Journal of Supramolecular Structure 7:15–27 (1977)
Molecular Aspects of Membrane Transport 379–391

Orientation of the Protonmotive Force in Membrane Vesicles of Escherichia coli

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Membrane vesicles of Escherichia coli can be produced by 2 different methods: lysis of intact cells by passage through a French pressure cell or by osmotic rupturing of spheroplasts. The membrane of vesicles produced by the former method is everted relative to the orientation of the inner membrane in vivo. Using NADH, D-lactate, reduced phenazine methosulfate, or ATP these vesicles produce protonmotive forces, acid and positive inside, as determined using flow dialysis to measured the distribution of the weak base methylamine and the lipophilic anion thiocyanate. The vesicles accumulate calcium using the same energy sources, most likely by a calcium/proton antiport. Calcium accumulation, therefore, is presumably indicative of a proton gradient, acid inside.

The latter type of vesicle, on the other hand, exhibits D-lactate-dependent proline transport but does not accumulate calcium with D-lactate as an energy source. NADH oxidation or ATP hydrolysis, however, will drive the transport of calcium but not proline in these vesicles. Oxidation of NADH or hydrolysis of ATP simultaneous with oxidation of D-lactate does not result in either calcium or proline transport. These results suggest that the vesicles are a patchwork or mosiac, in which certain enzyme complexes have an orientation opposite to that found in vivo, resulting in the formation of electrochemical proton gradients with an orientation opposite to that found in the intact cell. Other complexes retain their original orientation, making it possible to set up simultaneous proton fluxes in both directions, causing an apparent uncoupling of energy-linked processes. That the vesicles are capable of generating protonmotive forces of the opposite polarity was demonstrated by measurements of the distribution of acetate and methylamine (to measure the ΔpH) and thiocyanate (to measure the $\Delta\psi$).

Key words: protonmotive force, active transport, energy transduction, E. coli

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Received April 12, 1977; accepted June 8, 1977.

Active transport of solutes in many organisms is a vectorial process in which the solute is concentrated in an unaltered form in 1 compartment of a 2 (or multi-) compartment system. We have been interested in the nature of the biochemical events which determine the directionality of active transport systems. Many bacterial transport systems are secondary in nature, that is, they couple the flow of 1 solute against an apparent electrochemical gradient to a previously established electrochemical gradient of another solute [see the recent reviews by Harold (1) and Rosen and Kashket (2)]. The primary sources of those electrochemical gradients in bacteria are the electron transport chains (3), Mg^{2+}-ATPase (or BF_0F_1) (4–6) and bacteriorhodopsin (7). Each of those is a primary active transport system for protons, converting either chemical or electromagnetic energy into electrochemical potential energy. Escherichia coli does not synthesize bacteriorhodopsin, but does create electrochemical proton gradients or protonmotive forces (8) presumably with the energy derived from ATP during hydrolysis via the BF_0F_1 or from NADH, D-lactate, or other reduced substrates during their oxidation via flavin-linked dehydrogenases coupled to electron transport chains (1–3, 9, 10).

Are transport carrier proteins symmetrical? Is the only factor which determines the direction of transport by secondary systems the orientation of the electrochemical ion gradient established by the primary system? Or are the carriers "different" on the 2 sides of the membrane, such that they could only work in 1 direction regardless of the orientation of the protonmotive force? In order to answer these question, we began a study of the active transport of calcium in membrane vesicles of E. coli. Our previous results had indicated that calcium was actively accumlated by everted membrane vesicles but not by "right-side-out" vesicles when energy was supplied by oxidation of reduced phenazine methosulfate (PMS) (11). Our data further indicated that the calcium transport system works via an antiport mechanism with protons (12), suggesting that an artificially imposed pH gradient (ΔpH), acid inside, should be capable of driving a transient accumulation of calcium in everted vesicles. This was experimentally verified (13). If, however, the carrier is capable of acting symmetrically, with the orientation of the ΔpH determining the direction of calcium transport, then it could be predicted that a reversal of the normal orientation of the ΔpH in "right-side-out" membrane vesicles, from acid outside to acid inside, would result in calcium transport in those vesicles.

We report here that so-called "right-side-out" vesicles do indeed transport calcium inwards in response to an artifically imposed ΔpH.* Burnell et al. (14) have similarly reported that an artifically imposed ΔpH will drive the transport of sulfate in vesicles of Paracoccus denitrificans regardless of the orientation of the vesicles.

However, these results are not necessarily indicative of symmetrical carriers: that interpretation depends on the vesicles having the assumed orientation. There is convincing evidence that vesicles prepared by lysis of intact cells of E. coli with a French press form a single population with membrane having an orientation everted relative to the inner membrane of the intact cell (15–18). Vesicles prepared by lysis of spheroplasts according to the method of Kaback (19) have been shown to be a single population (17, 18, 20–22) and have been assumed to have a right-side-out orientation (20, 21, 23). However, evidence from a number of laboratories shows that some proteins which exist solely on the cytoplasmic surface of the inner membrane in vivo are found on both surfaces in these vesicles

*A preliminary account of a portion of this work was presented at the Annual Meetings of the American Society for Microbiology, Atlantic City, New Jersey, 1975.

