A NOVEL TECHNIQUE FOR THE PREPARATION OF TRANSPORT-ACTIVE MEMBRANE VESICLES FROM PSEUDOMONAS AERUGINOSA: OBSERVATIONS ON GLUCONATE TRANSPORT[1]

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SUMMARY: Membrane vesicles were prepared from glucose-grown Pseudomonas aeruginosa by osmotic lysis of cells treated with LiCl and lysozyme. These vesicles accumulated gluconate by coupling active transport with electron flow via FAD-linked L-malate dehydrogenase or D-glucose dehydrogenase. Glucose was not transported as the free sugar; instead, it was first oxidized to gluconate which was then transported by the gluconate transport system. Evidence was presented that suggested that a component(s) of the glucose transport system was lost during vesicle preparation.

A milestone in the study of bacterial transport mechanisms was the description of an isolated membrane vesicle preparation from Escherichia coli which retained transport activity and which was largely devoid of cytoplasmic enzymes (1). Isolated membrane vesicles have been used extensively since then to study bacterial transport mechanisms. This has been the subject of a recent excellent review by Kaback (2).

Efforts have been unsuccessful to produce transport-active membrane vesicles from Pseudomonas aeruginosa by the Kaback method (3). This technique involves the osmotic lysis of spheroplasts formed by exposure of gram-negative bacterial cells to ethylenediaminetetraacetate (EDTA), tris(hydroxymethyl)aminomethane (Tris) buffer and lysozyme (EC 3.2.1.17). P. aeruginosa, however, demonstrates extreme sensitivity to EDTA in the presence of Tris buffer (4). Thus, the absence of transport activity of membrane vesicles from P. aeruginosa, when prepared by the Kaback technique, may be due to the effects of EDTA and Tris.

We have now developed a method to prepare transport-active membrane

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vesicles from P. aeruginosa which avoids the use of EDTA and Tris buffer. This paper describes the technique. Preliminary data on the respiration-coupled transport of gluconate by these vesicles are also presented.

MATERIALS AND METHODS

Preparation of vesicles: P. aeruginosa strain H-1 was used for these studies. The cells were grown in a basal salts-glucose medium for 12 h at 30 C on a rotary shaker as previously described (5). The cells were harvested by centrifuging at 10,000xg for 10 min at 30 C. The pellet was suspended in 2/3 the original culture volume in a solution containing, in final concentration, 2.5% LiCl, 0.75 M sucrose, 10 mM potassium phosphate (pH 7), 10 mM $MgSO_4$ and 500 μg lysozyme/ml. This suspension was incubated for 1 h at 30 C on a rotary shaker. The osmotically fragile rods were then harvested by centrifuging at 10,000xg for 30 min at 4 C. The pellet was suspended with a Waring blendor in the smallest volume possible of 2.5% LiCl in 0.75 M sucrose at 4 C, then added rapidly to 50 volumes of ice-cold 10 mM potassium phosphate (pH 6.6) containing 1 mM $MgSO_4$ and blended for 10 sec. DNase and RNase were added to a final concentration each of 20 μg/ml and the mixture was incubated at 25 C with gentle stirring for 30 min. The suspension was centrifuged at 40,000xg for 30 min at 4 C. The pellet was suspended by means of a loose-fitting glass homogenizer in a 1:4 (v/v) ratio in ice-cold 100 mM potassium phosphate (pH 6.6) containing 10 mM $MgSO_4$. Whole cells and large fragments were removed by centrifuging at 800xg for 30 min at 4 C. The supernatant fluid was centrifuged at 40,000xg for 30 min at 4 C. The pellet was washed twice with 100 mM potassium phosphate (pH 6.6) containing 10 mM $MgSO_4$. The final pellet, which was composed of membrane vesicles, was suspended in the same buffer system to a final concentration of 5 mg of protein/ml, sealed in glass vials and stored in liquid nitrogen.

