Cation/Proton Antiport Systems in Escherichia coli

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The calcium/proton antiporter of Escherichia coli was characterized by measurement of the effect of divalent cations on the respiration-coupled quenching of the fluorescence of quinacrine or 9-aminoacridine in everted membrane vesicles. Energy-linked quenching of the fluorescence of aminoacridines is dependent on proton uptake into everted vesicles and provides an indirect assay for measurement of the transmembrane pH gradient. Fluorescence quenching was partially reversed by addition of the divalent cations Ca2+, Mn2+, Sr2+, or Ba2+. The antiporter exhibited sigmoidal kinetics when the initial rate of fluorescence change was measured as a function of cation concentration. For each substrate, the value of the Hill coefficient approached 2. The S0.5 values indicate that the order of affinity of the antiporter for substrates was Ca2+ > Mn2+ > Sr2+ > Ba2+. Neither Mg2+ nor La3+ were found to be substrates, but both inhibited the reversal of the quenching of quinacrine fluorescence produced by substrates of the antiporter, inhibiting by about 90% at 50 mM MgCl2 or 50 μM LaCl3. In the presence of lower concentrations of MgCl2 or LaCl3, the Hill coefficients for Ca2+ and Sr2+ were reduced from values approaching 2 to values approaching 1. These data suggest an allosteric mechanism in which both homotropic and heterotropic effects occur.

An artificially imposed ΔpH, acid inside, was found to drive calcium transport, consistent with a Ca2+/H+ antiport (3). West and Mitchell (7) had shown that Na+ could affect proton fluxes in Escherichia coli, and they postulated a Na+/H+ antiporter. We therefore began a study of the effect of cations on ΔpH to determine whether antiport systems could be studied through their effect on ΔpH. Instrumentation for the direct measurement of internal pH are not available for bacterial systems. Indirect methods include measurement of the distribution of lipophilic weak acids and bases (8). For the indirect assay of ΔpH oriented acid interior, aminoacridines have proven useful. The fluorescence of such amines is quenched by the formation of a ΔpH, acid interior, although the exact relation between ΔpH and the degree of fluorescence quenching is not clear (9). Thus, we have used the quenching of fluorescence of aminoacridines as a qualitative measure of ΔpH. In this way, we have shown that there are three major cation antiporters, the Ca2+/H+ antiporter, the K+/H+ antiporter, and the Na+/H+ antiporter (10). Some of the properties of the NHA system have also been reported by Schuldiner and Fishkes (11).

In this communication, we report the effect of addition of calcium and other divalent cations on the energy-linked quenching of quinacrine fluorescence. This assay has allowed for determination of properties of the CHA system which could not be obtained with the previous assay, which involved measurement of [35]CaCl2 uptake (1-3). These properties include 1) substrate specificity, 2) kinetics, 3) anion specificity, and 4) requirement for a membrane potential. The results suggest that the CHA system transports Ca2+, Mn2+, Sr2+, or Ba2+ in an apparently cooperative manner, with Mg2+ or La3+ acting as an allosteric regulator.

MATERIALS AND METHODS

Preparation of Everted Membrane Vesicles—E. coli strain 7 (12) was grown to a stationary phase in a basal salts medium (13) supplemented with 54 mM glycerol as carbon source. Everted membrane vesicles were prepared as described previously (10, 14) by lysis of intact cells with a French press at 4000 p.s.i. Vesicles were stored at −20°C in 55% glycerol until use.

Fluorescence Assays—Qualitative changes in ΔpH were estimated from the energy-linked quenching of quinacrine fluorescence. The assay medium consisted of 10 mM Tris-HCl, pH 8.0, containing 140 mM choline chloride, 140 mM KCl, a combination of 90 mM KC1 and 50 mM KSCN, or a combination of 90 mM choline chloride and 50 mM choline thiocyanate, as noted. Quenching was initiated by addition of α-lactate adjusted to pH 8.0 with Tris base. The final concentration of lactate was 10 mM. In the assays reported in Fig. 6 the assay medium and lactate were adjusted to the indicated pH.

