Determination of the Absolute Number of *Escherichia coli* Membrane Vesicles That Catalyze Active Transport*†

(vinylglycolate/2-hydroxy-3-butenate/D-lactate/radioautography/electron microscopy)

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ABSTRACT Transport of vinylglycolate (2-hydroxy-3-butenic acid) via the lactate transport system is the limiting step for covalent labeling of membrane vesicles prepared from *E. coli* ML 308-225. Thus, the rate and extent of vinylglycolate labeling is stimulated about 10-fold by ascorbate-phenazine methosulfate, and stimulation is abolished by 2,4-dinitrophenol and by phospholipase treatment, neither of which affect the rate of vinylglycolate oxidation. [³H]Vinylglycolate of high specific activity has been prepared, and vesicles have been labeled with this compound in the presence of ascorbate-phenazine methosulfate. Examination of these preparations by high resolution radioautography in the electron microscope demonstrates that virtually all of the vesicles are labeled. The experiments provide a strong indication that most, if not all, of the membrane vesicles in these preparations catalyze active transport.

Active transport of many metabolites by isolated cytoplasmic membrane vesicles prepared from *Escherichia coli* is coupled primarily to the oxidation of D-lactate or reduced phenazine methosulfate (1-3). In many cases, the activity of the transport systems in isolated membrane vesicles approaches that of whole cells (1-4). It has been emphasized, however, that quantitative comparisons between vesicles and whole cells are difficult to interpret (1-5), especially when the activity manifested by intact cells towards a particular transport substrate may be a composite result of more than one uptake system or when one component of a transport system is not tightly bound to the membrane. Despite evidence to the contrary (1-3, 6, 7), a number of workers have argued that a significant number of membrane vesicles become inverted during preparation (7-12). Such inverted vesicles would not be expected to catalyze active transport, but they might oxidize certain electron donors, such as NADH, to which the membrane is presumably impermeable. This possibility is not trivial because the apparent high degree of specificity of D-lactate as an electron donor for active transport has important implications for the mechanism of energy-coupling in this system (1-4, 13).

Previous experiments demonstrate that vinylglycolate (VG, 2-hydroxy-3-butenic acid) inactivates Enzyme I of the

phosphoenolpyruvate-phosphotransferase (EC 2.7.3.9) system in whole cells and membrane vesicles of *E. coli* (14, 15). The experiments to be presented demonstrate that the rate of inactivation of the phosphotransferase system in isolated membrane vesicles is enhanced when VG transport is stimulated by addition of ascorbate-phenazine methosulfate (PMS), and that VG transport is the limiting step for covalent labeling of many membrane proteins. Based on these findings, [³H]VG of high specific activity has been prepared and used to label vesicles in the presence of ascorbate-PMS. The vesicles were then examined by high resolution radioautography in the electron microscope. Each vesicle that has catalyzed active VG transport is overlaid with silver grains, and about 85-95% of the vesicles are so labeled. The experiments provide direct evidence for the conclusion that the great majority of the membrane vesicles in these preparations catalyze active transport, and, therefore, cannot be inverted.

METHODS

Growth of Cells and Preparation of Membrane Vesicles. *E. coli* ML 308-225 (*i*⁻*z*⁻*y*⁺*a*⁺) was grown on medium A with 1.0% (w/v) sodium succinate (hexahydrate), and membrane vesicles were prepared (16). For radioautography experiments, vesicles were labeled with [³H]VG before they were frozen in liquid nitrogen.

