Characterization of an Active Transport System for Calcium in Inverted Membrane Vesicles of *Escherichia coli*

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The energy-dependent uptake of calcium by inverted membrane vesicles of *Escherichia coli* was investigated. Methods for preparation and storage of the vesicles were devised to allow for the maximal activity and stability of the calcium transport system. The pH and temperature optima for the reaction were observed to occur at pH 8.0 and 30°, respectively. The effects of various ions on the transport reaction were determined. It was found that the extent of the reaction depended on the presence of phosphate or oxalate. Phosphate was found to enter the vesicles at a rate slower than that of calcium. A \( \text{Ca}^{2+}:\text{P} \) ratio of approximately 1.5 was found, suggesting formation of \( \text{Ca}_4(\text{PO}_4)_2 \). Monovalent cations stimulated calcium uptake, with the order of effectiveness being \( \text{K}^+ > \text{Na}^+ > \text{Li}^+ > \text{NH}_4^+ \). Inhibition was found with certain divalent cations, but these cations also inhibited the electron transport chain. Of the divalent cations examined only \( \text{Mg}^{2+} \) and \( \text{Sr}^{2+} \) inhibited calcium transport without a corresponding inhibition of respiration.

Calcium transport exhibited biphasic kinetics, with a low affinity system and a high affinity system. The low affinity system showed a \( K_m \) of 0.34 mM and a \( V_{max} \) of 85 nmol/min/mg of protein. The kinetic constants of the high affinity system were 4.5 mM and 2 nmol/min/mg of protein.

The energy for calcium transport could be derived from the electron transport chain by oxidation of NADH, d-lactate, and succinate, in order of their effectiveness. Respiration-driven calcium transport was inhibited by inhibitors of the electron transport chain and by uncouplers of oxidative phosphorylation. ATP could also be used to supply energy for calcium transport. The ATP-driven reaction was inhibited by inhibitors of the \( \text{Mg}^{2+} \text{ATPase} \) and by an antiserum prepared against that protein, demonstrating that that enzyme is involved in the utilization of ATP for active transport in inverted vesicles.

Membrane vesicles prepared by the osmotic lysis of spheroplasts of *Escherichia coli* and other bacteria have been used extensively for the study of active transport (1). These vesicles accumulate a variety of amino acids, sugars, and ions when various respiratory substrates (1) or artificial electrical gradients (1, 2) are used to supply energy. The sidedness of lysed spheroplast vesicles is open to question. Some evidence suggests that these vesicles retain the orientation of the original cells (3, 4), while other data indicate that the vesicles are a mixture of right-side-out and inverted membranes after freeze-thawing (4) or even when freshly prepared (5, 6). It is generally agreed that active transport of sugars and amino acids is measured only in right-side-out vesicles (1, 3, 6). The question of sidedness has led to further controversy over the nature of energy coupling for active transport and other energy-requiring membrane processes. Respiratory substrates such as d-lactate and succinate are effective energy donors (1). NADH is much less effective when supplied exogenously but can be used to drive transport when generated internally (7). ATP is ineffective for driving transport when supplied externally (1, 8), and its ability to supply energy when supplied internally (9) has been questioned (8). Although ATP can drive transport in whole cells (10), it has been suggested that an indirect mechanism exists, rather than direct coupling via the \( \text{Mg}^{2+} \text{ATPase} \) (1, 8).

In order to investigate these questions, we have devised a procedure to measure active transport of calcium in vesicles prepared by lysis with a French pressure cell. Such vesicles have been shown to have the opposite orientation as the whole cell, that is, they are inverted vesicles (4, 5). Active transport of calcium has subsequently been reported to occur in inverted vesicles of *Bacillus megaterium* (11). The purpose of this report is to extend the observations described in our earlier paper (12) and to investigate the conditions for preparation and storage of inverted vesicles, and of calcium transport in these vesicles.
MATERIALS AND METHODS

Growth of Cells—Escherichia coli strain 7 (13) cultures were grown in a basal salts medium (14) supplemented with 68 mM glycerol as a carbon source.

Chemicals—CaCl₂ (1.3 to 1.4 Ci/mmol) and carrier-free ³²P, were purchased from New England Nuclear Corp. The uncoupler carbonyl cyanide p-trifluoromethoxyphenylhydrazone was a gift of Dr. P. G. Heylter of the E. I. Dupont de Nemours Co. All other chemicals were reagent grade and purchased from commercial sources.

