosphate dehydrogenase mixed with deficient membrane vesicles (Kaback, 1972) was found to function as a part of the oxidase system and was also observed to support the transport of amino acids.

Reeves *et al.* (1973) pointed out that reconstituted vesicles of this type differ from "natural" vesicles in their response to the D-lactate dehydrogenase inhibitor, oxamate. This may be due to the fact that the enzyme was bound to the outside of the vesicles, which may not be the normal site. In the present study, "natural" and reconstituted vesicles, both containing glycerol-3-phosphate dehydrogenase, were compared with respect to sensitivity to ferricyanide. This impermeant agent would act as a competing electron acceptor if it gained access to the dehydrogenase and therefore inhibit respiration and transport driven by glycerol 3-phosphate. The results suggested that glycerol-3-phosphate dehydrogenase in "natural" vesicles from normally induced cells had partly moved to the outside during preparation of membrane vesicles. By contrast, D-lactate-driven transport by reconstituted vesicles was only slightly inhibited by ferricyanide. Part of the reason for this is that ferricyanide is a poor electron acceptor for D-lactate dehydrogenase.

Materials and Methods

Bacteria and Growth Conditions. E. coli dld 3 (D-lactate dehydrogenase negative) derived from *E. coli* ML 308-225 ($i^-z^-y^+a^+$) was a generous gift from Dr. H. R. Kaback (Hong

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and Kaback, 1972). *E. coli* strain 27 (K12), constitutive for glycerol-3-phosphate dehydrogenase, was kindly supplied by Dr. E. C. C. Lin. All cultures were grown aerobically in a synthetic medium (Tanaka *et al.*, 1967) supplemented with 0.5% succinate (ML 308-225, dld 3, strain 27) or 0.5% glycerol (ML 308-225). Physiologically young cells (Birdsell and Cota-Robles, 1967) were harvested by centrifugation and twice washed with cold 0.01 M Tris-HCl buffer (pH 8).

Membrane vesicles were prepared essentially as described by Kaback (1971).

Partial Purification of Glycerol-3-phosphate Dehydrogenase. A membrane fraction was obtained from 36 g (wet weight) of *E. coli* strain 27 using lysozyme–EDTA followed by treatment with DNase and RNase as previously described (Weiner and Heppel, 1972; Futai, 1973). The following steps were carried out at 0–4°. The membrane fraction was suspended in 90 ml of 0.01 M Tris-Cl (pH 7.4) containing 0.02% β-mercaptoethanol and 100 mg/l. of phenylmethylsulfonyl fluoride. After sonication in the Raytheon Model DF101 at 10 kc for 7 min, the suspension was centrifuged at 10,000*g* for 10 min. To 100 ml of the supernatant 17.6 g of solid ammonium sulfate was added and the mixture was stirred for 1 hr. The precipitate obtained by centrifugation at 48,000*g* for 30 min was suspended in 100 ml of 0.05 M Tris-Cl (pH 8.0) containing 15 mM glycerol 3-phosphate. Sodium perchlorate (3 M) was added to a final concentration of 0.45 M followed by stirring for 20 min. To 100 ml of supernatant obtained after centrifugation at 78,000*g* for 90 min, 31 g of solid ammonium sulfate was added. After the mixture was stirred for 10 min, a precipitate was obtained by centrifugation at 10,000*g* for 10 min. This was then dissolved in 1 ml of 0.01 M Tris-Cl (pH 7.4), 0.02% β-mercaptoethanol, and dialyzed against the same buffer overnight. The dialyzed solution was centrifuged at 100,000*g* for 60 min. The supernatant fraction contained most of the enzyme activity (5.0 units/ml). Recovery and specific activity were 60% and 0.70 unit/mg of protein, respectively. The final purification was about tenfold, as the specific activity of the membrane fraction was 0.06 unit/mg of protein.

Reconstitution of Oxidation and Transport Dependent on D-*Lactate or Glycerol 3-Phosphate.* Purified D-lactate dehydrogenase (about 0.4 unit) and glycerol-3-phosphate dehydrogenase (about 0.6 unit) were incubated in 0.1 M potassium phosphate buffer (pH 6.6) with membrane vesicles (about 1 mg of protein) from dld 3 or ML 308-225, grown on succinate. After incubation at 37° for 10 min, the mixture was centrifuged at 90,000*g* for 20 min. The pellet was resuspended in 1.0 ml of 0.1 M potassium phosphate buffer (pH 6.6), using the Potter–Elvejehm homogenizer. In some experiments this centrifugation was omitted, as time required for it affected the conditions tested. For the reconstitution of D-lactate-dependent oxidation and transport, the concentration of Triton X-100 was kept below 4 μg/mg of total protein, unless otherwise specified.

Transport by Membrane Vesicles. Transport was assayed at 23° by a procedure essentially the same as that used by Kaback (1971). The incubation mixture contained 10 mM D-lactate or glycerol 3-phosphate, 10 μM amino acid, and membrane vesicles (100–200 μg of protein).

