J. Membrane Biol. 15, 15–28 (1974)
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Orientation of Membrane Vesicles from *Escherichia coli* Prepared by Different Procedures

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Received 27 July 1973; revised 11 October 1973

Summary. The orientation of membrane vesicles prepared from *Escherichia coli* by either French press, sonication or ethylenediamine tetraacetate (EDTA)-lysozyme was examined. The following procedures were used to determine orientation: (1) accessibility of the impermeable ferricyanide ion to the respiratory chain; (2) inhibition of membranal ATPase by specific antiserum; (3) binding of ATPase to the membrane. Data with spheroplasts indicated that ATPase, ATPase binding sites and ferricyanide reductase activities were localized on the inner part of the cytoplasmic membrane. Thus, there was no demonstrable NADH-ferricyanide reductase activity, low ATPase activity, no inhibition of ATPase by antiserum and no binding of purified ATPase by spheroplasts. In the case of membrane vesicles prepared by French press or sonication, the ATPase activity, the ATPase binding site and the site where ferricyanide takes electrons from the respiratory chain all appeared to be on the outside of the vesicles, suggesting that they are inverted. In the case of EDTA-lysozyme vesicles, which are widely used for transport studies, about half of the ATPase binding sites and ferricyanide reactive sites were exposed to the outside. Sixty percent of the ATPase activity was sensitive to antiserum. The two most probable explanations for these data are: (1) partial inversion of EDTA-lysozyme vesicles in the course of preparation; (2) movement of marker enzymes within the membrane vesicles during their isolation.

A study by Weiner (1974) has indicated that L-glycerol-3-phosphate dehydrogenase is located on the inner surface of the cytoplasmic membrane in intact cells of *Escherichia coli.* The two major pieces of evidence are: (1) a starved mutant, unable to transport L-glycerol-3-phosphate, could not use this compound to drive the transport of amino acids; (2) using the non-penetrating ion, ferricyanide, as electron acceptor, no dehydrogenase activity could be detected with either whole cells or spheroplasts unless permeability barriers were destroyed by toluene. However, when spheroplasts were lysed and converted to membrane vesicles according to Kaback (1971), approximately one-half of the total dehydrogenase activity for L-glycerol-3-phosphate as well as D-lactate was accessible to ferricyanide even before toluenization. This suggested the possibility that the localization

of half of the dehydrogenases in vesicles differs from that of spheroplasts. It then became of interest to examine the orientation of various types of membrane vesicles using enzymatic and immunochemical techniques.

In the present report we have studied the localization of ferricyanide reductase, and of ATPase and its binding sites, in membrane vesicles, in an effort to elucidate their orientation. Vesicles were prepared by sonication, by a French press procedure and by the method of Kaback (1972).

Materials and Methods

Experimental Procedures

Bacteria and Growth Conditions. E. coli Strain 27 (L-glycerol-3-phosphate dehydrogenase constitutive), a derivative of K 12 Hfr Cavalli, was the gift of Dr. E. C. C. Lin. *E. coli* DL 54 (ATPase negative) and its parent, ML 308–225 (i^-, z^-, y^+, a^+) were kindly supplied by Dr. R. D. Simoni (Simoni & Schallenberger, 1972). All cultures were grown in a synthetic medium (Tanaka, Lerner & Lin, 1967) supplemented with 0.5% succinate (Strain 27) or 0.5% glucose (ML 308–225 and DL 54), unless otherwise specified. Physiologically young cells (Birdsell & Cota-Robles, 1967) were used in all experiments. Thus, cells were grown with vigorous aeration for 12 hr at 37 °C to stationary phase in the above medium and then were diluted with the same medium to an optical density of 0.5 at 600 nm (about 5×10^8 cells per ml). After one generation of growth they were harvested by centrifugation, washed twice with ice cold 0.01 M Tris HCl, pH 8.0, and used for the preparations described below. Frozen cells of *E. coli* B, grown in peptone medium and harvested in late exponential phase, were obtained from the General Biochemical Co.

Preparation of Spheroplasts. This procedure is essentially the same as that of Birdsell and Cota-Robles (1967). One gram of wet cells was suspended in 80 ml of 0.033 M Tris HCl, pH 8.0, containing 20% sucrose. After addition of ethylenediamine tetraacetate (EDTA) and lysozyme, to a final concentration of 0.01 M and 0.25 mg/ml, respectively, the mixture was incubated at 23 °C for 30 min. The spheroplasts were then chilled at once and used for further experiments without washing. As a test to measure completeness of spheroplast formation, a sample was diluted 10-fold in water. The optical density at 600 nm decreased by more than 90%, suggesting nearly complete conversion to spheroplasts.