Enzyme assays: D-Glucose dehydrogenase (EC 1.1.99.a) was assayed according to the procedure described by Hauge (6). Particulate L-malate dehydrogenase (FAD-linked) (EC 1.1.3.3) was assayed by the same procedure but 10 nmoles of FAD was added to the assay system. Succinate dehydrogenase (EC 1.3.99.1) and D-lactate dehydrogenase (EC 1.1.2.4) were assayed as described by Arrigoni and Singer (7). Assays for glucose 6-phosphate dehydrogenase (EC 1.1.1.49) (8) and for isocitrate dehydroge-

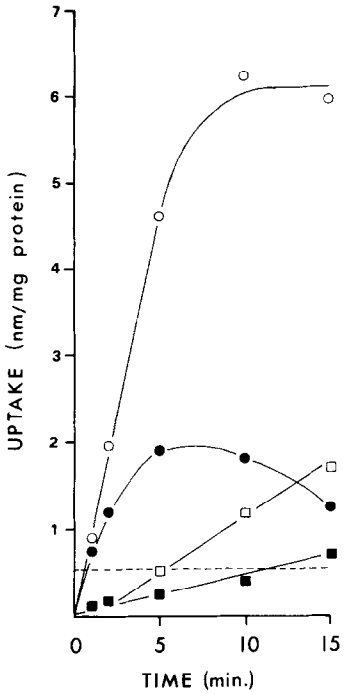

FIG. 1. Uptake of gluconate and glucose by membrane vesicles of P. aeruginosa. The accumulation of radioactivity was determined at 30 C on a reciprocal shaking water bath. Each vessel contained 50 μmoles of potassium phosphate (pH 6.6), 1 mg of membrane vesicle protein, 80 nmoles of [^{14}C]gluconate (3.7 nCi/nmole) or of [^{14}C]glucose (12.5 nCi/nmole), 12 μmoles $MgSO_4$, and, unless otherwise indicated, 20 μmoles of ascorbate and 0.1 μmole of PMS in a total volume of 1 ml. Each vessel was continuously gassed with oxygen. The reactions were started by the addition of vesicles. At intervals, 0.1 ml samples were withdrawn, diluted into 20 volumes of 0.1 M LiCl, 25 C, overlaying a Millipore filter membrane (25 mm diameter, 0.45 μm pore size), filtered instantaneously, and immediately washed with another 20 volumes of 0.1 M LiCl. The radioactivity remaining on the filters was determined as previously described (13). Open and closed circles, gluconate uptake in the presence and absence respectively of ascorbate and PMS; open and closed squares, glucose uptake in the presence and absence respectively of ascorbate and PMS. The horizontal broken line represents equilibration of substrate with the external concentration.

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nase (EC 1.1.1.42) (9) were followed by measuring the reduction of NADP spectrophotometrically. Protein was estimated by the method of Hartree (10).

RESULTS

Characterization of the vesicles: Membrane vesicles prepared from P. aeruginosa as described herein were free of whole cells as judged by phase

contrast and electron microscopy. The vesicles had high levels of activities of membrane-associated enzymes (glucose, succinate, D-lactate and L-malate [FAD-linked] dehydrogenases) but very low levels of the cytoplasmic enzymes, glucose 6-phosphate and isocitrate dehydrogenases (data not shown).

Uptake of gluconate and glucose: Membrane vesicles prepared from glucose-grown P. aeruginosa retained the capacity to accumulate [^{14}C]gluconate (Fig. 1). These vesicles also accumulated radioactivity from [^{14}C]glucose after a 2-min lag. The artificial electron donor system of ascorbate-reduced phenazine methosulfate (PMS) stimulated uptake of radioactivity from both [^{14}C]gluconate and [^{14}C]glucose.

Identification of accumulated substances: Radioactive materials extracted from membrane vesicles after incubation with [^{14}C]gluconate or [^{14}C]glucose appeared to be identical as revealed by radiochromatography (Fig. 2). Gluconate was the major product that was found in the extracts and no phosphate esters were detected. 2-Ketogluconate may also have been present as a minor component. No free glucose was found in the extract from vesicles that were incubated with [^{14}C]glucose. We conclude that glucose was first converted by membrane-bound glucose dehydrogenase to gluconate which, in turn, was taken up by the gluconate transport system.

Inhibition of uptake of radioactivity from [^{14}C]glucose by gluconate: Gluconate was a strong inhibitor of the uptake of radioactivity from [^{14}]glucose. Accumulation of radioactivity from [^{14}C]glucose by vesicles was reduced by 98% during 5 min of incubation in the presence of 10 mM gluconate.

