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# Active transport of calcium in *Neurospora* plasma membrane vesicles

(electrogenic ATPase/electrochemical proton gradient/calcium/proton antiport/vanadate/nigericin)

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**ABSTRACT** **Functionally inverted plasma membrane vesicles isolated from the eukaryotic microorganism *Neurospora crassa* catalyze $Mg^{2+}$/ATP-dependent $Ca^{2+}$ uptake. Inhibitor-induced efflux studies and isotope-exchange experiments indicate that the $Ca^{2+}$ is accumulated inside the vesicles against a concentration gradient of about 40-fold, and that the majority of the transported $Ca^{2+}$ is present essentially in free solution. Comparisons of $Mg^{2+}$/ATP-driven $^{45}Ca^{2+}$ uptake and [$^{14}$C]$SCN^-$ uptake with respect to the $Mg^{2+}$/ATP concentration dependence, the effects of inhibitors, and the nucleotide and divalent cation specificities indicate that the energy for $Ca^{2+}$ accumulation is derived from ATP hydrolysis catalyzed by the electrogenic plasma membrane ATPase. Energized $Ca^{2+}$ uptake is stimulated by the permeant anion $SCN^-$ to a degree that varies reciprocally with the ability of this anion to dissipate the membrane potential, and is inhibited by $K^+$ in the presence of nigericin. All of these data point to the conclusion that the active transport of $Ca^{2+}$ across the *Neurospora* plasma membrane takes place via a $Ca^{2+}/H^+$ antiporter, which functions to pump $Ca^{2+}$ out of the intact cell.**

An earlier report from this laboratory (1) described a novel method for the isolation of plasma membranes from the eukaryotic microorganism, *Neurospora crassa*, in high yield and purity. The method involves (*i*) coating the surface of intact cells of a cell wall-less mutant with concanavalin A to stabilize the plasma membrane against fragmentation and vesiculation upon osmotic lysis of the cells, (*ii*) separating the large, concanavalin A-stabilized plasma membrane sheets (ghosts) from other cellular constituents by low speed centrifugation, and (*iii*) converting the topologically open plasma membrane sheets to closed vesicles by removing the bulk of the concanavalin A with α-methylmannoside. Subsequent communications (2, 3) described the biochemical properties of a $Mg^{2+}$-ATPase in the isolated *Neurospora* plasma membranes, and another (4) demonstrated that the plasma membrane ATPase is an electrogenic pump, probably a proton pump. ATP hydrolysis catalyzed by the plasma membrane ATPase in functionally inverted plasma membrane vesicles gives rise to the generation of a transmembrane electrical potential ($\Delta\psi$, interior positive) that can be monitored by [$^{14}$C]$SCN^-$ uptake or anilinonaphthalene sulfonate fluorescence enhancement. The advantages of the isolated *Neurospora* plasma membrane vesicles as a new experimental system for investigating eukaryotic surface membrane structure and function have been reviewed (5).

At present, our major interests are in the molecular mechanisms of the electrogenic ATPase and any other transport systems that may be present in the *Neurospora* plasma membrane vesicles. In view of the widespread occurrence of energy-dependent $Ca^{2+}$-extrusion systems localized in plasma membranes and the fact that significant numbers of the isolated *Neurospora* plasma membrane vesicles are functionally inverted, it seemed likely that these vesicles would possess an energy-dependent $Ca^{2+}$-accumulation system which functions physiologically to extrude $Ca^{2+}$. The experiments presented in this paper demonstrate that the *Neurospora* plasma membrane does indeed contain such an active transport system, in which the concentrative uptake of $Ca^{2+}$ in functionally inverted vesicles is coupled, via a $Ca^{2+}/H^+$ antiporter* (6), to a transmembrane pH gradient generated by the plasma membrane ATPase.

## METHODS

**Preparation of *Neurospora* Plasma Membrane Vesicles.** Plasma membrane ghosts were isolated in large quantities by a scaled-up modification of the original method (1) and stored at −20°C as a suspension in 60% (vol/vol) glycerol. As needed, portions of the ghosts were converted to vesicles by treatment with α-methylmannoside. The details of this technically simplified procedure for the routine preparation of *Neurospora* plasma membrane vesicles have been published (7).

**Transport Assays.** [$^{14}$C]$SCN^-$ (60 Ci/mol; 1 Ci = $3.7 \times 10^{10}$ becquerels) and $^{45}Ca^{2+}$ (5–10 Ci/mol) uptakes by the plasma membrane vesicles were measured essentially as described (4). Vesicles were suspended in 10 mM 2-(*N*-morpholino)ethanesulfonic acid, adjusted to pH 7.3 with Tris base, and were assayed at 23°C. When necessary, the pH of additional components of the transport assays was adjusted before addition to maintain the final pH of the assay at 7.3. Except for the data in Figs. 1, 2, and 5, corrections for nonspecific $Ca^{2+}$ binding were made by carrying out each $^{45}Ca^{2+}$ transport assay in the presence of 50 μM $Na_3VO_4$ and subtracting these values from the appropriate values obtained without this inhibitor. Nonspecific $Ca^{2+}$ binding was about 10% of the maximum value of energized $Ca^{2+}$ uptake in these experiments. In all $Ca^{2+}$ uptake studies, $Ca^{2+}$ was present at a final concentration of 100 μM, which is near the $K_m$ for $Ca^{2+}$ uptake. An accurate determination of the $K_m$ was beyond the scope of these studies and will require a more detailed investigation, due to problems arising from inherent interactions among $Ca^{2+}$, $Mg^{2+}$, ATP, and the membranes.