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The abbreviations used are: ΔpH, chemical gradient of protons; Δε, membrane potential; CHA, Ca2+/H+ antiporter; NHA, Na+/H+ antiporter; KHA, K+/H+ antiporter.
values. Each assay contained 40 to 90 μg of membrane protein. Fluorescence was measured with an Amino-Bowman spectrofluorometer with excitation at 420 nm and emission at 500 nm. When 1 μM 9-aminoacridine was substituted for quinacrine, the wavelengths were 400 nm for excitation and 460 nm for emission. All other conditions were the same as above.

Assay of "Ca" Transport—Uptake of "Ca" by everted membrane vesicles was measured by a filtration method (2, 14). Uptake assays were performed at pH 8.0 using 0.5 mM "CaCl₂ with 5 mM NaADP as an energy source and 3 mM potassium phosphate, pH 8.0, as the source of the precipitating co-ion.

Protein Determinations—Protein concentrations were determined by a microdetermination of the method of Lowry et al. (15) using bovine serum albumin as standard.

Reagents—"CaCl₂ (1.3 to 1.4 Ci/mmol) was purchased from New England Nuclear Corp. Nitrocellulose filters (0.45-μm pore size) were obtained from Matheson-Higgens Inc., Woburn, MA. Quinacrine dihydrochloride and 9-aminoacridine were purchased from Sigma Chemical Co. Choline thiocyanate was prepared by equilibrating a Dowex 1-X8 column with KSCN followed by exchange with 3 M choline chloride. The thiocyanate concentration was calculated from the absorbance of Fe(SCN)₃⁻ at 460 nm after titration with FeCl₃. The molar extinction coefficient of Fe(SCN)₃⁻ was estimated to be 1.72 × 10⁵ cm⁻¹ M⁻¹. The ionophore A23187 was the generous gift of Dr. Robert Hamill, Eli Lilly and Co. The ionophores N-PR and c-C-PR were generously provided by Dr. Robert Bittman of Queens College, City University of New York, New York. All other chemicals were reagent grade and obtained from commercial sources.

RESULTS

Substrate Specificity of the CHA System—The fluorescent signal of either quinacrine or 9-aminoacridine was quenched by respiring everted membrane vesicles (energized vesicles), reflecting uptake of protons and formation of ΔpH, acid interior. As shown in Fig. 1, addition of certain divalent cations to energized vesicles caused an immediate enhancement of fluorescence, reflecting efflux of quinacrine and, hence, of protons. A new steady state level of fluorescence was attained within 15 s after addition of cation. None of the ions tested affected the fluorescence of quinacrine alone, but in the presence of nonenergized vesicles addition of any of the cations produced a 4 to 8% quenching of the fluorescent signal (data not shown). This quenching may be due to binding phenomena and is not believed to be related to the fluorescence enhancement observed in energized vesicles.

Ca²⁺, Mn²⁺, Sr²⁺, and Ba²⁺ were each capable of partial dissipation of ΔpH (Fig. 1). Addition of Ca²⁺ prior to any of the other ions prevented response of ΔpH to those ions. Further, in the presence of any of the cations the vesicles were not able to form as large a ΔpH as in their absence, as demonstrated with Mn²⁺ in Fig. 1A, Curve 1. Because Mn²⁺, Sr²⁺, and Ba²⁺ also prevented dissipation of ΔpH by Ca²⁺, it is likely that the CHA system catalyzes exchange of any of those four cations for H⁺. Ruthenium red, which acts as a noncompetitive inhibitor of calcium transport in mitochondria (16), was without effect on the CHA system at concentrations up to 75 μM.

Na⁺, Li⁺, K⁺, or Rb⁺ each caused dissipation of ΔpH (10), but addition of any of them did not prevent dissipation of ΔpH by subsequent addition of substrates of the CHA system (data not shown). As seen in Fig. 1, A and B, Na⁺ was capable of further dissipation of ΔpH following partial dissipation by Ca²⁺, Mn²⁺, or Sr²⁺, defining the CHA system as a divalent/proton antiporter. It should be pointed out that 0.5 mM NaCl caused a slight decrease in the maximal effect produced by Ca²⁺. KCl had no effect on the fluorescence enhancement produced by Ca²⁺.

