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Isolation of Membrane Vesicles With Inverted Topology by Osmotic Lysis of Azotobacter vinelandii Spheroplasts

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Membrane vesicles were prepared from Azotobacter vinelandii spheroplasts by lysis in either potassium phosphate (pH 7.0) or Tris[1]-acetate (pH 7.8) buffers. These 2 types of preparations differ considerably in their properties: 1) Examination by scanning electron microscopy reveals that the Pi vesicles consist primarily of closed structures 0.6–0.8 μm in diameter with a rough or particulate surface similar to that of spheroplasts. The Tris vesicles are significantly smaller, 0.1–0.3 μm in diameter, and have a much smoother surface structure. 2) Antisera from rabbits immunized with A. vinelandii lipopolysaccharide antigen will agglutinate Pi vesicles but not Tris vesicles. 3) Tris vesicles have a fourfold higher specific activity of latent H^+-ATPase than Pi vesicles. After exposure to Triton X-100 similar ATPase activities are observed for both types of vesicles. 4) Pi vesicles transport calcium in the presence of ATP or lactate at less than 30% of the rates observed for Tris vesicles. 5) Tris vesicles have less than 22% of the transport capacity of Pi vesicles for accumulation of labeled sucrose and less than 3% of the capacity for valinomycin-induced uptake of rubidium observed during respiration. 6) Quinacrine fluorescence intensity is reduced by 30% during lactate oxidation and 20% during ATP hydrolysis by Tris vesicles. Under similar conditions, fluorescence in Pi vesicles is quenched by only 7% and less than 2%, respectively. These findings suggest that Pi vesicles have the normal orientation of the intact cell whereas Tris vesicles have an inverted topology.

Key words: calcium transport, quinacrine fluorescence, rubidium transport, sucrose transport, lipopolysaccharide antibody, scanning electron microscopy, topology, membrane vesicles, Azotobacter vinelandii

Cytoplasmic membrane vesicles isolated from bacteria according to the techniques pioneered by Kaback (1) have proven to be a powerful system for elucidation of active transport mechanisms. The topological orientation or sidedness of such preparations and the homogeneity of topology in the vesicle population are key factors in interpretation of solute transport experiments, especially if results are to be evaluated in chemiosmotic terms. Kaback has cited considerable evidence (1–3) in support of his claim that Escherichia coli membrane vesicles prepared by the established technique have a normal or right-side-out orientation, i.e., identical to that of the cell. Perhaps most convincing are freeze-cleave electron microscopic studies, repeated in several laboratories (1, 4, 5), which

[1] Abbreviations: CCP – Chlorocarbonylcyanide phenylhydrazone; LPS – Lipopolysaccharide; Tricine – N-tris-(hydroxymethyl)-methylglycine; Tris – Tris-(hydroxymethyl)-aminomethane.

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confirm that preparations of the Kaback type are almost completely right-side out. However, extensive examination of certain membrane markers known to be located at the internal face of the cytoplasmic membrane in intact cells and spheroplasts is not consistent with this interpretation. For example, it has been shown that ATPase, NADH dehydrogenase, succinic-ferricyanide reductase, and α-glycerol-P ferricyanide reductase are located on the inner surface of the plasma membrane of E. coli spheroplasts (6–9). These enzyme systems in spheroplasts are inaccessible to impermeant substrates (ATP, NADH, ferricyanide) or to specific antibodies, but become accessible when the permeability barrier is destroyed. In spheroplast vesicles (kabackosomes), however, about 50% of the total activity is accessible to impermeant substrates for these marker enzymes (8, 9) and to the ATPase antibody (6, 7). But other markers, such as D-lactic dehydrogenase, remain inaccessible in spheroplast vesicles (3). The most widely accepted interpretation of these conflicting findings is that the E. coli spheroplast vesicles have a normal gross topology, but that certain internal components become externalized during the isolation procedure. The topology of such preparations would thus be functionally heterogenous.

In the course of our studies with Azotobacter vinelandii we observed that vesicles prepared by lysis of spheroplasts in Tris acetate buffer transport calcium in the presence of oxidizable substrates (10, 11) or ATP (12) but show very low transport activity for respiration-coupled uptake of glucose or sucrose. These latter solutes are accumulated by A. vinelandii membrane vesicles (13, 14) prepared by lysis of spheroplasts in the potassium phosphate buffer employed by Kaback. Since most bacterial cells actively extrude calcium (15) and inverted vesicles from E. coli accumulate calcium (16), it appeared that our Tris preparations everted during isolation. In light of the foregoing discussion, however, it was not clear whether only selected components (i.e., ATPase and dehydrogenase) became inverted or the entire surface became everted. In this paper we report further studies which support the latter conclusion.