**Estimation of Protein.** Protein was estimated by the method of Lowry *et al.* (8) with bovine serum albumin as a standard.

**Materials.** $^{45}CaCl_2$ and [$^{14}$C]KSCN were obtained from New England Nuclear. ATP (Tris salt, low in vanadate) was from Sigma, carbonylcyanide *m*-chlorophenylhydrazone (CCCP) was from Calbiochem, and sodium vanadate (ortho) was from Fisher. Due to the uncertainty in the water content of the commercial sodium vanadate, the amount of vanadium in this product was determined by permanganate titration (9). Ni-

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Abbreviation: CCCP, carbonylcyanide *m*-chlorophenylhydrazone.
* The term $Ca^{2+}/H^+$ does not imply a specific stoichiometry.

gericin was the generous gift of J. Berger of Hoffman-La Roche. All other chemicals were reagent grade or of the highest purity obtainable from commercial sources.

## RESULTS

**$Ca^{2+}$ Uptake by *Neurospora* Plasma Membrane Vesicles.** The data presented in Fig. 1 demonstrate the salient features of energized $Ca^{2+}$ uptake catalyzed by the *Neurospora* plasma membrane vesicles. The amount of uptake seen in the presence of $Mg^{2+}$/ADP is essentially the baseline control. The $Mg^{2+}$ serves to eliminate a significant amount of nonspecific $Ca^{2+}$ binding to the membranes, and the $Mg^{2+}$ and ADP together serve as osmotic controls. In the presence of $Mg^{2+}$/ATP there was a marked increase in $Ca^{2+}$ uptake above the baseline control, and the rate and extent of $Mg^{2+}$/ATP-dependent $Ca^{2+}$ uptake were approximately doubled by the addition of 10 mM NaSCN. The same effect was seen upon the addition of KSCN but not $K_2SO_4$ (data not shown), which indicates that the stimulation is due to SCNsupn−. The effect of $SCN^-$ is pertinent to the mechanism of $Mg^{2+}$/ATP-dependent $Ca^{2+}$ uptake and will be elaborated upon below. Taking the value of 3 μl of intravesicular space per mg of protein (unpublished results) and assuming that the vesicles are all inverted and active, the concentration of $Ca^{2+}$ accumulated inside the vesicles in the presence of $SCN^-$ and $Mg^{2+}$/ATP is about 4 mM. This indicates an apparent accumulation of about 40-fold over the $Ca^{2+}$ concentration in the incubation medium. If significant numbers of the vesicles are right-side-out, the concentration ratio is even greater. The effects of CCCP and vanadate on $SCN^-$-stimulated $Mg^{2+}$/ATP-dependent $Ca^{2+}$ uptake are also presented in Fig. 1. The proton conductor CCCP (10) markedly inhibited energized $Ca^{2+}$ uptake, and vanadate, a potent inhibitor of the *Neurospora* plasma membrane ATPase (11) and consequently an inhibitor of $Mg^{2+}$/ATP-dependent generation of $\Delta\psi$ in the vesicles (unpublished results), was also a potent inhibitor of $SCN^-$-stimulated $Mg^{2+}$/ATP-dependent $Ca^{2+}$ uptake. It should be pointed out that CCCP and vanadate also inhibit $Mg^{2+}$/ATP-dependent $Ca^{2+}$ uptake to a similar degree in the absence of $SCN^-$ (data not shown).

The above results alone suggest that $Ca^{2+}$ uptake catalyzed by the *Neurospora* plasma membrane vesicles is concentrative, is energized by the electrogenic plasma membrane ATPase, and operates via a $Ca^{2+}/H^+$ antiport mechanism. Additional support for each of these propositions will be provided in the following paragraphs.

