Efflux of Ca^{2+} and Mn^{2+} from Rat Liver Mitochondria†

Thomas E. Gunter,* Karlene K. Gunter, Jerome S. Puskin, and Phyllis R. Russell

ABSTRACT: Net efflux of Ca^{2+} and Mn^{2+} from previously loaded rat liver mitochondria has been studied. Several agents have been found which affect Ca^{2+} efflux and Mn^{2+} efflux differently. Rapid efflux of either Ca^{2+} or Mn^{2+} follows addition of sufficiently large amounts of an uncoupler such as carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) to loaded mitochondria. Under these conditions, ruthenium red greatly retards Mn^{2+} but not Ca^{2+} efflux. Under similar conditions a chelating agent such as ethylene glycol bis(β-aminoethyl ether)-*N,N'*-tetraacetic acid (EGTA) greatly retards Mn^{2+} but not Ca^{2+} efflux. Where very small amounts of CCCP are added to loaded mitochondria, on the other hand, Ca^{2+} efflux in the presence of EGTA can be significantly retarded. Furthermore, when rapid spontaneous Ca^{2+} efflux occurs, it is sometimes slowed or almost completely prevented by addition of small amounts of CCCP. The amount of CCCP which maximally inhibits Mn^{2+} efflux is approximately a factor of 20 higher than the amount which maximally inhibits Ca^{2+} efflux. Mn^{2+} efflux in the presence of EGTA or ruthenium red is greatly inhibited by metabolic inhibitors such as KCN while Ca^{2+} efflux is not. It is concluded that influx and efflux of Ca^{2+} and Mn^{2+} take place primarily through distinguishable mechanisms, and that the mechanisms facilitating the efflux of Ca^{2+} and Mn^{2+} are also distinct. Evidence is also presented supporting the hypothesis that Mn^{2+} efflux and perhaps Ca^{2+} efflux as well take place at least in part by means of an active mechanism.

Ca^{2+} transport in mitochondria is usually thought of as being mediated by an electrogenic carrier complex responding to a membrane potential. (For recent review, see Bygrave, 1976). Evidence supporting the existence of a Ca^{2+} carrier falls into three categories: (1) inhibition by small amounts of lanthanide ions or ruthenium red (Mela, 1967, 1968; Scarpa and Azzone, 1970; Reed and Bygrave, 1974a,b; Moore, 1971; Vasington et al., 1972); (2) the saturation behavior of uptake kinetics (Bygrave et al., 1971; Reed and Bygrave, 1975; Scarpa and Graziotti, 1973; Vinogradov and Scarpa, 1973; Sordahl, 1974); and (3) the existence of a small ionophore-like component which binds Mn^{2+} and Gd^{3+} as evidenced by nuclear magnetic resonance (Case, 1975).

Mitochondrial uptake of Mn^{2+} has been shown to be mediated by the Ca^{2+} carrier (Chappell et al., 1963; Chance and Mela, 1966; Vainio et al., 1970). The latter also demonstrated that the same system transported other divalent cations with the specificity $Ca^{2+} > Sr^{2+} > Mn^{2+} > Ba^{2+}$ but did not transport Mg^{2+}.

In the presence of metabolic inhibitors, the efflux of endogenous K^+ through valinomycin can be used to induce accumulation of Ca^{2+} (Scarpa and Azzone, 1970). This has been taken as an indication that Ca^{2+} uptake occurs in response to an internally negative membrane potential. This conclusion is further supported by swelling measurements of Selwyn et al. (1970) showing that, under deenergized conditions, uptake was over either a simple facilitated diffusion mechanism (uniport), or through an electrogenic Ca^{2+} for K^+ exchange diffusion mechanism (antiport), either of which could be driven by an internally negative membrane potential.

Initial efforts seemed to indicate that Ca^{2+} was transported by a uniport mechanism alone (Rottenberg and Scarpa, 1974); however, this was found to be inconsistent with later results which indicate that no single passive mechanism can account for either Ca^{2+} or Mn^{2+} transport in liver mitochondria (Puskin et al., 1976; Massari and Pozzan, 1976; Azzone et al., 1976; Crompton et al., 1976).

There are two major problems with the uniport model of Ca^{2+} transport. First, when corrections are made for ion binding, the potentials calculated from steady state Ca^{2+} or Mn^{2+} distributions generally disagree with those calculated from the distribution of permeable monovalent cations (e.g., Rb^+, in the presence of valinomycin; Puskin et al., 1976; Massari and Pozzan, 1976; Azzone et al., 1976, 1977). The concentration distribution ratios of Ca^{2+} and Mn^{2+} ions relate through a Nernst equation (assuming a uniport mechanism) to membrane potentials of 90 to 100 mV, after binding corrections. This number is not constant but varies with conditions over a range of 30 mV or more. If a uniport mechanism were present, and if the actual membrane potential were higher than that indicated by the distribution ratios for Ca^{2+} or Mn^{2+}, as is indicated by the Rb^+ distribution in the presence of valinomycin, then the uniport mechanism would necessarily transport more divalent cations inward than outward. It could then be thought of as a "net influx" mechanism. The ion distribution data would then imply that some other mechanism exists which balances fluxes under steady-state conditions.

Second, data on both Ca^{2+} uptake in the presence of small amounts of ruthenium red (Puskin et al., 1976) and on ruthenium red induced Ca^{2+} efflux (Vasington et al., 1972; Sordahl, 1974; Puskin et al., 1976) indicate the existence of at least two "separate" Ca^{2+} transport mechanisms. Those results also strongly suggest that one of these transport mechanisms operates primarily as an influx mechanism and the other primarily as an efflux mechanism (Sordahl, 1974; Puskin et al., 1976).

