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Calcium Uptake and Membrane Potential in Mitochondria†

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ABSTRACT: The effect of uncouplers and the ionophores A23187, X-537A, nigericin, and valinomycin on calcium concentration gradients in rat liver mitochondria has been investigated. The calcium gradients under various conditions were compared with rubidium concentration gradients. Calcium concentration gradients were estimated from the extent of uptake as measured by changes in murexide absorption or by ^{45}Ca distribution and the matrix water content, under conditions in which Ca^{2+} binding and internal precipitation inside mitochondria are minimized. All the tested ionophores in very high concentration cause release of calcium. However, in low concentrations A23187 is without effect, valinomycin causes Ca^{2+} release (which depends on external potassium), and nigericin increases the rate and extent of Ca^{2+} uptake. This stimulation is observed only at low nigericin concentrations, low external calcium concentrations, and in the absence of anions such as acetate or phosphate. In the presence of valinomycin, $^{86}Rb^+$ distribution shows good correlation with Ca^{2+} distribution over a wide range of values which were obtained by varying uncoupler concentration or potassium concentration. The Ca^{2+} concentration ratio is always higher than the Rb^+ ratio, approximately obeying the relation $\log([Ca^{2+}]_{in}/[Ca^{2+}]_{out}) = 2 \log([Rb^+]_{in}/[Rb^+]_{out})$. Assuming that rubidium distribution in the presence of valinomycin is governed by membrane potential obeying the Nernst equation, these results are interpreted as evidence that calcium uptake in mitochondria is an electrogenic process driven by membrane potential with a net charge transfer of 2.

The reaction of calcium ions with mitochondria has been the subject of investigation from various standpoints for over a decade. Mainly from the studies of the groups of Lehninger (Lehninger *et al.,* 1967) and Chance (Chance, 1965), it is now well established that Ca^{2+} accumulation by mitochondria is an energy-linked process in which the energy can be derived from electron transport or ATP hydrolysis. More recently, attention has been focused on the existence of a specific Ca^{2+} carrier localized in the inner membrane of mitochondria. The Ca^{2+} binding properties of the proposed carrier (Reynafarje and Lehninger, 1969), its inhibition by lanthanides (Mela, 1968), and its kinetic behavior (Vinogradov and Scarpa, 1973) have been studied in great detail.

However, the nature of the driving force for the Ca^{2+} accumulation against concentration gradients in mitochondria has not been established. Several models have been suggested to describe the coupling between the energy producing metabolic reactions and the energy utilizing transport process. The earlier models were based on direct coupling between the metabolic reactions and the transport process, thus forming a calcium pump (Chance, 1965). However, various other models were considered in which the coupling is not direct but mediated through the transport of other ions, either directly or electrically (for recent discussion, see Lehninger, 1973).

A gradient of major importance, which is formed in energized mitochondria, is that of the proton electrochemical potential which is composed of a membrane potential ($\Delta\psi$) and a concentration gradient (ΔpH). An important role for this gradient for energy conversion in mitochondria has

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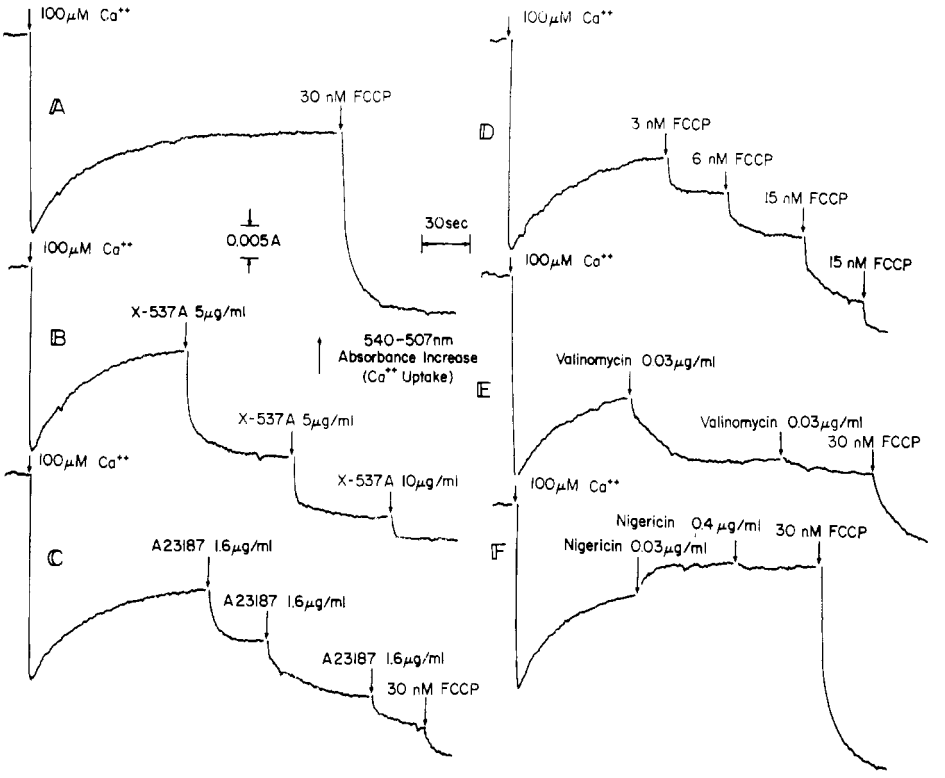