**$Ca^{2+}$ Uptake Is Concentrative.** As mentioned above, *Neurospora* plasma membrane vesicles appear to accumulate $Ca^{2+}$ against a considerable concentration gradient. However, because accumulated $Ca^{2+}$ in mitochondria (12), submitochondrial particles (13), inverted bacterial plasma membrane vesicles (14, 15), sarcoplasmic reticulum vesicles (16), and plasma membrane vesicles of *Halobacterium halobium* (17) can be bound or present as a precipitate, the nature of the $Ca^{2+}$ accumulated by the *Neurospora* plasma membrane vesicles was investigated to determine whether the uptake was truly concentrative. In most of the above experimental systems, $Ca^{2+}$ uptake was carried out in the presence of anions, such as phosphate or oxalate, to promote the formation of $C^{2+}$ precipitates in the intramembranous space. Because no such anion was provided in the experiments presented here, extensive precipitation is unlikely, although it is conceivable that inorganic phosphate liberated from ATP by the plasma membrane ATPase could enter the vesicles and precipitate the transported $Ca^{2+}$. If precipitation of the transported $Ca^{2+}$ or tight binding of $Ca^{2+}$ to the inner surface of the vesicles were the sole reason for the apparent accumulation against a concentration gradient, then the accumulated $Ca^{2+}$ should neither be readily released from poisoned vesicles nor be easily exchangeable with extravesicular $Ca^{2+}$. Fig. 2 demonstrates the effects of CCCP, vanadate, and CCCP plus vanadate on the $Ca^{2+}$ content of vesicles preloaded with $Ca^{2+}$. It can be seen that the addition of either inhibitor or a combination of both elicited the rapid loss of

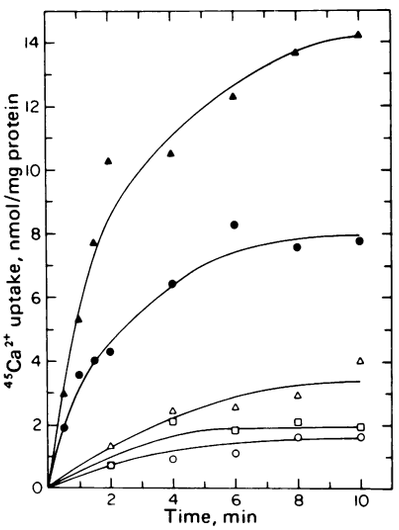


FIG. 1. $Mg^{2+}$/ATP-dependent $^{45}Ca^{2+}$ uptake by *Neurospora* plasma membrane vesicles. Aliquots of a membrane vesicle suspension (50 μl, containing 25 μg of protein) were preincubated for 30 sec, and the indicated additions were then made to give a total volume of 53 μl. Immediately thereafter, 2 μl of 2.5 mM $^{45}CaCl_2$ was added. Additions were made to give the following final concentrations: ▲, ATP, $MgSO_4$, and NaSCN at 10 mM; ●, ATP and $MgSO_4$ at 10 mM; △, ATP, $MgSO_4$ and NaSCN at 10 mM, CCCP at 50 μM, and ethanol at 0.25%; □, ADP, $MgSO_4$, and NaSCN at 10 mM; ○, ATP, $MgSO_4$, and NaSCN at 10 mM and $Na_3VO_4$ at 50 μM. Ethanol at the above concentration had no effect on $^{45}Ca$ uptake. Points indicate the average of duplicate determinations.

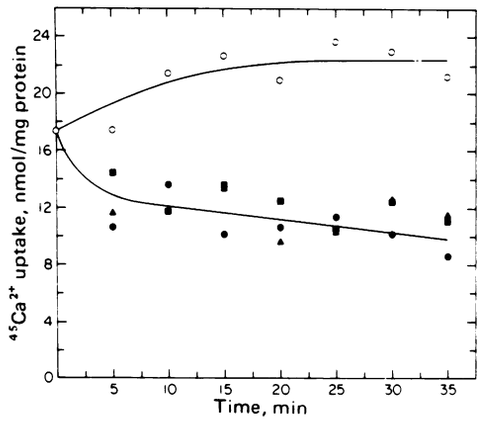


FIG. 2. Efflux of $^{45}Ca^{2+}$ from preloaded *Neurospora* plasma membrane vesicles. Aliquots of a membrane vesicle suspension (50 μl, containing 14 μg of protein) were preincubated for 30 sec, and ATP, $MgSO_4$, and NaSCN were then added to give final concentrations of 10 mM in a total volume of 53 μl. Immediately thereafter, 2 μl of 2.5 mM $^{45}CaCl_2$ was added. After 5 min of incubation, the following additions were made: ○, control (no addition); ■, 0.5 μl of 5 mM $Na_3VO_4$; ▲, 0.5 μl of 5 mM CCCP in ethanol; ●, 0.5 μl of 5 mM $Na_3VO_4$ and 0.5 μl of 5 mM CCCP in ethanol. Ethanol at the above concentration had no effect on $^{45}Ca^{2+}$ efflux. Points indicate the average of duplicate determinations. Corrections for nonspecific $Ca^{2+}$ binding were made by carrying out the above control assay in the presence of 50 μM $Na_3VO_4$ and subtracting these values from those obtained without this inhibitor.