METHODS

Membrane vesicles were isolated from Azotobacter vinelandii strain O after growth on 1% glucose or 1% sucrose by a procedure described earlier (12, 13).

Samples for scanning electron microscopy were immediately fixed in 2% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) containing 0.1 M sucrose at 4°C for at least 1 h. The samples were then rinsed with cold buffer and dehydrated in a graded series of alcohol followed by dehydration in amyl acetate. The samples were further processed by critical point drying in a Denton DCP1 apparatus using oil-free liquid CO_2 as an intermediate fluid. Specimens were immediately coated with 50–70 Å of a 60:40 alloy of gold palladium with a DC sputtering module in a Kinney KSM-2 evaporator at 2×10^{-5} torr. Specimens were examined with a 100C JEOL electron microscope equipped with ASID at 40 kV using 30–50° tilting angle in a side entry goniometer. Images were recorded on Polaroid 105 N/P film from a 2000 line CRT. Photographic enlargement did not exceed 1.6 diameters.

Antigenic lipopolysaccharide was isolated from Azotobacter vinlandii cells by the method of Boivin and Mesrobeanu as described by Staub (17). Aqueous solutions of LPS were sterilized by filtration and stored at 4°C. Rabbits were injected with an aliquot of this solution equivalent to 250 μg dry weight of LPS and then received a booster injection of 500 μg on day 20 and 2.5 mg on day 30. Blood was collected on day 35, incubated at 0°C for 2 h, and then centrifuged at 48,000 × g for 30 min. The supernate was adjusted

to 50% saturation of ammonium sulfate and the precipitate collected by centrifugation. The pellet was dissolved in 0.1 M sodium phosphate buffer (pH 7.0) and stored at −20°C. Precipitating antibody was detected by discontinuous counterimmunoelectrophoresis (18). A weak but distinct precipitin line was observed between immune globulin and LPS antigen but no reaction occurred with normal rabbit globulin. For antibody agglutination experiments, membrane vesicles were pelleted by centrifugation and resuspended in 0.15 M sodium chloride buffered with 10 mM potassium phosphate (pH 7.0). Membrane vesicles (500 μg protein) were incubated in a final volume of 60 μl with 100 μg globulin at 0°C for 1 h. These incubations were inspected by phase contrast microscopy using a Leitz Orthoplan microscope and photographed with a Leitz Orthomat camera on KB-14 film (ASA 100).

ATPase activity of membrane vesicles was activated with trypsin and assayed as previously described (12). The methods for measuring transport of calcium (12), sucrose (14), and rubidium (19) were also reported earlier.

For fluorescence measurements, vesicles were pelleted by centrifugation and resuspended in a buffer prepared by titration of 0.10 M Tricine with Tris base to pH 8.0. The assay mixture (2 ml) contained this buffer solution plus 1 μM quinacrine hydrochloride, 1 mM $MgSO_4$, and 0.2 mg/ml vesicle protein. Quinacrine fluorescence was recorded as described (19) at excitation and emission wavelengths of 425 nm and 500 nm, respectively.

RESULTS

Membrane vesicles were prepared by osmotic lysis of lysozyme-EDTA spheroplasts from Azotobacter vinelandii strain O. Two different buffers were employed as the lysis medium and in subsequent washing of the vesicles. Preparations referred to as Pi vesicles were lysed and washed in potassium phosphate (pH 7.0), whereas Tris vesicles were prepared in Tris-acetate (pH 7.8). Both the spheroplasts and derived vesicles were examined by scanning electron microscopy as shown on Figs. 1 and 2. The Pi vesicles consist primarily of closed structures 0.6–0.8 μm in diameter which have a rough or particulate surface topology similar to that of the spheroplasts. In contrast, the Tris preparations contain a much higher content of small vesicles, 0.1–0.3 μm in diameter. These latter preparations have a much smoother surface structure than is observed for spheroplasts or Pi vesicles. Although extensive aggregation of vesicles is observed in the electron photomicrographs this probably represents an artifact due to fixation in glutaraldehyde. Little aggregation of vesicles is observed by phase contrast light microscopy in untreated preparations but extensive aggregation is noted after incubation in 4% glutaraldehyde (not shown).

In order to further examine the surface components of these vesicles, lipopolysaccharide antigen was extracted from A. vinelandii and injected into rabbits. Immune globulin gave a precipitin line with purified LPS in discontinuous counterimmunoelectrophoresis but none was observed using preimmune globulin. The LPS antibody produced extensive agglutination of Pi vesicles as shown in the phase contrast micrographs of Fig. 3. This effect was blocked by addition of excess LPS; no agglutination was produced by preimmune globulin. However, incubation of LPS antibody with Tris vesicles failed to produce significant agglutination.