FIGURE 1: The effect of FCCP and the ionophores X-537A, A23187, valinomycin, and nigericin on calcium release from rat liver mitochondria. The reaction mixture contained 50 mM KCl, 100 mM sucrose, 10 mM Mops (pH 7.2), 5 mM sodium succinate, 1 μM rotenone, 3 mM $MgCl_2$, 100 μM murexide, and 0.8 mg/ml of mitochondrial protein. The other additions are indicated in the figure. Temperature, 24°.

been suggested by Mitchell (1961, 1966), and its possible role in ion movements in mitochondria and submitochondrial particles was discussed by Chance and Montal (1971). It has been proposed that the driving force in the uptake of monovalent cations is the membrane potential which is formed in energized mitochondria (Mitchell, 1968; Rottenberg, 1973). It has been recently shown (Rottenberg, 1973) that, when the membrane becomes permeable to both sodium and potassium (as in the presence of gramicidin), the concentration gradients of these cations in steady state are identical, suggesting that both gradients are being held by equivalent membrane potential.

In the present study, we present measurements of calcium concentration gradients in mitochondria as affected by uncouplers and ionophores and we compare these gradients with rubidium gradients. A careful use of uncouplers and ionophores resulted in either a stimulation of Ca^{2+} release or uptake, which could be correlated with the expected effect of these compounds on energization, ΔpH, and membrane potential. The results of these studies are interpreted as an indication that calcium uptake by mitochondria is driven by a membrane potential and that the net charge transfer during this electrogenic process is 2.

Materials and Methods

Mitochondria were prepared from rat liver homogenates, as described previously (Vinogradov *et al.*, 1972) in an isolation medium containing 0.25 mM sucrose and 1 mM sodium ethylenediaminetetraacetate (pH 7.2). The same medium was used for the first two washings and a 0.25 M sucrose solution, which was deionized and contained less than 0.2 μM Ca^{2+}, was used for the last washing and final suspension of mitochondria. Calcium content in this preparation ranged from 10 to 15 nmol of calcium/mg of protein. Protein was measured by the biuret method with crystalline bovine serum albumin used as a standard.

Ca^{2+} uptake and release by isolated mitochondria were measured either radiochemically or spectrophotometrically. In the latter case, kinetics of Ca^{2+} movements were followed by recording the absorption changes of the metallochromic indicator murexide through an Aminco DW2 dual wavelength spectrophotometer (540–507 nm), as described previously (Scarpa, 1972).

Water content (^3H_2O) and $^{45}Ca^{2+}$ and $^{86}Rb^+$ distribution were calculated from the relative activities of these isotopes in the pellets and supernatants after centrifugation of mitochondria through a Coleman 8-611 desk microcentrifuge. ^{14}C-Sucrose distribution was used to correct the water volume of the total pellet from that of the nonmatrix space. This analysis was carried out as described previously in great detail (Padan and Rottenberg, 1973), except that the pellet was dissolved overnight in 2 N formic acid.

Calcium-45 (18 Ci/g), rubidium-86 (5 Ci/g), tritium-labeled water (1 mCi/g), and [^{14}C(U)]sucrose (3 Ci/mol) were purchased from New England Nuclear, Boston, Mass. Murexide was obtained from K and K Chemicals, Plainview, N. J., and was crystallized twice. Valinomycin was from Calbiochem, Los Angeles, Calif., and rotenone from Sigma Co., St. Louis, Mo. FCCP[1] (carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone) was a gift from Dr. P. G. Heytler of Du Pont Co., Wilmington, Del.; X-537A and nigericin were from Dr. J. Berger of Hoffman-La Roche, Nutley, N. J.; A23187 was from Dr. R. Hamill of Eli Lilly and Co., Indianapolis, Ind. All the ionophores were dissolved in ethanol and the volume of ethanol added for each experiment did not exceed 3 μl/ml of reaction mixture.

[1] Abbreviations used are: FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; Mops, morpholinopropanesulfonic acid.