Preparation of Membrane Vesicles. EDTA-lysozyme vesicles were obtained by lysing cells with EDTA and lysozyme, followed by washing the membrane fractions with 0.1 M potassium phosphate buffer, pH 6.6, containing 10 mM EDTA (Kaback, 1971). Vesicles that had been washed 4 times at 4 °C were suspended in 0.1 M potassium phosphate buffer, pH 6.6. *Sonic vesicles* were obtained as follows: One gram of wet cells was suspended in 80 ml of 0.01 M Tris HCl, pH 7.4, containing 10 mM $MgCl_2$, and 10 μg per ml each of DNase and RNase, and sonicated at 10 kc for 7 min. The following procedures were done at 4 °C. After removing undisrupted cells by centrifugation at $8{,}000 \times g$ for 10 min, the supernatant fraction was centrifuged at $100{,}000 \times g$ for 30 min. The precipitate was washed once and suspended in 0.01 M Tris HCl, pH 7.4 – 5 mM $MgCl_2$. *French press vesicles* were obtained at 4 °C by suspending one gram of bacteria (wet

weight) in 5 ml of 0.01 M Tris – 5 mM $MgCl_2$, pH 7.4, containing 10 µg per ml each of DNase and RNase, passing the mixture through a French press (pressure set at 8,000 lbs) and centrifuging at 8,000 × *g* for 10 min. The supernatant fraction was removed and recentrifuged at 100,000 × *g* for 90 min. The pellet was suspended in 0.01 M Tris HCl, pH 7.4 – 0.01 M $MgCl_2$.

Each membrane fraction was used at once for the experiments described below, although essentially the same results were obtained using membrane vesicles stored at –90 °C for as long as one week. It is worth mentioning that examination of negatively stained samples or thin sections in the electron-microscope reveals that these membranes are vesicles surrounded by a single membrane. These pictures were taken by Dr. John N. Telford.

Assays

Measurement of ATPase Activity. The standard ATPase reaction mixture contained 2.5 mM γ-^{32}P-ATP (disodium), 20 mM Tris HCl, pH 9, 2 mM $MgCl_2$ and 10% sucrose in a total volume of 1.0 ml. After incubation at 37 °C for 15 min, the reaction was stopped with 0.1 ml of 50% trichloroacetic acid. The mixture was centrifuged at 3,000 × *g* for 3 min, after which the supernatant solution was mixed with 4 ml of 1.25% ammonium molybdate containing 3.8% HCl and extracted with isobutanol-benzene-acetone (1500:1500:500 v/v). Samples of both the organic and aqueous layers were dried and measured for radioactivity in a gas flow counter. In the assay of spheroplasts, 10 µliters of the suspension were used so that the final concentration of EDTA in the reaction mixture was 0.1 mM, which had no effect on enzyme assay. For measurement of sensitivity to N,N′-dicyclohexylcarbodiimide (DCCD), the pH of the Tris HCl was adjusted to 7.4. Ten µliters of ethanol solution containing different concentrations of DCCD were added to each incubation mixture. Only ethanol was added to the control. ATPase activity was also measured in a total volume of 0.5 ml containing 0.05 M potassium phosphate, pH 7.0, and 2 mM $MgCl_2$. In this case, 0.5 ml of distilled water was added before mixing with trichloroacetic acid. Centrifugation was done at 10,000 × *g* for 3 min when serum was added to the reaction mixture.

Measurement of Ferricyanide Reductase Activity. The reduction of ferricyanide was measured in the presence of 0.08 M Tris HCl, pH 7.4, 9 mM KCN, 1.0 mM NADH and 0.7 mM ferricyanide. The substrate-dependent reduction of ferricyanide was followed at 420 nm ($\varepsilon = 1.0\ \text{mM}^{-1}\ \text{cm}^{-1}$) in a Gilford spectrophotometer. In some experiments the Tris buffer was replaced by 0.1 M potassium phosphate, pH 6.6. When the activity of spheroplasts was tested, 20% sucrose was added to the reaction mixture.

Assay of Binding of ATPase to Membranes. Membrane fractions from *E. coli* DL 54 (0.4 mg protein) were incubated with varying amounts of purified ATPase in 10 mM $MgCl_2$ and 0.05 M potassium phosphate, pH 6.6, in a volume of 0.4 ml for 15 min at 37 °C. In the case of spheroplasts, 20% sucrose was also present. After a further addition of 5 ml of the phosphate buffer, the mixture was centrifuged at 100,000 × *g* for 30 min. The precipitate was suspended in the same buffer and ATPase activity was measured as described above.

Other Procedures. Transport assays were done essentially the same as described (Kaback, 1971). Toluenization was done as follows: membrane vesicles (20 mg protein/ml) or spheroplasts (2.5 mg protein/ml) were treated with 1% toluene and incubated at 37 °C for 10 min. Each preparation was in buffer as described above. Protein was measured by the procedure of Lowry, Rosebrough, Farr and Randall (1951).

Materials

ATPase was purified from *E. coli* B by the method of Hanson and Kennedy (1973) or Kobayashi and Anraku (1972). The former preparation appeared to contain four bands, believed to be subunits, when subjected to polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (Weber & Osborn, 1969). It was injected into rabbits to produce antiserum. The latter preparation, which, in our hands, was less pure but easier to obtain, was used to study binding of ATPase to membranes. γ-^{32}P-ATP was kindly supplied by Drs. C. Yocum and D. Deters. The following materials were obtained from commercial sources: lysozyme, RNase and NADH, from Sigma Chemical Co.; DNase, from Worthington Chemical Co.