These mechanisms are described as "separate" in the sense that they can be experimentally distinguished from one another in terms of some characteristic, e.g., mechanism of energization, sensitivity to a specific inhibitor, coupling of divalent

† From the Department of Radiation Biology and Biophysics, the University of Rochester School of Medicine and Dentistry, Rochester, New York 14642. *Received September 21, 1977.* This work was performed under contract with the United States Energy Research and Development Administration at the University of Rochester Biomedical and Environmental Research Project and has been assigned Report No. UR-3490-1165. This work was supported in part by U.S. Public Health Service Grant No. GM 21664.

0006-2960/78/0417-0339$01.00/0

cation flux to the flux of another ion, etc. It is still possible that they share some common molecular components.

The divalent cation transport mechanisms are referred to below, for convenience, as the "influx mechanism" and the "efflux mechanism". This usage is not meant to imply that the mechanisms are not reversible under some conditions, but only that in most in vitro experiments, and probably under usual in vivo conditions, as well, the energetics are such that one of them operates primarily as an influx mechanism and the other primarily as an efflux mechanism.

The discussion here of these mechanisms of mitochondrial divalent cation transport reflects the view that the usual potential across the energized mitochondrial inner membrane is greater than 90 to 100 mV (inside negative). While considerable support may be found in the literature for this view (Mitchell and Moyle, 1969; Rottenberg, 1973; Rottenberg and Scarpa, 1974; Nicholls, 1974; Rottenberg, 1975; Puskin et al., 1976), differing opinions are also found (Pressman and Fields, 1976; Tedeschi, 1975; Kinnally and Tedeschi, 1977).

Since the work of Vainio et al. (1970), the transport of Sr^{2+}, Mn^{2+}, and Ba^{2+} has been assumed to be mediated by the same system which mediates Ca^{2+} transport. Since those experiments were performed under conditions of net influx, they should now be regarded as indicating a common influx mechanism for those ions. Little is known about divalent cation efflux, specifically, whether Mn^{2+} and Ca^{2+} share the same efflux mechanisms and how divalent cation efflux varies with such factors as concentration of ruthenium red, metabolic inhibitors, or uncouplers.

We present evidence here indicating that, while the Ca^{2+} and Mn^{2+} influx mechanisms appear to be the same, their efflux mechanisms are distinct. In addition we provide data relevant to further considerations of possible models of Ca^{2+} and Mn^{2+} transport.

Materials and Methods

Rat liver mitochondria were prepared, either as described by Schnaitman and Greenwalt (1967) or by Lowenstein et al. (1970). EDTA[1] (1 mM) was included in the homogenization step. The results reported below were independent of the preparation technique used. The final resuspension medium contained 3 mM sodium succinate, 24 mM Hepes (pH 7.2), 1 mM $MgCl_2$, 1 mg/mL bovine serum albumin, and 10 mM NaOAc and sufficient mannitol/sucrose (3:1) to bring the medium to 300 mosM unless otherwise noted. Mitochondrial protein was assayed using the biuret method.

Mitochondria were suspended at either 1 mg/mL or 3 mg/mL, respectively, depending on whether filtration or centrifugation was to be employed in separating the mitochondria. $CaCl_2$ or $MnCl_2$ was added while the aliquot was being vigorously vortexed, at a time labeled "time zero", at a concentration of 80 nmol/mg of mitochondrial protein (except as otherwise noted). The aliquots under study were stirred frequently during the course of the experiment to ensure good oxygenation of the sample. After 4 or 5 min had been allowed for uptake, various substances were added to the sample aliquots as indicated in the Results section below. Controls were obtained by adding volumes of suspending medium, water and/or alcohol, equal to those in which the added substances were dissolved, to the mitochondrial suspensions. Small amounts of the sample aliquots (1 or 2 mL) were withdrawn periodically and either filtered or rapidly centrifuged.

When filtration was used, the samples were put through both a glass depth filter and an EH 0.45-μm Millipore filter in a syringe type filtration system. The filtration process was initiated at the proper time by pressuring the system with the syringe plunger. The filtrates were counted or assayed for Ca^{2+}, Mn^{2+}, or K^+.

When centrifugation (9000g for 1 min) was used, the supernatant was counted. $^{54}Mn^{2+}$ was assayed by γ counting in a Picker Nuclear Autowell II or a Beckman Biogamma Counter. Ca^{2+} was assayed by treating each supernatant or filtrate with an equal volume of trichloroacetic acid (50% w/v, e.g., 50 g of Cl_3CCOOH powder diluted to 100 mL with water). 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 in a Beckman LS-230 or LS-100C liquid scintillation counter. K^+ was assayed by treating each filtrate with an equal volume of trichloroacetic acid (50% w/v). Insoluble components were centrifuged out and the supernatant was assayed for $[K^+]$ on an Instrumentation Laboratory flame photometer (Model 143).

Valinomycin and CCCP were dissolved in ethanol and added to samples as indicated. 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.

Results

Effects of Uncouplers on Efflux. EGTA, which does not permeate the mitochondrial transport membrane, is a powerful chelator of both Ca^{2+} and Mn^{2+}, having a log K_d for complexation with Ca^{2+} and Mn^{2+} of -11.0 and -12.3, respectively (Anderegg, 1964). Consequently, when a sizable molar excess of EGTA over divalent cation is added to mitochondria which have previously taken up a divalent cation, the chemical activity of the cation in the suspending medium is reduced to the point where influx becomes negligible. Under these conditions, net flux is equivalent to unidirectional efflux. Ruthenium red, in sufficient concentration, may also be used to block influx.

