Evidence for More than One Ca^{2+} Transport Mechanism in Mitochondria†

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ABSTRACT: The active transport and internal binding of the Ca^{2+} analogue Mn^{2+} by rat liver mitochondria were monitored with electron paramagnetic resonance. The binding of transported Mn^{2+} depended strongly on internal pH over the range 7.7–8.9. Gradients of free Mn^{2+} were compared with K^+ gradients measured on valinomycin-treated samples. In the steady state, the electrochemical Mn^{2+} activity was larger outside than inside the mitochondria. The observed gradients of free Mn^{2+} and of H^+ could not be explained by a single "passive" uniport or antiport mechanism of divalent cation transport. This conclusion was further substantiated by observed changes in steady-state Ca^{2+} and Mn^{2+} distributions induced by La^{3+} and ruthenium red. Ruthenium red reduced total Ca^{2+} or Mn^{2+} uptake, and both inhibitors caused release of divalent cation from preloaded mitochondria. A model is proposed in which divalent cations are transported by at least two mechanisms: (1) a passive uniport and (2) an active pump, cation antiport or anion symport. The former is more sensitive to La^{3+} and ruthenium red. Under energized steady-state conditions, the net flux of Ca^{2+} or Mn^{2+} is inward over (1) and outward over (2). The need for more than one transport system in regulating cytoplasmic Ca^{2+} is discussed.

Two classes of models have been proposed to describe respiration-linked divalent cation uptake in mitochondria. In the chemically coupled (pump) models, transport proceeds against an electrochemical gradient, and is driven by a coupling of transport to the stoichiometric dissipation of a high energy chemical intermediate generated from electron transport (Chance, 1965). In the second class of models Ca^{2+} moves "passively" in response to other ion gradients and/or a membrane potential, established through active proton extrusion and other passive ion movements (Mitchell, 1968). The passive models can be subdivided into: (a) the movement of Ca^{2+} alone in its fully charged form (electrogenic uniport), (b) the exchange of Ca^{2+} for a K^+ or H^+ ion (electrogenic antiport), and (c) the exchange of Ca^{2+} for two monovalent cations (neutral antiport). Other passive models involving, e.g., exchange for Mg^{2+}, are unlikely to be the primary mode for uptake (Lehninger, 1972).

Passive uniport down an electrochemical gradient appears to play the dominant role in Ca^{2+} uptake by mitochondria. First of all, this contention is supported by considerable evidence that respiring mitochondria maintain a sizeable inside-negative potential gradient across the inner membrane. This evidence includes: (a) the ability of mitochondria to take up lipophilic cations but not lipophilic anions, while the situation is reversed with inside-out particles (for review, see Skulachev, 1972); (b) the ability of nitrate and thiocyanate to penetrate inhibited, but not respiring, mitochondria (Mitchell and Moyle, 1969b; Lehninger, 1974); (c) the distribution of K^+ (or Rb^+) ions in the presence of valinomycin (Pressman, 1965; Mitchell and Moyle, 1969a; Rottenberg, 1973, 1975). Valinomycin is generally believed to create a passive uniport for K^+ across the inner mitochondrial membrane (see Discussion). Under this assumption, and neglecting binding and activity coefficient corrections, the potential difference in the presence of valinomycin is given by the expression:

$$\Delta\psi = (60\ \text{mV}) \log \{[K^+]_{in}/[K^+]_{out}\}^{-1}$$

where $\Delta\psi$ is taken to be $\psi_{out} - \psi_{in}$. Membrane potentials, estimated in this way on energized mitochondrial suspensions, tend to fall in the range of 100–200 mV (for review, see Rottenberg, 1975). Evidence for either a passive Ca^{2+} uniport or Ca^{2+}/K^+ antiport has been obtained by Selwyn et al. (1970) through observations of calcium uptake by nonenergized mitochondria in the presence of various anions. The relevance of this finding to energized conditions is strengthened by the fact that small amounts of Pr^{3+}, a specific inhibitor of active Ca^{2+} transport in mitochondria, also blocked uptake in the inhibited samples. Since active transport of divalent cations is not accompanied by efflux of K^+ (Lehninger, 1972), passive uniport is more likely to be the dominant uptake mechanism than is K^+ antiport.

Further support for electrogenic uniport has been provided by Rottenberg and Scarpa (1974), who measured steady-state Ca^{2+} and K^+ gradients in respiring, valinomycin-treated mitochondria. As expected with independent uniport mechanisms of K^+ AND Ca^{2+} transport, the ratio $[Ca^{2+}]_{in}/[Ca^{2+}]_{out}$ was found nearly equal to $[K^+]_{in}^2/[K^+]_{out}^2$ over a range of conditions.

The data of Rottenberg and Scarpa suggest that Ca^{2+} ions may distribute themselves across the membrane so as to reach equal electrochemical activities on the two sides, as expected from a uniport model of transport. However, the finding that ruthenium red induces release of Ca^{2+} from mitochondria is in conflict with this simple picture and has led to the suggestion that there are two separate Ca^{2+} transport systems in mito-

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[1] Abbreviations used are: EPR, electron paramagnetic resonance; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N'*-tetraacetic acid; CCCP, *m*-chlorocarbonyl cyanide phenylhydrazone; ADP, adenosine 5'-diphosphate. When enclosing the chemical symbol for an ion, brackets indicate concentration, parentheses indicate chemical activity, and braces indicate electrochemical activity.

chondria, only one of which is ruthenium red sensitive (Sordahl, 1974).

In this paper we explore the characteristics of steady state cation gradients in energized mitochondria. Mn^{2+} is taken up by mitochondria over the Ca^{2+} transport system (Vainio et al., 1970). The characteristics of Mn^{2+} uptake by mitochondria are generally very similar to those of Ca^{2+} uptake. Mn^{2+} uptake is slower than Ca^{2+} uptake but is activated by small amounts of Ca^{2+} in the medium. Using Mn^{2+} as a paramagnetic Ca^{2+} analogue (Chappell et al., 1963; Gunter and Puskin, 1972), EPR measurements of free Mn^{2+} concentration gradients were made on valinomycin-treated mitochondria and compared with K^+ and Rb^+ gradients measured on the same samples. The EPR technique helps to eliminate errors due to internal binding or precipitation of the divalent cation (Puskin and Gunter, 1973; Gunter and Puskin, 1975). Second, the influence of inhibitors, especially ruthenium red, on steady-state divalent cation gradients was investigated further. Both sets of results are shown to be inconsistent with a passive electrogenic uniport system acting alone. Instead, the evidence from this and other studies favors: (a) a passive uniport tending to pull Ca^{2+} in along an electrochemical activity gradient and (b) another system(s) that can operate to transport ions outward against the activity gradient.

