Calcium Transport Driven by a Proton Gradient in Inverted Membrane Vesicles of *Escherichia coli*

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Calcium transport into inverted vesicles of *Escherichia coli* was observed to occur without an exogenous energy source when an artificial proton gradient was used. The orientation of the proton gradient was acid inside and alkaline outside. Either phosphate or oxalate was necessary for transport, as was found for respiratory driven or ATP driven uptake (Tsuchiya, T., and Rosen, B. P. (1975) J. Biol. Chem. 250, 7687-7692). Phosphate accumulation was found to occur in conjunction with calcium accumulation. Calcium transport driven by an artificial proton gradient was stimulated by dicyclohexylcarbodiimide, an inhibitor of the Mg\(^{2+}\)ATPase (EC 3.6.1.3). Valinomycin, which catalyzes electrogenic potassium movement, stimulated calcium accumulation, while nigericin, which catalyzes electroneutral exchange of potassium and protons, inhibited both artificial proton gradient-driven transport and respiratory-driven transport. Other properties of the proton gradient-driven system and the previously reported energy-linked calcium transport system are similar, indicating that calcium is transported by the same carrier whether energy is supplied through an artificial proton gradient or an energized membrane state. These results suggest the existence of a calcium/proton antiport.

An important aspect of the active transport of solutes across biological membranes is the mechanism of energy coupling. However, it is still not clear how energy is transduced from metabolism to carrier proteins. Of the several models which have been proposed to explain energy coupling (1, 2), the chemiosmotic hypothesis of Mitchell (2, 3) appears to us the most likely. According to this model, the driving force for active transport is the electrochemical gradient of protons established either by the electron transport chain during the oxidation of substrates or by the energy-transducing Mg\(^{2+}\)ATPase (EC 3.6.1.3) of the oxidative phosphorylation pathway during the hydrolysis of ATP. Under anaerobic conditions specialized electron transport chains, using nitrate as the terminal electron acceptor, may fill this role (4). Evidence supporting this hypothesis has come from numerous laboratories. Harold and co-workers (5, 6) reported that proton conductors inhibited the transport of sugars, amino acids, and ions. The results of West (7) suggested the existence of a proton/galactoside symport, and this concept has been supported by others (8-10). An electrochemical gradient of protons implies a membrane potential. Hirata et al. (11, 12) showed that a membrane potential could drive active transport in membrane vesicles of *Escherichia coli*. Kashket and Wilson (9) showed directly that a proton gradient could drive the uptake of galactosides in whole cells of *Streptococcus lactis*. Further, there was a linear correlation between the protonmotive force and the logarithm of the concentration ratio of sugar (9, 13).

We have previously reported that inverted vesicles of *Escherichia coli* actively transport calcium with energy derived from the oxidation of respiratory substrates or from the hydrolysis of ATP by the Mg\(^{2+}\)ATPase (14, 15). Moreover, calcium transport was inhibited by proton conductors. The experimental findings of Kashket and Wilson (9) suggested a more direct test of Mitchell's hypothesis by determining if an artificial proton gradient could drive the transport of calcium in inverted membrane vesicles of *E. coli*.

Vesicles made by the French press method have been shown to be predominantly inverted, that is, the surface of the cytoplasmic membrane which faces the cytosol in whole cells faces the outer medium in such vesicles (16, 17). In intact bacterial cells the direction of proton translocation catalyzed by the electron transport chain is outward (18, 19), that is, acid outside and alkaline inside. Presumably, ATP hydrolysis by the Mg\(^{2+}\)ATPase also causes proton extrusion from bacterial cells (18). Vesicles of *E. coli* prepared by osmotic lysis of spheroplasts, likewise establish a gradient which is acid outside (20), and such vesicles are believed to be predominantly right side out, that is, they retain the same orientation of the cytoplasmic membrane as found in whole cells (21). In contrast, an inward flow of protons was found in vesicles prepared by lysis of cells in a French press during NADH oxidation or ATP hydrolysis (99). These results suggest that an artificial proton gradient alkaline outside and acid inside should be used with inverted vesicles in order to drive calcium uptake. We now report that the establishment of such a proton...
gradient can directly cause the uptake of calcium in inverted vesicles of E. coli. The uptake is inhibited by ionophores which dissipate the proton gradient but not by ionophores which dissipate only the membrane potential. These observations suggest that calcium transport occurs via a calcium/proton antport.