Results

Uptake of Proline by Various Types of Membrane Vesicles

Because of the vectorial nature of the mechanism, transport of substrates is one indication of the orientation of membrane vesicles. EDTA-lysozyme vesicles prepared from *E. coli* ML 308–225 or DL 54 transported proline in the presence of 20 mM D-lactate at essentially the same rate as previously reported (Simoni & Shallenberger, 1972): the rates for glucose-grown cells were 0.89 and 0.37 nmoles per mg protein per min, respectively. No significant uptake was observed in the case of French press vesicles or sonic vesicles, though vesicles were retained on the filter judging from the protein assay.

Ferricyanide Reductase of Various Preparations

In spheroplasts prepared from Strain ML 308–225, ferricyanide reduction in the presence of NADH was increased 40-fold by toluenization (Table 1), suggesting that the site where ferricyanide obtains electrons from the respiratory chain is on the internal membrane surface. This confirms Weiner's observation with Strain 27, a K12 derivative, and suggests that ferricyanide reductase activity can serve as a marker of sidedness of membrane vesicles. As shown in Table 2, toluenization had no effect on the rate of reduction of ferricyanide by vesicles obtained by French press or sonication. With EDTA-lysozyme vesicles, however, the activity was increased about twofold after toluenization, as already reported by Weiner (1974), suggesting that in half of the vesicles the enzyme activity is accessible to ferricyanide. The various preparations differ somewhat in specific activity, perhaps due to partial loss of some component of this complex reaction. This effect of toluenization on ferricyanide reductase in the presence of NADH or L-glycerol-3-phosphate was confirmed using three preparations from *E. coli* Strain 27.

Table 1. NADH-ferricyanide reductase and ATPase activity of spheroplasts

Enzyme	Activity (µmole/mg/min)	
	No treatment	After toluenization
NADH-ferricyanide reductase	0.019	0.800
ATPase	0.038	0.58
ATPase (+ antiserum)	0.025	0.046

Each enzyme activity was assayed using a suspension of spheroplasts containing 50 µg protein. Conditions for assay are as described in "Experimental Procedures" except that the incubation mixture also contained 20% sucrose. The amount of ATPase antiserum used was 340 µg protein per mg spheroplast protein. Since a spheroplast suspension containing EDTA and lysozyme was directly used in the above assays, the concentration of cell protein was corrected for the amount of lysozyme added.

Table 2. NADH-ferricyanide reductase of various membrane vesicles prepared from *E. coli* ML 308–225

Membranes	Activity (µmole/mg/min)	
	No treatment	After toluenization
Sonic vesicles	0.68	0.61
EDTA-lysozyme vesicles	0.35	0.80
French press vesicles	0.58	0.52

NADH-ferricyanide reductase was measured as described in the text. Essentially the same results were obtained using 0.1 M potassium phosphate buffer, pH 6.6, except that the activities were 70% of the values observed at pH 7.4.

ATPase Activity of Various Preparations

The ATPase activity of spheroplasts increased more than 15-fold after toluenization, suggesting that this enzyme is localized at the inner membrane surface (Table 1). This ATPase activity present in toluenized spheroplasts was almost completely inhibited by antiserum against the pure ATPase. More than 80% of the activity in sonic extracts of whole cells was also inhibited by the same antiserum. These results suggest that the bulk of the ATPase activity is due to an enzyme immunologically indistinguishable from the purified ATPase. The ATPase activity of three membrane vesicle preparations is shown in Table 3. Activity was increased about 30% after treating EDTA-lysozyme vesicles with toluene, cholate or Triton X-100, while the activity of sonic vesicles or French press vesicles was unchanged (Table 3).

Table 3. Effect of various treatments on membranal ATPase activity

Membrane	Treatment	ATPase activity (μmole/mg/min)
EDTA-lysozyme vesicles	None	0.51
	1% triton X-100	0.66
	1% cholate	0.62
	1% toluene	0.64
Sonic vesicles	None	0.48
	1% triton X-100	0.48
	1% toluene	0.50
French press vesicles	None	0.57
	1% toluene	0.62
	1% cholate	0.53
	1% triton X-100	0.58

Each membrane preparation (6 mg/ml) was treated with 1% toluene, 1% triton X-100 or 1% cholate at 37 °C for 10 min. Aliquots of 10 μliters were used for assay of ATPase activity. For details *see text.*

Inhibition of Membranal ATPase by Specific Antiserum

For further characterization of ATPase activity in various membrane vesicles, the sensitivity to antiserum was measured. As shown in Fig. 1, the activity of purified enzyme and of vesicles prepared by French press or sonication was completely inhibited by specific antiserum, although normal serum was without effect. The ATPase activity of inner membranes prepared by fractionation of French press vesicles (Schnaitman, 1970) was also completely inhibited by specific antiserum (data not shown). The inhibition was not affected by the presence of 0.05 M potassium phosphate or 1 mM EDTA. This antiserum inhibited the ATPase preparation of Kobayashi and Anraku (1972) completely, although it was obtained after injecting enzyme purified by the method of Hanson and Kennedy (1973). The ATPase activity of EDTA-lysozyme vesicles was inhibited only to the extent of 60% by the antiserum. Because of reports that vesicles become leaky at higher temperatures (Shechter, Gulik-Krzywicki & Kaback, 1972), the titration was also done at 23 °C, resulting in 70% inhibition. This partial inhibition was confirmed under a variety of conditions, such as in the presence of 20 mM Tris buffer, pH 7 or 9, containing 0.1 M NaCl or 10% sucrose, or in 0.05 M potassium phosphate buffer, pH 7.0 or 6.6, containing 10 mM $MgCl_2$. The latter buffer mixture is routinely used for transport experiments with these vesicles. The ATPase activity of the vesicles became almost completely sensitive to antiserum after treating them with detergents. As shown in Fig. 2, ATPase activity of vesicles pretreated with Triton X-100 or cholate

