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Pathway for Uncoupler-Induced Calcium Efflux in Rat Liver Mitochondria: Inhibition by Ruthenium Red†

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ABSTRACT: The rate of uncoupler-induced Ca^{2+} efflux from rat liver mitochondria is increased by acetate and decreased by phosphate. This effect depends on a shift of the apparent K_m, which is increased by phosphate and decreased by acetate, while the V_{max} is not modified. The modification of the apparent K_m by permeant anions presumably reflects changes in the concentration of matrix free Ca^{2+}. A major part of uncoupler-induced Ca^{2+} efflux is sensitive to Ruthenium Red, the specific inhibitor of the Ca^{2+} uniporter, but an apparent insensitivity is observed when the H^+ permeability is rate limiting in the process of Ca^{2+} efflux. The titer of uncoupler required for maximal stimulation of Ca^{2+} efflux increases with the Ca^{2+} load and may be 1–2 orders of magnitude higher than that required for maximal stimulation of respiration. On the other hand, when the uncoupler concentration is raised above 10^{-6} M, the process of Ca^{2+} efflux becomes again Ruthenium Red insensitive. The Ruthenium Red inhibition of uncoupler-induced Ca^{2+} efflux is time dependent, in that the degree of inhibition exerted by low amounts of Ruthenium Red increases with the incubation time. Since the inhibition of the rate of Ca^{2+} influx undergoes a parallel relief, it is possible that Ruthenium Red moves from the cytosolic to the matrix side of the inner membrane. It is concluded that, in native mitochondria, uncoupler-induced Ca^{2+} efflux occurs via reversal of the uniport Ca^{2+} carrier, and not through activation of an independent pathway.

The inhibition of Ca^{2+} transport by lanthanides (Mela, 1968, 1969; Vainio et al., 1970; Scarpa & Azzone, 1970; Reed & Bygrave, 1974; Lehninger et al., 1978) and Ruthenium Red (Moore, 1971; Vasington et al., 1972) has been taken as evidence that Ca^{2+} transport across the inner mitochondrial membrane occurs through a specific carrier [for recent reviews, see Saris & Akerman (1980) and Nicholls & Akerman (1982)]. The inhibition is competitive for La^{3+} (Scarpa & Azzone, 1970; Reed & Bygrave, 1974) and noncompetitive for Ruthenium Red (Reed & Bygrave, 1974) and Pr^{3+} (Mela, 1969). Due to the specificity of its inhibitory effect, Ruthenium Red has become an important tool to define the pathways for Ca^{2+} transport. Addition of Ruthenium Red to aerobic mitochondria, where Ca^{2+} uptake has reached a steady state, results in Ca^{2+} release (Puskin et al., 1976; Pozzan et al., 1977). This has been interpreted as evidence for the existence of two independent pathways for Ca^{2+} transport (Carafoli, 1979; Nicholls & Crompton, 1980): the Ruthenium Red sensitive, electrophoretic pathway for Ca^{2+} uptake and the Ruthenium Red insensitive, presumably electroneutral pathway for Ca^{2+} efflux, activated by Na^+ in excitable tissues [Crompton et al., 1978; for reviews, see Nicholls & Akerman (1982)] and by high $\Delta\psi$[1] in liver (Bernardi & Azzone, 1982, 1983). Besides the Ruthenium Red sensitivity and the nature of the driving force, the two pathways differ significantly in the kinetics, since the rate of Ruthenium Red sensitive Ca^{2+} uptake is 2–3 orders of magnitude higher than the Ruthenium Red insensitive Ca^{2+} efflux.

In 1972, Vasington et al. observed that the very rapid Ca^{2+} release induced by uncouplers is not inhibited by Ruthenium Red. This observation has been repeatedly confirmed in subsequent studies [Puskin et al., 1976; Pozzan & Azzone, 1977; Pozzan et al., 1977; Zoccarato & Nicholls, 1982; cf., however, Sottocasa et al. (1977), Luthra & Olson (1977), and Rigoni et al. (1980)] and attributed to a release of Ruthenium

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[1] Abbreviations: EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; FCCP, carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone; Mops, 4-morpholinepropanesulfonic acid; $\Delta\psi$, membrane potential; Tris, tris(hydroxymethyl)aminomethane.