Transport Assays. Phosphoenolpyruvate-dependent glucose uptake was assayed as described (16). Rates of inactivation of phosphoenolpyruvate-dependent glucose uptake by VG were also described in an earlier publication (14). For [¹⁴C]-VG uptake experiments, unless indicated otherwise, aliquots of ML 308-225 membrane vesicles (25 μ l containing 0.1 mg of membrane protein) were diluted to a total volume of 50 μ l, containing, in final concentrations, 50 mM potassium phosphate (pH 6.6) and 10 mM magnesium sulfate. The samples were then incubated at 25°C under oxygen as described (17, 18). After about 1 min, ascorbate, PMS, and [¹⁴C]VG (60 mCi/mmol) were added at final concentrations of 20 mM, 0.1 mM, and 0.1 mM, respectively. The incubations were continued at 25°C under oxygen for given periods of time, the reactions terminated, and the samples assayed as described (16-18).

High Resolution Radioautography in the Electron Microscope. Membrane vesicles (0.5 ml; 4 mg of protein per ml) were diluted to a total volume of 1.0 ml, as described above, in a 15-ml centrifuge tube. The samples were incubated at 25°C under oxygen for about 1 min, and ascorbate, PMS, and [³H]VG (25,000 Ci/mole) were added at 20 mM, 0.1

Abbreviations: VG, vinylglycolate; PMS, phenazine methosulfate.

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mM, and 0.1 mM, respectively. The incubations were continued at 25°C under oxygen for 3 min. At this time, the reactions were terminated by the addition of 10 ml of cold 0.1 M potassium phosphate (pH 6.6), and immediately centrifuged at $40,000 \times g$ for 20 min. The supernatant was discarded, the pellet was resuspended in 12 ml of 0.1 M sodium cacodylate (pH 6.8), and the suspension was centrifuged at $40,000 \times g$ for 20 min. The pellet was then washed by resuspension and centrifugation in cacodylate buffer until the radioactivity recovered in the supernatant reached a constant value. A total of four washes was required. The membrane pellet was then resuspended in 0.1 M sodium cacodylate (pH 6.8) to a concentration of 1 mg of membrane protein per ml. Aliquots (5–10 μ l) of the membrane suspension were placed on carbon-collodion-coated 400 mesh or carbon-formvar-coated 150 mesh copper grids, stained with 1% uranyl acetate in water for 10 sec, and washed with distilled water (droplet technique). The grids were then passed through a graded series of ethanol-water (20%, 40%, 60%, 80%, and 100% ethanol) and, subsequently, Freon 113-ethanol (10%, 20%, 30%, 50%, and 100% Freon 113) solutions. The sample was placed in each solution for about 5–7 sec. The samples (under Freon 113) were then placed in the pressure chamber of a Bomar SPC-900 critical point drying apparatus. The pressure chamber contained Freon 13 cooled to 10°C. The chamber was flushed twice with Freon 13, and drying was performed at a pressure of about 850 lbs./inch² at 42°C. The grids were shadowed with platinum and carbon at an angle of 45° in a Balzers freeze-etch apparatus (BA 360M). The grids were then coated with photographic emulsion as described (19), incubated in the dark for 8 days, developed, and examined in a Siemens I electron microscope. Controls were obtained by carrying out the same procedure with membrane vesicles that had not been incubated with VG.

In order to determine the maximum number of radioactive vesicles that can be visualized by these techniques, vesicles were labeled nonspecifically with [³H]acetic anhydride in the following manner: ML 308-225 membrane vesicles (4 mg of membrane protein per ml) suspended in 0.1 M potassium phosphate (pH 6.6) were diluted 10-fold with 0.1 M sodium acetate (pH 7.0), collected by centrifugation at $40,000 \times g$ for 20 min, and resuspended in 0.1 M sodium acetate (pH 7.0) to a concentration of 4 mg of membrane protein per ml. The suspension (1.0 ml) was then added to a reaction vessel containing 0.5 Ci of [³H]acetic anhydride (5,000–10,000 Ci/mole) (1.1 ml total volume) and incubated at room temperature for 20 min. At this time, the reaction mixture was diluted with 9.0 ml of cold 0.1 M sodium acetate (pH 7.0), and centrifuged at $40,000 \times g$ for 20 min. The supernatant was decanted and discarded, and the pellet was washed three times by resuspension and centrifugation in 0.1 M sodium acetate (pH 7.0). Subsequently, the labeled vesicles were washed three times in 0.1 M sodium cacodylate (pH 6.8), and resuspended in 0.1 M sodium cacodylate (pH 6.8) to a concentration of 1 mg of membrane protein per ml. The sample was then prepared and processed for electron microscopic autoradiography as described above for [³H]VG-labeled membrane vesicles, except that uranyl staining was omitted, and the emulsion was exposed for 2 days prior to development.