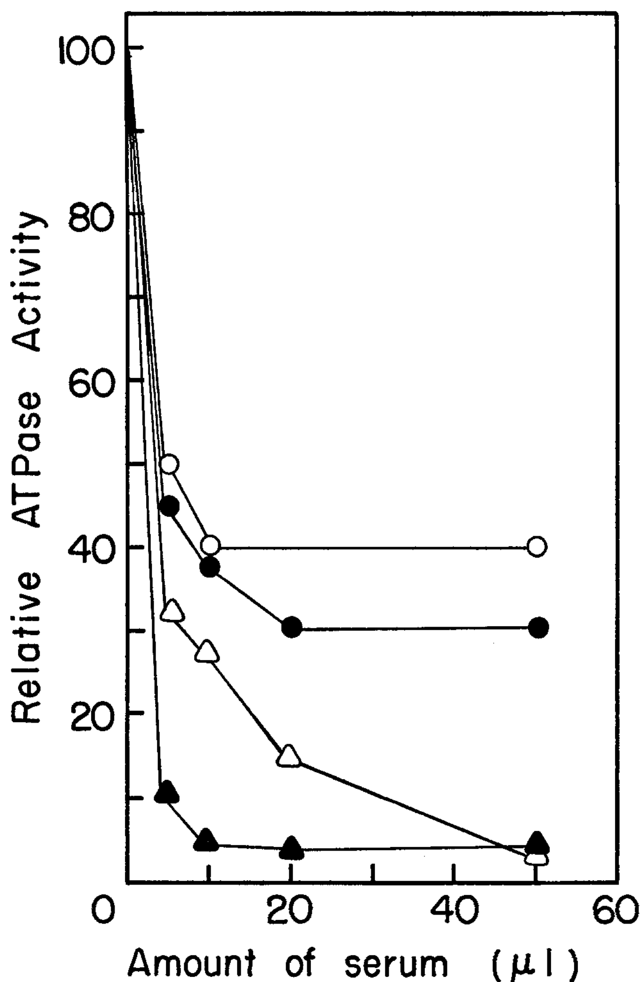

Fig. 1. Effect of specific antiserum on ATPase activity of different membrane vesicles from *E. coli* ML 308–225. ATPase activity of EDTA-lysozyme vesicles (○——○), sonic vesicles (△——△) and French press vesicles (▲——▲) was measured in the presence of different amounts of antiserum under standard assay conditions. ATPase activity of EDTA-lysozyme vesicles was also measured at 23 °C in the presence of antiserum (●——●). Activity at 23 °C was 79 % of that at 37 °C without antiserum. Five μliters of this antiserum inhibited 10 μg of ATPase. The protein content of each sample of membrane vesicles was 0.1 mg. The specific activity of each preparation is shown in Tables 1 and 3. Normal serum had no effect on ATPase activity of any of these vesicles or of the pure preparation

was inhibited by 85 and 95 %, respectively, while pretreatment of vesicles with toluene had only a slight effect. These results were essentially confirmed using vesicles prepared from Strain 27. In this case, the ATPase activity of EDTA-lysozyme vesicles was inhibited by 40 % with excess antiserum.