approximately one-third of the accumulated $Ca^{2+}$, followed by a slower rate of loss of the remaining $Ca^{2+}$. This experiment indicates that at least one-third of the accumulated $Ca^{2+}$ was not precipitated or tightly bound inside the vesicles and must therefore be present essentially in free solution. However, from this experiment the nature of the other two-thirds of the accumulated $Ca^{2+}$ remains equivocal. To investigate the nature of the accumulated $Ca^{2+}$ in another way, isotope exchange experiments were carried out. If precipitation or essentially irreversible binding is the driving force for $Ca^{2+}$ accumulation in the vesicles, after the plateau level of uptake is reached, no further influx of $Ca^{2+}$ should take place and little, if any, isotope exchange is expected. However, if the plateau value represents a steady-state balance between influx and efflux of $Ca^{2+}$, then the kinetics of isotope exchange at the steady state should mimic those of the initial $Ca^{2+}$ uptake. The results of an isotope exchange experiment are presented in Fig. 3. In the first control, plasma membrane vesicles were allowed to accumulate $Ca^{2+}$ present as a mixture of $^{40}Ca^{2+}$ (100 $\mu$M) and $^{45}Ca^{2+}$ (trace amounts). It can be seen that the uptake approaches a plateau after about 5 min, at which time approximately 7% of the $Ca^{2+}$ in the incubation mixture has been taken up. As a second control, identical assays were carried out, except that the $Ca^{2+}$ isotope mixture was added to the vesicles after 5 min of incubation. This control serves to demonstrate that the $Ca^{2+}$ transport activity of the vesicles decreases (for unknown reasons) by about one-third during the first 5 min of incubation and delimits the maximum possible extent of isotope exchange. To determine the extent of isotope exchange at the plateau level, vesicles were allowed to accumulate $^{40}Ca^{2+}$ (100 $\mu$M) for 5 min, after which time the $^{45}Ca^{2+}$ tracer was added. It can be seen that the $^{45}Ca^{2+}$ was taken up in a manner similar to the second control, indicating extensive $Ca^{2+}$ influx and efflux at the uptake plateau. The results of the experiments described in Figs. 2 and 3 together indicate that neither $Ca^{2+}$ precipitation nor essentially irreversible binding provides the driving force for $Mg^{2+}$/ATP-dependent $Ca^{2+}$ accumulation, although a mechanism involving extensive and rapidly reversible binding is not totally eliminated. Thus, in the light of the present information, we conclude that the majority of the transported $Ca^{2+}$ present inside the vesicles is essentially in free solution, and that $Ca^{2+}$ accumulation is therefore the result of concentrative uptake. The fact that CCCP and vanadate do not induce rapid and complete efflux of the accumulated $Ca^{2+}$ is presumably a reflection of the carrier mechanism.

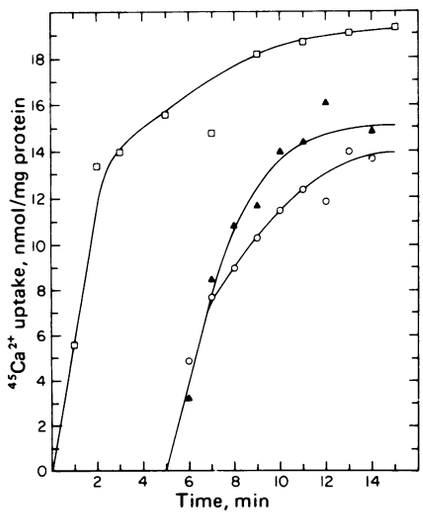


FIG. 3. Isotope exchange of preloaded $^{40}Ca^{2+}$ with tracer $^{45}Ca^{2+}$ in *Neurospora* plasma membrane vesicles. Aliquots of a membrane vesicle suspension (50 $\mu$l, containing 18 $\mu$g of protein) were preincubated for 30 sec, and ATP, $MgSO_4$, and NaSCN were then added to give final concentrations of 10 mM in a total volume of 53 $\mu$l. The following additions were then made: □, 2 $\mu$l of 2.5 mM $^{45}CaCl_2$ (0.025 $\mu$Ci) immediately (zero time); ▲, 2 $\mu$l of 2.5 mM $^{45}CaCl_2$ (0.025 $\mu$Ci) at 5 min; ○, 2 $\mu$l of 2.5 mM $^{40}CaCl_2$ at zero time and 1 $\mu$l of 78 $\mu$M $^{45}CaCl_2$ (0.025 $\mu$Ci) at 5 min.

**$Ca^{2+}$ Uptake is Energized by the Electrogenic Plasma Membrane ATPase.** The inhibitory effects of CCCP and vanadate on $Mg^{2+}$/ATP-dependent $Ca^{2+}$ uptake (Fig. 1) suggest that the electrogenic plasma membrane ATPase provides the energy for $Ca^{2+}$ accumulation by the *Neurospora* plasma membrane vesicles. As previously shown, CCCP inhibits the $Mg^{2+}$/ATP-dependent generation of a protonmotive force catalyzed by the electrogenic plasma membrane ATPase (4), presumably by facilitating transmembrane proton movements, and has no effect on ATP hydrolysis catalyzed by the ATPase (2). Therefore, the inhibition of $C^{2+}$ uptake by CCCP implicates a protonmotive force and thus the electrogenic ATPase in the mechanism of $Ca^{2+}$ uptake. On the other hand, vanadate inhibits ATP hydrolysis catalyzed by the plasma membrane ATPase and as a result abolishes electrogenic pumping by this enzyme. Thus, vanadate inhibition of $Ca^{2+}$ accumulation by the vesicles also implicates the electrogenic ATPase in the energetics of $Ca^{2+}$ accumulation.