MATERIALS AND METHODS

Growth of Cells—Escherichia coli strain 7 (23) cultures were grown to midexponential phase in a basal salts medium (24) supplemented with 10 mM glycerol as a carbon source.

Chemicals—[^CaCl] (1.3 to 1.4 Ci/mmol) and carrier-free ^32P, were purchased from New England Nuclear Corp. Valinomycin was obtained from Sigma Chemical Co. Nigericin was the gift of Dr. L. H. Frank of this department. All other compounds were reagent grade and purchased from commercial sources.

Preparation of Inverted Membrane Vesicles—Membrane vesicles were prepared as described previously (15) by lysis of cell suspension with a French press in 0.1 M potassium phosphate buffer, pH 6.6, containing 10 mM EDTA. Depending on the experiment, the membranes were washed and resuspended in various solutions. For the experiments described in Fig. 1, the vesicles were washed once with and resuspended in an unbuffered solution consisting of 0.14 M KCl, 2 mM 2-mercaptoethanol, and 10% glycerol (v/v). For the experiments described in Figs. 2 to 5A, the vesicles were washed once and resuspended in the same solution, except that 1 mM Tris-maleate was added to adjust the pH. The pH of the Tris-maleate buffer was 5.6 except in the experiments described in Fig. 2A, where the pH was varied as described in the text. For the experiments described in Fig. 5B, the previously described method (15) was used. All vesicle suspensions were 1 to 3 mg/ml of protein.

Transport Assays—Transport assays were performed as described previously (15) in a buffer consisting of 10 mM Tris-Cl, 10 mM potassium phosphate, and 0.14 M KCl, with modifications depending on the assay. The experiments described in Fig. 5B were performed as described previously (15). In assays using an artificial proton gradient, the pH of the assay and wash buffer was 8.5 instead of 8.0, except in the experiments described in Fig. 2A, where the pH was varied as described in the text. In the experiments described in Figs. 1 to 5A, the reaction was initiated by a 10- to 20-fold dilution of the vesicle suspension into transport assay buffer containing ^4CaCl, rather than by addition of ^4CaCl,. In control experiments vesicles were added to the transport assay buffer 10 min prior to the initiation of the assay by the addition of ^4CaCl,. The concentration of ^4CaCl, was 0.5 mM in all assays, except that 0.05 mM ^4CaCl, was used when oxalate was present in the assay. The concentration of vesicles in the assays was 0.1 to 0.24 mg/ml of membrane protein.

Protein Assays—Protein assays were performed according to a micro modification of the method of Lowry et al. (25).

RESULTS

Calcium Transport Driven by Proton Gradient—Vesicles were washed and resuspended in an unbuffered medium as described under "Materials and Methods" and then transferred to buffered media at various pH values in the presence of ^4CaCl,. As shown in Fig. 1, calcium transport occurred only when the outer pH was alkaline (pH 8.5). Neutral (pH 7.3) and acidic (pH 6.5) buffers were not effective. In control experiment vesicles were allowed to remain in alkaline buffer for 10 min prior to the addition of calcium in order to allow the pH gradient to dissipate, and no calcium uptake was observed. Thus, inward flow of calcium was found concomitant with the establishment of a proton gradient. Presumably there would be an outward flow of protons, although this is not directly measured.