Materials. VG was synthesized by procedures described

(20). [¹⁻¹⁴C]VG (60 mCi/mmol) was prepared from acrolein and [¹⁴C]cyanide on a microscale version of the standard procedure by the Radiation Synthesis Section of Hoffmann-LaRoche under the direction of Dr. Arnold Liebman. The material was radiochemically pure, as determined by thin-layer chromatography on silica gel G with either water-saturated ether-formic acid (7:1, v/v) or methanol-ethylacetate (6:4, v/v) as solvent systems.

[³H]VG was synthesized by Mr. David Malarek and Dr. Arnold Liebman of the Radiation Synthesis Section of Hoffmann-LaRoche. 2-Hydroxy-3-butyric acid (1.0 mg), synthesized as described (21), was dissolved in 0.5 ml of ethylacetate, and 11.0 mg of Lindlar-vitamin A catalyst plus 0.01 ml of 5% quinoline in ethylacetate were added. The reaction mixture was then stirred in an atmosphere of 0.55 ml of tritium gas containing a total of 1.42 Ci for 16 hr at room temperature. After filtration, labile tritium was removed under reduced pressure, with methanol as a solvent, and the product was dissolved in water. This material was radiochemically pure by thin-layer chromatography carried out as described above, and had a specific activity of 25,000 Ci/mole.

[³H]Acetic anhydride (5,000–10,000 Ci/mole) and [^{U-14}C]glucose (192 mCi/mmol) were obtained from the New England Nuclear Corp. (Boston, Mass.); D-[¹⁻¹⁴C]lactate (9.1 mCi/mmol) was obtained from Amersham-Searle (Arlington Heights, Ill.). Freon 113 (Freon TF, genetron) and Freon 13 were supplied by duPont de Nemours (Wilmington, Del.). All other materials were of analytical grade obtained from commercial sources.

RESULTS

Effect of Ascorbate-PMS on the Rate of Vinylglycolate Inactivation of Phosphoenolpyruvate-Dependent Glucose Uptake. As demonstrated (14, 15), incubation of membrane vesicles in the presence of VG causes time-dependent, irreversible inactivation of phosphoenolpyruvate-dependent glucose uptake due to inactivation of Enzyme I of the phosphoenolpyruvate-phosphotransferase system. The results presented in Fig. 1 demonstrate that the rate of inactivation is enhanced when VG transport is stimulated by the addition of ascorbate-PMS to the incubation mixtures. In the absence of ascorbate-PMS, phosphoenolpyruvate-dependent glucose transport is inactivated by 20 μ M VG with a half-time of 4–5 min, while in the presence of ascorbate-PMS, the half-time of inactivation is 1–2 min. The effect of ascorbate-PMS on the uptake of [¹⁻¹⁴C]VG is also shown in the same figure. In the absence of ascorbate-PMS, VG uptake is essentially linear until about 7 min, when the rate of uptake begins to decrease. When ascorbate-PMS is added to the reaction mixtures, there is a marked stimulation of VG uptake, which corresponds to the increased rate of inactivation of glucose uptake by VG under identical conditions. The difference in VG uptake in the presence and absence of ascorbate-PMS is less striking here compared to that shown in subsequent experiments (see below) because the concentration of VG is about 5-fold below the K_m of the transport system.