Previous studies from this laboratory established that Tris vesicles isolated from A. vinelandii contain a trypsin-activated, dicyclohexylcarbodiimide-sensitive ATPase which catalyzed proton uptake (12). The F_1 subunit of this enzyme is thought to be attached to

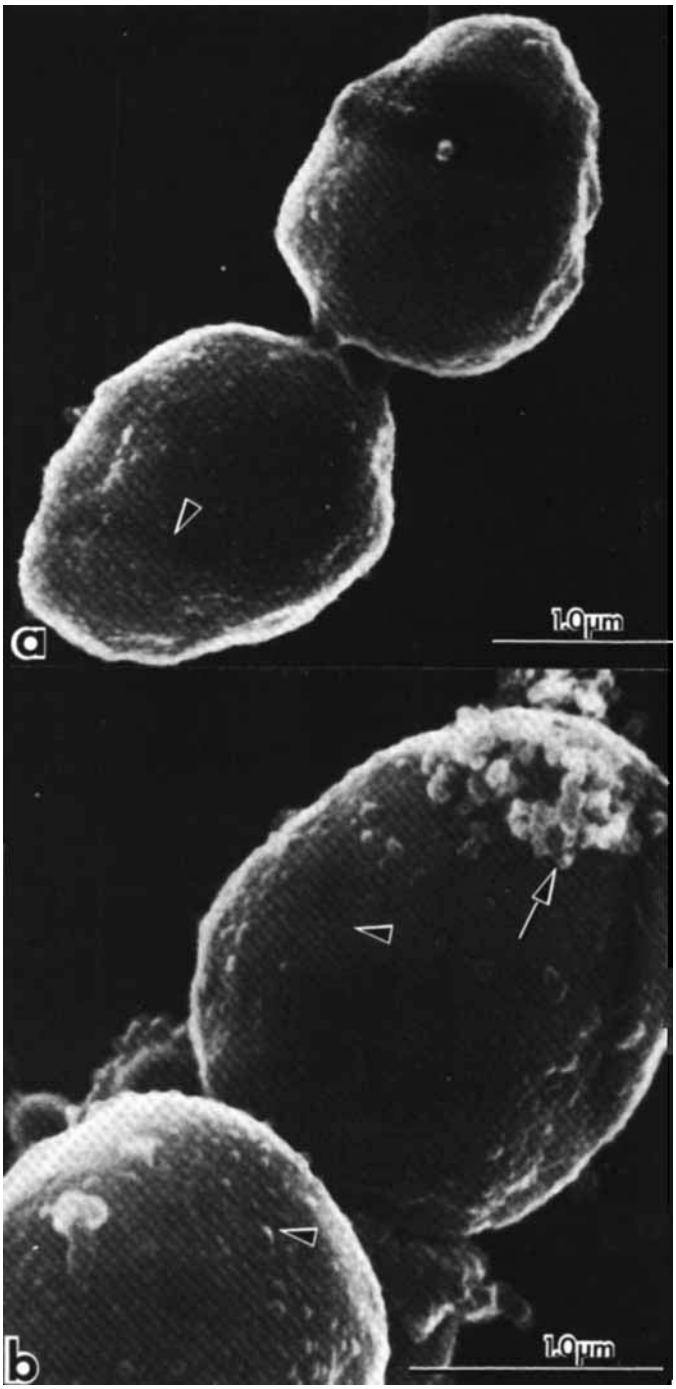

Fig. 1. Scanning electron photomicrographs of A. vinelandii cells (a) and spheroplasts (b). Specimens were prepared and examined as described under Methods.