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Red from its binding sites due to the uncoupler-induced collapse of $\Delta\psi$ (Pozzan et al., 1977).

If one postulates that, in Ruthenium Red treated mitochondria, Ca^{2+} fluxes occur only through uniport-independent pathways, a major problem arises: mitochondria should possess two pathways, for Ca^{2+} influx and efflux, both operative at very high rate with inevitable futile Ca^{2+} cycling and energy drain. Since this is not the case, it must be assumed that the high-rate efflux pathway is normally silent but is activated by uncouplers either directly or indirectly through the collapse of $\Delta\psi$ (Zoccarato & Nicholls, 1982). This, however, contrasts with the observation that the efflux pathway is depressed rather than activated at low $\Delta\psi$ (Bernardi & Azzone, 1982, 1983).

The present research aims to clarify the nature of the pathway for uncoupler-induced Ca^{2+} efflux, in native mitochondria, and to assess its sensitivity to Ruthenium Red. It will be shown that (i) the rate of uncoupler-induced Ca^{2+} efflux depends on the concentration of intramitochondrial free Ca^{2+}, (ii) the apparent sensitivity of the uncoupler-induced Ca^{2+} efflux to Ruthenium Red depends on whether the rate-limiting step is the transport of Ca^{2+} through the uniport carrier or of H^+ through the uncoupler, (iii) at high uncoupler concentrations the sensitivity of Ca^{2+} efflux to Ruthenium Red tends to disappear, (iv) under conditions of maximal sensitivity of Ca^{2+} efflux to Ruthenium Red, the inhibitor titers for the uncoupler-induced Ca^{2+} efflux and for the $\Delta\psi$-driven Ca^{2+} influx are similar, and (v) the extent of inhibitory effect of Ruthenium Red in the processes of Ca^{2+} influx and efflux depends on the incubation time, which is compatible with a slow movement of the inhibitor toward the matrix. It is concluded that the uncoupler-induced Ca^{2+} efflux occurs through the uniport carrier, and a mechanism is discussed for the Ruthenium Red inhibition.

Materials and Methods

Rat liver mitochondria were prepared in 0.25 M sucrose, 10 mM Tris-HCl, pH 7.4, and 0.1 mM EGTA according to Massari et al. (1972). The last washing was carried out in an EGTA-free medium, and mitochondrial protein was assayed with the biuret method, with bovine serum albumin as a standard.

Ca^{2+} transport was followed with the metallochromic indicators Murexide, Antipyrilazo III, and Arsenazo III (Scarpa et al., 1978; Scarpa, 1979) with an Aminco DW 2A dual-wavelength spectrophotometer, equipped with magnetic stirring and thermostatic control (final volume 2 mL, 27 °C). Wavelength pairs were 507 minus 540 nm for Murexide, 720 minus 790 nm for Antipyrilazo III, and 650 minus 690 nm for Arsenazo III. Calibration was routinely performed by addition of known amounts of $CaCl_2$ and/or by back-titration with known amounts of EGTA in the presence of 0.4 μM FCCP at the end of each experiment or set of experiments.

Antipyrilazo III was purified according to Scarpa et al. (1978), and Ca^{2+} contamination was minimized by passing both Arsenazo III and Antipyrilazo III through a Chelex X-100 ion-exchange column. Ruthenium Red was purified according to Luft (1971). The solutions of the dye were prepared daily, and the concentration of Ruthenium Red was determined spectrophotometrically on the basis of an ϵ of 68 $\times$ mM^{-1} cm^{-1} at 533 nm (Fletcher et al., 1964). Oxygen consumption was measured with a Clark oxygen electrode (Yellow Springs Instruments, Yellow Springs, OH) in a closed 2-mL chamber equipped with magnetic stirring and thermostatic control, at 27 °C.

The incubation media are specified in the figure legends. Ruthenium Red, Antipyrilazo III, and Arsenazo III were purchased from Sigma (St. Louis, MO). Murexide was purchased from Carlo Erba (Milano). All chemicals were analytical grade.