(16, 18, 22, 24). This is not consistent with a strictly right-side-out orientation, but suggests that the proteins of the inner membrane become in some way scrambled during lysis of spheroplasts (but not during lysis of intact cells by a French press!). Recent results from our laboratory have suggested that the translocated proteins remain functional components of the larger proton-translocating complexes with which they are associated in vivo (18). In this report we confirm and extend our earlier results with the observation that such vesicles produce protonmotive forces of opposite orientations depending on what energy donor is used during the establishment of the force.

MATERIALS AND METHODS

Preparation of Membrane Vesicles

Escherichia coli K12 strain 7 (25) was grown with shaking at 37°C in a basal salts medium (26) supplemented with 68 mM glycerol as a carbon source. Everted membrane vesicles were prepared by lysis of intact cells with a French press as described previously (27). Production of membrane vesicles by osmotic lysis of spheroplasts was performed according to the method of Kaback (19) as modified by Adler and Rosen (18).

Transport Assays

Calcium transport assays were performed at pH 8.0 with 0.5 mM $^{45}CaCl_2$ as described previously (27). Calcium transport driven by an artificially imposed ΔpH was performed as described previously (13). Flow dialysis was performed using a modification (26) of the apparatus described by Colowick and Womack (29). Conditions for measurement of the uptake of weak acids and bases by flow dialysis were as described by Ramos et al. (9).

Assay of NADH Dehydrogenase Activity

NADH dehydrogenase activity was measured during a calcium transport assay in which calcium uptake was supported by 5 mM NADH, as described above. At various times duplicate 0.02 ml samples were withdrawn and diluted into 1 ml of ice water to terminate the reaction. The absorbance at 340 nm of each sample was measured and plotted against time; the slope of the resulting line was taken as a measure of NADH dehydrogenase activity. When the effect of D-lactate on that activity was measured, 20 mM D-lactate was added to the vesicle suspension 2.5 min prior to the addition of NADH.

Protein Determinations

Protein concentrations were determined by a micromodification of the method of Lowry et al. (30).

Chemicals

$^{45}CaCl_2$ (1.3–1.4 Ci/mmol), sodium [^{3}H]acetate, (686 mCi/mmol) and [^{3}H]methylamine (34 mCi/mmol) were purchased from New England Nuclear Corporation (Boston, Massachusetts). Sodium [^{14}C]thiocyanate (8.0 mCi/mmol) was obtained from ICN Pharmaceuticals (Irvine, California). Valinomycin was supplied by Sigma Chemical Company (St. Louis, Missouri). Nigericin was the generous gift of Dr. L. Frank of this department. All other compounds were reagent grade and obtained from commercial sources.

TABLE I. Sources of Energy for Calcium Transport in Everted Membrane Vesicles

Energy source	Calcium uptake
	nmol/30 min/mg membrane protein
None	26
5 mM NADH	175
20 mM Lithium D-lactate	120
20 mM Sodium succinate	85
0.1 mM PMS + 20 mM potassium ascorbate	65
5 mM ATP + 5 mM $MgCl_2$	81
5 mM ADP + 5 mM $MgCl_2$	12

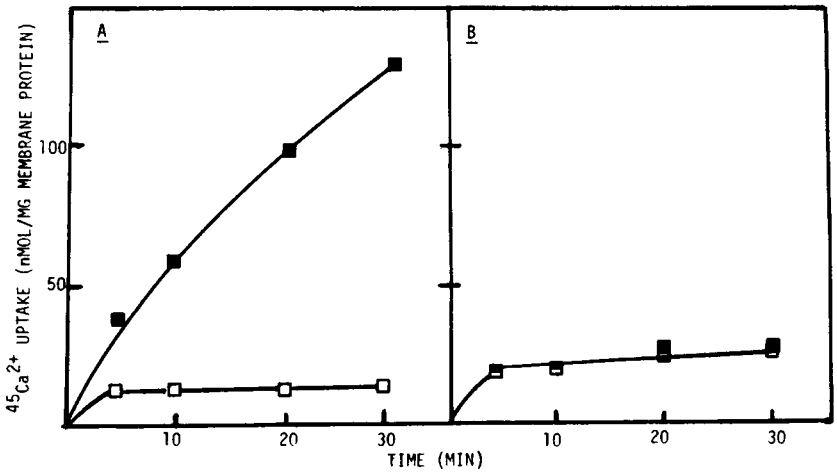

Fig. 1. D-lactate dependent calcium transport in membrane vesicles. Assays were performed as described under Methods. A) everted membrane vesicles prepared by slysis of intact cells with a French press. B) membrane vesicles prepared by osmotic lysis of spheroplasts. Open symbols) +20 mM D-lactate; closed symbols) no exogenous energy source.