Materials and Methods

Rat liver mitochondria were prepared as described by Schnaitman and Greenawalt (1967) except that 1 mM EDTA was included in the homogenization step. The mitochondria were resuspended at about 15 mg/ml in 135 mM mannitol, 45 mM sucrose, 15 mM sodium succinate, 24 mM Na-Hepes (pH 7.2), and 1.5 mg/ml of bovine serum albumin. Respiratory control ratios were found to lie in the range 6.5–8. Protein was assayed with the biuret method.

Valinomycin and CCCP were dissolved and added to samples in ethanol (6.25 μl/ml of sample). Ruthenium red stock solutions were prepared by adding the crude product obtained from Sigma to water. Only the soluble portion was kept and diluted, but the nominal concentrations employed were calculated directly from the grams of dry powder/ml of water.

Measurements of free Mn^{2+} gradients were carried out on a Varian E12 EPR spectrometer using dual cavity techniques to obtain more accurate quantitation (Gunter et al., 1975). An aliquot of the mitochondrial preparation was pipetted into a test tube containing the proper bathing solution. Each sample contained 15 mM Na-Hepes (pH 7.2), 10 mM sodium succinate, 1 mg/ml of bovine serum albumin, varying amounts of NaOAc and KCl, 1 mM $MgCl_2$ to reduce external K^+, Ca^{2+}, or Mn^{2+} binding (Vinogradov and Scarpa, 1973), and sufficient mannitol/sucrose (3:1) to bring the medium to ~300 mosM. After allowing it to reach room temperature, the sample was bubbled with O_2, and $MnCl_2$ was added slowly. The sample (total volume, 4 ml) was allowed to attain a steady-state distribution of Mn^{2+} ions during a 15-min incubation period (controls showed that the ratio of internal to external Mn was normally constant to within 30% over the interval from 10 to 25 min following manganese addition). The sample was reoxygenated and then: (1) an aqueous flat cell was filled with a small volume of the sample and immediately transferred to the EPR spectrometer, where the first two lines of the Mn^{2+} hyperfine sextet were recorded. This signal was a superposition of signals from internal and external free Mn^{2+}. (2) Concurrently, a 2-ml sample aliquot was being rapidly centrifuged. The supernatant was saved and the sextet intensity was later recorded to be used as a measure of the free external $[Mn^{2+}]$. (3) After the first spectrum had been recorded, the flat cell was cleaned. The remainder of the sample was reoxygenated and enough EDTA was added to chelate all external Mn^{2+}. The flat cell was refilled and the first two lines of the sextet were rapidly recorded. This spectrum arises from internal free Mn^{2+}. The entire procedure was completed within 22 min after Mn^{2+} addition.

Determinations of the internal water compartment were carried out on duplicate samples (from the same mitochondrial prep) containing [^{14}C]sucrose. Fifteen minutes after Mn^{2+} was added, the samples were spun at 8000g for 3 min at 20 °C. Both the supernatants and the pellets were saved. The latter were weighed, dried, and reweighed to obtain pellet water and then extracted in: 0.5 ml of 50% trichloroacetic acid + 0.5 ml of cold resuspension medium. A 0.5-ml aliquot of each supernatant was combined with an equal volume of 50% trichloroacetic acid. Insoluble components were centrifuged out, a 0.1-ml sample was taken from each trichloroacetic acid-treated fraction, solubilized in Beckman Bio-Solv, then mixed with scintillation fluid, and counted. The external pellet water was calculated from the ratio of pellet to supernatant counts. The internal (matrix) water was defined then to equal pellet minus external water.

The procedure for K^+ determination follows closely that for internal volume but was carried out on the same 2-ml aliquots as was the external $[Mn^{2+}]$ measurement. Again the mitochondrial pellets were dried and weighed to determine total pellet water, V_p, and then extracted in 1 ml of 25% trichloroacetic acid. An aliquot of the extract and an aliquot of the untreated supernatant were assayed for $[K^+]$ on an Instrumentation Laboratory flame photometer (Model 143). The standardized reading on the supernatant was used as a measure of $[K^+]_{ext}$. Internal $[K^+]$ was calculated from:

$$[K^+]_{in} = \{[K^+]_p(1\text{ ml}) - [K]_{ext}(V_p - V_{in})\}/V_{in}$$

where $[K^+]_p$ is the potassium concentration in the pellet trichloroacetic acid extract and V_{in} is the matrix water from [^{14}C]sucrose measurements. $^{86}Rb^+$ distribution measurements were carried out on duplicate aliquots analogous to the K^+ determinations except that concentrations were deduced from the number of counts in the supernatants and pellets.

In time studies of radioactive $^{45}Ca^{2+}$ and $^{54}Mn^{2+}$ uptake or release, 1-ml aliquots were removed sequentially from a treated mitochondrial suspension, the aliquot was centrifuged at 7000g for 1 min, and the supernatants were counted.

Determination of Free Mn^{2+} Gradients. As summarized above, three separate determinations of the free Mn^{2+} sextet were made on each sample after the mitochondria had been incubated with Mn^{2+} for 15 min: (a) on the whole sample with no further treatment (internal and external free Mn^{2+}); (b) on the EDTA-treated sample (internal free Mn^{2+}: external Mn^{2+} is chelated and produces only a broad signal without a sextet pattern); (c) on the supernatant after centrifugation (external free Mn^{2+}).

The following procedure was employed in calculating the internal free Mn^{2+} in a sample: (1) the amplitude (peak-to-peak height of the first line) of the supernatant signal (c) was subtracted from the amplitude of the sample signal (a) to obtain the amplitude of the internal free Mn^{2+} signal; (2) the width of the internal signal was derived from the observed widths of the lines in the EDTA treated aliquot (b); (3) the resulting spectrum was matched against that of a standard composed of 0.5 mM $MnCl_2$ in water/glycerol showing nearly the same line width. A comparison of the amplitudes and spectrometer gains was then used to calculate the total free

TABLE I: Comparison of Mn and K Potentials.[a]

Added Mn^{2+} (nmol/mg of protein)	Added KCl (mM)	Mitochondrial $[Mn^{2+}]$		$\Delta\psi_{Mn}'$ (mV)	$\Delta\psi_{Mn}$ (mV)	$\Delta\psi_{K}'$ (mV)
		Total (mM)	Free (mM)			
130	0.0	55	12.9	114	95	159
	0.3	47	10.5	111	92	157
	1.0	32	8.7	106	89	156
	3.0	26	7.8	88	72	97
	10.0	22	5.9	72	55	65
260	0.0	72	31	111	100	160
	0.3	62	25	108	96	143
	1.0	47	23	101	93	132
	3.0	54	15	104	87	86
	10.0	42	18	72	61	69
390	0.0	81	37	76	64	71
	1.0	53	28	72	63	65
	10.0	66	32	71	61	63

[a] The samples were prepared and assayed as described under Methods. Each sample contained 25 mM NaOAc, of 0.16 μg/ml valinomycin, plus the ingredients listed in Methods and in the Table. The mitochondrial protein concentration was 3.2 mg/ml.

internal Mn^{2+} in the sample. The result was divided by the intramitochondrial volume to obtain the internal free $[Mn^{2+}]$.