Transport Assay for Spheroplasts. Spheroplasts were prepared from glycerol grown cells as described previously (Futai, 1974). After incubation with lysozyme and EDTA, 0.04 M $MgCl_2$ was added to minimize the effect of EDTA during the transport experiment. The uptake of amino acids was done in a total volume of 0.5 ml containing 0.01 M $MgCl_2$, 0.04 M potassium phosphate (pH 6.6), and 20% sucrose with or without 10 mM glycerol 3-phosphate. The reaction was begun by addition of amino acid to a final concentration of 10 μM. At various times after incubation at 23°, 0.2-ml samples were removed, filtered on 25-mm nitrocellulose filters (0.45 μ), and washed with 10 ml of solution containing 20% sucrose, 0.01 M $MgCl_2$. The rest of the procedure was essentially the same as for transport by intact cells (Berger, 1973). This assay was done within 1 hr after the formation of spheroplasts, as transport activity was reduced by about 50% after storage for 4 hr.

Other Assay Procedures. Oxygen uptake was measured in 0.05 M potassium buffer (pH 7.0) containing 1 mM $MgCl_2$ at 23°, using the Gilson oxygraph. D-Lactate dehydrogenase and glycerol-3-phosphate dehydrogenase were assayed by following the phenazine methosulfate mediated reduction of 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide, as described previously (Weiner and Heppell, 1972; Futai, 1973). One unit of each enzyme was defined as the amount of enzyme which reduces 1 μmol of the above tetrazolium dye per minute. Triton X-100 was estimated by its absorbancy at 275 nm. Difference spectra of cytochromes and other membrane pigments were taken at 77°K using the Aminco Model DU-2 spectrophotometer with low-temperature adaptor. Protein was determined by the method of Lowry *et al.* (1951).

Method for Concentrating D-*Lactate Dehydrogenase and Glycerol-3-phosphate Dehydrogenase.* Purified D-lactate dehydrogenase that had been dialyzed against 0.05 M sodium phosphate (pH 7.1) containing 1% Triton X-100 and 0.02% β-mercaptoethanol was applied to a DEAE-cellulose (DE 52, Whatman) column (0.5 × 4 cm) equilibrated with the same buffer. The column was washed with a small portion of the same buffer and the enzyme was eluted with 0.2 M NaCl in the same buffer. The fraction containing most of the activity (about 0.5 ml) was dialyzed against the above buffer for 4 hr to remove NaCl. Purified glycerol-3-phosphate dehydrogenase that had been dialyzed against 0.01 M Tris-Cl (pH 7.3) containing 0.2% Brij-58 and 0.05% β-mercaptoethanol was applied to a DEAE-cellulose column (0.5 × 4 cm) equilibrated with this buffer. The column was washed and the enzyme was eluted with 0.2 M NaCl in above buffer followed by dialysis as above.

Materials. D-Lactate dehydrogenase was purified as described previously (Futai, 1973). Purified glycerol-3-phosphate dehydrogenase was a gift from Weiner and Heppel (1972). [^{14}C]Proline, [^{14}C]serine, [^{14}C]lysine, and [^{14}C]lactose were from New England Nuclear Co. The isotopes were diluted with nonradioactive material to a final specific activity of 20–25 Ci/mol. Triton X-100 was from Rohm and Haas Co. Bovine serum albumin and glycerol 3-phosphate were purchased from Sigma Co. D-Lactate was a product of Calbiochem Co. Other chemicals not listed here were either described previously (Futai, 1973, 1974) or were reagent grade from commercial sources.

Results

Effect of Detergents on the Reconstitution. Both D-lactate dehydrogenase and glycerol-3-phosphate dehydrogenase were solubilized from the membranes and purified with the aid of Triton X-100 and Brij-58, respectively (Futai, 1973; Weiner and Heppel, 1972). As attempts to remove these neutral detergents from enzyme preparations without appreciable loss of activity were unsuccessful, the solutions were concentrated so that the associated detergent had no effect on the reconstitution experiments. Under "standard conditions," vesicles