RESULTS

Calcium Transport in Everted Membrane Vesicles

As shown in Table I, calcium transport in everted membrane vesicles is energized by the substrates of 3 different dehydrogenases, each of which is a component of the electron transport chain as well as reduced PMS, which couples directly to the cytochrome chain. In addition, ATP also drives calcium uptake (Table I), with energy transduced through the BF_0F_1 (6). We have shown previously that reduced PMS will not energize calcium transport in vesicles prepared by osmotic lysis of the spheroplasts (11), and, as shown in Fig. 1, oxidation of D-lactate likewise does not support calcium transport in such vesicles, although the energy from that oxidation is conserved by transport of calcium in everted vesicles.

Calcium Transport in Membrane Vesicles Driven by an Artificially Imposed ΔpH

Everted vesicles have been shown to transport calcium in response to the imposition of a pH gradient, acid inside (13). Figure 2 demonstrates that vesicles formed by osmotic lysis of spheroplasts respond in a similar manner. Alkalinization of the external medium

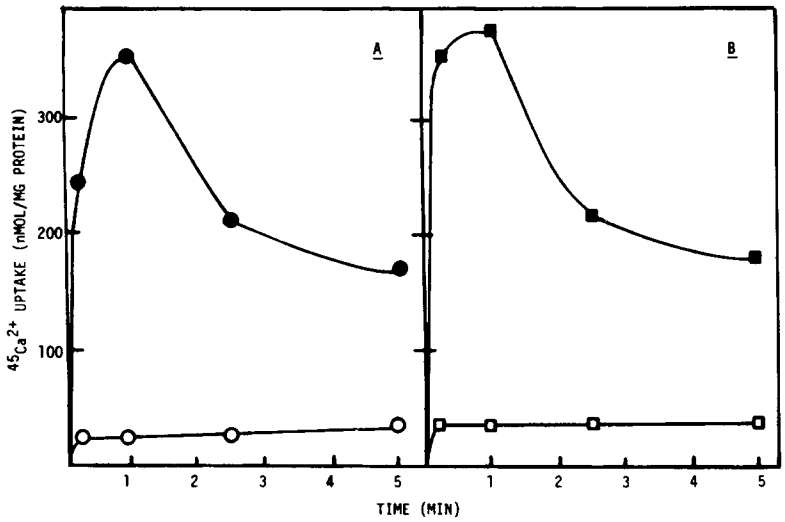

Fig. 2. Calcium transport driven by an artificially imposed ΔpH. Assays were performed as described under Methods. A) everted membrane vesicles; B) vesicles prepared by osmotic lysis of spheroplasts. Open symbols) chemical gradient of protons imposed by a shift in pH from 5.6 to 8.5. Closed symbols) no imposed ΔpH.

produces a transient uptake of calcium which is identical in both types of vesicles. This result would suggest free reversibility of the calcium carrier if the 2 types of vesicles were of the opposite orientation. However, the fact that some inner membrane proteins have been shown to translocate during lysis of spheroplasts (16, 18, 22) obscures the conclusion which could be drawn from this experiment. If a portion of the calcium carriers translocated during the lysis of spheroplasts, then the observed calcium uptake could be due to that fraction of the carriers. It was of interest, therefore, to determine whether other translocated proteins were functional.

Energy-dependent Calcium Transport in Vesicles Prepared by Osmotic Lysis of Spheroplasts

Two enzymes measurable on the external surface of vesicles derived from spheroplasts are NADH dehydrogenase and Mg^{2+}-ATPase. Each is a component of a proton-translocating complex. If the complexes were still functional but now in the opposite direction, it might be possible that a proton gradient, acid inside, would be formed during the oxidation of NADH or hydrolysis of ATP. (Note that the portion of those enzymes located on the inner surface of the membrane would not be active in this in vitro situation, since the membrane of E. coli is relatively impermeable to adenine nucleotides such as ATP or NADH.) Since calcium is transported in the direction of the acid component of the ΔpH, the ability of NADH oxidation of ATP hydrolysis to support calcium uptake could be used as a qualitative measure of the ability to form a ΔpH, acid inside. As shown in Fig. 3, vesicles prepared by osmotic lysis of spheroplasts were capable of coupling energy derived either from oxidation of NADH or hydrolysis of ATP to the uptake of calcium.