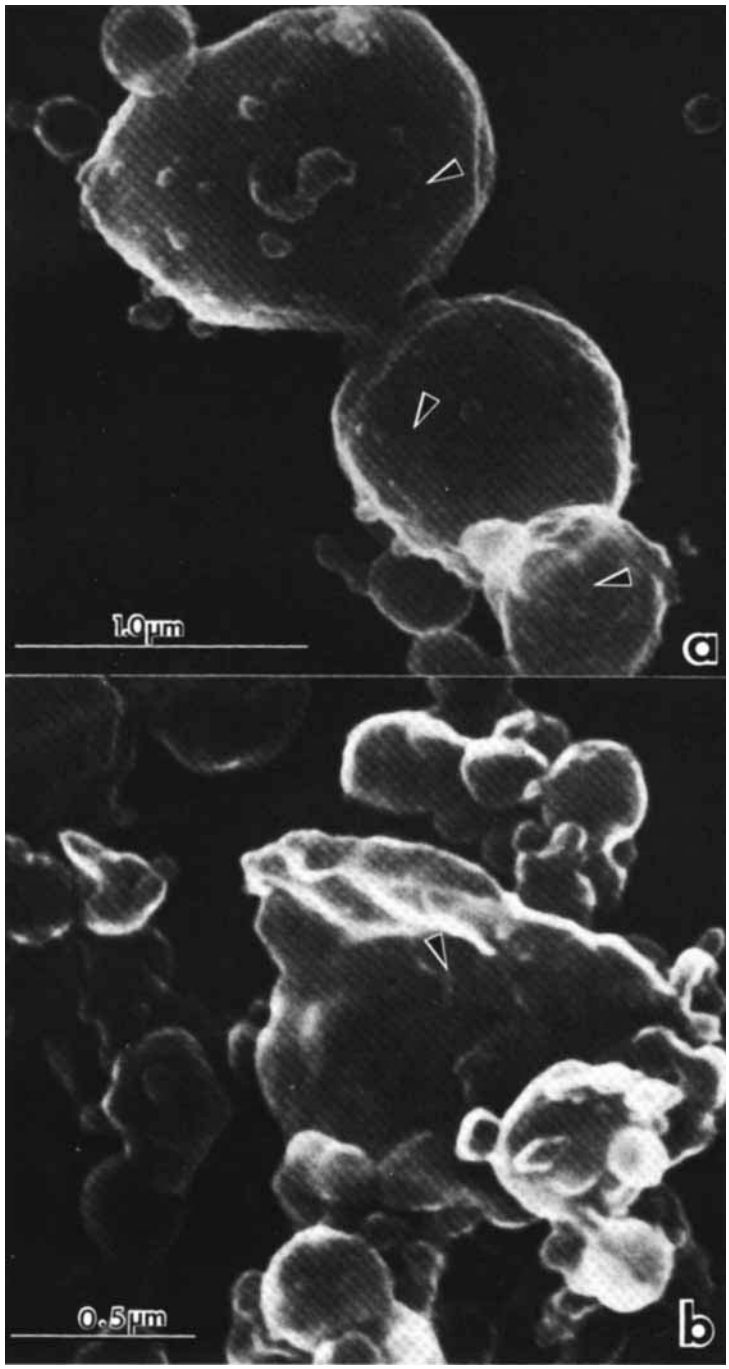

Fig. 2. Scanning electron photomicrographs of Pi vesicles (a) and Tris vesicles (b) from A. vinelandii. Specimens were prepared and examined as described under Methods.