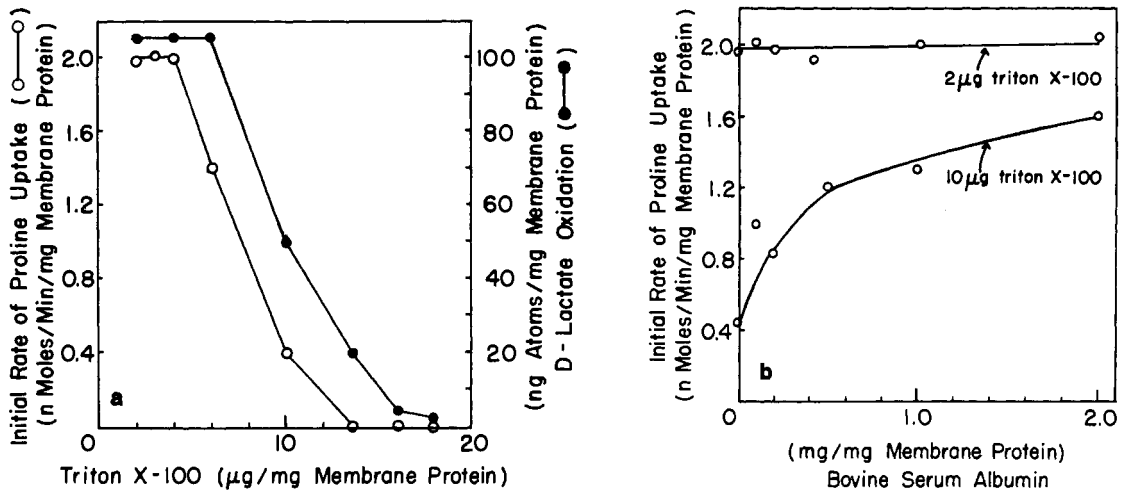

FIGURE 1: (a) Effect of Triton X-100 on the reconstitution of D-lactate dependent oxidation and uptake of proline. Membrane vesicles (1.0 mg of protein) from *E. coli* dld 3 (D-lactate dehydrogenase negative) were incubated with D-lactate dehydrogenase (0.4 unit) in the presence of different amounts of Triton X-100 in 0.1 M potassium phosphate (pH 6.6). After incubation at 37° for 10 min the incubation mixture was mixed with 4.0 ml of 0.1 M potassium phosphate (pH 6.6) followed by centrifugation. The precipitate obtained was suspended in 1.0 ml of the above buffer and proline transport (○) and oxidation (●) dependent on D-lactate were assayed. For other details see text. (b) Effect of bovine serum albumin on the restoration of transport of proline. Membrane vesicles (1.0 mg of protein) from dld 3, different concentrations of bovine serum albumin and D-lactate dehydrogenase (0.4 unit) were preincubated as described in the legend of Figure 1a in the presence of 2 μg (upper curve) or 10 μg (lower curve) of Triton X-100. Transport of proline was then assayed. For details see text.

from dld 3 preincubated with D-lactate dehydrogenase recovered D-lactate-dependent oxidation and transport of amino acid if the concentration of Triton X-100 in the preincubation mixture was below 6 μg/mg of membrane protein. With increase in amount of detergent, the extent of reconstitution became less, and no reconstitution of transport was observed in the presence of 14 μg of Triton X-100/mg of membrane protein (Figure 1a). It is noteworthy that reconstitution of respiration was slightly less sensitive to Triton X-100. With the addition of bovine serum albumin to the preincubation mixture, reconstitution of transport in the presence of 10 μg of Triton X-100/mg of protein increased about threefold (Figure 1b). This is in accord with the finding of Makino *et al.* (1973) that bovine serum albumin binds Triton X-100 with high affinity. However, reconstitution was not increased by the addition of bovine serum albumin if the concentration of Triton X-100 was kept below 4 μg/mg of membrane protein. In the present study Triton X-100 was kept below this concentration.

In the presence of comparable amounts of Brij-58, glycerol-3-phosphate-dependent oxidation or transport could not be reconstituted using purified enzyme and membrane vesicles from ML 308-225 uninduced for this inducible dehydrogenase. This could be explained by the fact that 1 and 5 μg of Brij-58 inhibited reconstitution by 30 and 100%, respectively. For the following experiments, partially purified glycerol-3-phosphate dehydrogenase made without the use of detergent was employed.

Reconstitution of Oxidation and Transport Dependent on Glycerol 3-Phosphate or D-Lactate. The optimum concentration and pH of potassium phosphate necessary for the reconstitution of D-lactate-dependent transport and oxidation were the same as for the glycerol 3-phosphate dependent reactions: 0.1 M potassium phosphate buffer (pH 6.5–7.0) was optimal for both systems. In 0.05 M of the same buffer, about half of the maximal reconstitution was observed (data not shown). The pH optimum for both reconstitution systems was broad, as at pH 8.5 about 50% of the maximum reconstitution was obtained. These conditions were also necessary for simple binding of enzymes to the deficient membrane as measured by centrifugation experiments. Under these optimal conditions, reconstitution of transport and oxidase driven either by D-lactate or glycerol 3-phosphate were a function of the amount of each enzyme added to the deficient membranes (Figures 2 and 3). The saturation curve for the reconstitution of transport was slightly different with each amino acid tested (Figures 2a and 3a). The saturation curve for the binding was approximately similar to that for the recovery of oxidation (Figures 2b and 3b). The optimum temperature for both reconstitution systems was 37° (Figures 4a,b). As a control, wild type vesicles were preincubated under the same conditions of temperature and pH, and this had no effect on subsequent measurements of transport or oxidase under standard assay conditions.

It is interesting that reconstitution of the glycerol 3-phosphate dependent reaction was more affected by the preincubation temperature than was the D-lactate-dependent reaction. The maximum reconstitution of glycerol 3-phosphate dependent oxidation or transport required incubation for 120 min at 25°, while 10 min at 37° was sufficient. At 0° an incubation time of 120 min was needed to obtain 50% of the maximum reconstitution of the glycerol 3-phosphate system, as compared with 15 min for the D-lactate system.