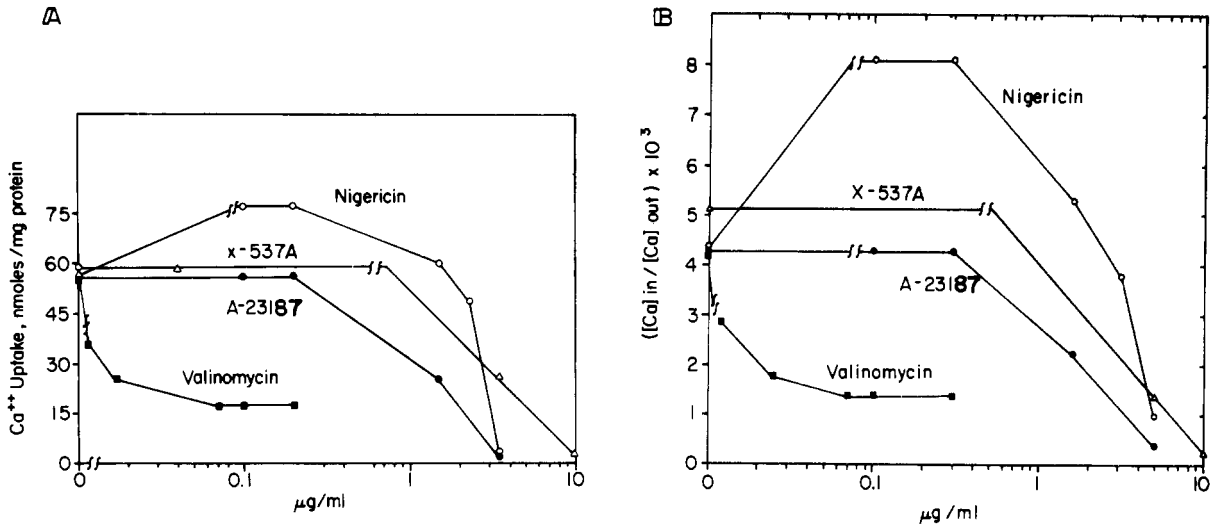

FIGURE 2: The effect of various ionophore concentrations on Ca^{2+} accumulation (A) and concentration gradients (B). The reaction mixture was identical with that of Figure 1. The calculation of calcium gradient is based on the concentration of calcium in the reaction mixture in steady state, the extent of Ca^{2+} uptake, and the water content of the mitochondrial matrix.

Results

The Effect of Ionophores on Mitochondrial Calcium Uptake and Release. The fact that the internal concentration of Ca^{2+} within energized mitochondria is much higher than the external Ca^{2+} concentration does not necessarily imply that the extent of Ca^{2+} uptake is limited by the concentration gradients. Mitochondria can reduce the external concentration of calcium ions to very low levels. In the presence of inorganic phosphate, the extent of mitochondrial Ca^{2+} uptake reaches very high values; however, under this condition, the extent of calcium uptake is not directly related to $[Ca^{2+}]$ gradients since the accumulation of large amounts of Ca^{2+} is accounted for by the internal precipitation of insoluble calcium salts (Greenawalt *et al.*, 1964). However, at relatively high external Ca^{2+} (about 1×10^{-4} M) and in the absence of phosphate, there is a limited extent of Ca^{2+} uptake, the rate of which depends on the Ca^{2+} concentration present in the reaction mixture. Since at this external $[Ca^{2+}]$ the rate of uptake is not limited by the carrier affinity (Vinogradov and Scarpa, 1973), the extent of Ca^{2+} uptake may be determined by the Ca^{2+} concentration gradients. Under these conditions, the calcium bound inside mitochondria may constitute a sizable fraction of the mitochondrial calcium. However, the fraction of calcium bound can be minimized by addition of an anion such as acetate which increases considerably the extent of uptake with no, or limited, internal precipitation. The fact that the mitochondria swell under these conditions indicates that the internal calcium acetate concentration can reach a very high value, similar in magnitude to the osmolarity of the medium. Furthermore, calcium aspecifically bound to mitochondria can be inhibited by the presence of Mg^{2+}, which competes with Ca^{2+} for the energy independent Ca^{2+} binding sites in mitochondria without interfering with the energy-linked Ca^{2+} transport (Scarpa and Graziotti, 1973).

Figure 1 shows several traces of Ca^{2+} uptake by rat liver mitochondria obtained by recording the changes in absorbance of the dye murexide using dual wavelength spectroscopy. The addition of $CaCl_2$ to mitochondria oxidizing succinate in the absence of other permeant anions produces an abrupt decrease in absorbance at 540 nm, due to the formation of the calcium–murexide complex. This absorbance decrease was followed by a slow increase in absorbance, related to the energy-dependent Ca^{2+} uptake by mitochondria. The content of mitochondria in the medium was adjusted to low levels, so that steady-state levels of Ca^{2+} accumulation were obtained when about half of the Ca^{2+} added was taken up by the mitochondria. When large concentrations of the uncoupler FCCP were added, all of the Ca^{2+} accumulated by mitochondria was released (Figure 1A). Figure 1B shows that a partial release of accumulated Ca^{2+} was obtained by the ionophore X-537A. Relatively high concentrations of X-537A are needed for this effect. Figure 1C shows the release of Ca^{2+}, induced by the divalent cation ionophore A23187. Here again, high concentrations are needed for the release; lower concentrations had no effect (see Figure 2). A gradual release of Ca^{2+} was also observed after addition of low concentrations of the uncoupler FCCP (Figure 1D) and at various concentrations of FCCP there was a well-defined steady-state level of Ca^{2+} accumulation. The fact that the mitochondrial Ca^{2+} content is related to the concentrations of FCCP suggests again that under these conditions, the extent of uptake is determined by an energetic factor, *i.e.*, the Ca^{2+} concentration gradients. Valinomycin (in the presence of 50 mM KCl) partially releases the accumulated Ca^{2+}, and this effect was saturated at very low valinomycin concentrations. Under such conditions, complete release was obtained after addition of FCCP (Figure 1E). In contrast, nigericin at low concentrations produces an additional accumulation of Ca^{2+}, so that higher steady-state levels of Ca^{2+} accumulation are obtained (Figure 1F).