D-lactate, which was shown above to be incapable of driving calcium transport in these vesicles (Fig. 1), actually inhibited the utilization of energy derived from NADH oxidation or ATP hydrolysis (Fig. 3). When ATP was used as an energy donor, it could be shown that inhibition of D-lactate oxidation by KCN prevented inhibition by D-lactate

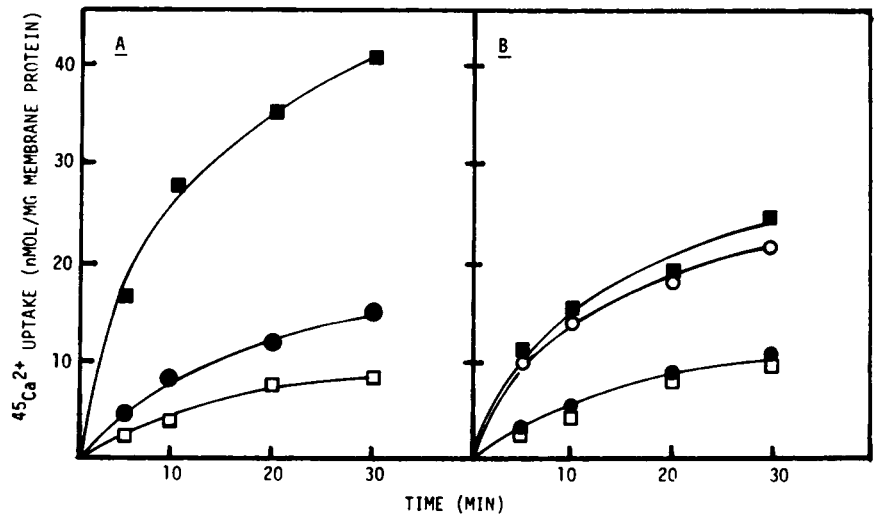

Fig. 3. NADH- and ATP-dependent calcium transport in membrane vesicles prepared by osmotic lysis of spheroplasts. Assays were performed as described under Methods. A) ■—■, +5 mM NADH; ●—●, +5 mM NADH and 20 mM D-lactate; □—□, no exogenous energy source. B) ■—■, +5 mM ATP; ●—●, +5 mM ATP and 20 mM D-lactate, ○—○, +5 mM ATP, 20 mM D-lactate, and 10 mM KCN; □—□, no exogenous energy source. D-lactate was added 2.5 min before the addition of NADH or ATP. KCN was added just prior to D-lactate. In assays utilizing ATP, 5mM $MgCl_2$ was present.

(Fig. 3). The analogous experiment could not be performed with NADH and D-lactate, since KCN inhibits oxidation of both compounds. Compounds such as oxalate and oxamate, which inhibit the D-lactate dehydrogenase, also could not be used, since they form insoluble complexes with calcium. However, the presence of D-lactate during the calcium transport assay did not significantly inhibit the oxidation of NADH (Fig. 4), suggesting that the inhibitory action of D-lactate is the result of an uncoupling of the energy derived from NADH oxidation or ATP hydrolysis, rather than a simple inhibition of the enzymes involved. Similarly, we have shown that D-lactate-dependent uptake of proline by these vesicles is inhibited by the oxidation of NADH or hydrolysis of ATP (18). [It should be pointed out that all of the above results were obtained using a K12 strain of E. coli at pH 8 because of the basic pH optimum of the Mg^{2+}-ATPase (6) and of the calcium transport system (12). Thus, these results may not be directly comparable with those of other laboratories, where proline transport is frequently measured in a ML strain at pH 6.6.]

Measurement of the Orientation of ΔpH and $\Delta\psi$ in Membrane Vesicles

A flow dialysis technique has recently been utilized by Ramos et al.(9) for the measurement of the transmembrane electrochemical gradient of protons. They have concluded that oxidation of D-lactate or reduced PMS results in the generation of a membrane potential, positive outside, which is independent of external pH over a wide range. A pH gradient, acid outside, is established at medium pHs which are neutral to acid, but no pH gradient is established if the external medium is basic.

We have found that everted membrane vesicles accumulate [^{3}H]methylamine or [^{14}C]thiocyanate during the oxidation of NADH, D-lactate, or reduced PMS or during the hydrolysis of ATP (T. Ichikawa and B. P. Rosen, unpublished results), showing that vesicles