Comparison of Reconstituted Vesicles and "Natural Vesicles." The rates of oxidation and transport by reconstituted vesicles and "natural" vesicles were compared in order to show that substantial restoration had been achieved. The maximal rate of oxygen consumption in the presence of glycerol 3-phosphate after reconstitution was 400 ng of atoms/mg of protein^{-1} min^{-1}, while the rate of oxidation of vesicles from normally induced cells was 230 ng of atoms mg^{-1} min^{-1}. Rates of proline uptake by reconstituted and "natural" vesicles were 2.0 and 1.3 nmol mg^{-1} min^{-1}, respectively. Glycerol-3-phosphate dehydrogenase activities of membranes from induced cells and of enzyme bound to deficient vesicles were 0.17 and 0.22 unit/mg of protein, respectively. These results suggest that bound enzyme is acting almost as efficiently as enzyme in membranes from induced cells.

When the D-lactate system was reconstituted, the levels of D-lactate-dependent oxidation and transport were about half

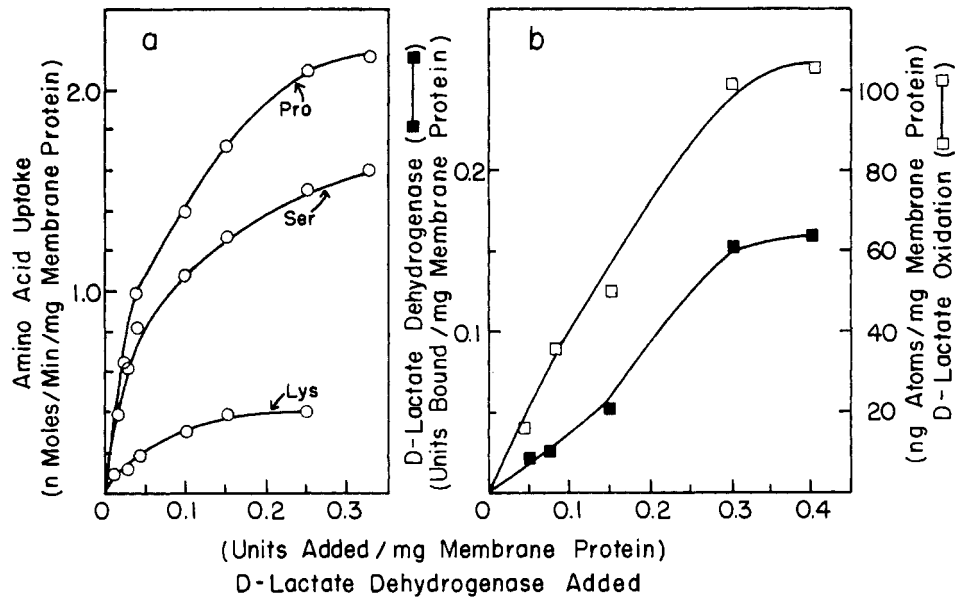

FIGURE 2: (a) Reconstitution of D-lactate dependent transport in dld 3 vesicles by purified enzyme. Membrane vesicles (1.0 mg of protein) from dld 3 were preincubated with increasing amount of D-lactate dehydrogenase. After, incubation amino acid transport was assayed in the presence of D-lactate. (b) Reconstitution of D-lactate-dependent oxidation and binding of D-lactate dehydrogenase in dld 3 vesicles by purified enzyme.