Qualitatively identical results were obtained using 9-aminoacridine as the fluorescence probe. Although 9-aminoacridine has been reported to be more amenable to quantification than quinacrine (9), the latter gives a more sensitive response to changes in ΔpH and was therefore used in most experiments.

Kinetics of the CHA System—When the effect of calcium on the initial rate of fluorescence enhancement was examined as a function of the calcium concentration, the resulting kinetic plot exhibited sigmoidicity (Fig. 2A). Sigmoidal kinetics were observed using either quinacrine or 9-aminoacridine. Since 9-aminoacridine is a monoamine, the fact that sigmoidal kinetics were observed with both probes indicates that results obtained with quinacrine are not artificial. The Hill plots obtained from initial rate data yielded coefficients (n) of 1.79 using quinacrine and 1.93 using 9-aminoacridine. The apparent V₅₀ values are expressed in arbitrary units and cannot be interpreted in terms of specific activity. It is obvious from Fig. 2A, however, that the apparent V₅₀ is much larger when quinacrine is used. The substrate concentration yielding 50% of V₅₀ (Eₛₒ₃) was approximately 0.1 mM with either probe and ranged from 0.1 to 0.5 mM in other experiments.

![Diagram](http://example.com/diagram.png)

**Fig. 1. Specificity of the CHA system.** Assay of quinacrine fluorescence was performed as described under "Materials and Methods." A, effect of Mn²⁺; Curve 1, 1 mM MnCl₂ added prior to initiation of the assay, 1 mM CaCl₂ and 0.5 mM NaCl added as indicated by the arrows; Curve 2, 1 mM MnCl₂ added as indicated by the arrows. B, effect of Sr²⁺; Curve 3, 5 mM SrCl₂ added first, followed by sequential addition of 0.5 mM CaCl₂ and 0.5 mM NaCl, as indicated; Curve 4, 0.5 mM CaCl₂ added first, followed by sequential addition of 5 mM SrCl₂ and 0.5 mM NaCl, as indicated; Curve 5, no addition. C, effect of Ba²⁺; Curve 6, 0.5 mM CaCl₂ added first, followed by 5 mM BaCl₂, as indicated; Curve 7, 5 mM BaCl₂ added as indicated; Curve 8, no addition.
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As shown in Fig. 3, addition of MgCl₂ to CaCl₂ resulted in a decrease in the effect of Ca²⁺ on ΔpH. Similar assays with saturating concentrations of calcium indicated that Mg²⁺ decreases the Vₘₐₓ of the antiprot reaction by 90% at 50 mM MgCl₂. Similar effects of Mg²⁺ were observed with other substrates of the CHA system. The nonlinear effect of increasing concentrations of MgCl₂ on the activity of the CHA system suggested cooperative inhibition.

It was of interest, therefore, to determine the effect of Mg²⁺ on the cooperativity of the CHA system. As seen in Fig. 4A for Sr²⁺ and in Fig. 4C for Ca²⁺; addition of 10 mM MgCl₂, which in this experiment was sufficient to decrease the Vₘₐₓ by 60%, resulted in a change from sigmoidal to hyperbolic kinetics. The Hill coefficients decreased from 1.69 to 0.81 with Sr²⁺ as substrate (Fig. 4B) and from 1.90 to 0.82 with Ca²⁺ as substrate (Fig. 4D). A slight decrease in Sₒ, was observed for each substrate: 1.86 mM to 1.02 mM for Sr²⁺ and 0.35 mM to

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**Table I**

<table>
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<th>Cation</th>
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<td>Mn²⁺</td>
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<td>Sr²⁺</td>
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<tr>
<td>Ba²⁺</td>
<td>5.60</td>
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Cooperative kinetics were also observed with each of the other substrates of the CHA system. The Hill coefficients for those substrates ranged from 1.0 to 1.9 (Table I). The values for Sₒ, suggested that the affinity of the CHA system is Ca²⁺ > Mn²⁺ > Sr²⁺ > Ba²⁺, which is similar to the order of ionic radii of those ions (17). In contrast to the sigmoidal kinetics observed with substrates of the CHA system, substrates of the NHA system produced simple hyperbolic kinetics.