Figure 2A summarizes the effect of various ionophore concentrations on the steady-state of Ca^{2+} accumulation by mitochondria. It is interesting that low concentrations of A23187 are without effect on steady-state levels of calcium accumulation. Since it is known that these concentrations of A23187 facilitate equilibration of Ca^{2+} gradients in mitochondria (Reed and Lardy, 1972), it appears that the efflux of Ca^{2+} induced by the ionophore is compensated for by increased Ca^{2+} uptake of mitochondria, indicating that under these conditions the rate of Ca^{2+} uptake is controlled by the Ca^{2+} concentration gradients. When the A23187 concentrations are increased, the Ca^{2+} transport mechanism of mitochondria cannot cope with Ca^{2+} efflux induced by the

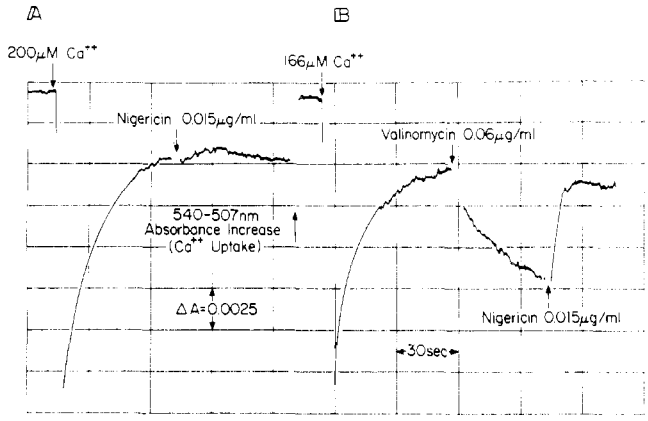

FIGURE 3: The effect of nigericin on calcium uptake. In Figure 3A the medium was as in Figure 1, except that 5 mM NaOAc was also present and the mitochondrial content was 0.75 mg of protein/ml. In Figure 3B the conditions were as in Figure 1 except for mitochondrial content which was 1.5 mg of protein/ml.

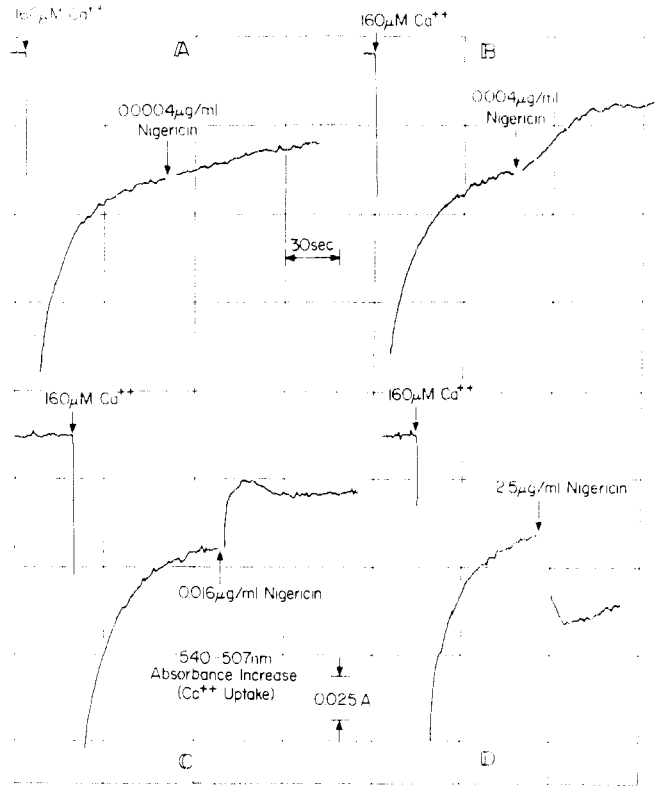

FIGURE 4: The dependence of the rate and extent of calcium uptake on nigericin concentration. Experimental conditions were the same as in Figure 3B.