Although a small error is introduced in subtracting two signals of differing widths, this procedure was adopted for calculating internal free Mn^{2+} because the sextet amplitude of the EDTA-treated sample systematically underestimates the internal free Mn^{2+}, typically by 10–20%, but sometimes by up to 50%. The major cause of this discrepancy is probably a slow net efflux of Mn^{2+} from mitochondria during the ~2 min elapsing between EDTA addition and recording of the spectrum. While controls showed $\lesssim$ 10% of the accumulated Mn^{2+} escapes in this time (see Figure 4), the ratio of internal free to internal bound Mn^{2+} may also decrease during this time period.

The calculations of internal free Mn^{2+} are based on the assumptions that the sextet signals arise from free Mn^{2+} and that the line broadening is a result of high intramitochondrial viscosity. As discussed in a previous publication (Puskin and Gunter, 1972), these assumptions are most reliable when the width of the sextet lines $\lesssim$35–38 Oe. Where the lines are broader than this, it becomes more likely that admixtures of signals from other manganese complexes might be contributing appreciably to the sextet, leading to an overestimate of internal free $[Mn^{2+}]$. It can be deduced from their probable line widths ($\gtrsim$50 Oe) that these admixtures contribute less than a factor of two error in the measurement of internal free Mn^{2+}.

Broad lines were observed only where permeant anion was absent or in low concentration. Under these conditions, the mitochondria in general also contain less free Mn^{2+}; consequently, the spectra are not only broader but possess poorer signal to noise, making it more difficult to accurately estimate line widths. The uncertainty in line width alone can, in extreme cases, result in up to 40% uncertainty in estimating free Mn^{2+}. The logarithmic dependence of $\Delta\psi_{Mn}$ on concentration gradient (Results: eq 3), however, implies that the uncertainty introduced by this source into $\Delta\psi_{Mn}$ calculations is <5 mV.

Results

Comparison of Mn^{2+} and K^+ Distributions. A passive uniport model of cation transport predicts a steady-state distribution:

$$\Delta\psi = (60/z) \log (M^{+z})_{in}/(M^{+z})_{out} \quad (1)$$

where $\Delta\psi$ is the electrical potential difference across the membrane, $\psi_{out} - \psi_{in}$, in mV. Thus, if passive K^+ uniport is induced with valinomycin, $\Delta\psi$ will equal the "potassium potential" defined by:

$$\Delta\psi_K \equiv 60 \log (K^+)_{in}/(K^+)_{out} \quad (2)$$

Moreover, if Mn^{2+} is transported purely by a passive uniport, $\Delta\psi$ should also equal the "manganese potential":

$$\Delta\psi_{Mn} \equiv 30 \log (Mn^{2+})_{in}/(Mn^{2+})_{out} \quad (3)$$

Therefore according to the passive uniport model of divalent cation transport, $\Delta\psi_{Mn} = \Delta\psi_K$ in the presence of valinomycin. In testing the validity of this relationship it must be noted that eq 2 and 3 refer to ratios of chemical activities. Usually, concentration gradients are measured. These fail to take into account activity-coefficient or binding corrections. The latter tend to cancel in comparing gradients of cations and cannot alter the conclusions reached below. The EPR technique removes the error due to Mn^{2+} binding. It is expected that K^+ binding should not influence the results significantly (see: Azzone et al., 1969, and the discussion below).

Free $[Mn^{2+}]$ gradients and total $[K^+]$ gradients were measured on valinomycin-treated mitochondrial samples as described under Methods. The gradients were converted to Mn^{2+} and K^+ potentials, $\Delta\psi_{Mn}$ and $\Delta\psi_K'$, using definitions 2 and 3. The prime has been used to indicate potentials in which the internal cation activity has been approximated by the total mitochondrial cation divided by the internal volume. Unprimed potentials refer to free concentration gradients.

The data summarized in Table I are not in agreement with the passive uniport model. $\Delta\psi_K'$ generally exceeded $\Delta\psi_{Mn}$, sometimes by $\gtrsim$60 mV. This difference decreased with added K^+ or Mn^{2+} so that excellent agreement between the two parameters was usually found when the mitochondria were swollen with large amounts of cation (in the presence of 25 mM acetate).

The question now arises as to whether the discrepancies between $\Delta\psi_K'$ and $\Delta\psi_{Mn}$ can be explained without modifying or abandoning the passive uniport model of divalent cation transport. First, it should be noted that the discrepancy is large,

TABLE II: Variation of Ion Gradients and Mn^{2+} Binding with Added Acetate.[a]

NaOAc (mM)	ΔpH	Mitochondrial [Mn^{2+}] Total (mM)	Mitochondrial [Mn^{2+}] Free (mM)	$\Delta\psi_{Mn}'$ (mV)	$\Delta\psi_{Mn}$ (mV)	$\Delta\psi_K'$ (mV)
0.2	1.68	142	2.4	110	57	117
1.2	1.67	124	4.0	112	68	124
3.2	1.57	71	6.0	107	75	118
10	1.13	31	6.3	96	75	126
30	0.75	40	10.8	87	70	89
50	0.48	54	17.3	94	79	90

[a] Each sample contained 4 mg of mitochondrial protein/ml, 127 nmol of Mn^{2+}/mg of protein, 3 mM KCl, 0.16 μg/ml of valinomycin.

a 60-mV change in $\Delta\psi_K'$ or $\Delta\psi_{Mn}$ representing a factor of 10 change in K^+ gradient or of 100 in Mn^{2+} gradient, respectively.

Errors in estimating matrix water cannot account for the discrepancy. The uncertainty in sucrose inaccessible water can produce at most a 5-mV change in $\Delta\psi_K' - \Delta\psi_{Mn}$ (corresponding to a 33% underestimate of internal volume). To account for ~60-mV differences, absurdly high internal water spaces (~100 μl/mg of protein) would have to be postulated. If, as contended by Bentzel and Solomon (1967), the free internal water is overestimated by the sucrose inaccessible volume, the differences between $\Delta\psi_K'$ and $\Delta\psi_{Mn}$ would have to be even larger.