As can be seen in Figure 1, where the membrane potential is dissipated by CCCP, net Mn^{2+} efflux is markedly affected by the presence of EGTA. When both the chelator and the uncoupler are present, the rate of efflux is less than that observed with the uncoupler alone. This is observed with Mn^{2+} efflux at all CCCP concentrations used (up to 50 μM). On the other hand, no such decreases are observed in the rates of Ca^{2+} efflux when strongly uncoupling concentrations of CCCP are used. Similar results may be obtained with another chelating agent (EDTA) substituted for EGTA, and other uncouplers (DNP, FCCP) substituted for CCCP.

The overall rate of a process is limited by the slowest step or steps. Consequently, to show a difference in the mechanisms of Ca^{2+} efflux and Mn^{2+} efflux, it is necessary to compare the efflux rates under conditions where some step in the efflux process itself is rate limiting. (One could not, for example, conclude that Ca^{2+} efflux after addition of uncoupler is unaffected by EGTA, if the rate of Ca^{2+} efflux is limited solely by the rate at which the membrane potential is decreasing.) EGTA, in the concentration range used here, has no effect on metabolism apart from its effects on Ca^{2+} flux and would not

[1] Abbreviations used: Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N'*-tetraacetic acid; CCCP, carbonyl cyanide *m*-chlorophenyl hydrazone; FCCP, carbonyl cyanide trifluoromethoxyphenylhydrazone; DNP, 2,4-dinitrophenol. When enclosing the chemical symbol for an ion, brackets indicate concentration and parentheses indicate chemical activity.

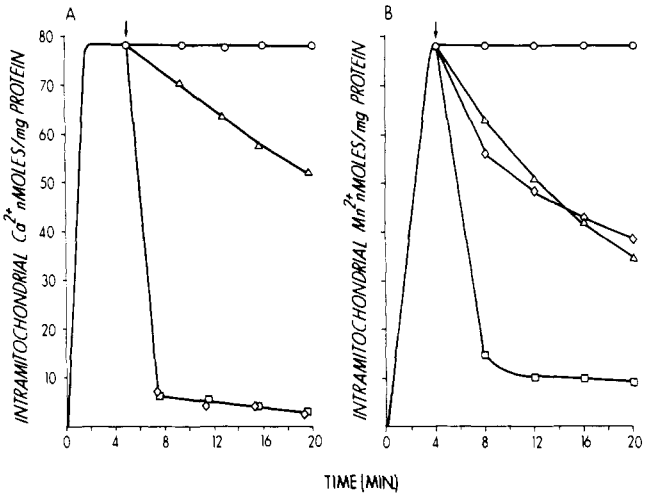

FIGURE 1: Efflux of Ca^{2+} and of Mn^{2+} in the presence of uncoupling concentrations of CCCP. (A) Mitochondria are suspended at 4.25 mg/mL in medium containing 10 mM NaOAc, 3 mM sodium succinate, 1 mg/mL bovine serum albumin, 24 mM Na-Hepes (pH 7.2), 1 mM $MgCl_2$, and enough mannitol/sucrose (3:1) to give an osmolarity of 300 mosM. At zero time Ca^{2+} (80 nmol/mg of protein) is added to the suspension while it is being vortexed. Compounds added at the arrow were: (○) control; (△) 0.5 mM EGTA; (□) 10 nmol of CCCP/mg of protein; (◇) both CCCP and EGTA added. EGTA has no effect on Ca^{2+} efflux in presence of 10 nmol of CCCP/mg of protein. (B) Conditions and symbols are similar to those in A, except that CCCP concentration is 12.5 nmol of CCCP/mg of protein and Mn^{2+} (80 nmol/mg of protein) is added at zero time instead of Ca^{2+}. EGTA slows Mn^{2+} efflux in the presence of the uncoupler CCCP to a rate slower than with CCCP alone. Mn^{2+} efflux seems inherently different from Ca^{2+} efflux.

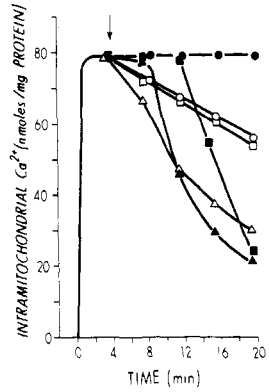

FIGURE 2: Efflux of Ca^{2+} under conditions where EGTA can slow Ca^{2+} release induced by uncoupler. Conditions are similar to those of Figure 1A except that 6 mM Na-Hepes (pH 7.2) was used. Compounds added at the arrow were: (●) control; (■) 0.75 μM CCCP; (▲) 1.0 μM CCCP; (○) 0.5 mM EGTA; (□) 0.75 μM CCCP plus EGTA; (△) 1.0 μM CCCP plus EGTA. Under these conditions where Ca^{2+} is held in the mitochondria for at least 20 min after uptake, there is a narrow CCCP concentration range where Ca^{2+} efflux in the presence of CCCP plus EGTA is slower than that with CCCP alone.

be expected to alter the rate at which the membrane potential decreases after addition of sufficient uncoupler. When efflux was monitored in suspensions containing K^+ (2 mM) and valinomycin (40 ng/mg of protein), it was found that added CCCP ($\gtrsim 1$ μM) induced a much more rapid efflux of K^+ than either Ca^{2+} or Mn^{2+}. Efflux of K^+ over the electrogenic mechanism provided by valinomycin would be expected to slow the rate of decrease of membrane potential because as each K^+ ion diffuses across the membrane, it leaves behind a negative counter ion which diffuses more slowly. Nevertheless, since net K^+ efflux is a reaction to the decrease in membrane potential, the collapse of the potential must occur at least as rapidly as that of the K^+ gradient. Since Ca^{2+} is transported outward much more slowly than K^+, its efflux must be limited, at least in part, by the velocity of the efflux mechanism itself. "Velocity of the mechanism" here refers not only to the "cycling time" of the carrier but also includes the time involved in binding to the carrier, release from the carrier and any other time consuming steps in the transport process.