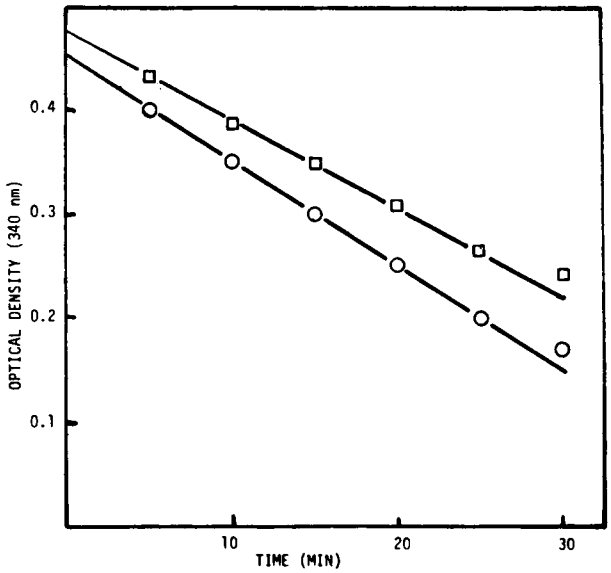

Fig. 4. Effect of D-lactate on NADH oxidation in membrane vesicles prepared by osmotic lysis of spheroplasts. Assays were performed in a manner identical to that utilized for calcium transport, except that nonradioactive $CaCl_2$ was used. At the indicated times samples (0.02 ml) were diluted into 1.0 ml of ice water, and the absorbance at 340 nm was measured. ○—○, +5 mM NADH; □—□, +5 mM NADH and 20 mM D-lactate.

prepared by lysis with a French press form a protonmotive force, acid and positive inside. Vesicles prepared by osmotic lysis of spheroplasts do not accumulate methylamine or thiocyanate during oxidation of D-lactate or reduced PMS, but do accumulate the weak acid acetate when assayed at pH 5.5 (Fig. 5), in confirmation of the results of Ramos et al. (9). At pH 8.0 no acetate uptake was observed, again in confirmation of the results of Ramos et al. (9). On the other hand, methylamine uptake was observed during the oxidation of NADH at pH 8 (Fig. 6) and during the hydrolysis of ATP (data not shown). The experiment shown in Fig. 5 was performed with approximately 3 times as much protein as that shown in Fig. 6. Thus, the pH gradients formed in the 2 experiments are similar in magnitude, but opposite in polarity. Little or no methylamine uptake occurred in the absence of an NADH-regenerating system when that energy source was utilized. Uptake of methylamine was not stimulated by the addition of valinomycin, as would be expected if the uptake of protons by the vesicles were electrogenic (valinomycin would catalyze movement of potassium out of the vesicles, dissipating any membrane potential which would have been formed due to electrogenic uptake of protons). Methylamine uptake was sensitive to nigericin, showing that the formation of a pH gradient had occurred. The reason for the lack of effect of valinomycin is unexplained, especially in light of the fact that NADH oxidation resulted in the uptake of the permeant anion thiocyanate, suggesting the formation of a membrane potential, positive inside. Perhaps the magnitude of the membrane potential is not large enough to create a significant back-pressure on the uptake of protons. It should be pointed out here that these results are being used as a qualitative indication of the orientation of the components of the protonmotive force; future experiments will be concerned with the magnitude of the forces.

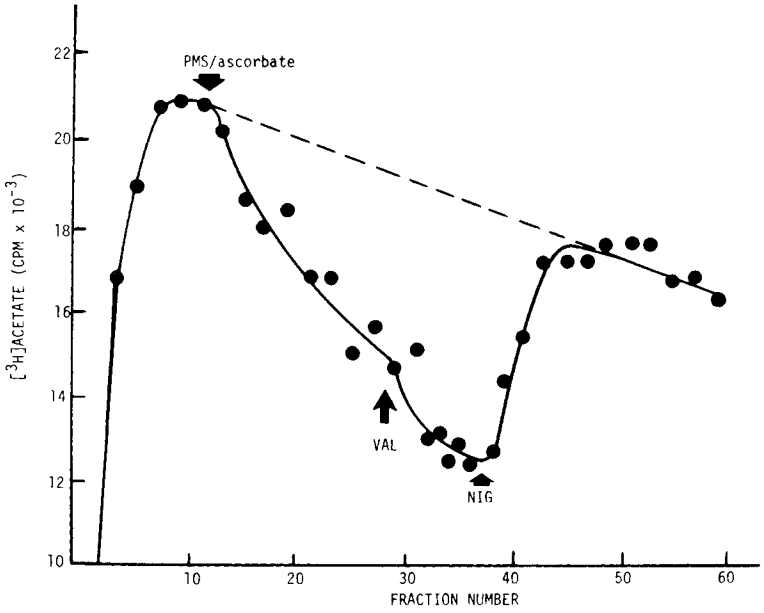

Fig. 5. Flow dialysis determination of the uptake of acetate by membrane vesicles produced by osmotic lysis of spheroplasts. [^{3}H]acetate was added to vesicles (10 mg of protein) at an initial concentration of 28 μM in a total of 1 ml of a buffer consisting of 0.1 M potassium phosphate, pH 5.5, containing 10 mM $MgSO_4$. The same buffer was passed through the lower chamber at a rate of 5 ml per min, with fractions of 1.67 ml collected. Portions (1.0 ml) were mixed with 10 ml of ACS (Amersham/Searle Corp., Arlington Heights, Illinois) and counted in a liquid scintillation counter. At the indicated times were added 0.1 mM PMS and 20 mM potassium ascorbate, pH 5.5, 1.35 μM valinomycin (VAL) and 1.35 μM nigericin (NIG).