Preparation of Membrane Vesicles— Cultures were harvested in midexponential phase by centrifugation. The cells were washed once with 0.1 M potassium phosphate buffer, pH 6.6, containing 10 mM EDTA, and resuspended in that buffer to 5 volumes/g of wet cells. The cells were lysed by a single passage through a French pressure cell using a pressure of 4,000 p.s.i. for most experiments. Reproducibility was found to be excellent when a pressure-regulated laboratory press was used (model J5-0598A, American Instrument Co., Silver Springs, Md.). The suspension was centrifuged at 27,000 x g for 10 min, and the pellet was discarded. The supernatant solution was centrifuged for 1 hour at 105,000 x g. The pellet was washed once with Buffer A (10 mM Tris-HCl, pH 7.3/0.14 M KCl/2 mM 2-mercaptoethanol and 10% glycerol (v/v)). The membranes were resuspended to 2 to 4 mg of protein/ml in Buffer A. All steps were performed at 4°. As noted under "Results," various constituents of Buffer A were omitted during the preparation of vesicles to determine the necessity for each component.

Transport Assays—Transport assays were performed by addition of 50 to 200 µg of membrane protein to 1 ml of a buffer consisting of 10 mM Tris-HCl, 10 mM potassium phosphate, and 0.15 M KCl, adjusted to pH 8.0. After 15 min at 23° an energy source was added, and the reaction was started by addition of CaCl₂. Samples at various times were filtered through nitrocellulose filters (0.45 µm pore size), followed by a wash with 5 ml of 10 mM Tris-HCl, pH 8.0, containing 0.14 M KCl. The filters were dried and counted in a liquid scintillation counter. With filters obtained from Millipore Corp., the assay was less reproducible and gave low specific activities. As noted under "Results," various modifications for both the extent of uptake and the initial rate. At 23 and 37° the extent of uptake is approximately 60 to 70% of that found at 30°. Again, it is difficult to be certain how much temperature affects calcium uptake per se as opposed to the utilization of the energy source.

Effect of Lysis Pressure on Calcium Transport in Inverted Vesicles—A major difficulty in the preparation of inverted vesicles is the low yield of membranes obtained using the French press at 4,000 p.s.i. As shown in Table I, the yield of membranes per g of wet cells could be increased linearly with increasing pressure up to a maximum of 14,000 p.s.i. The constant specific activity of Mg⁺⁺-ATPase suggests that inversion of the vesicles has occurred to the same extent at 4,000 as at 14,000 p.s.i. since an increase in the proportion of inverted to right-side-out vesicles would lead to an increase in accessible Mg⁺⁺-ATPase activity (5). The pressure of lysis did not affect the initial rate of calcium uptake in the resulting vesicles, but the extent of uptake decreased with increasing pressure (Table I). A possible explanation for this effect is a decrease in vesicle size with increasing pressure.

Conditions for Storage of Inverted Vesicles—Vesicles prepared from cells lysed in Buffer A lost transport activity after about 8 hours (12). Various methods of storage were tried in an attempt to increase the stability of the vesicles. The most satisfactory method found was dilution of the vesicles with an equal volume of glycerol, followed by storage in liquid nitrogen or at -70°. Under these conditions the vesicles retained nearly full activity over a period of at least 3 months. Vesicles diluted with glycerol could be stored up to a week at -25° with little loss of activity; at 4° substantial losses occurred after several days.

Effect of pH and Temperature on Calcium Transport—Vesicles prepared from cells lysed in Buffer A and assayed without added phosphate were shown to have a pH optimum for calcium transport at 8.5 (12). Using the conditions described under "Materials and Methods," a pH optimum of approximately 8.0 was obtained. The pH optimum may also depend on the energy source since the rate of calcium transport would depend on the rate of utilization of the energy source.

Effect of Temperature on the extent of transport was investigated. The optimum temperature is approximately 30° for both the extent of uptake and the initial rate. At 23 and 37° the extent of uptake is approximately 60 to 70% of that found at 30°. Again, it is difficult to be certain how much temperature affects calcium uptake per se as opposed to the utilization of the energy source.