Vinylglycolate Uptake is markedly stimulated by ascorbate-PMS, and virtually all of the radioactivity taken up by the vesicles is covalently bound (Fig. 2). As shown, VG uptake in the absence of ascorbate-PMS is essentially linear over the time course of the incubation. On addition of ascorbate-PMS, VG uptake is markedly stimulated, and about 15

FIG. 1. Effect of ascorbate-PMS (ASC-PMS) on the rate of VG inactivation of phosphoenolpyruvate-dependent glucose uptake and VG uptake. Inactivation of phosphoenolpyruvate-dependent glucose uptake by 20 μ M VG as a function of time was determined as described (14) in the presence (O) or absence (●) of ascorbate (20 mM) and PMS (0.1 mM) and oxygen (17, 18). [U - 14 C]Glucose (192 mCi/mmol) was used at a final concentration of 40 μ M, and the reactions were carried out at 46°C. VG uptake was assayed in the following manner: aliquots (25 μ l) of ML 308-225 membrane vesicles containing 0.1 mg of membrane protein were diluted to a total volume of 50 μ l containing (in final concentrations) 50 mM potassium phosphate (pH 6.6), 10 mM magnesium sulfate, 300 mM lithium chloride, and 100 mM phosphoenolpyruvate. Samples were incubated for 5 min at 46°C, and then placed in an atmosphere of oxygen for about 1 min. At this time, ascorbate (20 mM) and PMS (0.1 mM) were added where indicated, and immediately thereafter, [14 C]VG (60 mCi/mmol) at a final concentration of 20 μ M. At the times indicated, the samples were assayed as described (16, 18). Δ , VG uptake in the presence of ascorbate-PMS; $\blacktriangle$, VG uptake in the absence of ascorbate-PMS.

FIG. 2. Effect of 2,4-dinitrophenol (DNP) on VG uptake in the presence and absence of ascorbate-PMS (ASC-PMS). VG uptake was assayed as described in *Materials and Methods*. Where indicated, dinitrophenol was added to the reaction mixtures in a final concentration of 1 mM. ●, VG uptake in the presence of ascorbate-PMS; ∇ , VG uptake in the presence of ascorbate-PMS and dinitrophenol; ■, VG uptake in the absence of ascorbate-PMS; □, VG uptake in the presence of dinitrophenol. At 5 min (indicated by an arrow), unlabeled VG (20 mM final concentration) was added to some of the samples incubated in the presence of ascorbate-PMS (Δ). Alternatively, samples were placed in boiling water for 1 and 2 min ($\diamond$).

FIG. 3. Exchange of external VG with D-lactate in the intravesicular pool. D- $[^{14}$ C]Lactate (9.1 mCi/mmol) transport was assayed at 0.1 mM D-lactate in the presence (●) or absence (O) of ascorbate-PMS (ASC-PMS) as described (14, 23). At 2 min (indicated by arrow), unlabeled VG (∇) or D-lactate (Δ) was added to some of the samples at a final concentration of 20 mM.

nmol/mg of membrane protein is taken up within about 3 min. When an excess of unlabeled VG is added to vesicles that have taken up VG, there is essentially no loss of radioactivity, indicating that VG taken up by the vesicles is in a nonexchangeable state. This conclusion is corroborated by observations demonstrating that boiling (Fig. 2), extraction with 10% trichloroacetic acid (data not shown), or extraction with chloroform-methanol (2:1 v/v) (data not shown) also do not result in loss of significant amounts of VG from the vesicles.

It is evident from the experiments shown in Fig. 2 that addition of 2,4-dinitrophenol, an uncoupling agent that has no effect on VG oxidation (data not shown), completely abolishes the stimulatory effect of ascorbate-PMS, but has relatively little effect in the absence of this electron donor system. Although not shown, treatment of the vesicles with carbonylcyanide *m*-chlorophenylhydrazone, phospholipase A, or mouse duodenal phospholipase AB also abolishes stimulation of VG uptake by ascorbate-PMS, but has no effect on VG oxidation. These experiments demonstrate that the limiting step for covalent labeling of the membrane vesicles is transport of VG.