It has been noted both in our laboratory and by Dr. P. V. Phibbs (personal communication) that glucose-grown cells of P. aeruginosa are induced to both glucose and gluconate transport while gluconate-grown cells are induced to gluconate, but not to glucose, transport. Glucose catabolic enzymes, including glucose dehydrogenase, are fully induced by growth on either substance. Using intact cells, glucose and gluconate did not inhibit the transport of one another. Thus, when our present data are considered, we conclude that a component of the glucose transport system was lost during the preparation of the vesicles.

Kinetics of gluconate transport: Gluconate transport by vesicles from P. aeruginosa was a saturable process. An apparent K_m of 20 μM was calcu-

TABLE 1. Effect of Various Electron Donors on Gluconate Transport by P. aeruginosa Membrane Vesicles

Additions (conc.)	Gluconate uptake (nmoles/mg protein/5 min)
None[a]	1.33
L-Malate, 20 mM; FAD, 10 μM	5.98
L-Malate, 20 mM; FAD, 50 μM	5.90
L-Malate, 10 mM; FAD, 50 μM	6.30
L-Malate, 20 mM	4.23
FAD, 50 μM	1.68
L-Malate, 20 mM; FAD, 50 μM; Mg^{2+}, 2 mM (final conc.)[b]	3.92
D-Glucose, 10 mM	5.17
2-Deoxy-D-glucose, 10 mM	6.03
Ascorbate, 20 mM; PMS, 0.1 mM	4.70
D-Malate, 10 mM; FAD, 50 μM	2.22
D-Lactate, 10 mM; FAD, 50 μM	1.77
Succinate, 10 mM; FAD, 50 μM	2.69
Pyruvate, 10 mM	2.22
Oxalacetate, 10 mM	2.05
ATP, 10 mM	1.29
NADH, 10 mM	2.26
NADPH, 10 mM	1.87
L-Malate, 20 mM; FAD, 50 μM; N_2 atmosphere	0.64

[a] The basal incubation mixture consisted of membrane vesicles (1 mg protein), 50 μmoles of potassium phosphate (pH 6.6), 12 mmoles of $MgSO_4$ and 80 nmoles of [^{14}C]gluconate in a total volume of 1 ml. Each reaction was continuously gassed with oxygen unless otherwise indicated. See also protocol to Fig. 1.

[b] This incubation mixture contained 2 mM $MgSO_4$ instead of 12 mM $MgSO_4$.

lated. This compares favorably with a value of K_m = 50 μm for gluconate transport by whole glucose-grown cells (data not shown).

Coupling of gluconate transport to D-glucose dehydrogenase or to L-malate dehydrogenase: Various oxidizable substrates were tested to determine their effect on gluconate transport by membrane vesicles. D-Glucose, 2-deoxy-D-glucose and L-malate stimulated gluconate transport by 4-5 fold (Table 1). D-Malate, D-lactate, succinate, pyruvate, oxalacetate, ATP, NADH and NADPH had little or no effect on gluconate transport. Maximal stimulation of gluconate transport also required 12 mM Mg^{2+}. When L-malate was used as the electron donor, 10 μM FAD was additionally required. Finally, the transport of gluconate under anoxia was severely limited.