Effect of Anions on Calcium Transport—We have previously reported that uptake of calcium is almost unmeasurable in the absence of phosphate (19). The effect of phosphate concentration was investigated. NADH-driven calcium uptake increased linearly with increasing phosphate concentration up to 10 mM phosphate, followed by a slower increase in activity up to 0.1 M phosphate. Energy-independent calcium uptake increased linearly up to 0.1 M phosphate. Since the optimal ratio of energy-dependent to energy-independent uptake occurred at 10 mM phosphate, that concentration was used in most assays.

Phosphate has been found to stimulate calcium transport in mitochondria (18) and sarcoplasmic reticulum (19). This effect is due in large part to the formation of insoluble calcium phosphate compounds (18). Calcium phosphate precipitation may also occur within inverted vesicles of Escherichia coli, as shown by the fact that a 10-fold excess of nonradioactive calcium does not cause efflux of the accumulated radioactivity (Fig. 1). About 15% of the radioactivity comes out after addition of FCCP ¹ (Fig. 1), suggesting that there is a free pool of calcium within the vesicles in a concentration greater than that of the external medium. It is not clear, however, why an excess of nonradioactive calcium did not also cause a small amount of efflux.

¹The abbreviations used are: FCCP, carbonyl cyanide p-trifluoromethoxyphenylhydrazone; CCCP, carbonyl cyanide m-chlorophenylhydrazone; p-CMB, p-chloromercuribenzoate.
What is the role of phosphate in the transport of calcium? Several possibilities exist: (a) activation of the calcium carrier; (b) co-transport of the 2 ions by the same carrier; (c) sequestration of calcium by formation of insoluble salts of phosphate inside of the vesicles; or (d) action as a permeant anion to balance the accumulation of positive charges. Our data neither advances or eliminates (a) as a possibility. It is unlikely, however, that co-transport of calcium and phosphate occurs by the same carrier. Vesicles incubated with 10 mM potassium phosphate accumulated essentially no $^{32}$P. Neither the addition of 1 mM CaCl$_2$ nor 5 mM NADH caused any phosphate accumulation. When vesicles were incubated with both CaCl$_2$ and phosphate at the above concentrations, subsequent addition of 5 mM NADH caused an immediate and rapid accumulation of $^{32}$P. Addition of CaCl$_2$ to vesicles incubated with phosphate and NADH, all at the above concentrations, gave the same effect. However, the rate of phosphate accumulation was slower than that of calcium accumulation. In experiments designed to measure the simultaneous accumulation of $^4$Ca and $^{32}$P, vesicles were incubated in the presence of both isotopes at the above concentrations for 5 min, followed by the addition of NADH at 5 mM. Samples then were filtered at various times after the addition of NADH, and the ratio of accumulated $^4$Ca to $^{32}$P was calculated. At times less than 5 min after the addition of NADH, the Ca$^{2+}$:P ratio varied between 2 and 3, indicating that the rate of calcium uptake was greater than that of phosphate. At later times the rate of uptake of both ions decreased, but the rate of calcium accumulation decreased faster than that of phosphate. The Ca$^{2+}$:P ratio from eight separate experiments was 1.70 $\pm$ 0.05 after 15 min and 1.42 $\pm$ 0.07 after 30 min. After 60 min the ratio was essentially the same as that at 30 min. Decreasing the concentration of phosphate below 10 mM increased the time necessary to attain a constant Ca$^{2+}$:P ratio, but the final ratio was between 1.4 and 1.5. These results suggest that calcium is actively accumulated, with a subsequent slower and passive influx of phosphate. Hirata et al. (2) have similarly found that phosphate slowly leaks out of right-side-out vesicles of E. coli. If the two anions were transported by the same system, the rate of transport of both would be the same. Since they are not, co-transport appears unlikely. Since the ratio of the two ions becomes constant and since efflux of calcium does not occur, the sequestration of calcium as a phosphate salt is more probable. A Ca$^{2+}$:P ratio of 1.4 to 1.5 is suggestive of deposition of Ca$_3$(PO$_4$)$_2$, similar to the value found for calcium phosphate accumulation in inverted vesicles derived from mitochondria (21).