DISCUSSION

Two types of E. coli membrane vesicles are currently used for biochemical and physiological studies of energy-linked functions. Everted vesicles, prepared by lysis of intact cells with a French press, have been used for the study of oxidative phosphorylation (3, 15, 33), transport of calcium (6, 11–13, 27), and other questions related to bioenergetics (3, 6, 15, 31–34). These vesicles have been shown to be everted by a number of criteria, including freeze-etch electron microscopy (17) and enzyme localization (16, 18). An everted character is consistent with the observation that substances which are transported outwards by intact cells are transported inwards by these vesicles. For example, primary active transport systems such as the electron transport chain and the BF_0F_1 normally catalyze the extrusion of protons from intact cells; in everted vesicles they catalyze the uptake of protons (15, 32, 35). This leads to the formation of an electrochemical proton gradient which we have found to be acid and positive inside by measurements of the uptake of the weak base methylamine and the lipophilic anion thiocyanate (T. Ichikawa and B.P. Rosen, unpublished results). Calcium, which is normally extruded from intact cells (36), is transported inwards in everted vesicles (6, 11–13, 27). Since the calcium transport system is a secondary system, most likely a proton/calcium antiport (12, 13), the inwardly acid orientation of the protonmotive force would be a prerequisite for inwardly directed calcium transport. Thus, any reaction which generates a proton-

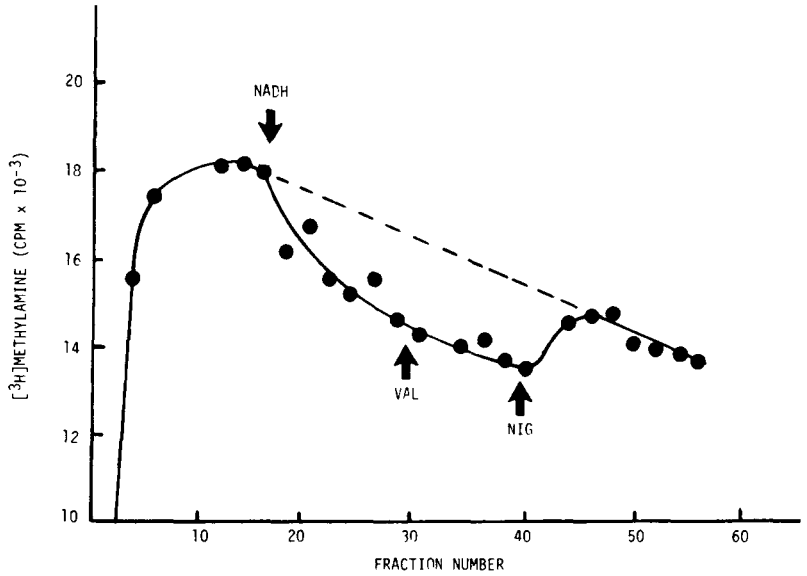

Fig. 6. Flow dialysis determination of the uptake of methylamine in membrane vesicle produced by osmotic lysis of spheroplasts. Conditions were as described in Fig. 5, except that the assay was performed at pH 8.0 with 5 mM NADH as an energy source for the uptake of [^{3}H] methylamine, 0.57 mM, initial concentration. The assay was performed with 3.6 mg of membrane protein.

motive force with that orientation might be expected to drive the accumulation of calcium. In everted vesicles substrates which result in the formation of a pH gradient acid inside include ATP and all tested electron donor substrates of the respiratory chain.

The other type of membrane vesicle derived from E. coli is that described by Kaback (23). Information gained by the use of these vesicles has proven to be enormously valuable for our understanding of the mechanisms of active transport, since this system provided the first in vitro method for the study of vectorial membrane functions. It has been suggested that these vesicles, produced by osmotic lysis of EDTA-lysozyme spheroplasts, on the one hand consist of a homogeneous population and, on the other hand, have an orientation of the membrane which is right-side-out with respect to the inner membrane of the intact cell. The first claim is undoubtedly warranted: freeze-etch electron microscopy (17, 20) and fractionation with antibody directed against membrane proteins (18, 22) have demonstrated only a single class of inner membrane vesicles. Perhaps the most convincing evidence is that each vesicle in the population is capable of transporting the D-lactate analogue 2-hydroxy-3-butenoate (21). However, there has been controversy concerning the second point, the orientation of the membrane of the vesicles. Primarily because the vesicles transport most solutes in the same direction as the intact cell, the claim that the vesicles are right-side-out seemed reasonable. But data concerning the localization of proteins normally associated with only 1 face of the inner membrane in vivo have led to the concept that the vesicles may be mosaics, with certain enzymes translocated from 1 face of the membrane to the other during the lysis event (17, 18, 22). Our data supports that notion, and extends it to include the possibility that translocated enzyme complexes can still be functional. Thus, a complex which establishes an electrochemical gradient of protons acid and positive outside in vivo may produce one of the opposite orientation in the