It was observed in this work that efflux rate is relatively insensitive to $[Ac^-]$. Since pH gradient across the inner mitochondrial membrane and the fraction of internal divalent cation which is bound have been found to be very dependent on $[Ac^-]$ (Gunter and Puskin, 1975), this suggests that the efflux process and not release of the intramitochondrial bound form is rate limiting.

Since the data in Figure 1 indicate differences in the response of Ca^{2+} and Mn^{2+} efflux to added CCCP, under conditions where the efflux process itself should be rate limiting, they strongly suggest that the two efflux systems are distinct.

The results in Figure 1 are strikingly similar to those published previously on the efflux of Ca^{2+} and Mn^{2+} in the presence of ruthenium red (Puskin et al., 1976; Figures 4 and 5). Further experiments substantiated the similarity between the behavior of efflux in the presence of EGTA and ruthenium red, respectively. Specifically, at uncoupler concentrations where the rates of divalent cation efflux were limited by their respective mechanisms of efflux, and not by the discharge of membrane potential, Mn^{2+} efflux in the presence of CCCP was greatly slowed by externally bound ruthenium red (4 nmol/mg of protein) while Ca^{2+} efflux in the presence of CCCP was not. Consequently, these observations further suggest that Ca^{2+} efflux and Mn^{2+} efflux take place primarily through separate mechanisms.

The effect of ruthenium red and of EGTA on Mn^{2+} efflux may indicate that Mn^{2+} efflux is activated by divalent cation binding to an external site, as Ca^{2+} and Mn^{2+} influx seem to be (Bygrave et al., 1971; Scarpa and Graziotti, 1973). Both EGTA, by chelation of external divalent ions, and ruthenium red by competition for binding sites and/or charge screening effects, could deny access of cations to external activation sites. Since the effect is still observed both when $[Mg^{2+}] = 0$ and when $[Mg^{2+}] > [EGTA]$, it appears that, if this activation hypothesis is valid, Mg^{2+} cannot activate the Mn^{2+} efflux mechanism.

Alternatively, it might be suggested that the efflux of Mn^{2+} is activated by divalent cation influx over the ruthenium red sensitive influx mechanism. Again, either EGTA or ruthenium red addition would remove this activation.

There seems to be some variation in the response of the Ca^{2+} efflux system to low concentrations of uncoupler, depending on the length of time which the mitochondria can hold Ca^{2+} before releasing it spontaneously in the absence of uncoupler. Specifically, where Ca^{2+} is held for at least 20 min before appreciable release occurs, there is a range of uncoupler concentration (just below the level which induces dissipation of membrane potential) within which efflux in the presence of CCCP plus EGTA is slower than that in the presence of CCCP alone. This point is illustrated by the results in Figure 2. There is normally some variability in the length of time during which Ca^{2+} will remain inside mitochondria after energy-linked uptake. The data in Figure 3 show that, in at least some cases where Ca^{2+} is spontaneously released soon after uptake, low levels of uncoupler can actually stabilize the mitochondrion against Ca^{2+} release. This is consistent with the observation of Stucki and Ineichen (1974) that Ca/O_2 ratios increase in the presence of low levels of uncoupler.

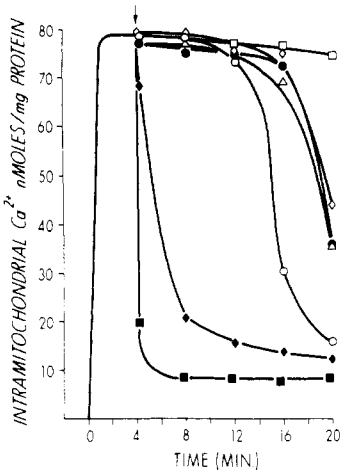

FIGURE 3: The effects of low levels of CCCP on mitochondria which show rapid spontaneous release of Ca^{2+}. The conditions are similar to those of Figure 1A. Compounds added at the arrow were: (○) control; (◇) 0.015 μM CCCP; (□) 0.05 μM CCCP; (△) 0.15 μM CCCP; (●) 0.5 μM CCCP; (◆) 1.5 μM CCCP; (■) 10 μM CCCP. Under some conditions where mitochondria do not hold Ca^{2+} for a very long time (spontaneous efflux), the loss of Ca^{2+} may be inhibited by addition of small amounts of uncoupler. This is consistent with the idea of inhibition of an outward pump.