Because electrogenic pumping by the plasma membrane ATPase is not affected by [ethylenebis(oxyethylenenitrilo)]-tetraacetic acid (EGTA) (data not shown), this enzyme is apparently not a $Ca^{2+}$ pump (utilizing trace amounts of $Ca^{2+}$, such as those present in many commercial ATP preparations, as the electrogenic species). However, the possibility was considered that the *Neurospora* plasma membrane contains a distinct $Ca^{2+}$-translocating ATPase similar to those found in erythrocytes (18), fibroblast microsomes (19), sarcoplasmic reticulum (20, 21), and *Streptococcus faecalis* (15), that is inhibited by CCCP and vanadate and is not detectable as a $Ca^{2+}$-stimulated ATP hydrolyzing activity. Several lines of evidence make this possibility unlikely. In Fig. 4, the initial rate of $Ca^{2+}$ uptake and the steady state of $[^{14}C]SCN^-$ uptake (the electrogenic pump assay) are compared as a function of the $Mg^{2+}$/ATP concentration. It can be seen that the two processes depend upon $Mg^{2+}$/ATP in a similar fashion. In a previous communication (4) it was shown that $[^{14}C]SCN^-$ uptake and ATP hydrolysis catalyzed by the *Neurospora* plasma membrane vesicles exhibit identical saturation profiles as a function of the $Mg^{2+}$/ATP concentration. Therefore, $Ca^{2+}$ accumulation, electrogenic pumping, and ATP hydrolysis catalyzed by these vesicles all depend upon the concentration of $Mg^{2+}$/ATP in a like manner, which argues for a cause–effect relationship among the three phenomena. Additional support for this conclusion comes from a quantitative comparison of the concentration dependence of several inhibitors on $Mg^{2+}$/ATP-dependent $Ca^{2+}$ accumulation and $[^{14}C]SCN^-$ uptake. Both processes are inhibited in parallel by increasing concentrations of vanadate, diethylstilbestrol,† and CCCP. Half-maximal inhibition of both processes is seen with 2–4 $\mu$M vanadate, 16–18 $\mu$M diethylstilbestrol, and 5–10 $\mu$M CCCP. Finally, the divalent cation and nucleotide specificities of both processes are similar. In the buffer system used in these experiments, divalent cations can substitute for $Mg^{2+}$ in both processes in the order $Mg^{2+} > Mn^{2+} > Co^{2+}$, and $Mg^{2+}$/GTP energizes neither. Thus, although a distinct $Ca^{2+}$-translocating ATPase still remains a remote possibility, all of the available information supports the

† Recently reported to inhibit the *Neurospora* plasma membrane ATPase (11).

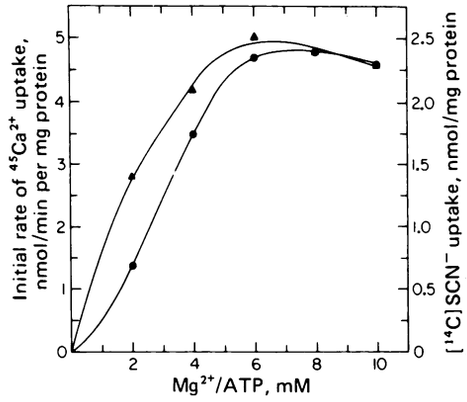


FIG. 4. $^{45}Ca^{2+}$ uptake and [$^{14}$C]SCN$^-$ uptake in *Neurospora* plasma membrane vesicles as a function of $Mg^{2+}$/ATP concentration. Aliquots of a membrane vesicle suspension (50 $\mu$l, containing 29 $\mu$g of protein) were preincubated for 30 sec, and ATP and $MgSO_4$ were then added to give final concentrations as indicated, in a total volume of 53 $\mu$l. ▲, Steady-state uptake of [$^{14}$C]SCN$^-$. At each $Mg^{2+}$/ATP concentration, uptake was measured at 1, 2, 3, and 4 min after adding 2 $\mu$l of 1 mM [$^{14}$C]KSCN. Steady-state uptakes are defined as the maximum values of the lines of best fit plotted from the uptake data; ●, initial rate of $^{45}Ca^{2+}$ uptake. At each $Mg^{2+}$/ATP concentration, uptake was measured 1 min after adding 2 $\mu$l of 2.5 mM $^{45}CaCl_2$, and initial rates of uptake were determined from these data after correction for nonspecific binding. Points indicate the average of duplicate determinations.