In mitochondrial and sarcoplasmic reticulum other anions increase the accumulation of calcium (18, 19). When the effect of other anions was examined, it was found that oxalate could substitute for phosphate but that arsenate, acetate, and citrate could not (Table II). That oxalate was more effective than phosphate again suggests that the mechanism of anion stimulation is via the formation of insoluble salts rather than by a co-transport mechanism. Since oxalate could not be used in assays containing 0.5 mM calcium because of the low solubility of calcium oxalate, phosphate was used in most other experiments.

The above experiments were designed to measure the effect of anions on the accumulation of calcium within the vesicles as opposed to an effect of the anions on the initial rate of calcium transport. Phosphate has been found to greatly stimulate the initial rate of calcium uptake. If calcium uptake were electrogenic, then in the absence of a permeant anion an electrical potential would form as calcium entered the vesicles. This potential would inhibit the further entry of calcium. Our preliminary results suggest that calcium transport is electrogenic and that phosphate participates by acting as a permeant anion, thus stimulating the initial rate of calcium transport. The results of these studies will be presented in a subsequent communication.

**Effect of Cations on Calcium Transport**—The specificity of the calcium transport system was determined by the effect of nonradioactive divalent cations on calcium uptake. The experiments were performed with 5 mM NADH as an energy source, 0.1 mM CaCl$_2$ as substrate, and 0.1 mM nonradioactive divalent cations. Higher concentrations of most of the divalent cations resulted in the formation of precipitates with phosphate. Under these conditions Mg$^{2+}$, Sr$^{2+}$, Ba$^{2+}$, Co$^{2+}$, Zn$^{2+}$, and Sn$^{2+}$ had no effect on calcium transport. The chloride salt of each cation was used, except for Zn$^{2+}$, Cu$^{2+}$, and Fe$^{2+}$, where the sulfate salt was used. MgCl$_2$ and SrCl$_2$ gave 40 to 60% inhibition at 1 mM. Cd$^{2+}$, Hg$^{2+}$, and Cu$^{2+}$ inhibited calcium uptake about 60 to 90%, but those ions had a similar effect on NADH oxidation, so it is reasonable to conclude that the effects were not due to competition with calcium. Fe$^{2+}$ and Mn$^{2+}$ stimulated calcium uptake by 90 and 40%, respectively. Fe$^{2+}$ also stimulated NADH oxidation. The effect of Mn$^{2+}$ is unexplained. LaCl$_3$ and ruthenium red were also without effect on calcium uptake when used at either 1 or 10 $\mu$M.

The effect of monovalent cations was also examined. Potassium, sodium, lithium, and ammonium each stimulated calcium uptake, with maximum stimulation occurring at between 0.1 and 0.2 mM monovalent cation. Higher concentrations of monovalent cation were inhibitory. In these experiments KCl was omitted from Buffer A and from the transport assay buffer, and 10 mM sodium phosphate buffer was used in the transport assays. No attempt was made to keep the ionic strength constant, so that the effects of the monovalent cations may be due in part to variations in this parameter. The effect of potassium, however, must be more specific, since it is consider-
**Experiment II**. The concentration of "CaCl₂, was 1 mM in Experiment I, addition of %CaCl₂. Energy was supplied in the form of 5 mM NADH, with various anions and incubated for 15 min at 23° prior to the vesicles were diluted into the transport assay buffer supplemented with the concentration of anions was 10 mM in Experiment I and 5 mM in Experiment II, and 0.1 mM in Experiment II.

The concentration of anions was 10 mM in Experiment I and 5 mM in Experiment II.

...73% when ATP was used (Table III). In either case 2-mercaptoethanol stimulated calcium uptake either in the presence or absence of p-CMB. In these experiments, 2-mercaptoethanol was omitted from Buffer A, which in itself decreased the stability of the vesicles. Thus, there may be an important sulfhydryl group (or groups) in the transport carrier...