ionophore, and there is a net release of Ca^{2+}. X-537A, another antibiotic which forms lipophilic complexes with Ca^{2+} and facilitates Ca^{2+} diffusion across membranes (Scarpa *et al.*, 1972; Pressman, 1973), at high concentrations prompts Ca^{2+} release from mitochondria. However, in this case, the lack of specificity of X-537A renders more complicated the interpretation of the results. On the other hand, valinomycin, which has no effect on Ca^{2+} movements *per se*, was able to release Ca^{2+} from mitochondria in the presence of potassium. Since valinomycin is known to affect mainly the membrane potential in a manner which is K^+ dependent (Rottenberg, 1970), it is reasonable to assume that the effect of valinomycin on calcium distribution is due to reduction of membrane potential. Low concentrations of nigericin, which markedly reduce mitochondrial ΔpH and apparently increase membrane potential, stimulate Ca^{2+} uptake and increase the intra-/extramitochondrial Ca^{2+} distribution ratio. Since nigericin has no direct effect on Ca^{2+} transport across the mitochondrial membrane, the increased steady states of Ca^{2+} accumulation must be due to an increase in membrane potential or a decrease in ΔpH, or both. If one tentatively assumes that the Ca^{2+} accumulated is free within the mitochondria, then under steady-state conditions of Ca^{2+} accumulation, the intra-extramitochondrial calcium distribution can be calculated. This is shown in Figure 2B. The values take into account the water content of the mitochondrial matrix which was measured separately as described in Materials and Methods. The calculation shows that the Ca^{2+} distribution ratio in steady-state conditions may reach very high values (8000 in the experiments reported and probably higher when Ca^{2+} added is lower). This ratio is significantly higher than the ratio obtained for monovalent cation distribution, which is around 100.

The effect of various ionophores on Ca^{2+} uptake and release was also studied in reaction mixtures containing 1 mM NaOAc. In the presence of a permeant acid, the mitochondria accumulate larger amounts of Ca^{2+}. Therefore, the amount of Ca^{2+} added was increased and the amount of mitochondria decreased, so that complete uptake of Ca^{2+} by mitochondria was prevented and the level of steady-state Ca^{2+} could be measured. Since under such conditions the mitochondria undergo extensive measurable swelling, the final intramitochondrial Ca^{2+} concentration and the Ca^{2+} concentration gradient are not significantly different from those observed in the absence of acetate. Figure 3A shows that the addition of nigericin does not stimulate additional Ca^{2+} uptake in the presence of acetate. Figure 3B shows that, in the absence of acetate, nigericin is effective even in the presence of valinomycin and can induce reaccumulation of the Ca^{2+} previously released by valinomycin.

Figure 4 shows the effect of various nigericin concentrations after Ca^{2+} uptake had reached steady-state levels. Although the extent of additional Ca^{2+} uptake induced by addition of nigericin was similar (see also Figure 2), the rate of the additional Ca^{2+} uptake was dependent on nigericin concentrations. Only a very high concentration of nigericin produces a release of accumulated Ca^{2+}, and this effect may be related to the reported uncoupling of mitochondria by these concentrations of nigericin (Ferguson *et al.*, 1971).

Figure 5 shows the effect of nigericin at various concentrations of added Ca^{2+}. Although the amount of Ca^{2+} accumulated per milligram of mitochondrial protein was similar, the steady-state concentration ratio decreases and the effect of nigericin is diminished when extramitochondrial $[Ca^{2+}]$ is high.

Comparison of Calcium and Rubidium Concentration Gradients. The distribution of monovalent cations in mitochondrial suspensions, in the presence of an ionophore that induces electrogenic transfer, is an equilibrium distribution in which the concentration gradient is balanced by a membrane potential according to the Nernst equation (Rottenberg, 1973). Therefore, a comparison of the distribution of rubidium, in the presence of valinomycin, with calcium distribution could provide quantitative information concerning the relation between membrane potential and calcium transport. In order to minimize the binding and internal precipitation of accumulated calcium, the experiments were carried out in the presence of acetate. Acetate increases the

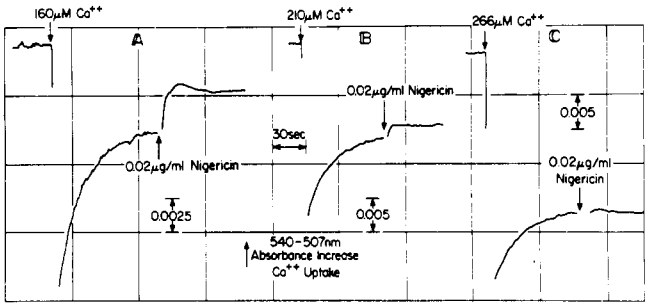

FIGURE 5: The dependence of the nigericin-induced calcium uptake on external calcium concentration. Conditions were the same as in Figure 3B.