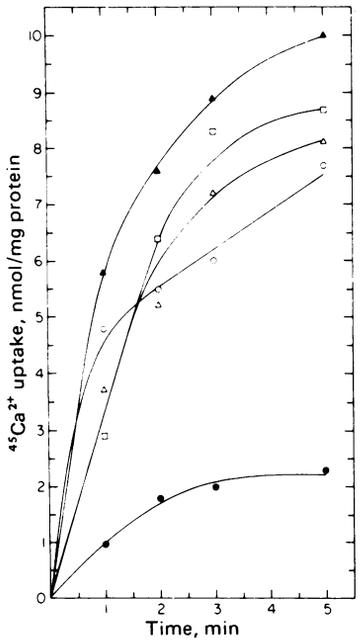


FIG. 5. Inhibition of $Mg^{2+}$/ATP-dependent $^{45}Ca^{2+}$ uptake in *Neurospora* plasma membrane vesicles by nigericin plus $K^+$. Aliquots of a membrane vesicle suspension (50 $\mu$l, containing 33 $\mu$g of protein) and the indicated additions were preincubated for 30 sec, and ATP and $MgSO_4$ were then added to give final concentrations of 10 mM in a total volume of 53 $\mu$l. Immediately thereafter, 2 $\mu$l of 2.5 mM $^{45}CaCl_2$ was added. Additions were made to give the following final concentrations and uptakes were performed in the order given: ▲, "no addition" control for beginning of experiment; □, 25 $\mu$M nigericin and 0.5% ethanol; △, 10 mM $K_2SO_4$; ●, 25 $\mu$M nigericin, 0.5% ethanol, and 10 mM $K_2SO_4$; ○, "no addition" control for end of experiment. Corrections for nonspecific $Ca^{2+}$ binding were made by carrying out the "no addition" control assays in the presence of 50 $\mu$M $Na_3VO_4$ and subtracting these values from those obtained without this inhibitor.

conclusion that $Ca^{2+}$ accumulation is energized via ATP hydrolysis catalyzed by the electrogenic plasma membrane ATPase.

**Evidence for a $Ca^{2+}/H^+$ Antiport Mechanism.** The facts that energized $Ca^{2+}$ uptake is driven by ATP hydrolysis catalyzed by the electrogenic plasma membrane ATPase and that this process is inhibited by CCCP strongly suggest that ATP hydrolysis and $Ca^{2+}$ transport are coupled via a $Ca^{2+}/H^+$ antiporter. However, because an unexpected mode of action of CCCP is possible (for example as a $Ca^{2+}$ ionophore), and as it has not yet been unequivocally demonstrated that the electrogenic plasma membrane ATPase is a proton pump, we sought additional evidence concerning the involvement of a $Ca^{2+}/H^+$ antiporter in the mechanism of $Ca^{2+}$ uptake.

Because the ionophore nigericin facilitates the electroneutral transmembrane exchange of $H^+$ and $K^+$ (22), nigericin in the presence of a concentration gradient of $K^+$ (out > in) should inhibit $Ca^{2+}$ accumulation by the vesicles if this process is driven by antiport of $Ca^{2+}$ for $H^+$. Fig. 5 demonstrates that $Mg^{2+}$/ATP-dependent $Ca^{2+}$ uptake was markedly inhibited by $K^+$ and nigericin together but was essentially unaffected by $K^+$ or nigericin alone. Although not shown, the degree of inhibition increased as a function of the $K^+$ concentration from 0 to 20 mM.

The effect of the permeant anion $SCN^-$ on $Mg^{2+}$/ATP-dependent $Ca^{2+}$ uptake provides additional support for a $Ca^{2+}/H^+$ antiport mechanism. It is clear from earlier results that ATP hydrolysis catalyzed by the plasma membrane ATPase leads to the generation of an interior positive $\Delta\psi$, and it is likely that $H^+$ is the electrogenic species (23). Depending upon various factors, including the anion permeability of the vesicle membrane, the size of the vesicles, and the intravesicular buffering capacity the enzyme may also generate, in addition to $\Delta\psi$ a chemical proton gradient, $\Delta$pH. The sum of $\Delta\psi$ and $\Delta$pH is the total electrochemical proton gradient (24). Because $Ca^{2+}$ accumulation is energized via the electrogenic ATPase, then $\Delta\psi$, $\Delta$pH, or both, must constitute the driving force for $Ca^{2+}$ uptake. $SCN^-$ can be used to determine which of these is coupled to $Ca^{2+}$ accumulation. Due to the charge and permeant nature of $SCN^-$, this ion can be used as a probe for monitoring $\Delta\psi$ (interior positive) when used at a sufficiently low concentration. Increasing amounts of $SCN^-$ will progressively dissipate $\Delta\psi$ by charge neutralization but should not collapse the transmembrane pH gradient. The effects of increasing concentrations of $SCN^-$ on $Mg^{2+}$/ATP-dependent $Ca^{2+}$ uptake and $Mg^{2+}$/ATP-dependent [$^{14}$C]$SCN^-$ uptake ($\Delta\psi$ generation) are shown in Fig. 6. It can be seen that the stimulation of $Mg^{2+}$/ATP-dependent $Ca^{2+}$ uptake as a function of the $SCN^-$ concentration correlates well with the dissipation of $\Delta\psi$. Although it is possible to envisage several mechanisms by which $SCN^-$ might stimulate energized $Ca^{2+}$ uptake, the precise nature of the effect is presently unresolved. In any case, the most important point made by this experiment is that $Mg^{2+}$/ATP-dependent $Ca^{2+}$ uptake is maximal in the absence of $\Delta\psi$. Thus, $\Delta\psi$ cannot be an obligatory component of the driving force for $Ca^{2+}$ uptake. Because $Ca^{2+}$ uptake is energized by the electrogenic ATPase and $\Delta\psi$ is not the driving force under the conditions of this experiment, $\Delta$pH must be. Taken together, the experiments described above constitute strong evidence that energized $Ca^{2+}$ uptake catalyzed by the isolated *Neurospora* plasma membranes operates via a $Ca^{2+}/H^+$ antiporter.