Previous experiments (14, 15) have demonstrated that VG is a competitive inhibitor of the lactate transport system in ML 308-225 membrane vesicles. As shown in Fig. 3, external VG exchanges with intravesicular D-lactate as effectively as D-lactate itself, providing further evidence that VG is transported via the lactate transport system. Other experiments, which will not be presented here, demonstrate that the apparent K_m for VG uptake in the presence of ascorbate-PMS is about 0.1 mM, a value that is very similar to that reported for D-lactate transport (14, 22).

Electron Microscopic Radioautography of [3 H]Vinylglycolate-Labeled Vesicles. Because of the properties of VG uptake described above, this compound can be used to estimate the transport activity of individual membrane vesicles on an all-or-none basis. As shown in Fig. 4, when membrane vesicles are incubated with [3 H]VG in the presence of ascorbate-PMS, about 200 μ Ci are bound per mg of membrane protein in 3 min, as opposed to about 20 μ Ci in the absence of ascorbate-PMS. When preparations incubated with [3 H]VG in the presence of ascorbate-PMS are prepared for high resolution radioautography and examined in the electron micro-

FIG. 4. Uptake of [^3H]VG. [^3H]VG (25,000 Ci/mole) uptake was assayed at 0.1 mM VG as described in *Materials and Methods* in the presence (●) or absence (○) of ascorbate-PMS (ASC-PMS) as indicated. Results are given in $\mu\text{Ci}/\text{mg}$ of membrane protein.

scope, the results shown in Fig. 5a are obtained. The micrograph shown is typical of over 100 random fields visualized. It is obvious from the figure that almost all of the vesicles, with the exception of a few small ones (arrows), are heavily overlaid with exposed silver grains. Between 85 and 95% of the vesicles are labeled. About 25–30% of the grains do not lie over structures that can be identified as vesicles. It should be emphasized that these are probably minimal and maximal estimates, respectively. The large vesicles are so heavily labeled that it is difficult to ascertain the exact number of grains over them, while the size of the smaller vesicles is such that their proximity to individual grains in the emulsion may be limiting. Autoradiographs of membrane vesicles incubated with [^3H]VG for 3 min in the *absence* of ascorbate-PMS contain far fewer grains, but essentially the same grain distribution as shown in Fig. 5a (data not shown).

The radioautograph in Fig. 5b shows a membrane preparation labeled with [^3H]acetic anhydride, a reagent that should label all of the vesicles nonspecifically. Allowing this assumption, the experiment provides an estimation of the maximum efficiency of the method. Essentially the same grain distribution and the same percentage of labeled vesicles are observed with [^3H]acetic anhydride as for [^3H]VG. These results suggest that labeling of 85–95% of the vesicles represents the maximum degree of labeling detectable by this method, and that in all likelihood, all of the vesicles incubated with [^3H]VG in the presence of ascorbate-PMS are labeled. Although not shown, control samples that were not incubated with [^3H]VG or [^3H]acetic anhydride exhibit essentially no grains.

DISCUSSION

The experiments presented in this paper are consistent with the model presented in Fig. 6. External VG gains access to the intravesicular pool via the lactate transport system where it

FIG. 5. Electron microscopic radioautography of ML 308-225 membrane vesicles labeled with [^3H]VG in the presence of ascorbate-PMS (a) or [^3H]acetic anhydride (b) ($\times 25,000$). Membrane vesicles labeled with [^3H]VG or [^3H]acetic anhydride were subjected to radioautography and electron microscopy as described in *Materials and Methods*. Vesicles labeled with [^3H]VG in the presence of ascorbate-PMS contained about 200 $\mu\text{Ci}/\text{mg}$ of membrane protein, and were incubated with photographic emulsion for 8 days in the dark. Samples labeled with [^3H]VG in the absence of ascorbate-PMS (not shown) contained about 20 $\mu\text{Ci}/\text{mg}$ of membrane protein. Samples labeled with [^3H]acetic anhydride contained about 450 $\mu\text{Ci}/\text{mg}$ of membrane protein, and were incubated with photographic emulsion for 2 days in the dark. Arrows signify unlabeled vesicles.