When the magnitude of the pH gradient was decreased, either by keeping the outer pH at 8.5 and raising the inner pH (Fig. 2A) or by keeping the inner pH at 6.6 and lowering the outer pH (Fig. 2B), the magnitude of calcium uptake was likewise decreased. As the outer pH was increased, the control level of calcium radioactivity associated with the vesicles increased, perhaps because of the lower solubility product of calcium phosphate at high pH. For that reason our standard assay utilized pH 8.5 for the outer pH. It is not clear why little accumulation was observed at pH 8.0, although the formation of a transient calcium phosphate precipitate may be necessary to observe the reaction. Although the level of calcium within the vesicles decreases with time as the pH gradient dissipates, we cannot be certain of the concentration of free calcium ion within the vesicles and consequently cannot be certain that accumulation against a gradient has occurred. However, the fact that the level of ^4Ca+ inside of the vesicles just after the change in pH is higher than that found after 30 min in the presence of oxalate (15) suggests that concentration against a gradient has occurred.

Anion Requirement for Proton Gradient-driven Calcium Transport—A requirement for phosphate or oxalate in the transport of calcium has been found in mitochondria (26), sarcoplasmic reticulum (27), and inverted vesicles of E. coli (14, 15). This requirement has been interpreted as a mechanism for trapping calcium inside as an insoluble precipitate or, by action as a counterion. As shown in Fig. 3, proton gradient-driven calcium uptake also requires phosphate or oxalate. With oxalate as the anion no efflux of calcium occurred after the pH gradient dissipates (Fig. 3B), while efflux was observed when phosphate was used (Fig. 3A). However, precipitation with phosphate must occur to some extent since the level of calcium within the vesicles never returns to the control level. As stated above, calcium may form a transient complex with phosphate even at early times. Since the solubility constant of calcium oxalate is much lower than that of calcium phosphate, the external concentration of calcium had to be reduced in assays using oxalate, accounting for the difference in the absolute level of calcium transport between assays with phosphate and those with oxalate. Neither acetate nor arsenate were effective anions.

We have previously reported the accumulation of ^4P, during the accumulation of calcium (15). Since phosphate can be replaced by oxalate, it is unlikely that the transport system is a calcium/phosphate symport. Phosphate was also accumulated concomitant with calcium accumulation in the proton gradi-
Proton-coupled Calcium Transport

FIG. 2. Effect of inner and outer pH on calcium transport driven by an artificial pH gradient. Vesicles and transport assays were performed as described under "Methods." Closed figures, reactions were initiated by dilution of vesicles into assay buffer containing \(^{45}\)CaCl\(_2\). Open figures, vesicles were added to the assay solution 10 min prior to the addition of \(^{45}\)CaCl\(_2\). A, vesicles prepared in buffers of the indicated pH values were diluted into assay buffer at pH 8.5 at a final concentration of 0.11 to 0.14 mg/ml of membrane protein; B, vesicles prepared in buffer at pH 5.6 were diluted into assay buffers of the indicated pH values at a final concentration of 0.2 mg/ml of membrane protein. The values used were the maximal values, taken at 15 s after initiating each reaction.

FIG. 3. Effect of phosphate and oxalate on calcium transport driven by an artificial proton gradient. Vesicles were prepared and assayed for transport as described under "Methods." Carrier-free \(^{32}\)P\(_i\) used in the transport assay was diluted to 10 mM final concentration with unlabeled potassium phosphate, pH 8.5. O—O, \(^{45}\)Ca\(^{2+}\) uptake; ■—■, \(^{32}\)P\(_i\) uptake; O—O, \(^{45}\)Ca\(^{2+}/^{32}\)P\(_i\) ratio. Vesicles were used at 0.23 mg/ml of membrane protein, final concentration.

Effect of Compounds Which Interfere with Energy Transduction on Proton Gradient-driven Calcium Transport—Inhibitors of the respiratory chain and of the Mg\(^{2+}\) ATPase and uncouplers of oxidative phosphorylation are inhibitors of many active transport systems, including the calcium transport system of E. coli (14, 15). The Mg\(^{2+}\) ATPase inhibitor DCCD\(^1\) has been shown to decrease the rate of proton flow through the membrane (10, 28). In mutants lacking the Mg\(^{2+}\)ATPase or in vesicles from which that enzyme was removed, DCCD stimulated energy-linked processes (10, 28-33), presumably by decreasing the rate of proton leakage. Likewise, DCCD stimulated proton gradient-driven calcium uptake in two ways: by increasing the amount of calcium accumulated at the peak by 10 to 30% and by decreasing the rate of calcium efflux at times after the peak (data not shown).