## DISCUSSION

The results presented in this communication demonstrate the existence of an active transport system for $Ca^{2+}$ in the plasma

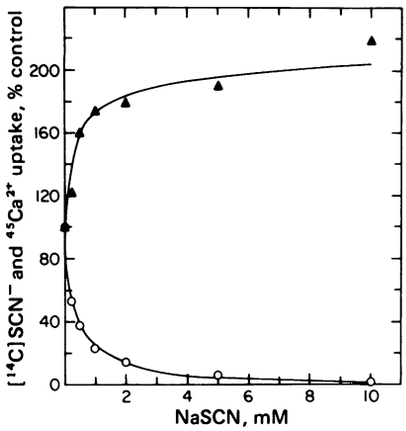


FIG. 6. Effect of $SCN^-$ on $Mg^{2+}$/ATP-dependent $[^{14}C]SCN^-$ uptake and $Mg^{2+}$/ATP-dependent $^{45}Ca^{2+}$ uptake by *Neurospora* plasma membrane vesicles. Aliquots of a membrane vesicle suspension (50 $\mu$l, containing 38 $\mu$g of protein) were preincubated for 30 sec, NaSCN was added to give the final concentrations indicated, and then ATP and $MgSO_4$ were added to give final concentrations of 10 mM in a total volume of 53 $\mu$l. Immediately thereafter, $^{45}CaCl_2$ or $[^{14}C]SCN^-$ were added as indicated. ▲, Steady-state uptake of $^{45}Ca^{2+}$. At each NaSCN concentration, uptake was measured at 7, 9, 11, and 13 min after adding 2 $\mu$l of 2.5 mM $^{45}CaCl_2$ and steady-state uptakes were determined from these data as described below, after correction for nonspecific $Ca^{2+}$ binding. Values are expressed as the percentages of the steady-state value obtained without NaSCN. ○, Steady-state uptake of $[^{14}C]SCN^-$. At each NaSCN concentration, uptake was measured at 2, 3, 4, and 7 min after adding 2 $\mu$l of 1 mM $[^{14}C]KSCN$, and steady-state uptakes were determined from these data as described below. Values are expressed as the percentages of the steady-state value obtained without NaSCN. Steady-state uptakes for $^{45}Ca^{2+}$ and $[^{14}C]SCN^-$ are defined as the maximum values of the lines of best fit plotted from the uptake data.

membrane of *Neurospora*. Functionally inverted plasma membrane vesicles isolated from a cell wall-less mutant of *Neurospora* catalyze $Mg^{2+}$/ATP-dependent $Ca^{2+}$ accumulation. Inhibitor-induced $Ca^{2+}$ efflux studies and isotope exchange experiments indicate that $Ca^{2+}$ is accumulated against a considerable concentration gradient. Comparisons of $Mg^{2+}$/ATP-driven $^{45}Ca^{2+}$ uptake and $[^{14}C]SCN^-$ uptake with respect to the $Mg^{2+}$/ATP concentration dependence, the effects of inhibitors, and the nucleotide and divalent cation specificities, indicate that the energy for $Ca^{2+}$ accumulation is derived from ATP hydrolysis catalyzed by the electrogenic plasma membrane ATPase. Because all of the available evidence (23) indicates that this enzyme is a proton-translocating ATPase, the observations that $Ca^{2+}$ accumulation is inhibited by CCCP and by $K^+$ plus nigericin, and is maximal in the presence of 10 mM $SCN^-$, indicate that $Ca^{2+}$ accumulation operates via a $Ca^{2+}/H^+$ antiporter. Because the energizable vesicles are functionally inverted, the $Ca^{2+}$ transport system must function *in vivo* as a $Ca^{2+}$ extrusion system.