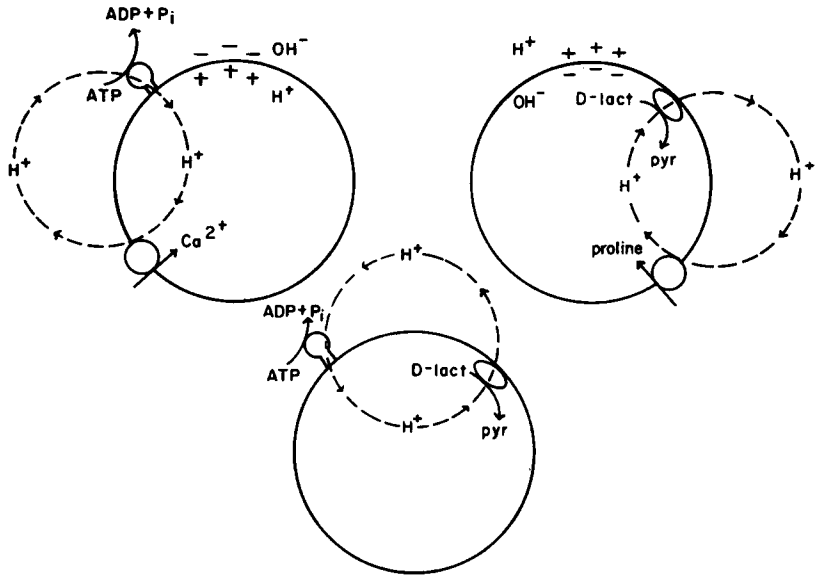

Fig. 7. Diagramatic representation of the proton circulation during the ATP-dependent transport of calcium (top left) and during the D-lactate-dependent transport of proline (top right). During the simultaneous hydrolysis of ATP and oxidation of D-lactate a completed proton circuit is formed resulting in apparent uncoupling (bottom).

in vitro situation following translocation. Again, a secondary solute/H^+ antiport which would catalyze extrusion of the solute in vivo would catalyze the uptake of that same solute in this particular in vitro situation. This appears to be the case with the NADH dehydrogenase and BF_0F_1, which catalyze the formation of protonmotive forces acid and positive inside in both types of membrane vesicles and drive calcium uptake in both types of vesicles. The D-lactate dehydrogenase, on the other hand, does not appear to be translocated during the lysis of spheroplasts (37, 38). Lactate oxidation catalyzes the formation of protonmotive forces of opposite orientation in the 2 types of vesicles, so that D-lactate driven calcium transport occurs in everted vesicles but not in those prepared by the method of Kaback (18).

The simultaneous functioning of 2 primary electrochemical pumps in opposite directions should lead to an apparent uncoupling of either from secondary active transport systems. The calcium transport system, as mentioned above, is linked to a protonmotive force acid and positive inside, while proline transport is linked to a protonmotive force of the opposite orientation. Thus, D-lactate oxidation drives proline but not calcium transport, and NADH oxidation or ATP hydrolysis drives calcium but not proline transport. The oxidation of NADH or hydrolysis of ATP simultaneous with the oxidation of D-lactate allows for neither calcium nor proline uptake, the result of the apparent uncoupling. The proton cycling presumed to occur is illustrated in Fig. 7.

These results in themselves are conceptually unremarkable; they simply reflect the creation of an artifact generated during the preparation of membrane vesicles. However, the fact that the artifact can exist raises certain interesting questions. For example, does the entire NADH oxidase system translocate? If not, how does the translocation of a por-