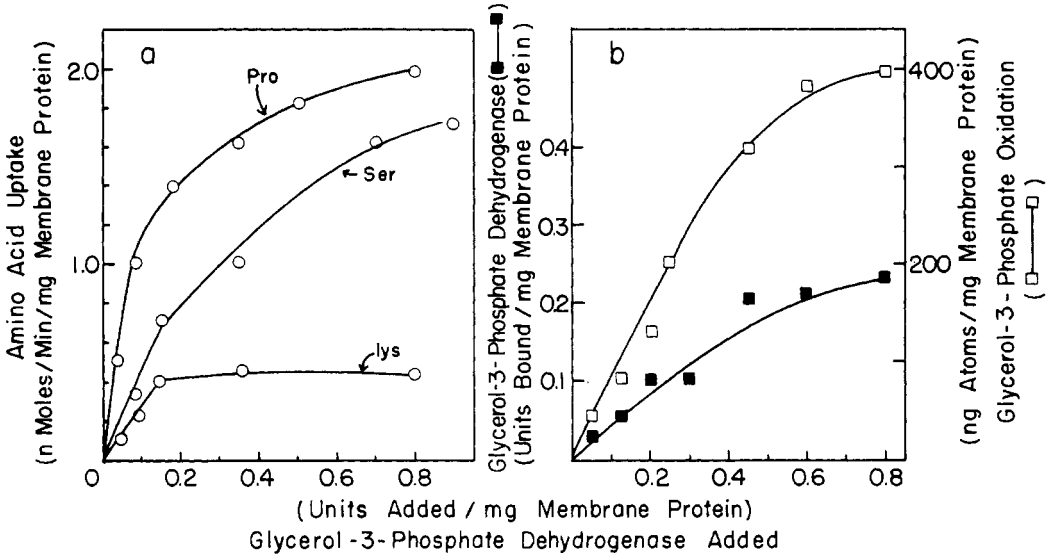

FIGURE 3: Reconstitution of glycerol 3-phosphate dependent oxidation and transport and binding of glycerol-3-phosphate dehydrogenase in uninduced membrane vesicles. Membrane vesicles (1.0 mg of protein) from *E. coli* ML 308-225 grown on succinate and having no glycerol-3-phosphate dehydrogenase activity were preincubated with increasing amount of partially purified glycerol-3-phosphate dehydrogenase in 0.1 M potassium phosphate (pH 6.6). After incubation at 37° for 10 min, the mixture was treated as in the legend of Figure 1a. Amino acid transport of the membranes suspended in 0.1 M potassium phosphate (pH 6.6) was measured in Figure 3a. Oxidation dependent on glycerol-3-phosphate (□) and activity of glycerol-3-phosphate dehydrogenase bound to the membrane (■) are shown in Figure 3b.

of the levels observed for vesicles from wild type cells. Thus, the oxidation rates by reconstituted vesicles and "natural" vesicles (from ML 308-225 grown on succinate) were 100 and 376 ng of atoms mg^{-1} min^{-1}, respectively. The corresponding rates of proline transport were 2.0 and 3.8 nmol mg^{-1} $min.^{-1}$ The level of D-lactate dehydrogenase bound to the deficient membrane was 0.15 unit/mg of protein, while the specific activity in "natural" vesicles was 0.17 unit/mg. Again, these results indicate a substantial degree of reconstitution.

Effect of Various Compounds on Transport and Respiration of Reconstituted Vesicles. Treatment with 4.5 mM KCN and 2×10^{-5} M hydroxyquinoline *N*-oxide inhibited glycerol 3-phosphate dependent oxidation of the reconstituted vesicles by 64 and 50%, respectively. The sulfhydryl reagent *N*-ethylmaleimide (1.0 mM) inhibited glycerol 3-phosphate dependent transport of reconstituted membranes by 90%, though inhibition of respiration was only 44%. Essentially the same results were obtained in D-lactate dependent reactions of reconstituted vesicles (dld 3). These results are similar to those of Reeves *et al.* (1973), in which reconstitution was done with dld 3 vesicles and guanidine-HCl extracts.

These results suggest that each dehydrogenase added to the deficient vesicles is donating electrons to some part of the respiratory chain. This was further confirmed by the difference spectrum (reduced by D-lactate or glycerol 3-phosphate *vs.* oxidized) of both reconstituted vesicles, which was qualitatively the same as that of vesicles from wild type cells or induced cells.

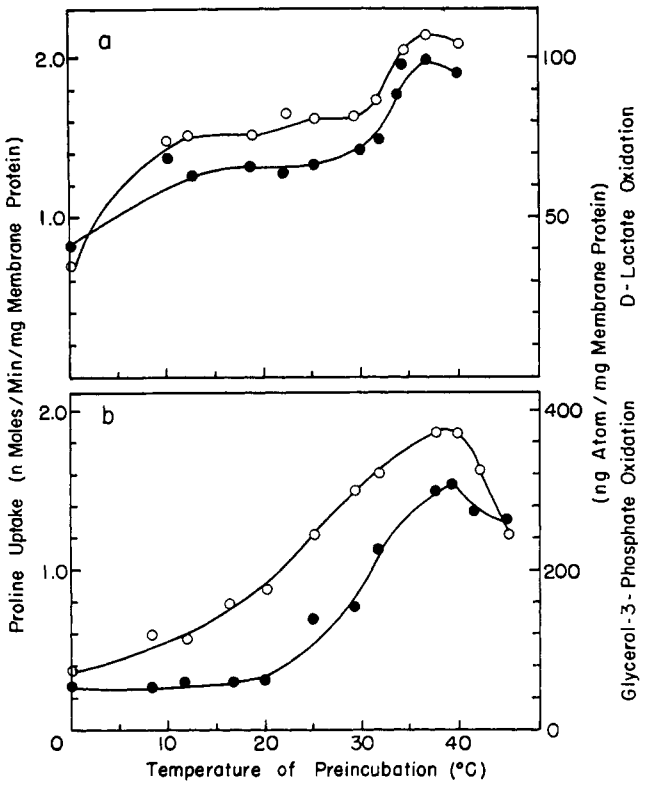

FIGURE 4: (a) Effect of temperature on the reconstitution of D-lactate-dependent oxidation and transport. Membrane from dld 3 was preincubated at different temperature for 10 min with purified D-lactate dehydrogenase. After preincubation, aliquots were taken and oxidation (●) and proline uptake (○) driven by D-lactate were assayed. In this experiment centrifugation of reconstituted vesicles was omitted to obtain more accurately the effect of temperature. (b) Effect of temperature on the reconstitution of gycerol 3-phosphate dependent oxidation and transport. Membranes from ML 308-225 grown on succinate were incubated at different temperatures for 10 min with partially purified glycerol-3-phosphate dehydrogenase. After preincubation, samples were taken and oxidation (●) and proline uptake (○) driven by glycerol 3-phopshate were assayed. Centrifugation of membranes was omitted in this experiment in order to measure temperature effects more accurately. For details see text.