Numerous energy-dependent cellular $Ca^{2+}$ extrusion systems have been reported (25–28), and in view of both the diversity of cell types now known to extrude $Ca^{2+}$ and the apparent intracellular toxicity of this ion (29), it seems likely that all cells possess a system for $Ca^{2+}$ extrusion (18, 27, 29). In many cell types, the maintenance of a low cytoplasmic concentration of $Ca^{2+}$ relative to that of the extracellular environment enables the transient entry of $Ca^{2+}$ into the cell to be used as a trigger or "second messenger" in a wide variety of cellular regulatory mechanisms (25, 27).

The discovery of an active transport system for $Ca^{2+}$ in *Neurospora* opens two important avenues for future research. First, it provides a useful system for investigating a specific molecular mechanism for ion translocation in a eukaryotic plasma membrane, and second, it raises the possibility that changes in the cytoplasmic concentrations of $Ca^{2+}$ play an important role in cellular regulatory processes in *Neurospora*.

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1. Scarborough, G. A. (1975) *J. Biol. Chem.* **250**, 1106–1111.
2. Scarborough, G. A. (1977) *Arch. Biochem. Biophys.* **180**, 384–393.
3. Bowman, B. J. & Slayman, C. W. (1977) *J. Biol. Chem.* **252**, 3357–3363.
4. Scarborough, G. A. (1976) *Proc. Natl. Acad. Sci. USA* **73**, 1485–1488.
5. Scarborough, G. A. (1978) *Methods Cell Biol.* **20**, 117–133.
6. Mitchell, P. (1966) *Biol. Rev.* **41**, 445–502.
7. Stroobant, P. & Scarborough, G. A. (1979) *Anal. Biochem.* **95**, 554–558.
8. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275.
9. Scott, W. W. (1939) in *Standard Methods of Chemical Analysis*, ed. Furman, N. H. (Van Nostrand, New York), pp. 1037–1038.
10. Harold, F. M. (1972) *Bacteriol. Rev.* **36**, 172–230.
11. Bowman, B. J., Mainzer. S. E., Allen, K. E. & Slayman, C. W. (1978) *Biochim. Biophys. Acta* **512**, 13–28.
12. Harris, E. J., Wimhurst, J. M. & Landaeta, I. (1974) *Eur. J. Biochem.* **45**, 561–565.
13. Christiansen, R. O., Loyter, A., Steensland, H., Saltzgaber, J. & Racker, E. (1969) *J. Biol. Chem.* **244**, 4428–4436.
14. Tsuchiya, T. & Rosen, B. P. (1975) *J. Biol. Chem.* **250**, 7687–7692.
15. Kobayashi, H., Van Brunt, J. & Harold, F. M. (1978) *J. Biol. Chem.* **253**, 2085–2092.
16. Inesi, G. (1972) *Annu. Rev. Biophys. Bioeng.* **1**, 191–210.
17. Belliveau, J. W. & Lanyi, J. K. (1978) *Arch. Biochem. Biophys.* **186**, 98–105.
18. Vincenzi, F. F. & Hinds, T. R. (1976) in *The Enzymes of Biological Membranes*, ed. Martonosi, A. (Plenum, New York), Vol. 3, pp. 261–281.
19. Moore, L. & Pastan, I. (1978) *Ann. N. Y. Acad. Sci.* **307**, 177–194.
20. Martonosi, A. N., Chyn, T. L. & Schibeci, A. (1978) *Ann. N. Y. Acad. Sci.* **307**, 148–159.
21. Martonosi, A. (1972) *Curr. Top. Membr. Transp.* **3**, 83–197.
22. Pressman, B. C. (1976) *Annu. Rev. Biochem.* **45**, 501–530.
23. Slayman, C. W. & Slayman, C. L. (1975) in *Molecular Aspects of Membrane Phenomena*, eds. Kaback, H. R., Neurath, H., Radda, G. K., Schwyzer, R. & Wiley, W. R. (Springer, New York), pp. 231–248.
24. Mitchell, P. (1976) *Biochem. Soc. Trans.* **4**, 399–430.
25. Scarpa, A. & Carafoli, E., eds. (1978) in *Calcium Transport and Cell Function*, Annals of the New York Academy of Sciences (New York Acad. Sci., New York), Vol. 307.
26. Silver, S. (1977) in *Microorganisms and Minerals*, ed. Weinberg, E. D. (Dekker, New York), pp. 49–103.
27. Rasmussen, H. & Goodman, D. P. B. (1977) *Physiol. Rev.* **57**, 421–509.
28. Gross, J. & Marmé, D. (1978) *Proc. Natl. Acad. Sci. USA* **75**, 1232–1236.
29. Kretsinger, R. H. (1978) in *Membrane Transport Processes*, eds. Tosteson, D. C., Ovchinnikov, Yu. A. & Latorre, R. (Raven, New York), Vol. 2, pp. 253–260.