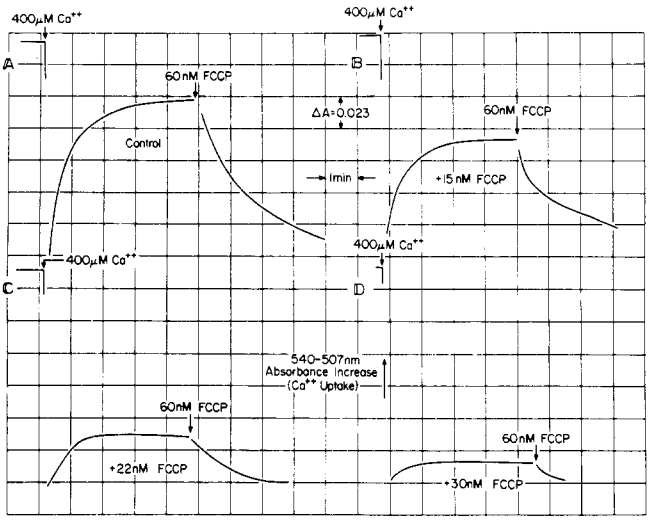

FIGURE 6: The dependence of the extent of calcium uptake on FCCP concentration. The medium was composed of 200 mM sucrose, 5 mM sodium succinate, 1 μM rotenone, 3 mM $MgCl_2$, 1 mM acetate, 10 mM Mops (pH 7.2), 5 mM KCl, 0.5 μg/ml of valinomycin, and 2.5 mg of mitochondrial protein/ml. The medium was saturated with oxygen prior to addition of mitochondria. In B, C, and D, the indicated concentrations of FCCP were added prior to the addition of calcium.

extent of calcium uptake, thus reducing the fraction of bound calcium, and reduces the internal pH, thus reducing the possibility of internal precipitation. Furthermore, the reaction mixture contained potassium and valinomycin, which are effective in diminishing the intramitochondrial Ca^{2+} accumulation. Under these conditions, the energy-dependent Ca^{2+} uptake and the subsequent release are shown in Figure 6A. In Figures 6B, C, and D, increasing concentrations of FCCP were added before addition of Ca^{2+}, resulting in a decrease in steady-state levels of Ca^{2+} accumulation. From these results and the parallel determination of water content of the mitochondrial matrix, the concentration ratio of calcium was calculated. In a parallel experiment, the distribution of ^{86}Rb between the mitochondrial matrix and the surrounding medium was measured after rapid centrifugation of the mitochondria and determination of the water content of the matrix (obtained through ^3H_2O space corrected for the nonmatrix space as measured by [^{14}C]sucrose. The results of these experiments are summarized in Table I, experiment 1, where the intra-/extramitochondrial calcium and rubidium ratios were measured in the presence of various FCCP concentrations. The calcium ratio falls from 230 to 12.6, whereas the rubidium ratio, which was smaller in all the conditions, decreases from 13.3 to 4.01. The two ratios are approximately related by the

TABLE I: Comparison of Rubidium and Calcium Distribution.

Medium[a]	$[^{86}Rb]_{in}/[^{86}Rb]_{out}$	Log Ratio	$\Delta\psi^b$	$[Ca]_{in}/[Ca]_{out}$	Log Ratio	$\Delta\psi^c$	Comments
Experiment I							
Control	13.3	1.24	67	230	2.36	71	Ca^{2+} distribution was calculated from the expt of Figure 6
+15 nM FCCP	5.87	0.77	46	95.2	1.98	59	
+22 nM FCCP	5.20	0.72	43	41.8	1.62	49	
+30 nM FCCP	4.01	0.60	36	12.6	1.10	33	
Experiment II							
1 mM KCl	42.0	1.62	97	2050	3.31	99	Ca^{2+} distribution was calculated from ^{45}Ca measurements
2 mM KCl	39.0	1.59	95	1810	3.26	97	
5 mM KCl	25.6	1.40	84	510	2.71	81	
Experiment III							
5 mM KCl	46	1.66	100	1840	3.26	98	Ca^{2+} distribution was determined as in expt II
10 mM KCl	18	1.25	75	322	2.50	75	

[a] Experiment I: The reaction mixture was similar to that of Figure 6, except that ^{86}Rb and ^3H_2O and 400 μM calcium were added to the mitochondrial suspension before precipitation. Mitochondrial preparation and concentration were identical with that of the experiment of Figure 6. Determination of matrix water volume was carried out in a parallel experiment under the same experimental conditions in the presence of [^{14}C]sucrose and ^3H_2O. Experiment II: Experimental conditions were the same as for experiment I except that the potassium concentration was varied as indicated and a parallel experiment with ^{45}Ca and ^3H_2O was also included. Experiment III: Experimental conditions were similar to those of experiment II, except that calcium concentration was 100 μM. [b] $\Delta\psi = (RT/F) \ln ([Rb]_{in}/[Rb]_{out})$. [c] $\Delta\psi = (RT/2F) \ln ([Ca]_{in}/[Ca]_{out})$.