Oxamate, a D-lactate dehydrogenase inhibitor, caused effux from reconstituted vesicles made from dld 3 vesicles and pure D-lactate dehydrogenase, which were preloaded with either proline or lactose. This confirms the observation made by Reeves *et al.* (1973).

Effect of Ferricyanide on Amino Acid Transport by Reconstituted Membrane Vesicles. Ferricyanide has been widely used in microbial, plant and mammalian systems as an impermeant electron acceptor. Since this compound can be used as an electron acceptor by dehydrogenases, it would be expected to compete with the endogenous electron acceptor when dehydrogenase was present on the outside face of the membrane. This competition might prevent the stimulation of transport into reconstituted vesicles in which the dehydrogenase had been added to deficient membrane vesicles from the outside.

As shown in Figure 5a, glycerol 3-phosphate dependent transport of amino acids by reconstituted vesicles was almost completely inhibited by ferricyanide. In the case of natural vesicles from cells induced for glycerol-3-phosphate dehydrogenase, inhibition was also obtained but it was only partial. The extents of inhibition of the transport of proline, serine, and lysine were 65, 55, and 30%, respectively (Figure 5b). D-

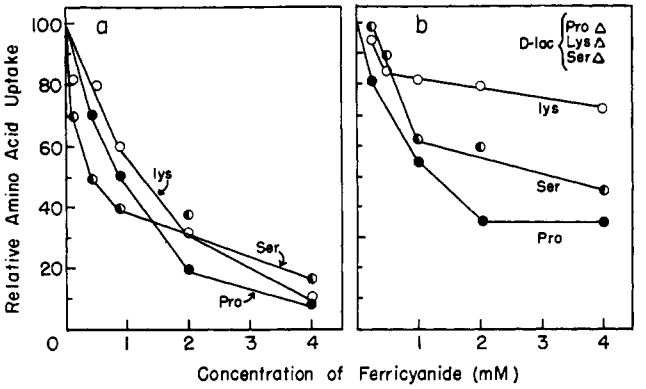

FIGURE 5: (a) Effect of ferricyanide on glycerol 3-phosphate dependent transport of amino acids by reconstituted vesicles. Membrane vesicles from ML 308-225 grown on succinate were incubated with partially purified glycerol-3-phosphate dehydrogenase. Reconstituted vesicles obtained as in the legend of Figure 3 was assayed for the transport of amino acids in the presence of ferricyanide. (b) Effect of ferricyanide on glycerol 3-phosphate dependent transport of amino acids by natural vesicles. Amino acid uptake by membrane vesicles from ML 308-225 grown on glycerol, which has glycerol-3-phosphate dehydrogenase, was assayed in the presence of ferricyanide (circles). D-Lactate dependent uptake of amino acids by the same membrane vesicles was also tested (triangles). Results were expressed as relative rates % of control (without ferricyanide). Control rates for glycerol 3-phosphate driven uptake were: proline, 0.70 nmol; serine, 0.99 nmol; lysine, 0.59 nmol, expressed per mg of protein. Control rates for D-lactate dependent uptake were: proline, 1.25 nmol; serine 1.16 nmol; lysine, 0.42 nmol, expressed per mg of protein. See text for details.

Lactate-dependent transport of these natural vesicles was only slightly inhibited (triangles in Figure 5b). The inhibition of transport by either reconstituted or natural vesicles was reversed simply by washing away ferricyanide (Table I). Ferricyanide inhibited glycerol 3-phosphate dependent oxygen uptake by reconstituted vesicles completely, while that of natural vesicles was inhibited only 50%.

TABLE I: Reversibility of the Inhibition by Ferricyanide of Transport by Vesicles.[a]

Vesicles	Addition: Preincubation	Addition: Transport Assay	Proline Transport (nmol min^{-1} mg^{-1})
Vesicles from induced cells (ML308-225 grown on glycerol)	None	None	0.75
	Ferricyanide	None	0.66
	None	Ferricyanide	0.24
Reconstituted vesicles	None	None	1.80
	Ferricyanide	None	1.70
	None	Ferricyanide	0.14

[a] Membrane vesicles (about 2.5 mg of each preparation) were preincubated with or without 4 mM ferricyanide in 5.0 ml of 0.1 M potassium phosphate (pH 6.6) containing 10 mM $MgSO_4$ and 10 mM glycerol 3-phosphate. After 5 min at 23°, the suspension was centrifuged at 90,000*g* for 20 min. The pellet was suspended in 5.0 ml of 0.1 M potassium phosphate buffer (pH 6.6) containing 10 mM EDTA and centrifuged as above. Precipitate was then suspended in 1.0 ml of 0.1 M potassium phosphate buffer (pH 6.6) and transport was assayed as already described.