We believe that the initial rate of enhancement of quinacrine fluorescence following addition of divalent cation is proportional to the initial rate of the antiporter itself. The extent of enhancement, representing the new steady state of the ΔpH, does not appear to be related specifically to the affinity of the antiporter. Although data from steady state levels have been used to approximate a Kₘ for antiprot reactions (11), the values are lower than those calculated from initial rate data.

Effect of Mg²⁺ and La³⁺—MgCl₂ did not dissipate ΔpH at any concentration examined up to 65 mM. A slight decrease in fluorescence was noted upon addition of MgCl₂ to energized vesicles, similar to that found with other divalent cations in unenergized vesicles. Again, this may represent a binding phenomenon. When MgCl₂ was added before quenching was initiated by addition of lactate, there was no effect on the extent of quenching, in contrast to what was observed when substrates of the various antiporters were added prior to initiation of quenching (cf. Fig. 1). These results suggest that Mg²⁺ is not a substrate of the CHA system or any other antipporter system.

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**FIG. 5.** Effect of LaCl₃ on the kinetics of the CHA system. A, rate of increase in quinacrine fluorescence as a function of CaCl₂ in the absence (○) or presence of LaCl₃ at 10 μM (▲) and 25 μM (■). B, Hill plots of the data.

**FIG. 6.** Effect of pH on the response of ΔpH to CaCl₂. Assays were performed in buffers adjusted to the indicated pH values.

0.14 mM for Ca²⁺. It is not clear whether those changes are significant.

The effects of LaCl₃, a potent and specific competitive inhibitor of the mitochondrial calcium transport system (16), were similar to those of MgCl₂ on the cooperativity of the CHA system. Addition of La³⁺ up to 100 μM caused a slight decrease in fluorescence in energized vesicles. At higher concentrations La³⁺ inhibited respiration and dissipated ΔpH. It is not clear whether La³⁺ is a substrate of the CHA system, but it is clear that La³⁺ is a potent inhibitor of the CHA system at concentrations which have no significant effect on respiration or ΔpH. As shown in Fig. 3, 50 μM La³⁺ almost totally abolished the response of ΔpH to Ca²⁺. At that concentration of LaCl₃, respiration was inhibited by about 10% but ΔpH was unaffected (data not shown). The nonlinearity of inhibition with La³⁺ also suggested a cooperative effect. As with Mg²⁺, the cooperativity of the kinetics of the CHA system was abolished in the presence of La³⁺ at concentrations three orders of magnitude less than those of Mg²⁺ (Fig. 5). The Hill coefficient was reduced from 1.86 in the absence of La³⁺ to 0.91 and 0.97 in the presence of 10 or 25 μM LaCl₃, respectively. In contrast to Mg²⁺, however, La³⁺ increased S₀.₅ from 0.51 mM to 0.84 mM and 2.39 mM in the presence of 10 or 25 μM LaCl₃, respectively. Thus, in addition to abolishing cooperativity, La³⁺ appears to act as a dead end inhibitor.

**Effect of pH**—The effect of Ca²⁺ on the quenching of

**FIG. 7.** Effect of SCN⁻ on the CHA system. Assays were performed either in a buffer consisting of 10 mM Tris-HCl and 140 mM KCl, pH 8.0 (Curves 1 and 3) or 10 mM Tris-HCl, 90 mM KCl, and 50 mM KSCN, pH 8.0 (Curves 2 and 4). ΔpH in Curves 2 and 4 were backtitrated with 0.75 mM NH₄OH. At the indicated times, 0.5 mM CaCl₂ (Curves 1 and 2) or 0.5 mM SrCl₂ (Curves 3 and 4) were added.