It might be suggested that EPR does not accurately reflect the internal free Mn^{2+} in a sample. The most important source of error in this regard probably arises from admixtures of broad sextet signals due to other Mn^{2+} complexes. As discussed earlier, this error is relatively small and is, moreover, in the wrong direction, i.e., it would lead to an overestimate of $\Delta\psi_{Mn}$. Although it is unlikely that estimates of $\Delta\psi_{Mn}$ are too high, it is illuminating to compute the distribution $\Delta\psi_{Mn}'$, substituting total mitochondrial [Mn^{2+}] for free internal [Mn^{2+}] (binding of Mn^{2+} in the bathing solution was neglected in this calculation). $\Delta\psi_{Mn}'$ then approximates the Mn potential that would be estimated from techniques not sensitive to the chemical state of mitochondrial divalent cations. It should be noted that $\Delta\psi_{Mn}'$ is still substantially less than $\Delta\psi_K'$ for most samples, although, not surprisingly, the inequality is reversed in several cases. Thus, errors in EPR quantitation of internal binding cannot possibly explain the observed differences between $\Delta\psi_K'$ and $\Delta\psi_{Mn}$.

Since $\Delta\psi_K'$ and $\Delta\psi_{Mn}$ approach equality with increasing internal cation content, it is tempting to attribute the differences to internal K^+ binding. That amount of K^+ binding is, however, highly improbable. As a reasonable upper limit to the K^+ binding, let the bound to free ratio for K^+ be the same as for Mn^{2+}. This would imply a hypothetical "free K^+ potential":

$$\Delta\psi_K \geq \Delta\psi_K' - 2(\Delta\psi_{Mn}' - \Delta\psi_{Mn}) \quad (4)$$

It follows from Table I that the values of $\Delta\psi_K$ must still exceed the corresponding values for $\Delta\psi_{Mn}$. Therefore, in order to account for the disagreements by K^+ binding, a large number of sites would have to be postulated which preferentially bind K^+ over divalent cations. It is unlikely that sufficient amounts of such binding sites (>100 nmol/mg of protein) would be found in mitochondria to explain the discrepancy.

The differences cannot be ascribed to a nonexchangeable pool of mitochondrial K^+. This is demonstrated by the existence of similar differences when $\Delta\psi_{Mn}$ is compared with $\Delta\psi_{Rb}'$, determined in analogy to $\Delta\psi_K'$, using Rb^+ isotope (results not shown).

High concentrations of acetate were usually employed because this improved the precision of EPR quantitation by narrowing the free signal and enhancing it relative to bound components. In Table II are shown data obtained at fixed Mn^{2+} and K^+ concentrations as a function of added acetate. $\Delta\psi_K'$ exceeded $\Delta\psi_{Mn}$ over the entire range; the differences tended to be maximal at low [Ac^-]. The functional dependences of $\Delta\psi_K'$ and $\Delta\psi_{Mn}$ on [Ac^-] were not strictly monotonic, probably reflecting changes in $\Delta\psi$, ΔpH, and internal volume accompanying uptake of acetate.

The amount of Mn^{2+} binding was strongly dependent on [Ac^-]. The percentage of mitochondrial Mn^{2+} that was free grew from 1.7 to 32%, as [Ac^-] was varied from 0.2 to 50 mM. The binding increased with the pH gradient (Table II) calculated from the distribution of [^{14}C]acetate, assuming the relationship (Rottenberg, 1973):

$$\Delta pH = pH_{in} - pH_{out} = \log [Ac^-]_{in}/[Ac^-]_{out} \quad (5)$$

Since the external pH was 7.2, the results indicate that the internal binding of Mn^{2+} increased sharply over the range of internal pH from 7.7 to 8.9. Precipitation of internal Mn^{2+} could also be important (Puskin & Gunter, 1973; Gunter et al., 1975).

So far, only a passive uniport model of divalent cation transport has been considered. According to this model, $\Delta\psi_{Mn} = \Delta\psi_K$ ($\simeq\Delta\psi_K'$). For any passive model of divalent cation transport, a relationship between the Mn^{2+} potential and other gradients can be derived. For example, in the presence of valinomycin, a passive Mn^{2+} H^+ antiport predicts:

$$\Delta\psi_{Mn} = \tfrac{1}{2}[\Delta\psi - 60(\Delta pH)] \simeq \tfrac{1}{2}[\Delta\psi_K' - 60(\Delta pH)] \quad (6)$$

However, $\Delta\psi_{Mn}$ is always larger (Table II) than the expression on the right of eq 6. This inequality was found to hold over a wide range of [K^+] and [Mn^{2+}] whenever Mn^{2+}, K^+, and H^+ gradients were determined on the same sample. In summary, the concentration gradient of free Mn^{2+} measured in valinomycin-treated mitochondria is less than predicted by the uniport model but more than predicted by an electrogenic proton antiport. This statement can be generalized (cf. Discussion) to say that: "no single passive mechanism (uniport or antiport) can provide a complete description of divalent cation transport".

A final criticism might be advanced against this conclusion based only on the data presented above. It might be questioned whether the divalent cation distributions approach a true steady state, or whether instead, the transport merely stops or becomes very slow. This interpretation, however, is very difficult to reconcile with the effects of inhibitors on divalent

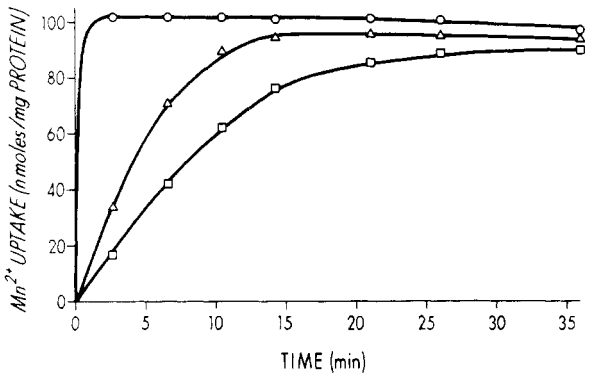

FIGURE 1: Effects of ruthenium red on Mn^{2+} uptake. Each sample contained 4.25 mg of mitochondrial protein/ml, 104 nmol of Mn^{2+}/mg of protein, 10 mM NaOAc. The ruthenium red added was 0(○), 0.3 (△), 0.45 (□) nmol/mg of protein.

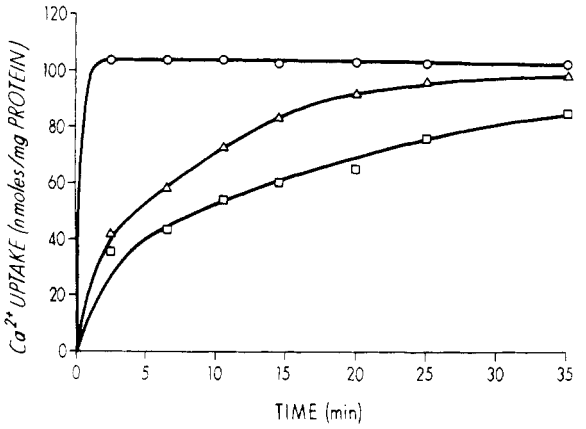

FIGURE 2: Effect of ruthenium red on Ca^{2+} uptake. Conditions were the same as in Figure 1 except that 104 nmol/mg of protein Ca^{2+} was added instead of Mn^{2+}. Ruthenium red was 0 (○), 0.3 (△), 0.45 (□) nmol/mg of protein, respectively.