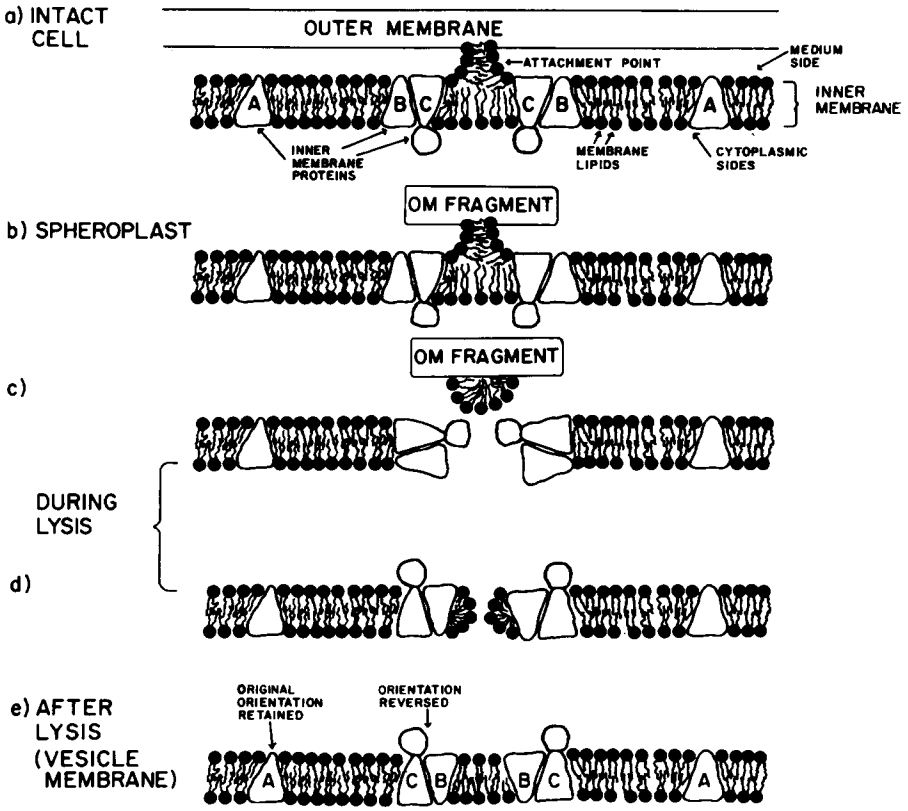

Fig. 8. Model for the translocation of specific inner membrane proteins during the osmotic lysis of spheroplasts. The model is patterned after that described by Alterndorf and Staehelin (17). a) Inner membrane is attached to outer membrane at specific points. Certain proteins, represented by A (an example of which might be the D-lactate dehydrogenase) are located away from the attachment points. Other proteins, represented by B and C (such as the NADH dehydrogenase and BF_0F_1) are localized adjacent to the attachment points. The model does not assume that every molecule of B and C are localized in that region, but that mosaic patches occur more frequently there. b) The outer membrane is degraded during the production of spheroplasts, but fragments still exist which constitute points at which lysis preferentially occurs during an osmotic shock, as in (c). When lysis occurs (c), transient pores are created. Since the inner and outer surfaces of the membrane are topologically identical during the life of the pores, there is no thermodynamic barrier to the lateral diffusion of proteins B and C around the pore as shown in (c) and (d). e) Phospholipids around the sites of the pores reassociate with each other, eliminating the pores, resulting in a reversal of the orientation of a portion of the B and C molecules. Protein A would retain its original orientation, as would the portion of B and C proteins not localized in the vicinity of the transient pore.

tion of the chain allow for the production of a protonmotive force with the opposite orientation? Our knowledge of the arrangement of the components of the electron transport chain and of the proton-translocating ability of the system as a whole is not sufficient at this time to answer this question. While it is obvious that we must know more about the respiratory chain as a whole, it should also be evident that the translocation process itself must be understood. This, then is another and perhaps more important question: how does translocation of enzymes occur; more, how does a specific translocation of certain proteins (such as the BF_0F_1) but not others (such as the D-lactate dehydrogenase) happen? Further-

more, only about half of the BF_0F_1 (and NADH dehydrogenase) activities appear to translocate. What governs this distribution? Related to these questions is the whole problem of how proteins traverse membraneous structures. Altendorf and Staehelin (17) have addressed this question and have postulated a movement of proteins around transient pores created during the lysis of the spheroplasts. Their model, however, did not take into account the apparent specificity of translocation. We have modified their suggestion to include the idea that the transient pores may be created at weak points in the inner membrane, with only specific enzyme complexes being localized near those weak points (Fig. 8). One such location for specific lysis could be points of attachment between the inner and outer membrane, attachments points known to exist in E. coli (39). Flagella are structures which extend through both the inner and outer membrane also (40). A possible structural association between an energy-requiring element such as the flagellar motor and energy-generating systems such as the BF_0F_1 would not be unreasonable from a functional point of view.* Whether such supramolecular structures exist in fact is worthy of further study.

ACKNOWLEDGMENTS

We thank Dr. H. R. Kaback, S. Ramos, and P. Stroobant for their discussions and criticisms. We are especially grateful to Dr. S. Ramos for demonstrating use of flow dialysis as a method for the measurement of the protonmotive force. This work was supported by National Science Foundation Grant PCM-7515896, Public Health Service Grant GM-21648 from the National Institute of General Medical Sciences, and a Basil O'Connor Starter Award from the National Foundation – March of Dimes.

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*DePamphilis and Adler (40) searched for ATPase activity associated with the flagella and with the membrane in the region of the flagellar attachment point. However, their assay conditions (2 mM ATP and 2 mM $MgCl_2$ at pH 7.0) were different from the optima associated with BF_0F_1 (6), so that those results may not be conclusive.

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