**FIG. 8.** Effect of A23187 on uptake of ⁴⁰Ca²⁺. Uptake of ⁴⁰Ca²⁺ by everted membrane vesicles was performed as described under "Materials and Methods" using 5 mM NADH (sodium salt) as an energy source. Vesicles were incubated with varying concentrations of A23187 for 5 min prior to addition of NADH and 0.5 mM "CaCl₂. Ionophore was added as an ethanolic solution, and all assays were adjusted to the same final concentration of ethanol. ▲, no NADH; ○, +NADH; ■, +NADH and 5 μM A23187. Inset, uptake as a function of A23187 concentration.
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Requirement for a Membrane Potential—When SCN⁻ was present in the assay mixture, a large increase in the quenching of quinacrine fluorescence occurred. Since SCN⁻ is a permeant anion, we interpret this effect to be the result of dissipation of $\Delta \psi$ through SCN⁻ influx. Similar results were obtained with NO₃⁻ and other permeant anions. In the experiment described in Fig. 7, $\Delta pH$ in the presence of SCN⁻ (Curves 2 and 4) was adjusted with NH₄Cl to the same level as in the absence of SCN⁻ (Curves 1 and 3). Thus, the effect of Ca²⁺ (Fig. 7A) or Sr²⁺ (Fig. 7B) on $\Delta pH$ could be measured in the presence and absence of $\Delta \psi$ with a constant $\Delta pH$. It is apparent that a membrane potential is required for the antipporter, as has been observed for the Na⁺/H⁺ antipporter. The requirement for $\Delta \psi$ was observed over the same pH range used in the experiment described in Fig. 6. Other permeant anions produced a similar effect. Potassium in the presence of valinomycin likewise reduced the effect of Ca²⁺, but not as reproducibly as did permeant anions. The requirement for $\Delta \psi$ is apparently a property of the CHA system, since the KHA system does not have that requirement. As shown below, Ca²⁺/H⁺ exchange catalyzed by the cationophore A23187 did not require a membrane potential (Fig 9), so that a potential is not necessary for Ca²⁺/H⁺ exchange per se.

Effect of Ionophores—A23187, which has been shown to catalyze proton exchange for cations in an electroneutral fashion (18), increased uptake of ⁴⁵Ca⁺ by everted membrane vesicles, with an optimum concentration of 5 μM ionophore (Fig. 8). At higher concentrations of ionophore an inhibition of ⁴⁵Ca⁺ uptake was observed. When the effect of A23187 on the energy-dependent quenching of quinacrine fluorescence was examined, it was found that concentrations of ionophore in excess of 1 μM produced a slow dissipation of $\Delta pH$, perhaps due to the ability of A23187 to carry choline (18). However, even at the limiting concentrations of ionophore consistent with the fluorescent assay, dissipation of $\Delta pH$ was observed in the presence of CaCl₂. As seen in Fig. 9, Curve 1, addition of CaCl₂ to everted membrane vesicles produced dissipation of $\Delta pH$ via the CHA system. After attainment of the new steady state, addition of 0.1 μM A23187 caused further dissipation of $\Delta pH$, resulting in a new steady state. When the assay was performed in the presence of SCN⁻, the effect of calcium via the CHA system was nearly eliminated, but the effect via the ionophore was unchanged; thus Ca²⁺/H⁺ exchange via the ionophore does not require a membrane potential. The neutral diamide ionophores N-PR and c-CPR, which catalyze electronegatic calcium movements but do not exchange calcium for protons (19), had no effect on $\Delta pH$ or on uptake of ⁴⁵CaCl₂ (data not shown).

**DISCUSSION**

By examination of the effect of cations on the energy-linked quenching of the fluorescence of the aminoacridine quinacrine, an indirect assay of $\Delta pH$, we have shown that E. coli has three major antiport systems for cations: the CHA system for divalent cations, the KHA system for monovalent cations, and the NHA system for Na⁺ and Li⁺ (10).