Results

Dependence of Uncoupler-Induced Ca^{2+} Efflux on the State of Intramitochondrial Ca^{2+} and on the H^+ Permeability. Addition of either uncouplers or respiratory inhibitors to Ca^{2+}-loaded mitochondria in state 4 results in a process of Ca^{2+} efflux. Although the rates of the observed Ca^{2+} efflux may differ widely, no systematic investigation of the parameters determining this process has yet been reported. For this purpose, the effects of the Ca^{2+} load, of the nature of weak acids, and of the uncoupler concentration on the rate of Ca^{2+} efflux were investigated. The dependence of the rate of uncoupler-induced Ca^{2+} efflux on the Ca^{2+} load was measured in the absence of added weak acids and in the presence of 1 mM P_i or of 10 mM acetate. The data may then be analyzed according to a double-reciprocal plot where, however, on the abscissa are reported the Ca^{2+} loads in nanomoles per milligram of protein instead of the Ca^{2+} concentrations in the medium. From the linear relations obtained in this type of plot, it is then possible to calculate both the V_{max} and the apparent K_m (in terms of Ca^{2+} load) for the efflux process. The plots indicated that at any given Ca^{2+} load, the rate of Ca^{2+} efflux was markedly dependent on the nature of the weak acid, acetate enhancing and P_i depressing the rate of Ca^{2+} efflux (not shown). The apparent V_{max} was 50 nmol of Ca^{2+} (mg of protein)$^{-1}$ s^{-1}, which is even higher than that reported for the influx process via the uniport carrier (Bragadin et al., 1979). P_i and acetate did not modify the V_{max} but shifted the apparent K_m of the efflux process. The apparent K_m corresponded to a Ca^{2+} load of 500 nmol (mg of protein)$^{-1}$ in the absence of added weak acid, and it was increased 10-fold by 1 mM P_i and 50% reduced by 10 mM acetate. Since it is well-established that in the presence of P_i divalent cations tend to form precipitates in the matrix (Lehninger, 1970; Pozzan et al., 1976; Lehninger et al., 1978), these data suggest that the effect on the apparent K_m reflects changes in the free matrix Ca^{2+} concentration due to P_i and acetate, rather than a real shift in K_m of the efflux pathway.

Figure 1 shows the effect of varying concentrations of P_i and acetate on the rate of uncoupler-induced Ca^{2+} efflux. Maximal rate of Ca^{2+} efflux in the presence of 50 μM Ca^{2+} was at about 10 mM acetate, while maximal depression was already at 0.3 mM P_i.

Figure 2 shows a titration of respiratory and Ca^{2+} efflux rates with FCCP. The concentration of FCCP required to induce a 50% stimulation of the rate of Ca^{2+} efflux was much higher than that required for a 50% stimulation of the respiration, even in the simultaneous presence of antimycin A, which enhanced the rate of Ca^{2+} efflux at low uncoupler concentrations. This explains why low rates of uncoupler-induced Ca^{2+} efflux can be obtained at uncoupler concentrations still causing full stimulation of the respiration. The results of Figure 2 are not unexpected if one considers that here there are two sources of $\Delta\psi$, one due to the H^+ pumps and the other due to Ca^{2+} diffusion, the latter being very efficient because of the high conductance of the uniport Ca^{2+} carrier. A much higher concentration of uncoupler is then required for the collapse of the $\Delta\psi$ in the presence of a large Ca^{2+} concentration gradient. The results of Figure 2 thus indicate that at low uncoupler concentrations and/or at high Ca^{2+} load the rate of Ca^{2+} efflux may be limited by the H^+ permeability rather than by the kinetics of Ca^{2+} efflux. This holds true also for the process of Ca^{2+} efflux induced by re-