**Effect of sulfhydryl reagents on calcium transport**

<table>
<thead>
<tr>
<th>Energy source and addition</th>
<th>Calcium uptake</th>
<th>% Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>NADH</td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>24</td>
<td>100</td>
</tr>
<tr>
<td>p-CMB</td>
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<td>29</td>
</tr>
<tr>
<td>2-mercaptopoethanol</td>
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</tr>
<tr>
<td>p-CMB + 2-mercaptopoethanol</td>
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</tr>
<tr>
<td>ATP</td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>13</td>
<td>100</td>
</tr>
<tr>
<td>p-CMB</td>
<td>2</td>
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</tr>
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</tr>
<tr>
<td>p-CMB + 2-mercaptopoethanol</td>
<td>16</td>
<td>123</td>
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</table>

Uptake driven by NADH is almost completely sensitive to cyanide, while ATP-driven uptake is insensitive to cyanide (Fig. 4). In fact, increasing concentrations of cyanide stimulate the ATP-driven uptake as much as 70%. We have also found ATP-dependent quenching of quinacrine fluorescence is slightly stimulated by KCN. The effect of cyanide on these ATP-dependent reactions is unexplained but an inhibition of reverse electron flow through the oxidized carriers of the electron transport chain is a possibility. ATP-driven uptake is sensitive to azide (data not shown) and N,N'-dicyclohexylcarbodiimide (12). Since these compounds are inhibitors of the Mg²⁺ATPase, it seems likely that the energy derived from ATP is transduced through the Mg²⁺ATPase. A more conclusive demonstration of the involvement of that enzyme is the effect of rabbit antiserum against purified Mg²⁺ATPase on calcium uptake (Fig. 5). ATP-driven uptake was completely inhibited by the Mg²⁺ATPase antiserum, but only partially inhibited by control serum from a nonimmunized rabbit. The inhibition by control serum may be nonspecific, since NADH-driven uptake...
FIG. 3. Effect of energy sources and CCCP on calcium transport in vesicles. Vesicles were prepared and assayed for calcium uptake as described under "Materials and Methods" using 0.5 mM \( \text{Ca}^{2+} \). Additions: \( \bullet \), 5 mM NADH; \( \square \), 20 mM L-lactate; \( \bigcirc \), 5 mM ATP + 5 mM MgCl\(_2\); \( \odot \), 20 mM sodium succinate; solid lines, no CCCP; ---, 20 \( \mu \)M CCCP.

Fig. 4. Effect of cyanide on NADH-driven and ATP-driven calcium uptake. Vesicles were prepared and assayed for calcium uptake for 10 min as described under "Materials and Methods" using 0.5 mM \( \text{Ca}^{2+} \). Cyanide was added to the transport assay buffer at the indicated concentrations. The energy source was either 5 mM ATP + 5 mM MgCl\(_2\) (upper curve) or 5 mM NADH (lower curve). The values for uninhibited vesicles were 15 nmol/mg of protein for ATP-driven transport and 48 nmol/mg of protein for NADH-driven uptake.

is inhibited to the same degree by either control or anti-Mg\(^{2+}\)ATPase sera.

**DISCUSSION**

Silver and co-workers (20) reported that calcium may be actively transported out of whole cells of *E. coli*. Active transport of calcium in inverted vesicles may occur by the same system since a mutant lacking calcium transport in inverted vesicles also lacks calcium efflux in whole cells. This mutant, however, is not sensitive to growth in the presence of high concentrations of calcium. Another mutant which is sensitive to growth in the presence of calcium does not exhibit reduced uptake of calcium in inverted vesicles. Thus, the physiological role of calcium transport in *E. coli* is unclear.

The effect of anions on calcium transport raises the question of whether active transport is occurring in these vesicles. The lack of efflux or exchange in the presence of phosphate suggests that calcium is being precipitated within the vesicles, as is the case in the mitochondrial calcium system (18, 21). The most likely reason for the low levels of accumulation in the absence of phosphate is that an inverted vesicle is quite small relative to a whole cell or a right-side-out vesicle. Altendorf and Staehelin (4) examined similar vesicles by freeze-cleave electron microscopy and concluded that the diameters of these vesicles were between 0.04 to 0.1 \( \mu \)m, compared to a diameter of 1 \( \mu \)m for right-side-out vesicles. If one cell were converted to one spherical right-side-out vesicle with a diameter of 1 \( \mu \)m or to \( 10^3 \) to \( 10^4 \) spherical inverted vesicles with diameters of 0.04 to 0.1 \( \mu \)m, then the ratio of the internal volume of total population of inverted vesicles to the right-side-out vesicle can be shown to be 0.04 to 0.1. That is, inverted vesicles from a gram of wet cells will have only 4 to 10% of the total volume of right-side-out vesicles from the same amount of cells. This calculation is not meant to be used to derive absolute numbers, but to point out a possible reason for the inability to see accumulation of calcium without some sort of trapping mechanism. If precipitation of calcium phosphate is occurring, then the concentrations of calcium and phosphate must have exceeded the solubility product of the complex within the vesicles but not outside, again suggesting a higher concentration of free calcium within the vesicles compared to the medium. Other possibilities cannot be excluded, such as a change in the chemical activity of calcium within the vesicle.