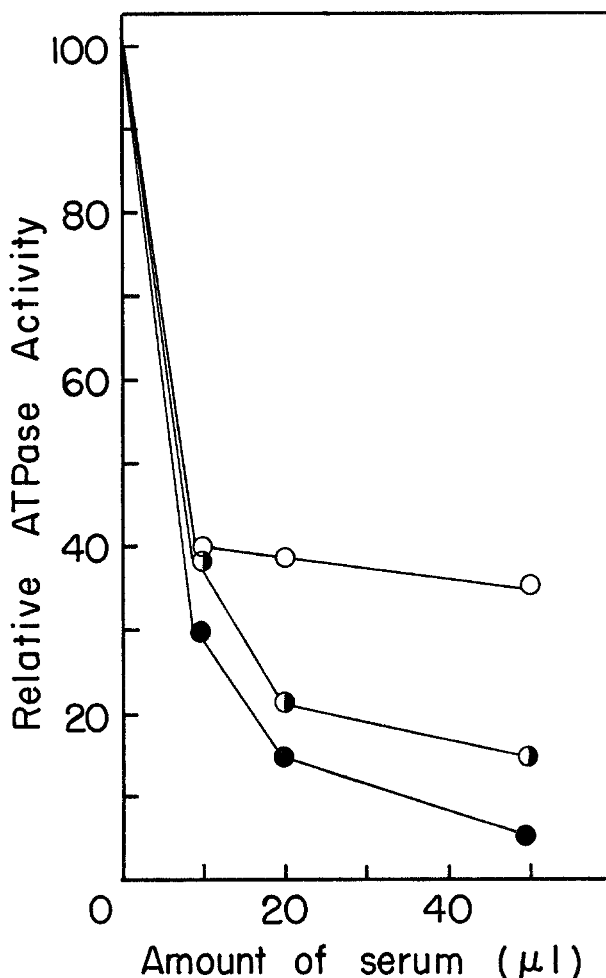

Fig. 2. Effect of various treatments on the sensitivity of ATPase in EDTA-lysozyme vesicles from *E. coli* ML 308–225 to specific antiserum. EDTA-lysozyme vesicles (6.0 mg/ml) were incubated with 1% toluene (○—○), 1% Triton X-100 (◑—◑) and 1% cholate (●—●) for 10 min at 37 °C in 0.1 M potassium phosphate, pH 6.6. After treatments, aliquots of 10 μliters were assayed in the presence of different amounts of antiserum. For further details see legend of Fig. 1 and text

DCCD Sensitive Binding of ATPase to Membranes

Further evidence on sidedness was obtained from ATPase binding studies. The purified ATPase did not bind significantly to spheroplasts made from Strain DL54 (ATPase negative), suggesting that the binding site is inside the membrane (Fig. 3). On the other hand, it was possible to bind ATPase to sonic vesicles and French press vesicles, and after maximal binding had occurred the specific activity in these vesicles derived from DL54 was almost equal to that of the parent ML308–225. The EDTA-

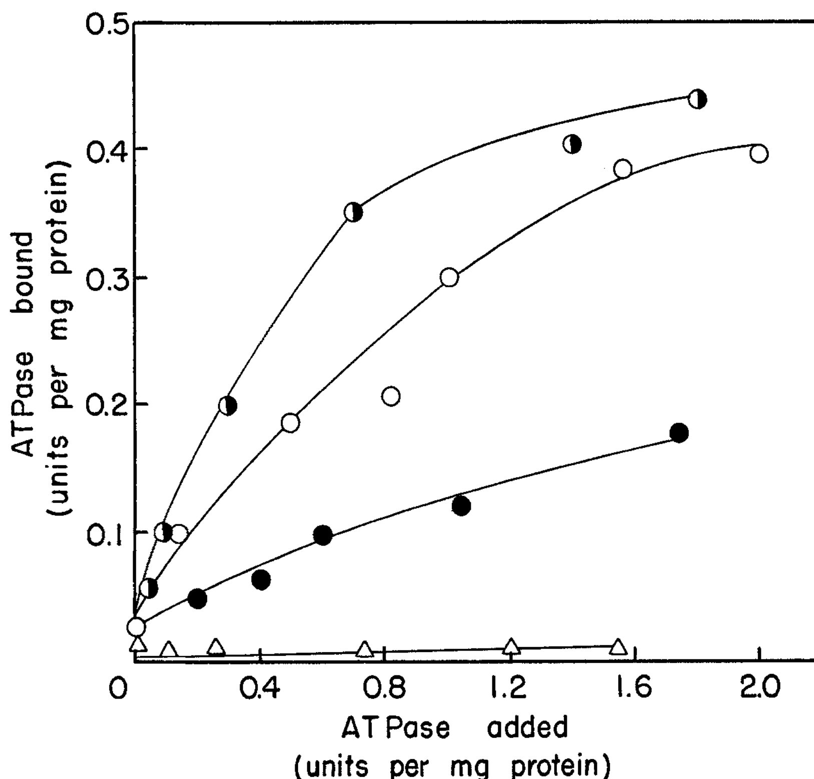

Fig. 3. Binding of ATPase to various types of membrane vesicles made from *E. coli* DL 54. EDTA-lysozyme vesicles (●——●), French press vesicles (◐——◐) and sonic vesicles (○——○) from the ATPase negative mutant DL 54 were incubated with different amounts of purified ATPase at 37 °C for 15 min. After centrifugation, the precipitate was suspended in 0.05 M potassium phosphate buffer, pH 6.6, and enzyme activity was measured. Spheroplasts (△——△) were incubated in buffers containing 20% sucrose; treatment was otherwise the same. The spheroplasts were freshly isolated. It was determined that lysozyme, EDTA and sucrose, required for spheroplast formation, had no effect on the binding of ATPase to sonic vesicles. One unit of ATPase was defined as the amount that under standard conditions hydrolyzed 1 μmole of substrate per min. For further details, *see text*

lysozyme vesicles bound about half as much ATPase per mg protein as did vesicles prepared by French press or sonication. In every case, preincubation in the presence of Mg^{++} was necessary to get maximal binding.