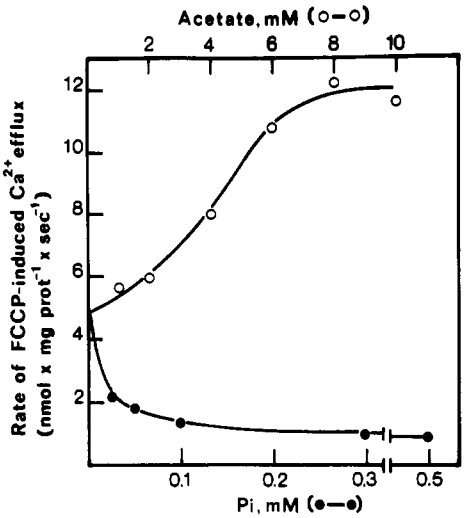

FIGURE 1: Concentration dependence of the effect of P_i and acetate on FCCP-induced Ca^{2+} efflux. The incubation medium contained 0.25 M sucrose, 10 mM Tris–Mops, pH 7.4, 5 mM succinate, 2 μM rotenone, 1 μg/mL oligomycin, 50 μM Antipyrilazo III, 50 μM $CaCl_2$, and the indicated concentrations of P_i (●) or acetate (○). The experiments were started by the addition of 1 mg/mL mitochondria. After attainment of steady-state Ca^{2+} distribution, Ca^{2+} efflux was started by the addition of 0.4 μM FCCP (chart speed 2–20 s/in.).

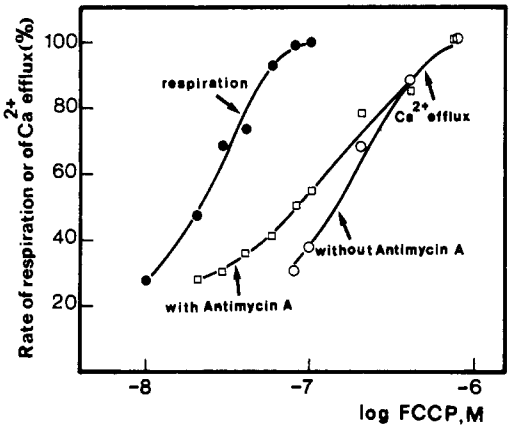

FIGURE 2: Dependence of respiratory and Ca^{2+} efflux rates on FCCP concentration. Experimental conditions are as in Figure 1, with 10 mM acetate. Oxygen electrode experiments (closed symbols). The experiments were started by the addition of 1 mg/mL mitochondria. After 1 min, the indicated concentrations of FCCP were added (●). Ca^{2+} efflux experiments (open symbols). Medium was supplemented with 50 μM $CaCl_2$. The experiments were started by the addition of 1 mg/mL mitochondria. After completion of Ca^{2+} uptake, Ca^{2+} efflux was started by the addition of the indicated concentrations of FCCP alone (○) or preceeded (about 1 s) by 2 μg of antimycin A (□) (chart speed 2–5 s/in.).

spiratory inhibitors where Ca^{2+} efflux depends only on the H^+ that leaks through the membrane.

Ruthenium Red Sensitivity of Uncoupler-Induced Ca^{2+} Efflux. Whether Ruthenium Red inhibits uncoupler-induced and respiratory inhibitor induced Ca^{2+} efflux is an intriguing question. In spite of the fact that in some studies a high sensitivity was reported (Sottocasa et al., 1977; Luthra & Olson, 1977; Rigoni et al., 1980), most investigations have concluded that this process is insensitive to Ruthenium Red (Vasington et al., 1972; Puskin et al., 1976; Pozzan & Azzone, 1977; Pozzan et al., 1977; Zoccarato & Nicholls, 1982). The reason for these discrepancies remains, however, unsettled.

Figure 3 analyzes the sensitivity of antimycin A induced Ca^{2+} efflux to Ruthenium Red at low (traces A and B) or high (traces C and D) Ca^{2+} load. Since the degree of inhibition was high (81%) at low Ca^{2+} load and much lower (29%) at