Nigericin, which catalyzes an electroneutral exchange of protons and potassium (2) and should thus dissipate the proton gradient, inhibited calcium transport whether driven by an artificial proton gradient (Fig. 5A) or by respiration (Fig. 5B). Valinomycin, which catalyzes an electrogenic movement of potassium (2), had no effect on lactate-driven calcium uptake (Fig. 5B) and slightly stimulated proton gradient-driven calcium uptake (Fig. 5A).

Respiratory-driven calcium uptake has been found to be inhibited by Hg\(^{2+}\) and Cu\(^{2+}\), and stimulated by Fe\(^{2+}\) and Mn\(^{2+}\) (15). Since these compounds can also affect the electron transport chain, it was not possible to determine if they acted directly on the calcium transport system. As shown in Table I, Hg\(^{2+}\) and Cu\(^{2+}\) had little effect on proton gradient-driven uptake, suggesting that their potent inhibition of respiratory driven calcium uptake was due to an inhibition of electron transport. Fe\(^{2+}\) and Mn\(^{2+}\) stimulated calcium uptake driven by a proton gradient just as was found for the respiratory driven uptake. Those two ions also increased the blank value slightly, so that the effect may be to promote the formation of an insoluble calcium complex, perhaps as a mixed salt.

Although cyanide was previously shown to inhibit respiratory driven calcium transport, it stimulated calcium transport

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\(^1\)The abbreviations used are: DCCD, dicyclocarbodiimide; pCMB, p-chloromercuribenzoate.
Effect of divalent cations, cyanide, and thiocyanate on calcium transport driven by an artificial proton gradient

Membrane vesicles were prepared and assayed for calcium transport as described under “Methods.” Each compound was added to both the vesicles and the assay mixture 10 min prior to the start of the assay. Divalent cations were used at 0.1 mM final concentrations. KCN and KSCN were used at 5 mM final concentrations. The values used are the maximal values, just prior to calcium efflux. This was usually after 15 or 30 s.

| Addition       | Calcium uptake | % Control |
|----------------|----------------|-----------|
| None           | 356            | 100       |
| HgCl₂          | 277            | 78        |
| CuCl₂          | 349            | 98        |
| FeSO₄          | 614            | 172       |
| MnCl₂          | 749            | 210       |
| KCN            | 896            | 232       |
| KSCN           | 404            | 113       |

| Addition                              | Calcium uptake | % Control |
|---------------------------------------|----------------|-----------|
| None                                  | 356            | 100       |
| pCMB                                  | 83             | 46        |
| 2-Mercaptoethanol                     | 100            | 98        |
| pCMB + 2-mercaptoethanol              | 201            | 99        |

DISCUSSION

Active transport of small molecules in E. coli has been shown to occur by two different mechanisms, one utilizing directly phosphate bond energy and the other utilizing the “high energy state of the membrane” (34, 35). The calcium transport system falls into the second category for several reasons: (a) it is present in membrane vesicles (14), while systems of the first type are not; (b) it can utilize the energy of oxidation by the respiratory chain even in the absence of the Mg⁺/ATPase (33), while phosphate bond-linked systems can use respiration only when the Mg⁺/ATPase is functional; and (c) it can utilize ATP only when the Mg⁺/ATPase is functional (15), while systems of the other type use the energy of ATP by some other, undefined mechanism.

The most likely form in which the “high energy membrane state” occurs is a protonotive force, consisting of a membrane potential and a chemical gradient of protons, both derived from the electrogenic extrusion of protons by the electron transport chain or by the Mg⁺/ATPase (2, 3). Mitchell has postulated that transport systems can use either or both of these component parts of the protonotive force to drive transport systems of the second type (3). In this proposal proton/substrate symports utilize protons directly, where proton movement through the symport could be driven by a chemical gradient of protons or a membrane potential, or both. Cations may also be extruded from whole cells by cation/proton antiports, as has been shown for sodium (36). If such cations are extruded from whole cells, then they should be accumulated by inverted vesicles. However, since the vesicles are inverted, the sign of the electrochemical gradient of protons must be the reverse of that found in whole cells, that is, protons must be translocated into inverted vesicles. Our results demonstrate that a gradient of protons, acid inside, causes the uptake of calcium into vesicles. Moreover, the uptake is dependent on the chemical gradient of protons, since it is inhibited by nigericin, but does not depend on the membrane potential directly, since valinomycin and thiocyanate do not inhibit.