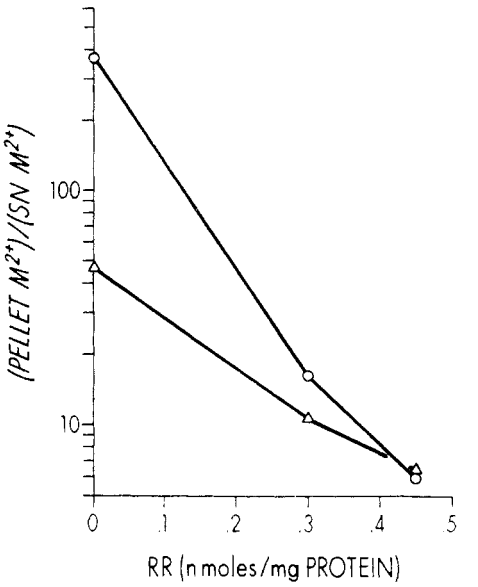

FIGURE 3: Effect of ruthenium red on steady state Mn^{2+} and Ca^{2+} gradients. The ratio of mitochondrial to medium Mn^{2+} (△) or Ca^{2+} (○) in the steady state is shown as a function of ruthenium red (RR) concentration. The data used was that shown in Figures 1 and 2.

cation gradients discussed below. In particular, when unidirectional influx was blocked with EGTA after uptake had been completed (Figures 4, 5) an easily detectable efflux ensued. Since the net flux was close to zero before EGTA addition, there existed a fairly rapid turnover of internal and external divalent cation (see also Drahota et al., 1965; Stucki and Ineichen, 1974) even after net uptake had stopped; this implies a true steady state.

Effect of Inhibitors on Steady State Ca^{2+} and Mn^{2+} Gradients. The strongest evidence for the existence of a specific Ca^{2+} carrier in mitochondria is the action of ions in the lanthanide series (including La^{3+}) and of the stain ruthenium red. In low concentrations these substances inhibit the uptake of divalent cations while apparently not interfering with other energized functions of the mitochondrion (Mela, 1969; Vainio et al., 1970; Moore, 1971; Vasington et al., 1972; Reed and Bygrave, 1974b).

Assuming that the transport proceeds exclusively through a passive uniport, such inhibitors should not alter the steady-state distribution of divalent cations. This is clear if one envisions the uniport schematically as a set of Ca^{2+}-selective pores and the inhibitors as plugs: stopping some of the pores will reduce the rate of flow, slowing the approach to equilibrium, but the net flow will cease at the same point, viz., when the Ca^{2+} (electrochemical) activities are equalized on the two sides of the membrane. Conversely, a finding that La^{3+} or ruthenium red disturb the steady-state distribution of Ca^{2+} or Mn^{2+} must be interpreted as evidence either against the passive uniport's encompassing a complete and correct description of divalent cation transport or against the idea that La^{3+} and ruthenium red act specifically on the transport system.

Effect of Inhibitors on Uptake. Figures 1-3 illustrate the effect of ruthenium red on mitochondrial Ca^{2+} and Mn^{2+} uptake. At each ruthenium red concentration, a different steady-state distribution of divalent cation was approached or attained and held for varying lengths of time. Both the uptake rates and the steady state Mn^{2+} gradients decreased in magnitude with increasing amounts of ruthenium red. At higher ruthenium red ($\gtrsim$1 nmol/mg of protein), it was impossible to reliably determine a steady-state distribution. In part, this was due to the slow rate of transport. Furthermore, in some of these samples there appeared to be a progressive loss of ruthenium red inhibition with time so that the uptake rate began to increase after ~30-40 min. The stimulation may take place in response to a loss of available external ruthenium red, e.g., through transport or metabolic breakdown. Alternatively, the mitochondria may overcome the ruthenium red inhibition by making available new transport sites.

Although the inhibition of divalent cation uptake by lanthanides bears many similarities to ruthenium red inhibition (Reed and Bygrave, 1974b), experiments analogous to those just described for ruthenium red were inconclusive with La^{3+}. The reasons were technical, relating to significant La^{3+} uptake (Reed and Bygrave, 1974a) during the approach to steady state. Perturbation of steady state cation gradients by La^{3+}, as well as by ruthenium red, were demonstrated in studies on the release of Ca^{2+} and Mn^{2+} from preloaded mitochondria described below.

The variation of steady state Ca^{2+} and Mn^{2+} distributions with added ruthenium red (Figures 1-3) is incompatible with a passive uniport model unless it is assumed that ruthenium red markedly decreased the membrane potential or mitochondrial divalent cation binding. Confirming previous reports (Moore, 1971; Vasington et al., 1972; Rossi et al., 1972), we found that the addition of up to ~5 nmol of ruthenium red/mg of protein had no sizeable effect on mitochondrial responses to substrate or ADP as measured by an O_2 electrode. This

TABLE III: Dependence of $\Delta\psi_{Mn}$ and $\Delta\psi_K'$ on Added Ruthenium Red.[a]

Ruthenium Red nmol/mg of protein)	Mitochondrial $[Mn^{2+}]$ Total (mM)	Free (mM)	$\Delta\psi_{Mn}'$ (mV)	$\Delta\psi_{Mn}$ (mV)	$\Delta\psi_K'$ (mV)
0.0	43	5.4	110	83	156
0.4	41	4.8	100	72	165
0.5	43	4.9	97	69	169
0.6	43	5.2	92	64	168

[a] Each sample contained 4.25 mg of mitochondrial protein/ml, 100 nmol of Mn^{2+}/mg of protein, 10 mM NaOAc, 1 mM KCl, 0.16 μg/ml of valinomycin. From this data and the definitions of $\Delta\psi_{Mn}$ and $\Delta\psi_K'$ it follows that upon addition of ruthenium red (0.6 nmol/mg of protein) the free $[Mn^{2+}]$ gradient decreased by over a factor of 4, while the $[K^+]$ gradient increased by ~58%.

finding strongly suggests that ruthenium red does not markedly perturb the membrane potential. A more direct demonstration of the same conclusion is provided by the results in Table III. Transport of Mn^{2+} into valinomycin-treated mitochondria was studied as a function of ruthenium red. At each ruthenium red concentration, the mitochondria were incubated with Mn^{2+} long enough for uptake to be completed (as predetermined by ^{54}Mn measurements). Then the free Mn^{2+} signals were observed with EPR, accompanied as before with volume and K^+ measurements. Table III shows that the steady-state free concentration gradient of $Mn(\Delta\psi_{Mn})$ decreased upon addition of the inhibitor. At the same time, the membrane potential (as measured by $\Delta\psi_K'$) actually increased slightly, perhaps due to a lower demand on mitochondrial energy stores (Stucki and Ineichen, 1974). Thus, ruthenium red enhanced the gap between $\Delta\psi_K'$ and $\Delta\psi_{Mn}$. Moreover, the ratio of bound to free internal Mn^{2+} was essentially unchanged by the addition of ruthenium red. In conclusion, the effects of ruthenium red on Ca^{2+} and Mn^{2+} uptake are incompatible with a model of divalent cation transport consisting of passive uniport alone.