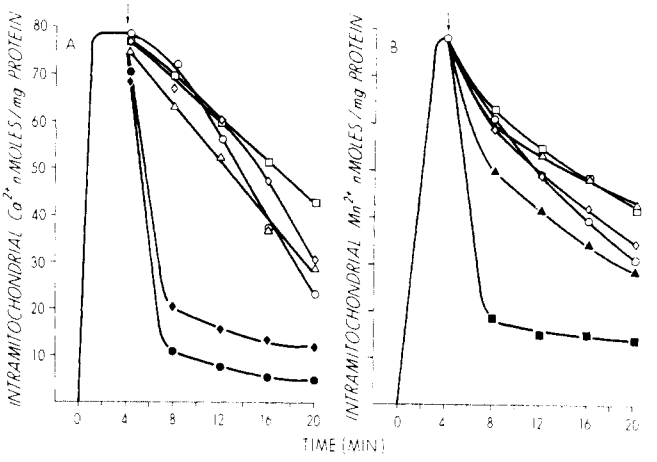

FIGURE 4: Efflux of Ca^{2+} and Mn^{2+} at low uncoupler concentrations. (A) Conditions are similar to those of Figure 1A. The symbols represent varying amounts of CCCP, EGTA, and CCCP plus EGTA added at the arrow. Compounds added were (○) 0.2 mM EGTA; (◇) 0.015 μM CCCP plus EGTA; (□) 0.05 μM CCCP plus EGTA; (△) 0.15 μM CCCP plus EGTA, (●) 0.5 μM CCCP plus EGTA; (◆) 0.5 μM CCCP alone. At very low concentrations of uncoupler Ca^{2+} efflux is inhibited by the presence of the uncoupler. Maximum inhibition is at around 0.05 μM CCCP. The mitochondrial preparation was the same as that depicted in Figure 3. As can be seen in Figure 3, this was a case where rapid spontaneous efflux did occur; consequently, the properties of Ca^{2+} efflux after addition of CCCP and EGTA are somewhat different from those shown in Figures 1 and 2. (B) Conditions are similar to those of Figure 1B. As in A, the symbols represent varying amounts of CCCP, EGTA, and CCCP plus EGTA added at the arrow. Compounds added were: (○) 0.2 mM EGTA; (◇) 0.15 μM CCCP plus EGTA; (□) 0.5 μM CCCP plus EGTA; (△) 1.5 μM CCCP plus EGTA; (▲) 18 μM CCCP plus EGTA; (■) 18 μM CCCP alone. Mn^{2+} efflux is also inhibited by low amounts of uncoupler but the maximum inhibition falls at approximately 1.0 μM. This is approximately 20 times higher than that for Ca^{2+} efflux. The Ca^{2+} efflux systems and the Mn^{2+} efflux systems behave as if they were different systems.

Under some conditions, added uncoupler slows efflux of Ca^{2+} or Mn^{2+} in the presence of EGTA to a value considerably less than that observed upon addition of EGTA alone (Figure 4). For Ca^{2+} (Figure 4A), this could only be observed in instances where spontaneous release of Ca^{2+} occurred within 20 min after uptake. These are the same cases in which low levels of CCCP slow efflux in the absence of EGTA. It seemed to be a general characteristic of Mn^{2+} efflux (Figure 4B). For either Ca^{2+} or Mn^{2+}, however, the effect was observed only over a narrow range of uncoupler concentrations, which was different for the two ions. This inhibition of efflux by the uncoupler cannot involve interference with an activation of efflux by external divalent cations, as discussed above, since the EGTA alone denies these cations access to the hypothetical activation sites or influx mechanism. The data are, however, consistent with a model of divalent cation transport consisting of a passive uniport in parallel with an outwardly directed pump (Puskin et al., 1976).

It is interesting to note that effects on Ca^{2+} efflux are observed at uncoupler concentrations approximately a factor of 20 smaller than those producing corresponding effects on Mn^{2+} efflux, as is shown in Figure 5. This is further evidence that separate efflux mechanisms are involved.

Effects of Metabolic Inhibitors and Inhibitors of ATP Hydrolysis on Transport. The data in Figure 6 further support the hypothesis of an outwardly directed pump for Mn^{2+}. When metabolic inhibitors such as antimycin A or cyanide are added along with EGTA to Mn^{2+} loaded mitochondria, the rate of efflux of Mn^{2+} is considerably slower than that observed when EGTA alone is added. In contrast, the rate of efflux of Ca^{2+} is not decreased by these metabolic inhibitors under similar conditions, again suggesting that Mn^{2+} and Ca^{2+} efflux take place largely over separate mechanisms. Again qualitatively similar results were obtained when ruthenium red was added in place of EGTA, both with respect to Ca^{2+} and Mn^{2+} efflux.

Neither oligomycin, an inhibitor of ATP hydrolysis by F_1, nor atractyloside, an inhibitor of membrane transport of ATP, show any effects on EGTA-induced Ca^{2+} or Mn^{2+} efflux. Moreover, the observed effects of other inhibitors or uncouplers on Mn^{2+} efflux are unaltered by these compounds. Likewise, neither additions of ADP nor ATP after uptake is complete, either by themselves or with uncouplers, EGTA or ruthenium red, show any effect on efflux other than those seen without the adenine nucleotides.

Mixed Aliquots Containing Both Ca^{2+} and Mn^{2+}. Finally, experiments were carried out in which 40 nmol Ca^{2+}/mg protein and 40 nmol Mn^{2+}/mg protein were both added to parallel aliquots of a mitochondrial suspension similar to those described above. These differed only in that a trace amount of ^{45}Ca was added to one aliquot while a trace amount of ^{54}Mn was added to the other. Efflux was then monitored in these "mixed" mitochondrial suspensions in the presence of various inhibitors. Neither the characteristics of Ca^{2+} nor of Mn^{2+} efflux seemed affected by the presence of the other ion under these conditions. Specifically, Mn^{2+} efflux in the presence of CCCP was inhibited by ruthenium red while that of Ca^{2+} was not. EGTA inhibited Mn^{2+} efflux in the presence of uncoupling concentrations of CCCP but not Ca^{2+} efflux. Cyanide inhibited Mn^{2+} efflux in the presence of EGTA but not Ca^{2+} efflux.