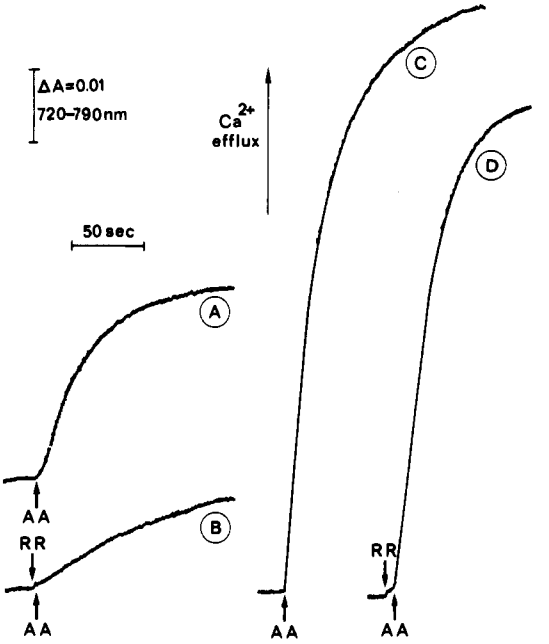

FIGURE 3: Ruthenium Red sensitivity of antimycin A induced Ca^{2+} efflux: dependence on the Ca^{2+} load. Experimental conditions are as in Figure 1, with 10 mM acetate. The experiments were started by the addition of 1 mg/mL mitochondria in the presence of 15 nmol of Ca^{2+} (mg of protein)$^{-1}$ (traces A and B) or of 65 nmol of Ca^{2+} (mg of protein)$^{-1}$ (traces C and D) (not shown). The recording was started after attainment of steady-state Ca^{2+} distribution. When indicated, 0.1 μg of antimycin A (mg of protein)$^{-1}$ (AA) and 0.7 μM Ruthenium Red (RR) were added.

high Ca^{2+} load, it appears that the size of the Ca^{2+} load is a key factor in determining the sensitivity of Ca^{2+} efflux to Ruthenium Red. However, even at low Ca^{2+} load a small fraction of the Ca^{2+} efflux is Ruthenium Red insensitive. A simple explanation for these phenomena is that the apparent Ruthenium Red sensitivity depends on the nature of the reaction that is rate limiting during the process of Ca^{2+} efflux. At low Ca^{2+} load, the rate of Ca^{2+} efflux is slow, due to the relatively low free–Ca^{2+} concentration in the matrix, and the process of Ca^{2+} efflux is not limited by the H^+ permeability. In this case, the Ruthenium Red sensitive fraction can be easily observed (Figure 3A,B). On the other hand, at high Ca^{2+} load the rate of Ca^{2+} efflux is fast, due to the higher free-Ca^{2+} concentration in the matrix, and the process of Ca^{2+} efflux is limited by the H^+ permeability rather than by the kinetics of Ca^{2+} efflux. In this case, the rate of antimycin A induced Ca^{2+} efflux is an underestimation of the true maximal rate, and the degree of inhibition appears to be much lower (Figure 3C,D). If this explanation is correct, one may predict that by increasing the rate of H^+ transport in antimycin A treated mitochondria, the Ruthenium Red sensitivity of Ca^{2+} efflux should increase at high Ca^{2+} load and remain relatively constant at low Ca^{2+} load.

A detailed analysis on the dependence of the rate of Ca^{2+} efflux on the FCCP concentrations at various Ca^{2+} loads and on the relative Ruthenium Red sensitivity is presented in Figure 4. Figure 4A shows that the increase of FCCP concentration from 10^{-8} to 10^{-6} M in the absence of Ruthenium Red enhanced the rate of Ca^{2+} efflux 2-fold at low Ca^{2+} load [from about 1 to 2 nmol of Ca^{2+} (mg of protein)$^{-1}$ s^{-1}] and about 6-fold at high Ca^{2+} [from 3 to about 20 nmol of Ca^{2+} (mg of protein)$^{-1}$ s^{-1}; Figure 4B). In the presence of Ruthenium Red, the rate of Ca^{2+} efflux was only slightly increased by the uncoupler concentration in the range 10^{-8}–10^{-6} M, both at high and low Ca^{2+} load; i.e., in this range of FCCP con-