TABLE II: Effect of Ferricyanide on Amino Acid Transport by Spheroplasts.[a]

Experiment	Amino Acid Transport (nmole min^{-1} mg of $protein^{-1}$)		
	Proline	Serine	Lysine
Expt 1			
Spheroplasts	3.82	5.18	0.88
Spheroplasts plus 4 mM ferricyanide	4.30	5.23	0.78
Spheroplasts lysed[b]	0.12		
Expt 2			
Cells[c]	4.52		
Cells[d] (lysis condition of spheroplasts)	4.34		

[a] Transport by spheroplasts was done in 20% sucrose containing 10 mM glycerol 3-phosphate, 0.01 M $MgCl_2$, and 0.04 M potassium phosphate (pH 6.6) and washed with 20% sucrose containing 0.01 M $MgCl_2$. Less than half of the above value was obtained by each transport system assayed in the absence of glycerol 3-phosphate; transport of proline, serine, and lysine by spheroplasts were 1.3, 2.5, and 0.38 nmol min^{-1} mg of $protein^{-1}$, respectively. [b] Spheroplasts were diluted 20-fold by 0.01 M Tris (pH 8.0) to get lysis and centrifuged at 40,000*g* for 30 min. The precipitate was suspended in original volume of 20% sucrose and assayed transport as above. [c] Cells were treated as for spheroplast formation except for addition of lysozyme. [d] Cells used in *c* were treated with 0.01 M Tris (pH 8.0) as in *b*. See text for details.

In contrast to the results with glycerol-3-phosphate dehydrogenase, D-lactate dependent transport of reconstituted vesicles was inhibited only 20% by ferricyanide, and "natural" vesicles showed no inhibition. The selective inhibitory action of ferricyanide, in affecting glycerol 3-phosphate but not D-lactate dependent transport, suggests that ferricyanide is not getting electrons from the respiratory chain beyond the dehydrogenase site. It is noteworthy that D-lactate dehydrogenase reduced ferricyanide much more slowly than did glycerol-3-phosphate dehydrogenase, when 1 mM ferricyanide replaced organic dyes in the standard assay mixture: 0.7 unit of purified D-lactate dehydrogenase reduced only 0.02 μmol of ferricyanide, while 0.06 unit of glycerol-3-phosphate dehydrogenase reduced 0.18 μmol. This partly explains the poor inhibition of D-lactate-stimulated systems, even in reconstituted vesicles. However, it is still possible that the location of D-lactate dehydrogenase in reconstituted or natural vesicles is different from that of glycerol-3-phosphate dehydrogenase.

Effect of Ferricyanide on the Uptake of Amino Acids by Spheroplasts. As shown in Table II, ferricyanide had no effect on lysine, proline, or serine transport by spheroplasts obtained from cells grown on glycerol. This transport of amino acids was shown to be due to spheroplasts and not contaminating cells because it was completely lost by dilution in 0.01 M Tris (pH 8.0). Dilution causes lysis of spheroplasts but has no effect on transport activity of cells treated in the same way except for addition of lysozyme.

Discussion

Oxidation and transport of amino acids driven by either D-lactate or glycerol 3-phosphate have been restored to deficient membrane vesicles prepared by use of a D-lactate dehydrogenase mutant (dld 3) or by using cells uninduced for glycerol-3-phosphate dehydrogenase. Conditions of pH, temperature, etc., necessary for maximal restoration of these activities may be required mainly for the interaction of enzyme with membrane vesicles, since these same conditions were also necessary for the binding of enzyme as measured in centrifugation studies. The effect of temperature on the reconstitution was not reported by Reeves *et al.* (1973) in their initial study. The fact that purified D-lactate dehydrogenase restored these activities may suggest that the detergents used in the purification did not change the enzyme significantly.

The artificial nature of this type of reconstitution was already suggested by the oxamate-induced efflux of lactose from reconstituted vesicles but not from "natural" vesicles (Reeves *et al.*, 1973). This is further suggested by the complete inhibition of amino acid uptake by ferricyanide reported here. Spheroplasts, which show large areas of apparently completely exposed inner membrane, show no inhibition of transport by ferricyanide.