Regardless of whether or not active accumulation is occurring,
active transport across the membrane does occur since the process is dependent on an energy source. With a V_max of 85 nmol/min/mg of protein, the calcium transport system is one of the most active in E. coli.

The calcium transport system of E. coli is quite different from the calcium systems of other organisms. It differs from the mitochondrial system in that it transports calcium in the opposite direction, although it might be argued that both transport calcium out of the cytosol of the cell. It also differs in specificity. The mitochondrial system is competitively inhibited by lanthanides and ruthenium red (22, 23) while the E. coli system is not. The two systems are similar in terms of which compounds can supply energy for calcium transport (18). The E. coli system differs from the calcium transport system of sarcoplasmic reticulum in its energy coupling. Only phosphate bond energy drives the uptake of calcium into the sarcoplasmic reticulum (19), while both respiratory energy and ATP can drive calcium transport in inverted vesicles of E. coli and in mitochondria (18).

Of interest is the mechanism by which energy is transduced in biological membranes. Energy derived from the electron transport chain has been shown to drive active transport both in whole cells and right-side-out vesicles (1). In right-side-out vesicles the D-lactate dehydrogenase has been postulated to have a special role in the coupling of respiratory energy to active transport in E. coli (1). NADH has been shown to be a poor energy source, although Futai (17) recently demonstrated that NADH generated within right-side-out vesicles is a better energy source than exogenously supplied NADH even though 50% of the NADH dehydrogenase activity can be found on the outer surface of right-side-out vesicles. In inverted vesicles NADH is consistently the most effective energy source for calcium transport even though it is added from the outside. These facts suggest that NADH must be accessible to its dehydrogenase from the inside of the membrane in right-side-out vesicles or from the outside in inverted vesicles. A similar proposition can be made for the role of ATP in supplying energy for active transport. Berger (10) has shown indirectly that ATP can supply energy for transport in whole cells of E. coli, with some systems receiving the energy via the Mg^2+-ATPase and others by an unknown mechanism. In right-side-out vesicles 50% of the Mg^2+-ATPase activity is exposed on the outer surface, yet there is only one report of ATP directly driving active transport (9). Moreover, in that report, the ATP must reach the inside of the vesicles to be effective. However, the conclusion from that experiment (9) has been questioned for several reasons (1, 8). It may be that NADH dehydrogenase and Mg^2+-ATPase cannot effectively couple to energy-requiring reactions when they are on the outer surface of right-side-out vesicles. ATP, which is impermeant, would not be able to reach the Mg^2+-ATPase on the inner surface, and would, therefore, not drive energy-requiring reactions. But in inverted vesicles ATP can directly drive the uptake of calcium via the Mg^2+-ATPase, as shown under "Results."

We believe that the ability to utilize ATP for transport is not unique to the calcium transport system. Aside from the direction of transport, the characteristics of the calcium transport system are little different from those of many sugars, amino acids and ions. Since Mg^2+-ATPase mutants of E. coli are unable to utilize glycerolysin alone for transport of many sugars and amino acids, while their parental strains are able to do so (10), it seems to us an unlikely proposition that ATP cannot be used to drive those systems in vesicles or that ATP utilization occurs by an indirect mechanism, as has been suggested (1, 8). The inability to demonstrate that effect (8) cannot be used as evidence for its nonexistence.

The discovery of a transport system for calcium in inverted vesicles provides a new method for the investigation of the mechanism of energy transduction in biological membranes. Besides demonstrating the effective utilization of NADH and ATP as energy donors for active transport, the calcium transport system offers a convenient way of directly investigating the function of the inner surface of the cytoplasmic membrane of E. coli, through such experiments as the removal and reconstitution of the Mg^2+-ATPase (15, 24).

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