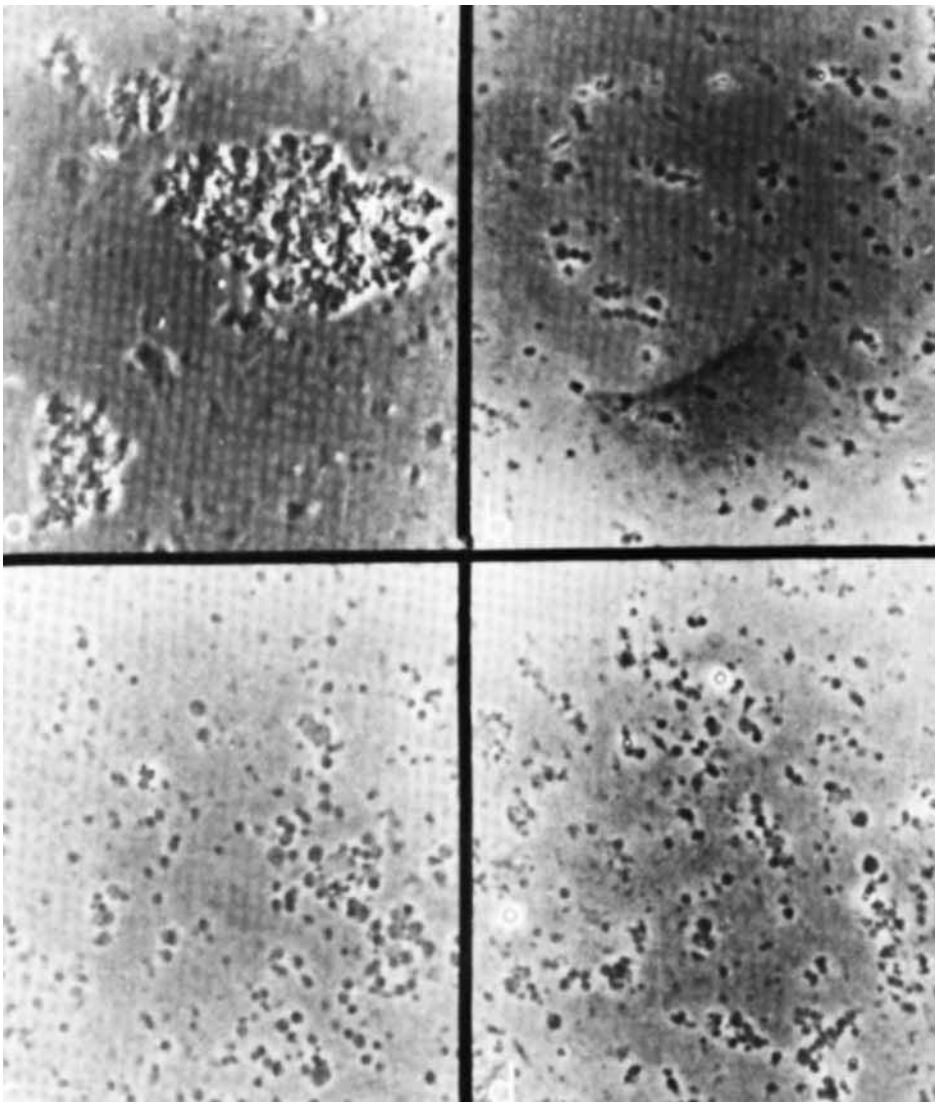

Fig. 3. Agglutination of membrane vesicles by lipopolysaccharide antibody. Vesicles were incubated with antibody and phase contrast photomicrographs obtained as described under Methods. a) Pi vesicles (500 μg protein) incubated with 100 μg LPS antibody; b) Pi vesicles (500 μg) incubated with 100 μg control (preimmune) globulin; d) Tris vesicles (500 μg) incubated with 100 μg LPS antibody.

the cytoplasmic face of the plasma membrane in bacteria (6, 9). Since ATP is not permeant, we sought to employ ATPase activity, unmasked by trypsin treatment, as an index of membrane sidedness in both Tris and Pi vesicles. These activities are shown in Table I. Both Tris and Pi vesicles have relatively low ATPase activity until exposed to a brief trypsin treatment. Trypsinization of Tris vesicles results in nearly a sevenfold increase in ATPase activity. This is presumably due to destruction of a polypeptide inhibitor as

TABLE I. ATPase Activity of A. vinelandii Membrane Vesicles*

Carbon source	Lysis buffer	Vesicle incubation		ATPase activity
		Trypsin	Triton	
				nmol/min/mg
Sucrose	Tris	–	–	17
		+	–	116
		+	+	134
Sucrose	Pi	–	–	16
		+	–	31
		+	+	118
Glucose	Pi	–	–	8
		+	–	19
		+	+	59

*Vesicles were isolated from cells grown on sucrose or glucose by spheroplast lysis in phosphate or Tris buffer (as indicated). Where indicated, latent ATPase was activated by exposure of vesicles to trypsin (62 μg/mg membrane protein at 0°C) in 0.1 M Tris-acetate (pH 7.8) for 2 min. Where indicated, the incubation medium for trypsinization contained 0.1% Triton X-100. After addition of soybean trypsin inhibitor, aliquots were removed for ATPase assay as described in Methods.

described for the oligomycin-sensitive ATPase in heart mitochondria (20). Trypsin treatment under these conditions does not make vesicles grossly leaky since they are able to retain accumulated calcium (cf., Table II). Exposure of Tris vesicles to trypsin in the presence of 0.1% Triton X-100 results in only a 13% further increase in ATPase activity.

On the other hand, exposure of Pi vesicles to trypsin yields only a twofold increase in ATPase activity. But the presence of Triton increases ATPase dramatically, i.e., up to a sevenfold overall increase. Values for ATPase activity are also reported for Pi vesicles from cells grown on glucose as sole carbon source. These latter preparations have been shown to extrude protons during respiration (19) and accumulate glucose actively (13). The same pattern is observed for Pi vesicles isolated from sucrose-grown cells although the ultimate ATPase levels are about twofold higher in preparations from the sucrose culture. It seems clear that intact Tris vesicles have much greater trypsin-activated ATPase activity (about

TABLE II. Rates of Energy-dependent Calcium Uptake by A. vinelandii Membrane Vesicles*

Carbon source	Lysis buffer	Calcium uptake rate	
		D-Lactate	ATP
		nmol/min/mg	nmol/min/mg
Sucrose	Tris	15.6	9.2
Sucrose	Pi	4.6	< 0.2
Glucose	Pi	2.3	< 0.2

*Vesicles were isolated from cells grown on sucrose or glucose by lysis in Pi or Tris buffer, as indicated. The vesicles were washed in 50 mM Tris-acetate (pH 7.8) and assayed in this buffer containing 2 mM $MgSO_4$, 50 μg membrane protein, 40 μM ^{45}Ca (36 Ci/g-atom), and either 20 mM D-lactate or 2 mM ATP, as described under Methods. Vesicles were trypsin treated (Table I) for assays of ATP-dependent uptake and untreated preparations were used for lactate-dependent uptake. All values for Ca uptake were corrected for the uptake by controls that lacked energy source.

fourfold) than the comparable Pi vesicles. However, after destruction of the permeability barrier with Triton, similar activities are observed.