The effect of ferricyanide is interesting and must be discussed in detail. As shown previously (Weiner, 1974; Futai, 1974) ferricyanide could not accept electrons from the inner membrane of spheroplasts. Thus, dehydrogenase activity when assayed with ferricyanide could not be measured until toluene was added to destroy the membrane permeability. This is in agreement with the present finding that ferricyanide had no effect on transport by spheroplasts. In both experiments, spheroplasts were made by the procedure of Birdsell and Cota-Robles (1967) and the inner membrane is the only visible barrier in the electron microscope, although the existence of another barrier which is invisible by electron microscope observation could not be excluded. Weiner (1974) showed that about 50% of total ferricyanide reductase activity of vesicles prepared by the method of Kaback was measurable without toluenization though no activity was measurable in intact spheroplasts. Futai (1974) confirmed this finding and extended it by demonstrating that 100% of this activity was measurable in inside-out vesicles made by a procedure developed by E. Hertzberg and P. Hinkle (unpublished). The present finding that amino acid uptake driven by glycerol 3-phosphate was completely inhibited by ferricyanide further confirms the notion that the glycerol-3-phosphate dehydrogenase supporting transport in reconstituted vesicles is on the outside surface of the membrane.

It would be desirable to supplement these data with other experiments using antisera against the two dehydrogenases as probes of enzyme localization. However, repeated injection into four rabbits failed to produce a useful antibody titre, using either purified glycerol-3-phosphate dehydrogenase or D-lactate dehydrogenase. This difficulty has also been reported by Short *et al.* (1974).

Oxidation of glycerol 3-phosphate or D-lactate from either side of the membrane must be coupled to energy conservation, since both types of oxidation can drive amino acid transport. This finding is not inconsistent with chemiosmotic hypothesis, since more than one site for proton translocation and energy conservation exists within the electron transport chain.

Since transport is a vectorial process the effects studied here involve only right-side out vesicles. The most probable explanation for ferricyanide inhibition of transport is that part of the glycerol-3-phosphate dehydrogenase changed its location during the preparation of the vesicles and this en-

zyme is still supporting transport just as the external enzyme functions in the reconstituted membrane.

Weiner (1974) showed that mutant cells lacking carrier for glycerol 3-phosphate could not transport amino acids using glycerol 3-phosphate in the medium as energy source, suggesting that this compound must be transported to stimulate uptake of another solute. However, he reproducibly observed that membrane vesicles from this mutant show amino acid transport stimulated by glycerol 3-phosphate, although its rate was about half that of vesicles from wild type cells. Thus, Weiner's observation supports the notion that a dehydrogenase may change its localization during preparation of vesicles and yet continue to support transport activity.

Leakiness of vesicles is another explanation of ferricyanide inhibition. This seems to be unlikely, as these vesicles can concentrate amino acids and sugars. However, it is difficult to exclude this possibility completely. It is noteworthy that the extent of inhibition of transport by ferricyanide differs with the amino acid tested. This might suggest different energy requirements for each transport system. Variation in the extent of inhibition with different transport systems was also observed in the case of other inhibitors of the respiratory chain (Berger and Heppel, 1974).

During the preparation of this paper, Thienen and Postma (1973) reported that vesicles prepared by the procedure of Kaback contain inside-out and right-side out vesicles. However, Kaback (1972) and recently Altendorf and Staehelin (1974) showed that the vast majority of the vesicles are right-side out as judged by freeze-etch electron micrographs. As we discussed here and earlier (Futai, 1974), the possibility that some of the marker enzymes changed their location could not be excluded. A model for translocation of enzymes during preparation of vesicles has already been presented (Altendorf and Staehelin, 1974).

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References

Altendorf, K. H., and Staehelin, L. A. (1974), *J. Bacteriol.* (in press).

Berger, E. A. (1973), *Proc. Nat. Acad. Sci. U. S. 70*, 1514.

Berger, E. A., and Heppel, L. A. (1974), *J. Biol. Chem.* (in press).

Birdsell, D. C., and Cota-Robles, E. H. (1967), *J. Bacteriol. 93*, 427.

Dietz, G. W. (1971), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 30*, 1062.

Futai, M. (1973), *Biochemistry 12*, 2468.

Futai, M. (1974), *J. Membrane Biol. 15*, 15.

Hong, J.-S., and Kaback, H. R. (1972), *Proc. Nat. Acad. Sci. U. S. 69*, 3336.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193*, 1392.

Kaback, H. R. (1971), *Methods Enzymol. 22*, 99.

Kaback, H. R. (1972), *Biochim. Biophys. Acta 265*, 367.

Kohn, L., and Kaback, H. R. (1973), *J. Biol. Chem. 248*, 7012.

Makino, S., Reynolds, J. A., and Tanford, C. (1973), *J. Biol. Chem. 248*, 4926.

Reeves, J. P., Hong, J.-S., and Kaback, H. R. (1973), *Proc. Nat. Acad. Sci. U. S. 70*, 1917.

Short, S. A., Kaback, H. R., and Kohn, L. D. (1974), *Proc. Nat. Acad. Sci. U. S.* (in press).

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

Thienen, G., van, and Postma, P. W. (1973), *Biochim. Biophys. Acta 323*, 429.

Weiner, J. H. (1974), *J. Membrane Biol. 15*, 1.

Weiner, J. H., and Heppel, L. A. (1972), *Biochem. Biophys. Res. Commun. 47*, 1360.