From these experiments, it can be concluded that the observed differences in Ca^{2+} and Mn^{2+} efflux do not arise because of any special state or condition of the mitochondria induced by the presence of one or the other of these ions.

Discussion

Separate Mechanisms for Ca^{2+} and Mn^{2+} Efflux. At least four significant differences in the behavior of Ca^{2+} efflux and Mn^{2+} efflux have been reported above. First, the rate of Ca^{2+} efflux after addition of uncoupler is unaffected by the presence

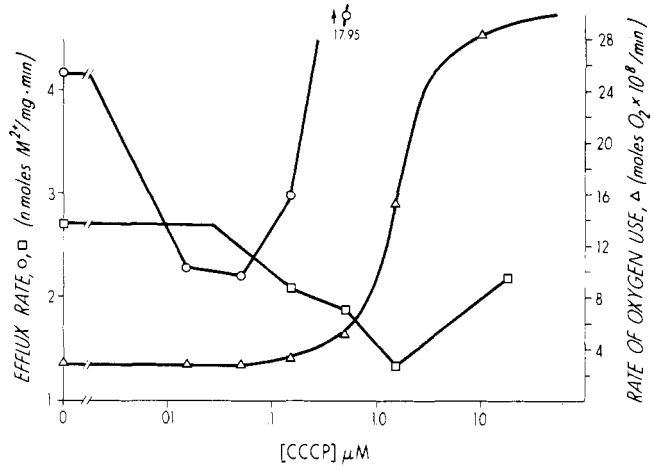

FIGURE 5: Rates of efflux and oxidation rates as a function of uncoupler concentration. Efflux rates (left scale) measured 8 min after addition of the test substances in the data shown in Figure 4 (i.e., at 12 min) are shown plotted against CCCP concentration. On the same plot O_2 consumption rates (right scale) measured with a Clark electrode on a separate but similar mitochondrial preparation are shown as a function of CCCP concentration.

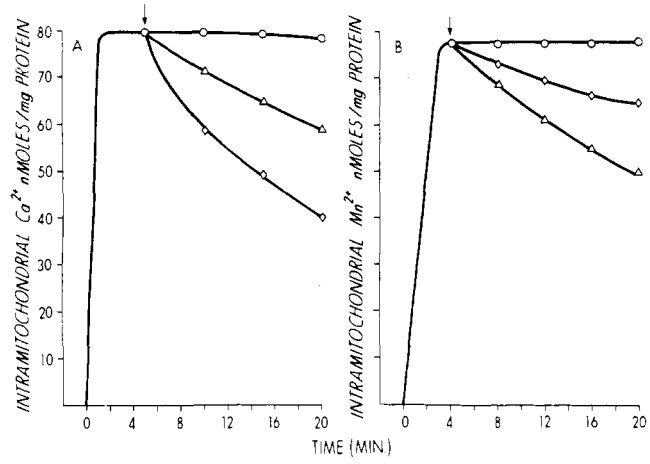

FIGURE 6: Efflux of Ca^{2+} and of Mn^{2+} in the presence of metabolic inhibition. (A) Conditions are similar to those of Figure 1A, except that 10 mM sodium succinate was used. Compounds added at the arrow were: (○) control; (△) 0.45 mM EGTA; (◇) EGTA plus 2 mM KCN. The rate of Ca^{2+} efflux is increased by the presence of metabolic inhibitors such as CN^-. (B) Conditions are similar to those of Figure 1B, except that 10 mM sodium succinate was used. The symbols have the same meaning as in 6A. The rate of Mn^{2+} efflux is greatly decreased by the presence of metabolic inhibitors such as CN^-.

of ruthenium red, while Mn^{2+} efflux under corresponding conditions is strongly inhibited. Second, the rate of Ca^{2+} efflux after addition of large amounts of uncoupler is unaffected by either the presence of a chelating agent such as EGTA or by ruthenium red, while Mn^{2+} efflux under corresponding conditions is strongly inhibited. Third, the amount of CCCP necessary to give minimum Ca^{2+} efflux in the presence of EGTA is approximately 20 times lower than the amount of CCCP necessary to give minimum Mn^{2+} efflux in the presence of EGTA. Fourth, Mn^{2+} efflux in the presence of EGTA or ruthenium red is markedly slowed by the presence of a metabolic inhibitor, while Ca^{2+} efflux under corresponding conditions is not. These observations strongly suggest that the outward transport of Ca^{2+} and Mn^{2+} takes place through separate mechanisms.

Exactly how different these mechanisms are cannot be ascertained from the data above. While it remains possible that the molecular complexes involved in Ca^{2+} efflux are completely distinct from those involved in Mn^{2+} transport, it may be that the complexes have some components in common. Perhaps these complexes differ only in a single component conveying the property of specific cation recognition to one or the other of the complexes.

The extent to which Mn^{2+} can be transported by the Ca^{2+} mechanism and vice versa might normally be ascertained through studies of competition kinetics. From the above data only the qualitative information that the characteristics of Ca^{2+} or Mn^{2+} efflux did not change significantly when the other ion was present can be extracted. Unfortunately, because the chemical activities of intramitochondrial Ca^{2+} and Mn^{2+} cannot be accurately controlled or, for Ca^{2+} even accurately measured ((Mn^{2+}) can be determined using electron paramagnetic resonance spectroscopy; see Gunter et al. (1975) and Gunter and Puskin (1975)), such kinetic studies with regard to efflux are not currently feasible.