Several species of bacteria have been shown to actively extrude calcium (15). Thus the proton-coupled accumulation of calcium by bacterial membrane vesicles can be an index of sidedness as well as the functional state of the ATP- and respiration-driven proton pumps. A comparison of rates for energy-linked calcium transport by A. vinelandii membrane vesicles is given in Table II. As indicated, Tris vesicles accumulate calcium at appreciable rates in the presence of D-lactate or ATP (after trypsin treatment). The rates shown have been corrected for the energy-independent uptake which is typically less than 5% of the coupled rate. In contrast, Pi vesicles in the presence of lactate transport calcium at less than 30% of the rate observed with Tris vesicles. In the presence of ATP, Pi vesicles do not accumulate significant amounts of calcium either before or after the usual trypsin treatment.

It was previously established that Pi vesicles isolated from A. vinelandii grown on glucose develop a membrane potential (inside-negative) and concentrate glucose by a proton-coupled mechanism (19). Since the Tris vesicles used in this study were derived from cells grown on sucrose, we examined the active accumulation of sucrose and Rb^+ (in the presence of valinomycin) in both vesicle types. Pi vesicles actively accumulate [^{14}C] sucrose from the medium during the oxidation of L-malate (Table III). Under similar conditions, Rb^+ is also accumulated but only after addition of valinomycin. This latter indicates that a transmembrane electric potential develops across the vesicles during respiration. The Tris vesicles, on the other hand, accumulate sucrose at only 20% of the level achieved by Pi vesicles. Furthermore, Tris vesicles fail to take up significant amounts of Rb^+ in the presence of valinomycin. These latter observations are not due to a gross defect in respiration since malate is rapidly oxidized by these preparations and drives calcium accumulation (11).

In order to study the development of an energized state during respiration or ATP hydrolysis by these preparations, the fluorescence of quinacrine hydrochloride was also examined. As shown on Fig. 4, lactate oxidation by Tris vesicles results in a 30% reduction in quinacrine fluorescence intensity. This quenching was reversed by addition of the uncoupler CCP. Quinacrine fluorescence was not significantly quenched by lactate addition to Pi vesicles derived from glucose-grown cells and was reduced only by 7% in Pi vesicles

TABLE III. L-Malate-dependent Accumulation of Sucrose and Rubidium (in the presence of valinomycin) by Vesicles*

Lysis buffer	Sucrose uptake	Rb^+ Uptake
	nmol/mg/10 min	nmol/mg/10 min
Pi	6.5	25.2
Tris	1.4	0.6

*For sucrose uptake vesicles were washed in 50 mM KPi buffer (pH 7.0) and assayed in that buffer containing 2 mM $MgSO_4$, 50 μM FAD, 20 mM L-malate, and 40 μM [^{14}C]-sucrose as described under Methods. For Rb uptake vesicles were washed in 50 mM NaPi (pH 7.0) and assayed in that buffer containing the above except that 40 μM $^{86}Rb^+$ and 1 μM valinomycin replaced sucrose.