The use of such indirect assays is widely accepted (20, 21). The advantage of indirect assays is that they allow visualization of the transport event in the absence of the formation of concentration gradients. In direct assays of accumulation, it is often difficult to separate translocation from accumulation. Our results suggest that the everted vesicles used in these assays are fairly leaky to the cations we have studied, so that only small gradients, if any, are formed. Whether the leaks are due to specific cation pores is currently under investigation. In previous studies, uptake of ⁴⁵Ca⁺ was observed only when the ion was trapped within the vesicle through the formation of insoluble phosphate salts (2), again an indirect assay. Only low levels of ⁴⁵Na⁺ uptake have been observed. No uptake of ⁸⁶Rb⁺ could be measured. Yet the experiments with uptake of ⁴⁵Ca⁺ (2) and ²²Na⁺ demonstrate that concentration gradients are formed, and the effect of these ions on $\Delta pH$ suggests that the fluxes of the ions are rapid. Calculations derived from experiments with 9-aminoacridine yield a $\Delta pH$, acid interior, of about 4 units during oxidation of lactate at pH 8. Ca²⁺, Mn²⁺, and Sr²⁺ were each capable of dissipating that gradient by about 10%, implying a high flux rate of cation. The fact that the $\Delta pH$ goes to a new steady state after addition of cation again suggests that the cations leak out of the vesicles. If calcium were accumulated to a thermodynamic equilibrium point, then, once that equilibrium had been attained, there would be no further drain on $\Delta pH$, and $\Delta pH$.


3 E. N. Sorensen and B. P. Rosen, unpublished results.
would be expected to return to its original value. To have a continuous drain on \( \Delta \phi \), there must be a continuous calcium current. Cation pores would allow for a rapid leak, which, in the presence of \( \Delta \phi \), would be accelerated, preventing the formation of concentration gradients. The nature of the leak is unknown, but the ability of the vesicles to maintain a \( \Delta \phi \) of about 4 units implies that the leak is not specific. There may in fact be specific electrogenic pores for certain cations. For example, the TrkF system has been reported to transport K\(^+\) and Rb\(^+\) driven by \( \Delta \phi \) (22). One of the immediate events in the action of calcium K has been postulated to be the opening of an ion channel (23). Considering the apparent cooperativity of the CHA system, a calcium pore which might be gated in vivo (but not in vitro) could be quite valuable for various regulatory phenomena.

The use of this indirect assay allows for the determination of properties of the CHA system which were unobtainable with the radioisotope assay. First, since phosphate was required for formation of insoluble calcium salts, it was never clear whether there was a special requirement for an ion for the transport event. Phosphate is neither required for nor affects dissipation of \( \Delta \phi \) caused by calcium (data not shown). Second, the specificity of the CHA system had not been defined. Competition experiments were difficult due to the formation of competitor phosphate precipitates outside of the vesicles. Using the present assay, it is quite clear that the specificity of the system is, in order of affinity, Ca\(^{2+}\) ≈ Mn\(^{2+}\) > Sr\(^{2+}\) > Ba\(^{2+}\). Third, the necessity for formation of calcium phosphate salts also made the results of alterations in the assay conditions difficult to interpret. Since the solubility of calcium phosphate is pH-dependent, the pH dependency of the transport reaction was difficult to decide. Using the fluorescence assay there is a clear pH optimum of about 8.2 for reversal of the quenching of quinacrine fluorescence by calcium. However, changes in the response of quinacrine fluorescence to \( \Delta \phi \) with pH make the interpretation of the pH optimum less clear.