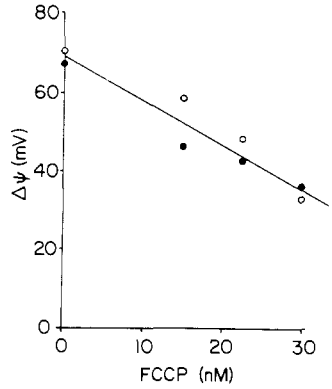

FIGURE 7: The effect of FCCP on membrane potential as calculated from rubidium and calcium distributions. Data for this figure are taken from Table I. Membrane potential was calculated from rubidium distribution (●) according to the equation $\Delta\psi = (RT/F)$ ln $([Rb]_{in}/[Rb]_{out})$ and from the calcium distribution (○) according to the equation $\Delta\psi = (RT/2F)$ ln $([Ca]_{in}/[Ca]_{out})$.

equation log $([Ca^{2+}]_{in}/[Ca^{2+}]_{out})$ = 2 log $([Rb^{+}]_{in}/[Rb^{+}]_{out})$. Since we assume that the rubidium ratio is governed by the membrane potential in accordance with the relation $\Delta\psi = (RT/ZF)$ ln (C_{in}/C_{out}), we can calculate membrane potential from these distributions. The same relation should hold true if the calcium transport is a simple electrogenic process. Since in this case $Z = 2$, for the same membrane potential the log of the Ca ratio should be twice the log of the rubidium ratio. This prediction was verified experimentally. Table I shows the membrane potential as calculated from rubidium distribution compared with that calculated from the calcium ratio (in which $Z = 2$). Figure 7 shows that a good agreement exists between the calculations for Ca^{2+} and Rb^{+}. The slightly higher values obtained from calcium distribution might be accounted for by the higher binding of Ca^{2+}, since no correction for binding was done in the calculations. Table I also shows that the same relation holds when the ratios are varied by changing the potassium concentration (Experiments II and III). In this experiment, the Ca ratio was calculated from the distribution of ^{45}Ca instead of from murexide absorption.

Discussion

The Effect of Ionophores on Ca Concentration Gradients in Energized Mitochondria. The interpretation of our results is based partly on the assumption that a large part of the Ca^{2+} accumulated by energized mitochondria is free in solutions. The fact that Ca^{2+} uptake in the presence of acetate and in a reaction mixture of several hundred milliosm produces an osmotic swelling of mitochondria indicates that the concentration of calcium acetate inside mitochondria must reach values of 10^{-1} M. Since this occurs at outside Ca^{2+} concentrations of 10^{-4} M, the intra-/extramitochondrial concentration ratio must reach high values. Although general agreement exists in the literature indicating that the Ca^{2+} accumulated in the presence of acetate is in solution within the mitochondria, only a limited amount of data is available on the state of Ca^{2+} accumulated in the absence of permeant anions. Results based on the electron paramagnetic resonance spectrum of the accumulated Mn^{2+} (Gunter and Puskin, 1972) and on sonication of mitochondria after Ca^{2+} uptake (Carafoli *et al.*, 1967) have suggested that, in the absence of permeant anions, Ca^{2+} may be bound to some energy-dependent sites. However, the indirect experimental approach may cast some doubts on these conclusions, and the evidence for Ca^{2+} binding to energy-dependent sites different from carrier sites appears inconclusive.

In the absence of acetate, there is no swelling, but the intra-/extramitochondrial Ca^{2+} ratio may reach values similar to those in the presence of acetate. In the absence of acetate, the ΔpH is quite high and succinate, which can be concentrated inside the mitochondria, can serve as a permeant acid (Rottenberg, 1973). The osmolarity of Ca^{2+} succinate is ⅔ that of $Ca(OAc)_2$ and this might account for the difference in swelling which in turn is responsible for the smaller extent of uptake. These considerations and the effect of various ionophores on the steady state of Ca^{2+} accumulation suggest a great part of the Ca^{2+} accumulated in the absence of permeant anions being free in solution rather than bound.

The addition of FCCP produces a graded release of the accumulated Ca^{2+} and the incubation of mitochondria with various concentrations of FCCP yields different steady-state levels of Ca^{2+} accumulation. These results only indicate that the concentration ratio of Ca^{2+} inside and outside the mitochondria depends on the coupling of mitochondria. On the other hand, the effect of various ionophores on Ca^{2+} uptake and release offers insight into the mechanisms responsible for Ca^{2+} accumulation against concentration gradients.