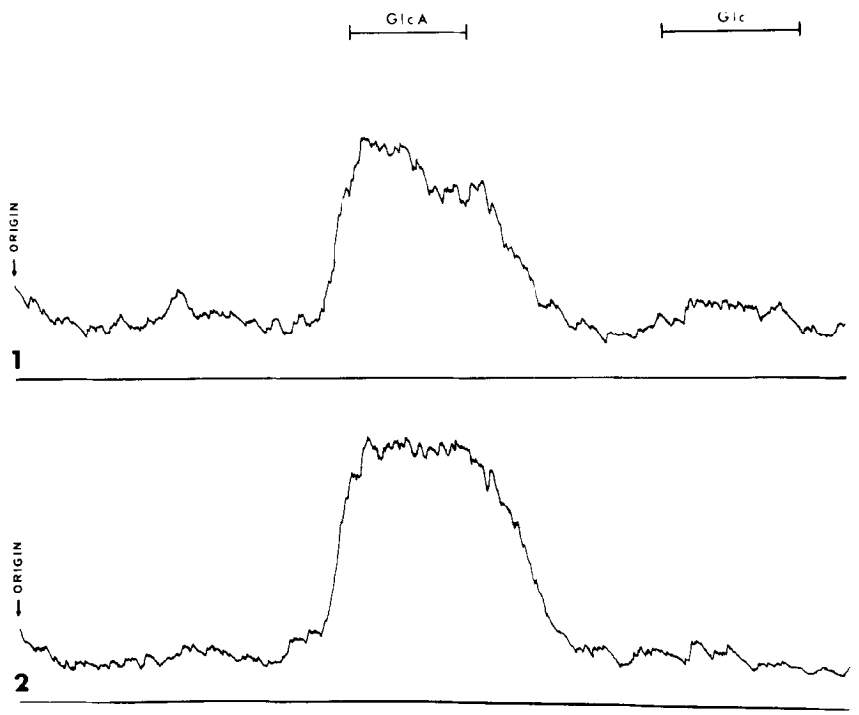

FIG. 2. Radiochromatograms of materials accumulated by membrane vesicles of P. aeruginosa after incubation with [^{14}C]gluconate (scan 1) or with [^{14}C]glucose (scan 2) for 5 min (see protocol to Fig. 1). The filters bearing the vesicles were extracted with hot water at 85 C for 15 min. The extract was centrifuged at 40,000xg for 30 min. The supernatant fluid was lyophilized to dryness, reconstituted with 0.1 ml of water, then applied to acid-washed Whatman no. 1 chromatography paper. Descending chromatography was performed for 20 h using a solvent of ethyl acetate:pyridine: acetic acid:water (5:5:1:3) according to the procedure of Fischer and Nebel (14). The paper was dried at room temperature, cut into strips and analyzed with a Packard radiochromatogram scanner model 7201 as previously described (5, 13). GlcA, gluconic acid; Glc, glucose. The shoulder on the gluconic acid peak may be 2-ketogluconic acid.

DISCUSSION

The results presented in this report indicate that membrane vesicles capable of active transport can be prepared from P. aeruginosa by a method which avoids the use of EDTA and Tris buffer. Vesicles so prepared were able to accumulate gluconate against a concentration gradient.

Active transport of gluconate was coupled to two equally effective membrane-bound dehydrogenases, i.e., FAD-linked L-malate dehydrogenase and D-glucose dehydrogenase. The artificial electron donor, PMS, was also ef-

fective in stimulating gluconate transport. Barnes (11) previously reported the presence of an active transport system for glucose by Azotobacter vinelandii membrane vesicles which was linked to L-malate oxidation by a FAD-dependent L-malate dehydrogenase. The presence of a similar system for gluconate uptake in P. aeruginosa, which, like A. vinelandii, is an obligate aerobe, is of considerable interest.

Eagon (12) previously suggested that carbohydrate transport of P. aeruginosa may be energized by glucose oxidation via D-glucose dehydrogenase. This report substantiates this suggestion in the case of gluconate transport and presents the first evidence for a physiological role for glucose dehydrogenase.

REFERENCES

1. Kaback, H. R. and E. R. Stadtman. Proc. Nat. Acad. Sci. U. S. A. 55: 920 (1966).
2. Kaback, H. R. Biochim. Biophys. Acta 265:367 (1972).
3. Kaback, H. R. In: W. B. Jakoby (ed.), Methods in Enzymology, Vol. 22, p. 99. Academic Press, Inc., New York. 1971.
4. Gilleland, H. E., Jr., J. D. Stinnett, I. L. Roth and R. G. Eagon. J. Bacteriol. 113, 417 (1973).
5. Eagon, R. G. and P. V. Phibbs, Jr. Can. J. Biochem. 49:1031 (1971).
6. Hauge, J. G. In: W. A. Wood (ed.), Methods in Enzymology, Vol. 9, p. 92. Academic Press, Inc., New York. 1966.
7. Arrigoni, O. and T. P. Singer. Nature 193:1256 (1962).
8. Hylemon, P. B. and P. V. Phibbs, Jr. Biochem. Biophys. Res. Commun. 48:1041 (1972).
9. Campbell, J. J. R. and R. A. Smith. Can. J. Microbiol. 2:433 (1956).
10. Hartree, E. F. Anal. Biochem. 48:422 (1972).
11. Barnes, E. M., Jr. Arch. Biochem. Biophys. 152:795 (1972).
12. Eagon, R. G. Can. J. Biochem. 49:606 (1971).
13. Phibbs, P. V., Jr. and R. G. Eagon. Arch. Biochem. Biophys. 138:470 (1970).
14. Fischer, F. G. and H. G. Nebel. Z. Physiol. Chim. 302:10 (1955).