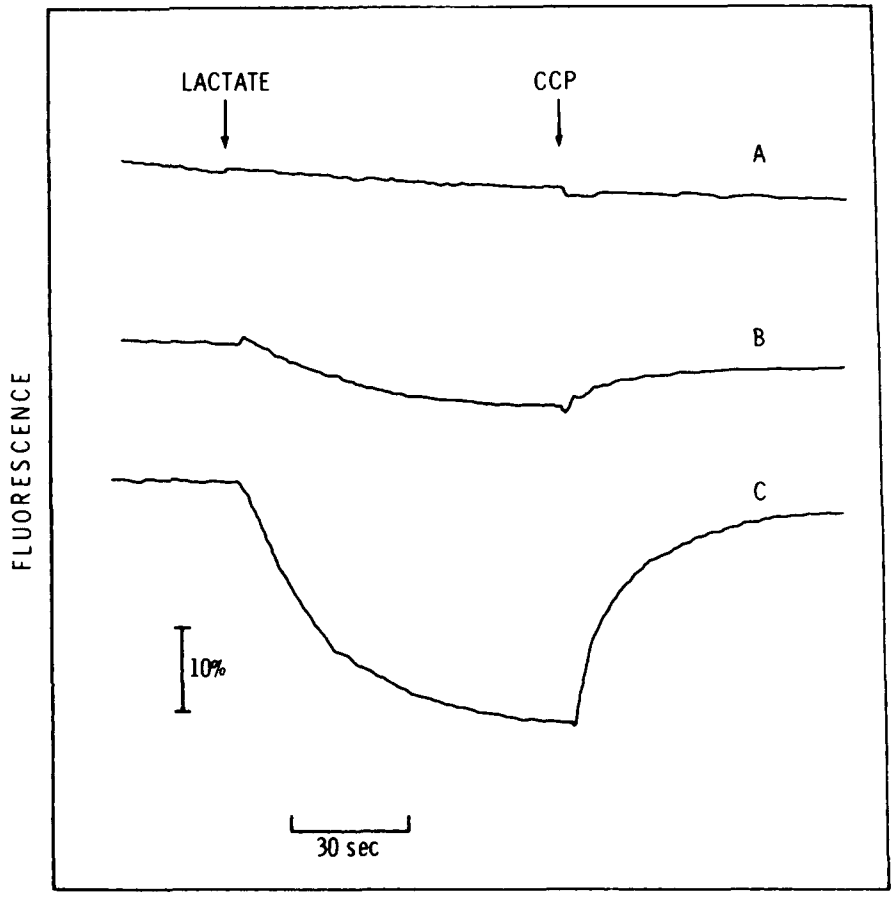

Fig. 4. Effect of lactate oxidation by membrane vesicles on quinacrine fluorescence. The assay mixture contained 0.10 M Tris-tricinate (pH 8.0), 1 mM $MgSO_4$, 0.2 mg/ml membrane protein, and 1 μM quinacrine hydrochloride. Fluorescence was recorded as described under Methods; the vertical bar indicates scale callibration equivalent to 10% of the initial fluorescence intensity. As indicated by the first arrow, DL-lactate (Tris salt) was added at a final concentration of 1 mM. At the second arrow, CCP was added at a final concentration of 1 μM. Trace A) Pi vesicles isolated from glucose-grown cells; B) Pi vesicles from sucrose-grown cells; C) Tris vesicles from sucrose-grown cells.

from sucrose-grown cells. Since quenching of quinacrine fluorescence reflects development of a proton gradient, inside-acid (21), these observations are consistent with the relative rates of calcium uptake given in Table II. Similar results were obtained on addition of ATP to trypsinized vesicles as shown in Fig. 5. ATP hydrolysis by Tris vesicles results in a 20% reduction of quinacrine fluorescence which was reversible by uncoupler. Addition of ATP to Tris vesicles before trypsin treatment gave no fluoresence quenching (data not shown). However, ATP hydrolysis by Pi vesicles, previously treated with trypsin, failed to produce any significant effect on quinacrine fluorescence intensity. This again is consistent with the effects of ATP on calcium transport.