Mitochondria have been shown to undergo a rapid change in configuration with accompanying changes in permeability upon Ca^{2+} uptake (Hackenbrock and Caplan, 1969; Hunter et al., 1976). For several reasons, however, it is unlikely that the observed differences in Ca^{2+} and Mn^{2+} efflux reflect differences in mitochondrial configuration. First, [^{14}C]sucrose permeability measurements similar to those carried out by Hunter et al. (1976) gave no indication of leakiness to [^{14}C]-sucrose when mitochondria were loaded with either Ca^{2+} or Mn^{2+} under the conditions described above. Second, no inorganic phosphate or arsenate was added to the mitochondria in this study, but Mg^{2+} was present (typically at a Mg^{2+}:Ca^{2+} ratio of $\simeq$12.5). No configuration transition has been reported under these conditions. Third, in the experiments in which both Mn^{2+} and Ca^{2+} were simultaneously added to mitochondrial suspensions, the qualitative behavior of the two separate efflux systems was identical with that found in those cases where the ions were added separately to separate suspensions. The mitochondrial configuration could hardly depend on which radioisotope was being employed.

What Is the Mn^{2+} Efflux Mechanism? Since Mn^{2+} is only found inside cells and mitochondria in trace amounts, it seems unlikely that the Mn^{2+} efflux mechanism discussed above evolved specifically for the transport of Mn^{2+}. It is more plausible that Mn^{2+} would be transported outward over a Mg^{2+} or Ca^{2+} efflux mechanism instead. Differences between Ca^{2+} and Mn^{2+} efflux, reported above, therefore, suggest that Mn^{2+} efflux takes place over a Mg^{2+} efflux system. This point is currently being pursued in experiments with submitochondrial particles.

Mn^{2+} resembles Mg^{2+} in such characteristics as charge, charge symmetry, ionic radius, and coordination symmetry. Thus, it might be employed as a substrate by a Mg^{2+} transport system. Mn^{2+} efflux has been shown here to be sensitive to inhibition by ruthenium red as Kun (1976) found Mg^{2+} flux to be. Also the observed velocities of Mn^{2+} efflux are in the same range as the V_{max}s for Mg^{2+} transport as reported by Kun (1976).

On the other hand, unlike Mg^{2+} transport, Mn^{2+} efflux did not seem to be affected by ATP or ADP concentration, or by the presence of atractyloside. Whether or not these differences imply separate mechanisms or merely differing experimental conditions remains to be determined.

The Case for Separate Influx and Efflux Mechanisms and for Active Efflux for Both Mn^{2+} and Ca^{2+}. The observation that Ca^{2+} uptake is greatly slowed by externally bound ruthenium red while Ca^{2+} efflux is not provides support for the idea that Ca^{2+} influx and efflux take place largely through

separate mechanisms (Puskin et al., 1976). It is surprising that, when the mitochondrial membrane potential is dissipated by an uncoupler, Ca^{2+} efflux through the usual influx mechanism, believed to be a ruthenium red sensitive, passive uniport (Selwyn et al., 1970; Rottenberg and Scarpa, 1974; Puskin et al., 1976), is not observed. While the second law precludes a mechanism which will preferentially transport Ca^{2+} in one direction without the expenditure of energy, it does not forbid a change in the permeability of a given ion whose transport is mediated by a facilitated diffusion mechanism, as conditions are altered. It is possible that a change in membrane potential, for example, would bring about such a permeability change. Such an effect could account for the above mentioned observations. A permeability change upon changing membrane potential has been observed for anion transport (Knauf and Marchant, 1977).

In contradistinction to Ca^{2+} efflux, Mn^{2+} efflux in the presence of metabolic inhibitors or uncouplers does appear to be ruthenium red sensitive. This could be taken as evidence that Mn^{2+} influx and efflux take place over a single mechanism. For several reasons, this is not likely to be the case.

First, measurements of steady-state Mn^{2+} gradients and their perturbation by ruthenium red have indicated that Mn^{2+} transport is not mediated by any single passive mechanism (Puskin et al., 1976).

Second, Ca^{2+} is transported in an inward direction more rapidly than Mn^{2+} by the influx mechanism common to both ions (Vainio et al., 1970). It would be surprising if, under action of an uncoupler and dissipation of the membrane potential, the velocity of Ca^{2+} transport by this mechanism decreased to near zero while the velocity of Mn^{2+} transport remained relatively high.

Third, the effects of uncouplers and metabolic inhibitors on Mn^{2+} efflux suggest a mechanism distinct from the influx mechanism, which appears to be passive and responsive to a membrane potential (Scarpa and Azzone, 1970; Selwyn et al., 1970).

As has been pointed out earlier (Puskin et al., 1976; Azzone et al., 1977) if the membrane potential is large enough (> 100 mV), an active efflux mechanism of some type is necessary to pump divalent cations outward against an electrochemical gradient. Whether this active efflux mechanism couples the flux of Ca^{2+} or Mn^{2+} to the flux of any other ion (antiport or symport) remains to be determined. It should be recognized that a simple, electrogenic efflux pump for Ca^{2+}, for example, would in operation enhance the internally negative membrane potential across the inner membrane. If membrane potential plays any vital role in mitochondrial function (e.g., in ADP phosphorylation) it would be useful to have a mechanism for Ca^{2+} efflux which does not require a decrease in membrane potential. An active efflux mechanism would be ideal from this point of view.

In this way, mitochondria could help regulate important cytoplasmic processes. For example, protein synthesis (among many other cellular processes) is stimulated by increasing the Mg^{2+} to Ca^{2+} ratio. Inhibition of an active Ca^{2+} efflux mechanism would increase the cytosolic Mg^{2+} to Ca^{2+} ratio and stimulate protein synthesis. The same result would be aided by active Mg^{2+} efflux as well.