Ruthenium Red and La^{3+} Induced Release. Although there is general agreement that ruthenium red and La^{3+} slow uptake of Ca^{2+}, some confusion has arisen over the effects these inhibitors have on divalent cation efflux. There have been reports that release is slowed by La^{3+} (Scarpa and Azzone, 1970) or ruthenium red (Reed and Bygrave, 1974c) while, on the other hand, it has been claimed that ruthenium red stimulates release (Sordahl, 1974) or has no effect on release (Vasington et al., 1972). In evaluating such experiments it is important to keep in mind whether one is considering: (a) unidirectional or net efflux, (b) slow or rapid efflux, (c) energized or deenergized conditions, or (d) complete or incomplete inhibition of efflux.

These points are illustrated in Figures 4 and 5. Mitochondria, preloaded with Ca^{2+} or Mn^{2+}, were treated with various combinations of inhibitors after 5 min. Release (net efflux) was monitored by the amount of isotope found in the supernatant at subsequent times. In agreement with Sordahl (1974), ruthenium red addition produced a slow loss of divalent cation from the mitochondria. This again demonstrates perturbation of steady-state gradients by ruthenium red in contradiction to a passive uniport model.

It is instructive to compare the effect of ruthenium red with that of EGTA. Since it chelates Ca^{2+} or Mn^{2+} but does not penetrate the inner membrane (Reed and Bygrave 1974b,c), EGTA should behave to first order as a block of unidirectional

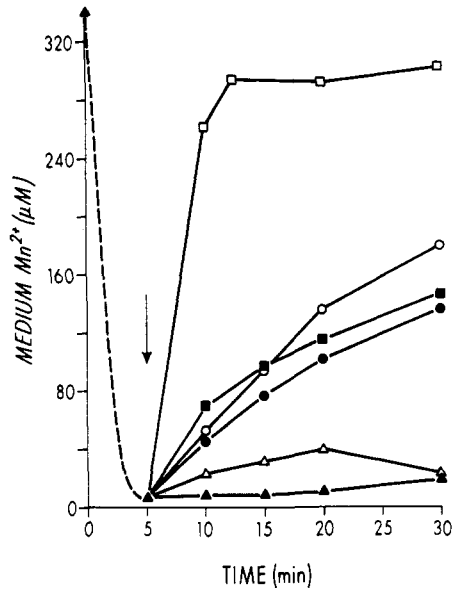

FIGURE 4: Inhibitor induced Mn^{2+} release after complete uptake. The mitochondrial and acetate concentrations were the same as in Figure 1. Inhibitors were added at 5 min as indicated: (●) ruthenium red; (○) EGTA, EGTA + ruthenium red (the release rates with EGTA and with ruthenium red + EGTA were very similar and are shown as a single curve); (△) La^{3+}; (□) CCCP; (■) CCCP + ruthenium red; (▲) control. The concentrations employed were 4 nmol of ruthenium red/mg of protein, 0.032 mM La^{3+}, 0.5 mM EGTA, and 0.018 mM CCCP.

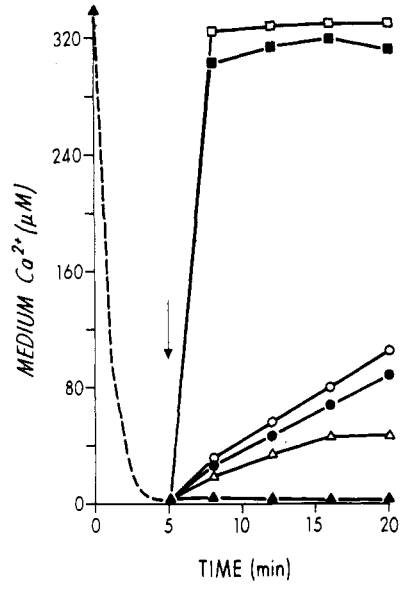

FIGURE 5: Inhibitor induced Ca^{2+} release after complete uptake. Conditions and symbols parallel to those in Figure 4 except that Mn^{2+} was replaced with Ca^{2+} and the La^{3+} was added at 0.083 mM.

influx. The efflux rate with EGTA was similar in magnitude but typically 20-40% faster than the net efflux produced by ruthenium red (Figures 4,5). Upon simultaneous treatment with both inhibitors, the efflux rate was nearly the same as with EGTA alone. The latter suggests that ruthenium red has no appreciable effect on unidirectional efflux from energized mitochondria. The small difference between ruthenium red- and EGTA-induced release can be explained in two ways: (a) some residual unidirectional influx in the presence of 4 nmol of ruthenium red/mg of protein; (b) some stimulation of unidirectional efflux by EGTA, perhaps through removal of external cations from transport sites.

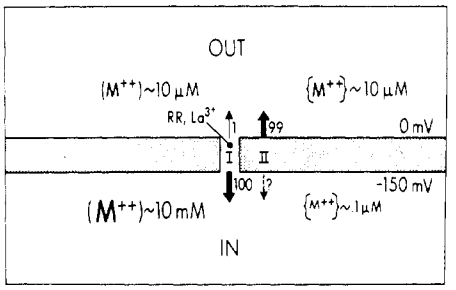

FIGURE 6: Model of mitochondrial divalent cation transport. Ruthenium red is abbreviated RR.

After steady state loading had been completed, release of divalent cations could also be brought about through administration of La^{3+} (Figures 4,5). Depending on the amount of La^{3+} added, the net efflux was often transient; after a certain length of time, the cations were reaccumulated in some instances. Generally, the release persisted for a longer time with increasing amounts of La^{3+}. This can be understood in light of the mitochondrion's ability to slowly accumulate La^{3+} (Reed and Bygrave, 1974a). It can be postulated that external La^{3+} blocks Ca^{2+} or Mn^{2+} uptake, but that accumulated La^{3+} fails to inhibit. This does not imply any asymmetry in the Ca^{2+} transport system, since the internal La^{3+} might be sequestered away from the transport sites. Both La^{3+} and ruthenium red also induced divalent cation release in the absence of permeant anion (results not shown).