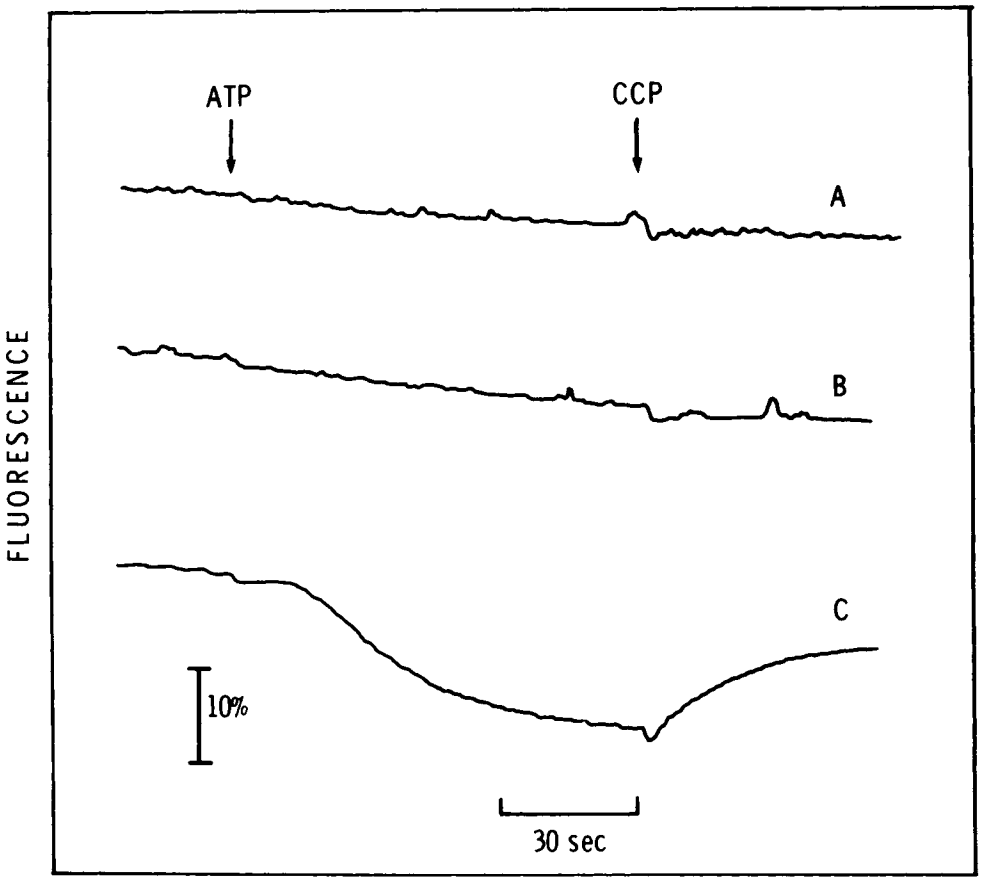

Fig. 5. Effect of ATP hydrolysis by membrane vesicles on quinacrine fluorescence. The assay conditions were identical to those described in the legend of Fig. 4 except that trypsin-treated vesicles were employed (Methods) and 0.3 mM ATP (Mg salt) replaced lactate. Trace A) Pi vesicles isolated from glucose-grown cells; B) Pi vesicles from sucrose-grown cells; C) Tris vesicles from sucrose-grown cells.

DISCUSSION

The results reported here clearly indicate topological differences in Pi and Tris vesicles isolated from A. vinelandii. The Pi vesicles, prepared by a method similar to Kaback's, appear to have properties (proton extrusion, sugar accumulation) similar to the intact cell (19). On the other hand, Tris vesicles clearly possess properties usually associated with an inverted (inside-out) topology, i.e., proton and calcium accumulation (13). The major point at issue, however, is whether: i) the Tris lysis of spheroplasts brings about inversion of selected membrane components leaving the gross topology normal or ii) Tris lysis causes a true everting of the membrane in a significant population of the vesicles. The findings presented here support the second interpretation; this rests on 7 lines of evidence.

1) Scanning electron micrographs indicate that the Tris vesicles are significantly smaller in diameter than Pi vesicles.

2) Pi vesicles have a rough or particulate topology, revealed by the scanning electron

microscope, which is similar to that of spheroplasts. Tris vesicles appear to have a much smoother surface.

3) Pi vesicles are readily agglutinated by LPS antibody, but Tris vesicles are not agglutinated. Using ferritin-conjugated LPS antibody, Shands (22) demonstrated the presence of LPS antigen on the outer surface of Salmonella typhimurium spheroplasts and plasma membranes obtained by osmotic lysis.

4) Tris vesicles have fourfold higher specific activity of trypsin-activated H^+-ATPase than intact Pi vesicles. After dissolution of the permeability barrier with Triton, similar ATPase activity is observed in both types of vesicles.

5) Tris vesicles rapidly accumulate calcium during oxidation of D-lactate or hydrolysis of ATP. Pi vesicles transport calcium at less than 30% of the rates observed for Tris vesicles during lactate oxidation and at negligible rates in the presence of ATP.

6) Tris vesicles have less than 22% of the transport capacity of Pi vesicles for the respiration-coupled accumulation of [^{14}C]sucrose, and less than 3% of the capacity for accumulating rubidium (in the presence of valinomycin) during respiration.

7) The fluorescence intensity of quinacrine is markedly reduced when lactate or ATP is added to Tris vesicles. Much less quenching is observed with Pi vesicles under identical conditions.

These findings support the interpretation that the Pi vesicles consist primarily of structures with the same topology as the intact cell, whereas the Tris preparations are principally composed of everted vesicles. It is difficult, however, to quantitate the extent to which each fraction is contaminated with vesicles of opposite topology. The electron photomicrographs, LPS-antibody studies, and fluorescence measurements cannot be stringently applied for quantitative purposes. Using ATPase and calcium transport activity as markers for inverted vesicles, we can estimate that Pi vesicles contain at most 29% and 27%, respectively, of inverted structures. Using sucrose and rubidium uptake as indices of right-side-out vesicles, we estimate the maximum contamination of Tris vesicles by right-side-out structures at 21% and 3%, respectively. Of course, these approximations rest on the assumptions that: i) these markers do not undergo transmembrane flip-flop and ii) the lysis and washing procedures do not cause marker activation. These possibilities cannot be rigorously excluded.

The mechanism of inversion is of great interest to us. Although lysis of A. vinelandii spheroplasts in Tris acetate buffer clearly promotes inversion, similar treatment of E. coli spheroplasts apparently does not. Such vesicles from E. coli do not accumulate significant amounts of calcium in the presence of D-lactate (H. R. Kaback, unpublished observations). A significant factor may be the network of internal membrane thought to be formed by invagination of the A. vinelandii plasma membrane (23, 24). Osmotic lysis of spheroplasts in a relatively permeant buffer such as Tris acetate may induce sealing and/or release of vesicles with inverted topology. Such structures may be present but trapped within the internal space of Pi vesicles. Further investigation is required to clarify this issue.

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