The rate at which the proton gradient dissipates must be related to the uptake of calcium. As the gradient dissipates,
calcium (and phosphate) efflux occurs (Fig. 4). Compared with the transport of galactosides driven by a proton gradient in whole cells (9), calcium uptake and efflux occurs very rapidly. This suggests a more rapid movement of protons in this system, perhaps because of the greater surface to volume ratio in inverted vesicles (15). DCCD, which decreases the rate at which the gradient disappears, stimulates uptake. Another factor which influences the proton permeability of the membrane is the Mg\(^{2+}\)ATPase, as shown for several Mg\(^{2+}\)ATPase mutants (32). In a previous communication we reported (37) that the membrane-bound Mg\(^{2+}\)ATPase has a function in maintaining the high energy state of the membrane, preventing dissipation of the proton gradient. In the absence of the Mg\(^{2+}\)ATPase, little respiratory driven calcium transport was observed (33), presumably because the membrane was unable to maintain the proton gradient established by the electron transport chain. This implies that an artificial proton gradient of sufficient magnitude would still drive calcium transport even in the absence of the Mg\(^{2+}\)ATPase. In fact, we have found that an artificial proton gradient will drive calcium transport in inverted vesicles lacking the Mg\(^{2+}\)ATPase, but the eubessequent efflux is much faster.\(^2\)

These results suggest the presence of a calcium proton antiport and support the postulates of Mitchell. The question remains as to the actual molecular mechanism of an antiport. According to our interpretation of Mitchell's hypothesis, the carrier protein of the calcium transport system would have two binding sites, one for calcium and one for protons. There are several alternative mechanisms which would be proposed for filling and emptying of the sites to account for the data, but it is probably not a useful exercise to examine them without a more detailed knowledge of the stoichiometry of the reaction. Also, other possibilities for coupling of the proton gradient to calcium transport cannot be excluded. For example, it may be that protonation of a protein other than the carrier causes a conformational change in the actual carrier, or even that changes in the lipid phase surrounding the carrier have a role in the coupling.

An observation which apparently does not support Mitchell's model is that attempts to induce sugar or amino acid transport either in inverted vesicles or vesicles prepared by osmotic lysis of spheroplasts using an artificial proton gradient have been unsuccessful. On the other hand, it is possible that our experimental conditions were not adequate to demonstrate proton gradient-driven sugar or amino acid transport.

Although the stoichiometry of the reaction is not yet known, there are several observations which should be considered: the requirement for phosphate and the stimulatory effects of valinomycin (and perhaps thiocyanate). If the reaction is an electroneutral exchange of protons and calcium, then there is no accumulation of net charge within the vesicles. In that case a compensating flow of anion inward or cation outward should not be required. If, on the other hand, the ratio of protons to calcium were less than 2 then the process would entail the accumulation of charge as well as chemical calcium and would require the flow of a compensating ion. This charge accumulation would provide a back-pressure of charge, slowing calcium uptake. An influx of phosphate or an efflux of potassium catalyzed by valinomycin might dissipate this back-pressure, stimulating calcium uptake, as has been observed. Therefore, it appears more likely that accumulation of calcium causes the formation of a membrane potential, positive inside.

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**Addendum**—After submission of this paper, the paper by Burnett et al. (38) appeared demonstrating the transport of sulfate driven by an indirectly formed artificial proton gradient in bacterial membrane vesicles. In that study either right side out or inverted vesicles were capable of sulfate transport when energy was supplied artificially, while only right side out vesicles transported sulfate when energy was supplied by respiration. In our case as well vesicles of either orientation transported calcium in experiments using artificial proton gradients.\(^4\)

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