In agreement with Vasington et al. (1972), ruthenium red had little effect on the rapid discharge of Ca^{2+} caused by treatment with an uncoupler (CCCP). Mn^{2+} release, however, was markedly slowed by ruthenium red under similar circumstances. The effect of La^{3+} on uncoupler-induced Ca^{2+} and Mn^{2+} release was similar to that of ruthenium red (data not shown). This puzzling difference between Mn^{2+} and Ca^{2+} release will be discussed further in the Discussion. However, it should be noted that the inhibition of Mn^{2+} release by ruthenium red and La^{3+} runs counter to the idea of separate unidirectional influx and efflux pathways for this ion, with only the former sensitive to these inhibitors.

Discussion

Comparison with Past Results. The internal free manganese concentrations reported here should be more accurate than past values based on rough estimates of matrix water (Puskin and Gunter, 1972, 1973; Gunter and Puskin, 1975). The methods employed in this study are also independent of the EDTA-induced efflux that introduced error into previous estimates. The values for free $[Mn^{2+}]_{in}$ are in reasonable agreement with those determined with an EPR flow technique (Bragadin et al., 1975) at pH 6.5. One difference between our observations and those of Bragadin et al. is that we find evidence for internal free Mn^{2+} even at very low $[Ac^-]$. However, as discussed earlier, the internal sextet is broad and reduced in amplitude under these conditions, so the identification of the signal with free Mn^{2+} is less certain than when acetate is present in higher concentrations.

The data in Table I show a strong positive correlation between membrane potential ($\Delta\psi_K'$) and Mn^{2+} gradient ($\Delta\psi_{Mn}$), consistent with the hypothesis that the membrane potential provides the immediate driving force for divalent cation uptake. Nevertheless, the differences between $\Delta\psi_K'$ and $\Delta\psi_{Mn}$ are inconsistent with a passive uniport model of Mn^{2+} transport. This negates the use of free Mn^{2+} gradients to measure membrane potential proposed in an earlier paper (Gunter and Puskin, 1975).

The differences between K^+ and Mn^{2+} potentials appear to conflict with results of Rottenberg and Scarpa (1974) who, following the notation employed in this paper, found $\Delta\psi_{Ca}' \simeq \Delta\psi_{Rb}'$. Several factors might be cited to reconcile the two sets of results: (1) the previous experiments failed to take into account internal Ca^{2+} binding. According to Table II, about 97% of the internal Mn^{2+} is bound at 1 mM acetate, the permeant anion concentration employed in the earlier study. If the same percentage of Ca^{2+} were bound under these conditions, $\Delta\psi_{Ca}$ would have been overestimated by ~45 mV. (2) Ca^{2+} is taken up more completely than Mn^{2+} (Figure 3) so that $\Delta\psi_{Ca}$ may exceed $\Delta\psi_{Mn}$ by up to 15–20 mV in some instances. (3) The internal volumes measured by Rottenberg and Scarpa at 1 mM acetate were considerably larger (~5 μl/mg of protein) than those measured in this study (~1 μl/mg of protein). This suggests some difference in mitochondrial preparations, which may be significant in light of the excellent agreement between $\Delta\psi_{Mn}$ and $\Delta\psi_K'$ in mitochondria swollen with large amount of cations (Table I).

Evidence for Two Transport Systems. The results of Selwyn et al. referred to in the Introduction strongly suggest a passive uniport mechanism for divalent cations and this conclusion is further strengthened by the near equality found between $\Delta\psi_{Mn}$ and $\Delta\psi_K'$ under some special conditions. Nevertheless, the large differences between $\Delta\psi_{Mn}$ and $\Delta\psi_K'$ in most valinomycin-treated samples and the perturbation of steady state Ca^{2+} and Mn^{2+} gradients by ruthenium red and La^{3+} demonstrate that the passive uniport picture cannot provide a complete description of divalent cation transport in mitochondria.

This dilemma can be resolved by postulating a second transport system in addition to a uniport system. (Logically, these two mechanisms could involve a common carrier, operating both as a uniport and as an antiport, for example.) To be concrete, consider the scheme depicted in Figure 6 under steady-state conditions. The chemical activity gradient $(Mn^{2+})_{in}/(Mn^{2+})_{out}$ was approximated from EPR measurements of free Mn^{2+} assuming that the membrane potential $\Delta\psi$ is approximately given by $\Delta\psi_K'$ (~150 mV, cf. Table I). The electrochemical activities inside and outside are given by:

$$\{M^{2+}\}_{out} = (M^{2+})_{out} \exp(2e\psi_{out}/kT)$$

$$\{M^{2+}\}_{in} = (M^{2+})_{in} \exp(2e\psi_{in}/kT)$$

From Figure 6 it is clear that although $(M^{2+})_{in}/(M^{2+})_{out} \gg 1$, the electrochemical gradient is reversed: $\{M^{2+}\}_{in}/\{M^{2+}\}_{out} \ll 1$. It follows that there is an imbalance in unidirectional fluxes through the uniport system I, depicted schematically as a pore. (This is done for illustrative purposes, the uniport could be a carrier instead.) The relative magnitudes of the fluxes are represented by the widths of the arrows and by the adjacent numbers.

In the steady state, such an inequality in unidirectional fluxes through I must be balanced by a net efflux through another pathway, denoted by II in Figure 6. The latter is shown as a unidirectional pump, but it could be a passive antiport or symport. Furthermore, II could represent two or more different transport mechanisms. The net effect of II, however, must be to move cations outward against an electrochemical gradient.

The steady state distribution of Ca^{2+} and Mn^{2+} in a particular case will depend on the magnitude of $\Delta\psi$, on the relative amounts of I and II and their respective transporting efficiencies for the divalent cation and, depending on the nature

of II, the distribution of other ions. For example, the difference between Ca^{2+} and Mn^{2+} gradients in Figure 3 (no ruthenium red) may be correlated with a higher transport affinity of I for Ca^{2+} (Vainio et al., 1970). Also, the close agreement between $\Delta\psi_K'$ and $\Delta\psi_{Mn}$ when the internal cation content is very high could reflect either an enhancement of I or an inhibition of II under these conditions.

Perturbations of steady-state divalent cation gradient by La^{3+} and ruthenium red underline the need to modify the passive uniport picture for Ca^{2+} as well as for Mn^{2+}. The effects of these inhibitors are consonant with the scheme of Figure 6 if one makes the assumption, indicated by previous work (Selwyn et al., 1970; Reed and Bygrave, 1972b), that La^{3+} and ruthenium red selectively block the uniport system. These agents, therefore, should slow influx more the efflux (see Figure 6), leading to a lower ratio of $(M^{2+})_{in}/(M^{2+})_{out}$ in the steady state. Taking into account La^{3+} transport, this is consistent with the observed tendencies of these inhibitors to reduce total uptake of Ca^{2+} or Mn^{2+} and to induce release from preloaded mitochondria.