It was observed that A23187 releases Ca^{2+} only at very high concentrations, whereas very low concentrations of valinomycin and nigericin stimulate Ca^{2+} release and Ca^{2+} uptake, respectively. A23187 forms specific hydrophobic complexes with divalent cations and is known to render mitochondrial membranes permeable to Ca^{2+} (Reed and Lardy, 1972). The fact that in energized mitochondria Ca^{2+} uptake is not affected by concentrations of A23187 which induce Ca^{2+} permeability indicates that, at steady-state levels of Ca^{2+} accumulation, the mitochondrial Ca^{2+} accumulation is limited by the concentration gradients. As long as the efflux mediated by the ionophore is limited and neutral, the carrier would be able to increase Ca^{2+} transport in order to maintain the concentration gradient that can be held by membrane potential. Since A23187 is supposed to catalyze a neutral Ca^{2+}–$2H^{+}$ exchange, its ability to release Ca^{2+} when added in high concentrations indicates that Ca^{2+} transport of mitochondria is driven by a force which does not directly affect the process mediated by the ionophore (for instance, electrical potential).

The finding that valinomycin plus potassium causes release of Ca^{2+} accumulated by mitochondria also indicates that Ca^{2+} uptake is driven by membrane potential. Since valinomycin does not directly transport Ca^{2+} across the biological membrane, its effect on energized mitochondria in the presence of K^{+} is best explained as resulting from a reduction of membrane potential (Rottenberg, 1970, 1973). The observation (Scarpa and Azzone, 1970) that calcium accumulation by respiration-inhibited mitochondria can be driven by potassium release induced by valinomycin can be similarly explained as resulting from a diffusion potential produced by the efflux of potassium.

Finally, nigericin in concentrations which are known to promote K^{+}–H^{+} exchange (Pressman, 1968) with consequent reduction of ΔpH and a slight increase of membrane potential (H. Rottenberg, unpublished observation) is shown to stimulate calcium uptake by mitochondria. The effectiveness of nigericin in partially converting ΔpH to $\Delta\psi$ is quite sensitive to the experimental conditions, and this ac-

counts for its lack of effect in increasing steady state of Ca^{2+} accumulation when acetate or high Ca^{2+} concentrations are present. Acetic acid accumulation considerably reduces the ΔpH consequent to Ca^{2+} accumulation and thus reduces the effectiveness of the ΔpH–$\Delta\psi$ conversion. On the other hand, high concentrations of Ca^{2+} probably decrease membrane resistance, thus limiting the enhancement of membrane potential. Thus, the effect of nigericin on Ca^{2+} uptake could be due to a decrease of ΔpH, an increase of $\Delta\psi$, or both. The release of Ca^{2+} observed in the presence of high concentrations of nigericin is probably an aspecific phenomenon which can be accounted for by the reported decrease of membrane potential and even the uncoupling of mitochondria at high nigericin concentrations. Likewise, the effect of X-537A is hard to interpret since the ionophore has poor ionic specificity (Pressman, 1973) and therefore its effect on Ca^{2+} movements cannot be discriminated from that of other cations.

Comparison of Rb^+ and Ca^{2+} Distribution. Valinomycin is known to catalyze electrogenic transfer of potassium and rubidium ions across various biological membranes, although the explanation of its effect on ion transport in mitochondria is still controversial (Pressman, 1970; Massari and Azzone, 1970; Rottenberg, 1973). If one accepts the interpretation that valinomycin effect in mitochondria is no different from its effect on other biological or artificial membranes, then membrane potential can be estimated from rubidium distribution in the presence of valinomycin as expressed by the Nernst equation. The relation between membrane potential and calcium distribution would depend on the mechanism of the carrier-mediated calcium transport. The fact that there is a very clear correlation between the calculated membrane potential and the Ca distribution ratio (together with the results of the effects of the ionophores) indicates that the calcium transport is an electrogenic process. Moreover, the fact that approximately the same values of ψ are calculated from Ca distribution by taking $Z = 2$ indicates that the net charge transfer associated with this process is 2. The calculations of $\Delta\psi$ presented in Table I were obtained from experiments carried out in the presence of valinomycin, potassium (1–10 mM), and calcium (0.1–0.4 mM). The values of $\Delta\psi$ (in the absence of FCCP) range from 100 to 70 mV, depending on the potassium and calcium concentrations. The experiments II and III of Table I show that both potassium in the presence of valinomycin and calcium reduce $\Delta\psi$. In the absence of valinomycin, the $\Delta\psi$ calculated from intra-/extramitochondrial ratios ranges from 110 to 120 mV. These values are still somewhat lower than the 120–140 mV previously observed and this may be accounted for by the relatively high concentration of Ca^{2+} present (100 μM). At lower extramitochondrial Ca^{2+} concentrations, reliable measurements of Ca^{2+} distribution ratios are more difficult to obtain.

In conclusion, the results indicate that calcium transport is an electrogenic process with a net charge transfer of 2. This can be accounted for either by a mitochondrial membrane which is highly permeable to Ca^{2+} (Selwyn *et al.*, 1970) or by the operation of an uncharged carrier for Ca^{2+} with no compulsory exchange with other anions. However, more complicated models for Ca^{2+} transport presented in the literature can be accommodated by the net charge transfer with or without modifications.

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