To determine if the binding was specific in nature, the sensitivity of the bound ATPase to DCCD was studied. The ATPase in either French press vesicles, sonic vesicles or EDTA-lysozyme vesicles was sensitive to DCCD, while the purified enzyme was insensitive (Fig. 4*a*). This inhibition of the membrane-bound ATPase was most striking between pH 6.5 and 7.5, and essentially no inhibition was observed at pH 9.0. The purified ATPase bound to each type of vesicle became sensitive to DCCD (Fig. 4*b*), and

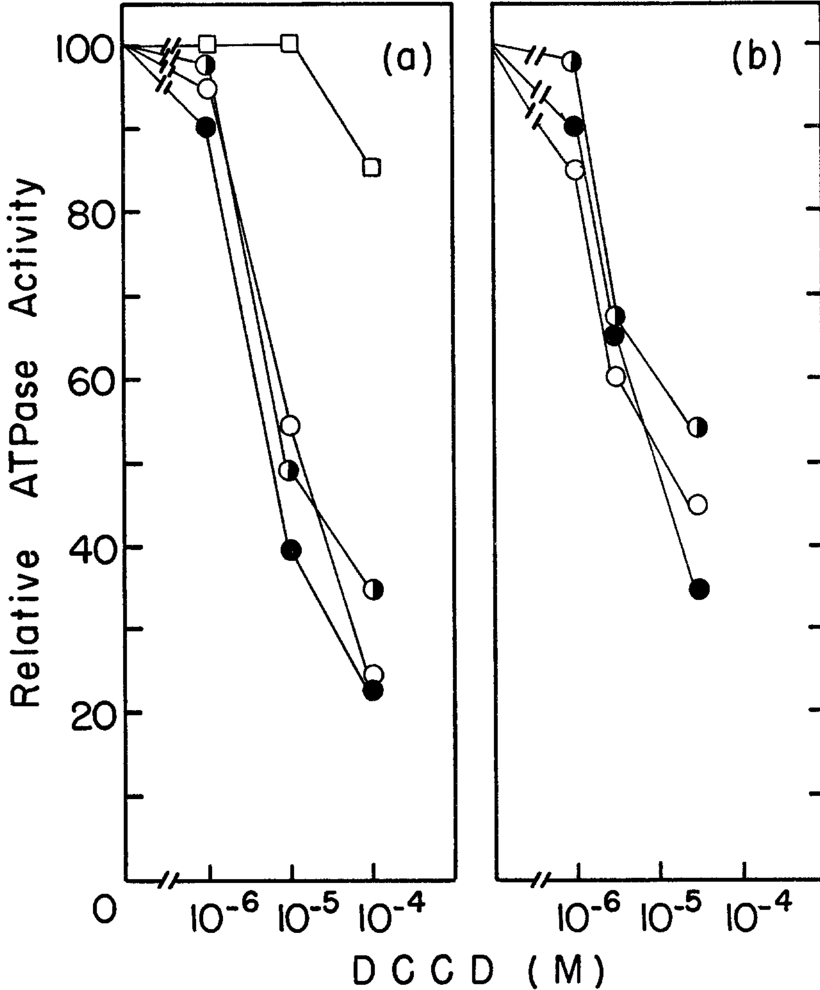

Fig. 4. Effect of DCCD on ATPase activity of various preparations. (*a*) Effect of DCCD on ATPase activity of membrane vesicles prepared from *E. coli* ML 308–225 and on pure enzyme. Two μg of pure ATPase (□—□), 110 μg of French press vesicles (●—●), 73 μg of sonic vesicles (◑—◑) or 45 μg of EDTA-lysozyme vesicles (○—○) were preincubated with different concentrations of DCCD at 23 °C for 3 min and ATPase activity was measured. For further details, *see text*. (*b*) Effect of DCCD on ATPase activity of membrane vesicles from *E. coli* DL 54 reconstituted with pure enzyme. French press vesicles (●—●), sonic vesicles (◑—◑) and EDTA-lysozyme vesicles (○—○) were incubated with 2 units ATPase/mg membrane protein. Samples of reconstituted vesicles (40 μg protein) were assayed in the presence of different concentrations of DCCD after preincubation as shown in (*a*). Further details are given in the text

the sensitivity was about the same as that of corresponding vesicles from the wild type parent strain. This increase in sensitivity together with the observation that spheroplasts did not bind suggests that the binding is specific in character.

Discussion

Results of the studies on spheroplasts suggested that ATPase and ferricyanide reductase used in this study as markers are localized at the inner surface of the cytoplasmic membrane of cells, since in intact spheroplasts they were inaccessible to antibody molecules or to the impermeable electron acceptor, ferricyanide. Another marker was based on the availability of binding sites for ATPase in membranes of DL54, which lacks the enzyme. Spheroplasts were unable to bind significant amounts of ATPase suggesting that the sites are on the inner surface of the cytoplasmic membrane.

In this study spheroplasts were made essentially according to Birdsell and Cota-Robles (1967). These preparations have large areas of naked inner membrane, visible in the electron-microscope. However, the existence of another barrier, invisible in the electron-microscope, in spheroplasts cannot be excluded, but is unlikely. This objection does not apply to the comparison of different types of vesicles.