Fourth, the question of stoichiometry and the related issue of electrogenicity could not be answered using the radioisotope assay. No definitive answer can be extracted from the results of the present study, but some results do bear on the question. Three possible types of stoichiometries exist: \( H^+ : Ca^{2+} < 2 \); \( H^+ : Ca^{2+} = 2 \) and \( H^+ : Ca^{2+} > 2 \). If less than two, then the system would be electrogenic in that more calcium charges would move in one direction than proton charges in the other. That sort of stoichiometry is unlikely from a purely thermodynamic point of view, since calcium charges would have to move against the membrane potential. But assuming that it were true, \( \Delta \phi \) would impede the reaction, and elimination of \( \Delta \phi \) would be expected to stimulate exchange. If the stoichiometry were two protons per calcium, then the process would be electroneutral, and \( \Delta \phi \) would be expected to be without effect, assuming no other role for \( \Delta \phi \) in the transport process (but see below). If \( H^+ : Ca^{2+} > 2 \), then the process would be electrogenic in the opposite direction to that mentioned above, that is, more proton charges would move out of everted vesicles (or into intact cells) than calcium charges into everted vesicles (or out of intact cells). This process would use the entire protomotive force as a driving force and would be the most efficient in terms of energizing calcium transport. Elimination of \( \Delta \phi \) would be expected to inhibit the reaction. As seen in Fig. 7, inhibition was observed. The fact that \( H^+ : Ca^{2+} \) exchange via A23187, which catalyzes exchange with a stoichiometry of \( H^+ : Ca^{2+} = 2 \), was not inhibited (Fig. 9) implies that the native exchanger has a different stoichiometry than the artificial antiporter. The absolute requirement for a potential is surprising and may indicate more than a simple electrogenic process is occurring. Recent experiments by Schuldiner et al. (24) have suggested that \( \Delta \phi \) might be required for activity of the lactose carrier. If a similar situation exists for the Ca\(^{2+}\)/H\(^+\) antiporter, then the inhibition caused by elimination of \( \Delta \phi \) may not bear directly on the stoichiometry of the reaction. This would be similar to a gating phenomenon, where a potential of a particular magnitude might be required to "open" or activate the antiporter. Thus, the data favor a stoichiometry of \( H^+ : Ca^{2+} > 2 \), but direct measurements of the stoichiometry should be made.

Another disadvantage of the radioisotope method of measuring calcium transport was that, in order to have precipitation of calcium phosphate salts, the concentrations of calcium and phosphate could be varied only over a very narrow range. This was sufficient to allow determination of a very approximate \( K_m \) (2). The lack of uptake of calcium at low concentrations of calcium was attributed to insufficient internal calcium to produce a precipitate with phosphate. The present assay has allowed a more extensive investigation of the kinetics of calcium transport. Uptake of each of the substrates of the CHA system was found to be associated with sigmoidal kinetics. In each case the value of the Hill coefficient approached two, suggesting a cooperative system with at least two interacting sites. The mitochondrial calcium transport system, although not an antiporter, exhibits similar cooperative effects (18). Mg\(^{2+}\) and La\(^{3+}\) do not appear to be substrates of the CHA system but apparently eliminate the cooperativity of the system for its substrates. In the presence of Mg\(^{2+}\) or La\(^{3+}\), the value of the Hill coefficient decreases from approximately 2 to 1. The model most consistent with these results is one in which the antiporter has two catalytic sites. Binding of substrate to the first increases the affinity of the second site. Mg\(^{2+}\) or La\(^{3+}\) could bind to one of the catalytic sites or to regulatory sites, resulting in a decrease in the Hill coefficient. It is more difficult to interpret the effects of La\(^{3+}\) and Mg\(^{2+}\) on the So.5 and Vmax values. While this model is consistent with the results, it is strictly a working hypothesis. It may be that the apparent cooperativity is the result of other unidentified factors. In the absence of knowledge of the function of the CHA system, it is difficult to envision the advantages of allosteric.

In paramecium, calcium fluxes provide a mechanism for primitive sensory transmission (25). Calcium has also been implicated in regulation of the chemotactic response of Bacillus subtilis (26). If such a function for calcium were the case for E. coli, as we have postulated in the past (1), then a fine regulation of calcium fluxes might be expected. One might, for example, postulate that filling of a chemoreceptor could activate a calcium pore and inhibit the CHA system, producing depolarization of the membrane. Recovery could involve inactivation of the channel and activation of the antiporter. Transient depolarization of the membrane has been shown to be associated with chemoreceptor in E. coli (27). In the absence of additional information, such a postulate is pure speculation. But the presence of an allosterically regulated system for the extrusion of calcium from intact cells would imply a more complicated function than simply to prevent the precipitation of intracellular phosphate.

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REFERENCES
R N Brey and B P Rosen