is then oxidized to 2-keto-3-butenate, a highly reactive compound due to its conjugated unsaturated system. This electrophile is attacked by sulfhydryl groups contained in Enzyme I and many other membrane proteins. Although there is considerable evidence supporting this sequence of events, only two points are critical to this discussion. *First*, VG transport is the limiting step for labeling of the membranes. Thus, the rate and extent of labeling are stimulated at least 10-fold by ascorbate-PMS, an electron donor system that drives the active transport of a variety of solutes. Moreover, stimulation of labeling by ascorbate-PMS is completely abolished by uncoupling agents and by treatment with phospholipases, both of which inhibit active transport, but do not affect the ability of the vesicles to oxidize VG. Since the K_m for D-lactate dehydrogenase is 1.4 mM (23)

FIG. 6. Proposed sequence of events in uptake and covalent binding of VG (2-hydroxy-3-butenate) by *E. coli* ML 308-225 membrane vesicles. D- and L-LDH, D- and L-lactate dehydrogenases; E II, Enzyme II of the phosphoenolpyruvate-phosphotransferase system; E I, Enzyme I of the phosphoenolpyruvate-phosphotransferase system; HPr, histidine-containing protein of the phosphotransferase system. Reaction of 2-keto-3-butenate with sulfhydryl groups exclusively on the inner surface of the membrane is a presumption at the present time.

and the external concentration of VG used in these experiments is 0.1 mM, the most reasonable explanation for these effects is that ascorbate-PMS causes VG to accumulate within the vesicles to concentrations that are saturating for the lactate dehydrogenases on the inside of the membrane. By this means, the electron donor system markedly accelerates the oxidation of VG, resulting in increased rates of labeling. In the absence of ascorbate-PMS, oxidation of VG is limited by the rate of passive diffusion of the compound through the membrane. *Second*, as opposed to the usual transport substrates, almost all of the radioactive VG taken up by the membranes is covalently bound to the vesicles. This conclusion is supported by experiments demonstrating that VG taken up by the vesicles cannot be displaced by an excess of unlabeled VG or removed by extraction with a variety of solvents. Given these observations, this system offers a unique opportunity to assess the number of membrane vesicles in the preparation that catalyze the active transport of VG. Such studies cannot be carried out with the usual transport substrates because they are not covalently bound by the vesicles, and are readily lost with even the most gentle manipulations.

Examination of vesicles incubated with high specific activity [^3H]VG in the presence of ascorbate-PMS by radioautography in the electron microscope reveals that virtually all of the vesicles are labeled. The experiment provides direct evidence that a high percentage of the vesicles in these preparations catalyze active transport. In view of this experiment and a number of other independent lines of evidence

(1-4, 6, 7), it also seems highly unlikely that a significant number of membrane vesicles are inverted in these preparations. In this respect, it is relevant that recent experiments (S. A. Short, H. R. Kaback, and L. D. Kohn, *J. Biol. Chem.*, submitted for publication) demonstrate that antiserum prepared against homogeneous preparations of D-lactate dehydrogenase does not inhibit either D-lactate oxidation or D-lactate-dependent active transport in ML 308-225 vesicles unless they are disrupted physically.

Finally, it is apparent that VG should provide an interesting approach to the study of membrane topology. Only those proteins accessible from the inside surface of the membrane should be labeled by VG under the conditions used in these experiments. On the other hand, generation of 2-keto-3-butenate in the external medium should label only those proteins accessible from the outside surface of the membrane.

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