It is concluded from this study that all of the ATPase, its binding sites, and the sites for reduction of ferricyanide are located on the external surface in vesicles prepared by French press or sonication, suggesting that they are inverted. Our results also suggest that these vesicles contain less than 5% of right-side out vesicles. This would account for the lack of transport by these vesicles in the presence of lactate. These findings confirm recent work of Hertzberg and Hinkle (*in preparation*) who postulated the inversion of their French press vesicles from studies of proton movement. Membranes of *M. phlei* were also shown to be inverted by sonication (Brodie, Hirata, Asano, Cohen, Hinds, Aithol & Kalva, 1972; Asano, Cohen, Baker & Brodie, 1973). Tsukagoshi and Fox (1971) found that hybrid vesicles were formed when a mixture of membranes of different origin was subjected to sonication. Thus, some of the vesicles prepared by sonication may have a "patch work" structure as suggested by Harold (1972). Kaback and Deuel (1969) showed that membranes that were sonicated or passed through a French press had transport activity, suggesting that these preparations still contain right-side out vesicles. It is noteworthy that the time of their sonication is much shorter than ours, and their conditions for passage of cells through the French pressure cell are different. In this regard Brodie *et al.* (1972) reported that proline uptake of intact ghosts from *M. phlei* was reduced as a function of time of sonication. Furthermore, the effect of these mechanical treatments on the orientation of membrane may be different when they are applied to intact cells rather than membrane vesicles or ghosts.

In the case of EDTA-lysozyme vesicles, interpretation is more difficult, as their response was intermediate between that of spheroplasts and other vesicles. Thus, EDTA-lysozyme vesicles from the mutant DL54 bound only 40% as much ATPase as did French press or sonic membrane vesicles. Furthermore, in EDTA-lysozyme vesicles from wild type strains, 40% of ATPase was inaccessible to antiserum, and treatment with detergents caused a 30% increase of ATPase activity. Finally, 50% of dehydrogenase activity was accessible to ferricyanide. This clearly suggests that the EDTA-lysozyme vesicles are not homogeneous, right-side out preparations like spheroplasts, though most of the vesicles are correctly oriented. The simplest explanation would be that about 50% of the vesicles are inverted. We find that relatively slow lysis of spheroplasts, by dilution of a 5% sucrose suspension into water, without washing or other treatment, made 40% of the dehydrogenase activity accessible to ferricyanide. This might suggest that during the resealing process inversion can occur. Against this notion is evidence of Kaback (1972) that freeze-etch preparations of the vesicles reveal only correctly oriented vesicles when examined by electron-microscopy. Another possibility is that within individual vesicles about half of each marker changed its location in the course of preparation of the vesicles. Histochemical studies such as the use of ferritin-labeled antibodies together with electron-microscopy or developing a method to separate two kinds of vesicles might give an answer. Such studies are planned.

In EDTA-lysozyme vesicles, only part of the ATPase activity was accessible to antiserum unless the vesicles were first treated with a high concentration of detergent, suggesting that the enzyme is on the inner surface or buried in the membrane in some unknown way. Since the uninhibitable fraction of activity is measurable, this means that it is accessible to ATP but not to antiserum. We cannot demonstrate a significant rate of transport of ATP into vesicles from DL54 (less than 0.1 nmole/min/mg of ATP [^{3}H] was transported in the same solution as used in the ATPase assay, when ATPase activity was 0.02 μmole/min/mg). It is difficult to reconcile these findings. The most reasonable interpretation to us is a combination of two events: (1) some inversion of vesicles; and (2) a fraction of the ATPase has changed its location so that it is cryptic to antiserum but accessible to ATP.

An interesting recent observation of Reeves, Kaback and Hong (1973) is that a D-lactate dehydrogenase preparation made by extracting wild type membranes with guanidine HCl could restore D-lactate-dependent transport of membrane vesicles made from a mutant lacking this enzyme. We have confirmed this observation using purified enzyme obtained as previously

described (Futai, 1973) and extended it to glycerol-3-phosphate dependent transport of amino acids (Futai, *in preparation*). Apparently, enzyme bound to the outside of the membrane vesicle is able to drive both respiration and transport, because other components of the electron transport chain are correctly oriented. Another possibility is that the bound enzyme has some mobility and manages to become correctly localized in most of the vesicles. For further elucidation of this problem, methods must be developed to separate different kinds of vesicles and to find out which ones are transporting. None of this argues against the proposed mechanism of transport itself (Kaback, 1972), if only the right-side-out vesicles are transporting.

The ATPase activity of spheroplasts after toluenization was as high as that of membrane vesicles (Tables 1 and 2). Since the membranes contain less than 20% of total cell protein, the specific activity of ATPase in the membranes should exceed that of spheroplasts if all of the activity is recovered in the membrane fraction. The actual recovery of ATPase in the membrane fraction was 30 to 40% in the procedure used here. Furthermore, some loss of activity was observed during washing. After washing EDTA-lysozyme vesicles 4 times with 10 mM EDTA, 32% of activity was lost from the vesicles. It is noteworthy that 0.5 mM EDTA solubilized ATPase (Davies & Bragg, 1972) but 10 mM EDTA did not (Kobayashi & Anraku, 1972). Loss of ATPase during washing of other types of membrane vesicles was about 10%. These factors could account for the low specific activity of ATPase in membranes.