If an uncoupler is added under the steady-state conditions of Figure 6, a large increase in $\{M^{2+}\}_{in}$ will accompany the discharge of membrane potential. Rapid efflux of M^{2+} through I should ensue, which according to Figure 6 ought to be slowed by ruthenium red or La^{3+}. It was noted that such ruthenium red and La^{3+} inhibition was observed with Mn^{2+}, but not with Ca^{2+} release. This point requires further study; however, it does not necessarily imply a failure in the proposed model. Movement of other ions must accompany M^{2+} release in order to preserve charge neutrality. Ruthenium red or La^{3+} will noticeably slow the release only if the efflux of M^{2+} through I becomes a rate-limiting step in the overall process. Another factor that may be significant is the more rapid efflux of Ca^{2+} (as compared with Mn^{2+}) induced by CCCP, i.e., the methods employed in this study may have been too slow to detect ruthenium red inhibition of Ca^{2+} efflux.

In summary, the ideas discussed here allow us to reconcile the evidence supporting: (1) Ca^{2+} uptake through a La^{3+} and ruthenium red sensitive passive uniport, driven by an inside-negative membrane potential (Selwyn et al., 1970), and (2) a ruthenium red insensitive pathway (Sordahl, 1974). Also suggesting a second transport mechanism are: (1) the differences found under some conditions between La^{3+} inhibition of Ca^{2+} uptake and Ca^{2+} efflux (Scarpa and Azzone, 1970), and (2) the reported uptake of Ca^{2+} into "inside-out" vesicles (Loyter, et al., 1969).

Mechanism of Ruthenium Red Insensitive M^{2+} Transport. To account for the data presented here, the (nonuniport) mechanism II must be able to transport divalent cations outward against an electrochemical gradient. Based on the close similarity between EGTA- and ruthenium red + EGTA-induced efflux rates, II is not strongly affected by ruthenium red. II could stand for an energized pump. This may appear to be a return to the earlier pump-leak model of Ca^{2+} transport (Chance, 1965). However, it should be noted that the pump proposed here is reversed and must now be directed outward, while the net "leak" through the uniport is normally directed inward. This is a simple consequence of the large electrical potential maintained across the inner membrane.

Alternatively, II might be some type of passive cation antiport or anion symport. It does not, however, appear to be an M^{2+}/K^+ or $M^{2+}/2\ K^+$ antiport. It can be shown that as long as K^+ movements occur primarily through a uniport mechanism, $\Delta\psi_{Mn} \simeq \Delta\psi_K'$ in any model consisting only of Mn^{2+} uniport and $Mn^{2+} - K^+$ antiports. Hence, the finding that $\Delta\psi_{Mn} < \Delta\psi_K'$ in valinomycin-treated samples indicates that uniport plus potassium exchange cannot account for divalent cation movements. The lack of a M^{2+}–potassium antiport mechanism in mitochondria is further indicated by the relative insensitivity of Mn^{2+} total uptake and ruthenium red-induced Mn^{2+} release to changes in external $[K^+]$ in the absence of valinomycin (unpublished results).

Experiments of Selwyn et al. (1970) appear to rule out $Ca^{2+}/2\ K^+$, $Ca^{2+}/2\ H^+$ and Ca^{2+}/H^+ transport mechanisms. This is not completely definitive, however, since the methods employed by the authors were incapable of detecting Ca^{2+}/H^+ antiport in the presence of a substantially larger Ca^{2+} uniport activity.

Some investigators have questioned the contention that valinomycin promotes an equalization of K^+ electrochemical activities across the inner mitochondrial membrane (Cockrell et al., 1966; Massari et al., 1972). In this view, the membrane potential may be less internally negative than indicated by $\Delta\psi_K'$. The observed effects of ruthenium red and La^{3+} on steady-state Ca^{2+} or Mn^{2+} gradients would nonetheless suggest two transport mechanisms.

If $\Delta\psi_{Mn}$ or $\Delta\psi_{Ca}$ were larger than the potential difference, $\psi_{out} - \psi_{in}$, the net flux of divalent cation through the uniport would then be outward. It would then be necessary to postulate an *inwardly directed* pump or some other mechanism to transport divalent cations inward against the electrochemical gradient.

Physiological Significance. In view of the dominant role probably played by the La^{3+}- and ruthenium red-sensitive uniport (I) in Ca^{2+} uptake, it might be suggested that the efflux of Ca^{2+} mediated by II serves no physiological purpose, reflecting perhaps some nonspecificity in a transport system for another ion. However, reasons for a second Ca^{2+} specific transport mechanism can be readily envisioned in the context of calcium's postulated role as an intracellular signal (Rasmussen, 1970), regulated perhaps by uptake and release from the mitochondria. For example, with the two transport systems outlined above, release of Ca^{2+} from the mitochondria into the cytoplasm could be induced: (a) by discharge of $\Delta\psi$ or ΔpH, (b) by inhibition of I or (c) by stimulation of II. Conversely, uptake could be enhanced by reversing these factors. Particularly significant in this light may be the capability for controlling Ca^{2+} movements independent of H^+ ion gradient and membrane potential.

Indeed, the simultaneous existence of a large membrane potential and a Ca^{2+} uniport system in mitochondria seems to necessitate a mechanism for extruding Ca^{2+} against an electrochemical gradient, even if mitochondrial Ca^{2+} uptake and release are not directly involved in intracellular signalling. To illustrate this point, consider a cell containing mitochondria with membrane potentials poised at 150–200 mV. The calcium phosphate solubility product makes it unlikely that intramitochondrial (Ca^{2+}) can exceed ~1 mM. Consequently, if only a uniport is available for transport, uptake might be expected to proceed until external (cytoplasmic) Ca^{2+} activity is reduced to 10^{-8}–10^{-10} M. However, before such levels can be reached, the plasma membrane pump would become inactivated (Vincenzi and Schatzmann, 1967), leading to net influx of Ca^{2+} from the extracellular fluid into the cytoplasm. This Ca^{2+} would then enter the mitochondria through the uniport. Eventually, the mitochondria will become uncoupled or the membrane potential will fall to a level where a steady state can be established at both the plasma and inner mitochondrial membranes. This process would certainly be wasteful of energy and could be damaging to the mitochondria. In order to

maintain a large membrane potential, perhaps necessary for efficient oxidative phosphorylation (Mitchell, 1968), and to use the potential for driving Ca^{2+} uptake through a passive uniport, the mitochondrion can extrude small amounts of Ca^{2+} against the electrochemical gradient. In this way, a steady state can be established where the membrane potential $\gtrsim 150$ mV and the cytoplasmic free $[Ca^{2+}] \sim 10^{-6}$–10^{-7} M.

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