The fact that metabolic inhibitors such as CN^- or antimycin A decrease Mn^{2+} efflux in the presence of a chelating agent can be taken as evidence for the existence of an active efflux mechanism which makes use of energy from electron transport to induce Mn^{2+} efflux.

The mechanism by which uncouplers inhibit Mn^{2+} efflux is not easily understood. Uncouplers show effects on Mn^{2+} efflux in the concentration range in which they are effective in uncoupling oxidation from phosphorylation. The uncoupler may simply provide a pathway for metabolic energy parallel to the use of this energy for Mn^{2+} efflux. This interpretation would suggest an active Mn^{2+} efflux mechanism. Alternatively, there could be a site specific inhibition of the transport system through binding of the uncoupler. While the former explanation may be more plausible in light of the fact that metabolic inhibitors (KCN or antimycin A) also slow Mn^{2+} efflux in the presence of a chelating agent, these hypotheses are not mutually exclusive, so both modes of inhibition may be simultaneously operative.

No indication of a Ca^{2+}:Na^+ antiport system in liver mitochondria has been found either in the current study or in the work of Crompton et al. (1976). The Ca^{2+} efflux data support a weaker case for an active Ca^{2+} efflux mechanism than the case for active Mn^{2+} efflux discussed above. The only observation suggesting active Ca^{2+} efflux is the retarding effect of low concentrations of uncoupler on Ca^{2+} efflux in the presence of EGTA. Since the effects of uncoupler on Ca^{2+} efflux occur at concentrations less than those causing dissipation of membrane potential and pH gradient, the data favor the site specific inhibitor hypothesis mentioned above in discussing the Mn^{2+} data. Under somewhat similar conditions, Stucki and Ineichen (1974) observed that low levels of CCCP increase the Ca/O_2 ratio. Interpreting our observation that low levels of CCCP can inhibit Ca^{2+} efflux in the light of this increased Ca/O_2 ratio suggests that CCCP is acting by inhibiting an efflux mechanism for Ca^{2+} which draws its energy from substrate oxidation.

If, as is indicated by measured K^+ gradients in the presence of valinomycin, the membrane potential is greater than around 100 mV [the potential predicted by Ca^{2+} concentration distribution data corrected roughly for Ca^{2+} binding (Puskin et al., 1976; Azzone et al., 1976)], then the data here support the inward leak–outward pump model (Puskin et al., 1976) for both the Ca^{2+} and Mn^{2+} transport systems. Alternatively, if the membrane potential is significantly less than 100 mV, these results support the inward pump–outward leak model for both Ca^{2+} and Mn^{2+} transport.

Acknowledgment

The authors wish to thank Dr. George Kimmich for stimulating discussions of this work.

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Magnetic Resonance Studies of the Binding Site Interactions between ^{19}F-Labeled Nitrophenyl Haptens and Specific Mouse Myeloma Immunoglobulin MOPC-315†

Dale A. Kooistra and John H. Richards*

ABSTRACT: The interactions between MOPC-315, a mouse myeloma protein with specificity for nitrophenyl haptens, and ^{19}F-substituted haptens have been investigated using nuclear magnetic resonance (NMR) spectroscopy. The haptens studied are mono- or dinitrophenyl derivatives of γ-aminobutyric acid, lysine, or glycine which have trifluoromethyl groups attached to the phenyl rings. Upon binding to immunoglobulin, the ^{19}F nucleus experiences a downfield shift whose magnitude depends on the position of the trifluoromethyl group on the phenyl ring but is independent of other structural changes in the hapten such as the number of nitro groups attached to the phenyl ring. Further, the chemical shift of bound hapten is not influenced by the amount of the constant region attached to the binding site; we accordingly conclude that the presence of the distal, constant regions of the immunoglobulin molecule does not influence binding site interactions.

As a part of a program aimed at gaining a molecular understanding of the specific recognition and binding of antigen by antibody (Goetze and Richards, 1977), we have used magnetic resonance techniques to study the interactions of the MOPC-315 myeloma protein, and its binding fragments, with specific haptens containing ^{19}F as a probe for magnetic resonance observation. In particular, trifluoromethyl groups have proved to be sensitive probes of their environment when used, for example, to study the allosteric changes which occur on ligation of hemoglobin (Huestis and Raftery, 1972) and to probe the binding site of chymotrypsin (Smallcombe et al., 1972a,1972b; Gammon et al., 1972; Maddox et al., 1975).

Mouse plasmacytoma MOPC-315 grows in Balb/c mice (Eisen et al., 1968) and produces an α class immunoglobin with a λ_2 light chain (Dugan et al., 1973). The antibody has a high affinity for ligands which incorporate a di- or trinitrophenyl group (Eisen et al., 1968; Johnston et al., 1974), and its binding specificity and affinity (Haimovich and Eisen, 1971; Johnston et al., 1974), amino acid sequence (Dugan et al., 1973; Francis et al., 1974), binding kinetics (Haselkorn et al., 1974), and fragmentation by proteolytic enzymes (Inbar et al., 1971, 1972; Hochman et al., 1973) have been previously studied. The nature of the binding packet of MOPC-315 has also recently received attention (Dwek et al., 1977; Padlan et al., 1977).

† Contribution No. 5495 from the Church Laboratory of Chemical Biology, Division of Chemistry and Chemical Engineering, California Institute of Technology, Pasadena, California 91125. *Received March 11, 1977.* This work was supported by the President's Fund of Caltech and by the National Institutes of Health Grant GM-16424.

0006-2960/78/0417-0345$01.00/0