We used the increased sensitivity of ATPase to DCCD as a measure of specificity of binding to membrane vesicles of the ATPase mutant DL54. Recently it was shown with the same mutant that the bound ATPase could be coupled to yield ATP-dependent transhydrogenase activity (Bragg & Hou, 1973). Restoration of oxidative phosphorylation has been demonstrated by Hertzberg and Hinkle (*in preparation*) when French press vesicles were washed to remove ATPase and then incubated with the purified enzyme. This restoration of phosphorylation was also shown by incubating sonic vesicles of the mutant DL54 with the purified enzyme (Futai, *in preparation*).

This work was done in the laboratory of Dr. Leon A. Heppel. I am most grateful to him for stimulating discussions and criticism and for help in the preparation of this paper. I am also thankful to Drs. P. L. Hanson and E. P. Kennedy for showing me their manuscript before publication, and to a number of members of the Section of Biochemistry for helpful discussions, in particular to Drs. E. Racker, D. B. Wilson, G. Schatz, C. F. Yocum and Mr. E. Hertzberg. I wish also to thank Miss Amy Shandell for her skillful technical assistance.

This work was supported by Grant No. 1178904 from the National Institutes of Health and Grant No. GB 27396X from the National Science Foundation.

References

Asano, A., Cohen, N. S., Baker, R. F., Brodie, A. F. 1973. Orientation of the cell membrane in ghost and electron transport particles of *Mycobacterium phlei*. *J. Biol. Chem.* **248**:3386

Birdsell, D. C., Cota-Robles, E. H. 1967. Production and ultrastructure of lysozyme and ethylenediamine tetracetate lysozyme spheroplasts of *Escherichia coli*. *J. Bacteriol.* **93**:427

Bragg, P. D., Hou, C. 1973. Reconstitution of energy-dependent transhydrogenase in ATPase-negative mutants of *Escherichia coli*. *Biochem. Biophys. Res. Commun.* **50**:729

Brodie, A. G., Hirata, H., Asano, A., Cohen, N. S., Hinds, T. R., Aithol, H. N., Kalva, V. K. 1972. The relationship of bacterial membrane orientation to oxidative phosphorylation and active transport. *In:* Biological Membranes. C. F. Fox, editor. p. 445. Academic Press Inc., New York

Davies, P. L., Bragg, P. D. 1972. Properties of a soluble Ca^{2+} and Mg^{2+} activated ATPase released from *Escherichia coli* membranes. *Biochim. Biophys. Acta* **266**:273

Futai, M. 1973. Membrane D-lactate dehydrogenase from *Escherichia coli*: Purification and properties. *Biochemistry* **12**:2468

Hanson, R. L., Kennedy, E. P. 1973. Energy-transducing adenosine triphosphates from *Escherichia coli:* Purification, properties and inhibition by antibody. *J. Bacteriol.* **114**:772

Harold, F. M. 1972. Conservation and transformation of energy by bacterial membranes. *Bact. Rev.* **36**:172

Kaback, H. R. 1971. Bacterial membranes. *In:* Methods in Enzymology. XXII. B. Jacoby, editor. p. 99. Academic Press Inc., New York

Kaback, H. R. 1972. Transport across isolated bacterial cytoplasmic membranes. *Biochim. Biophys. Acta* **265**:367

Kaback, H. R., Deuel, T. F. 1969. Proline uptake by disrupted membrane preparations from *Escherichia coli*. *Arch. Biochem. Biophys.* **132**:118

Kobayashi, H., Anraku, Y. 1972. Membrane-bound adenosine triphosphatase of *Escherichia coli*. I. Partial purification and properties. *J. Biochem. Tokyo* **71**:387

Lowry, O. H., Rosebrough, N. J., Farr, A. L., Randall, R. J. 1951. Protein measurement with the folin phenol reagent. *J. Biol. Chem.* **193**:265

Reeves, J. P., Kaback, H. R., Hong, J. 1973. Reconstitution of D-lactate dependent transport in membrane vesicles from D-lactate dehydrogenase negative mutants. *Fed. Proc.* **32**:599

Schnaitman, C. A. 1970. Protein composition of the cell wall and cytoplasmic membranes of *Escherichia coli*. *J. Bacteriol.* **104**:890

Shechter, E., Gulik-Krzywicki, T., Kaback, H. R. 1972. Correlations between fluorescence, x-ray diffraction and physiological properties in cytoplasmic membrane vesicles isolated from *Escherichia coli*. *Biochim. Biophys. Acta* **274**:466

Simoni, R. D., Shallenberger, M. K. 1972. Coupling of energy to active transport of amino acids in *Escherichia coli*. *Proc. Nat. Acad. Sci.* **69**:2663

Tanaka, S., Lerner, S. A., Lin, E. C. C. 1967. Replacement of a phosphoenol-pyruvate dependent phosphotransferase by a nicotinamide adenine dinucleotide-linked dehydrogenase for the utilization of mannitol. *J. Bacteriol.* **93**:642

Tsukagoshi, N., Fox, C. F. 1971. Hybridization of membranes by sonic irradiation. *Biochemistry* **10**:3309

Weber, K., Osborn, M. 1969. The reliability of molecular weight determination by dodecyl sulfate polyacrylamide gel electrophoresis. *J. Biol. Chem.* **244**:4406

Weiner, J. H. 1974. The localization of glycerol-3-phosphate dehydrogenase in *Escherichia coli*. *J. Membrane Biol.* **15**:1