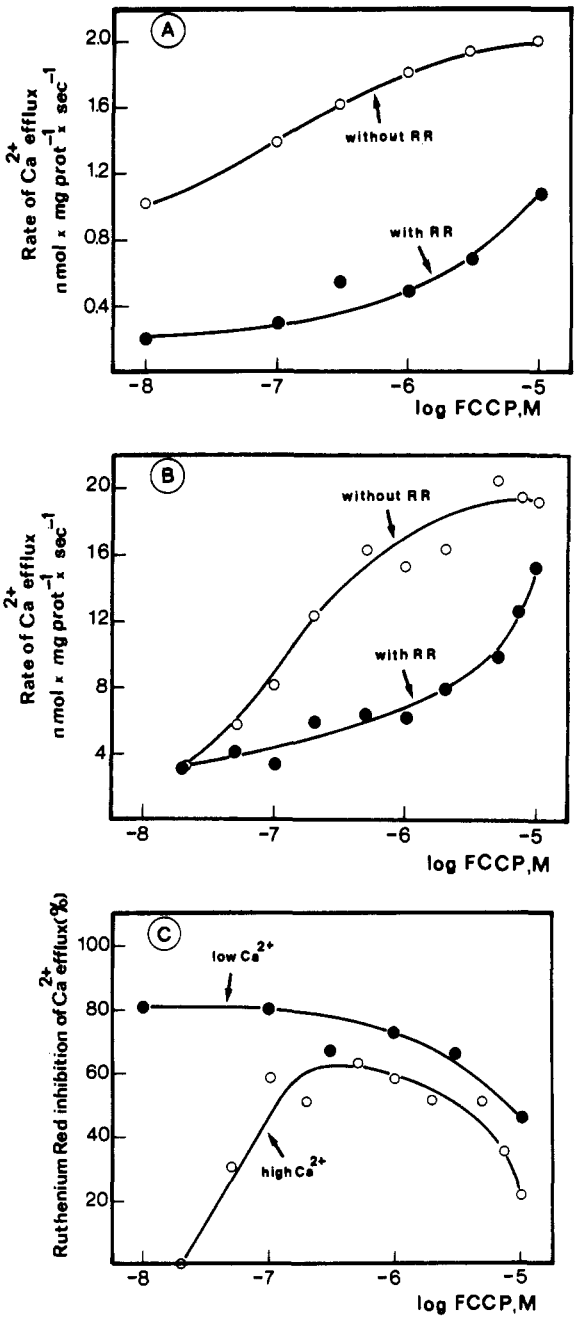

FIGURE 4: Ruthenium Red inhibition of FCCP-induced Ca^{2+} efflux: dependence on FCCP concentration. Experimental conditions are as in Figure 1, with 10 mM acetate. The experiments were started by the addition of 1 mg/mL mitochondria. After completion of Ca^{2+} uptake, Ca^{2+} efflux was initiated by the addition of 2 μg of antimycin A followed (about 1 s) by the indicated concentrations of FCCP (O). Ruthenium Red (0.6 μM) was added about 2 s before antimycin A (●). The Ca^{2+} load (including endogenous Ca^{2+} released by FCCP) was 15 nmol (mg of protein)$^{-1}$ (panel A) or 65 nmol (mg of protein)$^{-1}$ (panel B). Panel C shows the percent inhibition by Ruthenium Red calculated on the data of panels A and B: (●) 15 nmol of Ca^{2+} (mg of protein)$^{-1}$; (O) 65 nmol of Ca^{2+} (mg of protein)$^{-1}$.

centrations the Ruthenium Red sensitivity remained large (close to 80%) at low Ca^{2+} load, while it increased exponentially from 0 to 63% at high Ca^{2+} load (Figure 4C). Figure 4 furthermore shows another phenomenon of interest, namely, that both at high and at low Ca^{2+} load, at FCCP concentrations above 10^{-6} M, there was a progressive increase of Ruthenium Red insensitive Ca^{2+} efflux (Figure 4A,B). At high Ca^{2+} load, the rate of Ca^{2+} efflux at 10^{-5} M FCCP reached values on the order of 15 nmol of Ca^{2+} (mg of protein)$^{-1}$ s^{-1}. The marked increase of the rate of Ca^{2+} efflux in Ruthenium

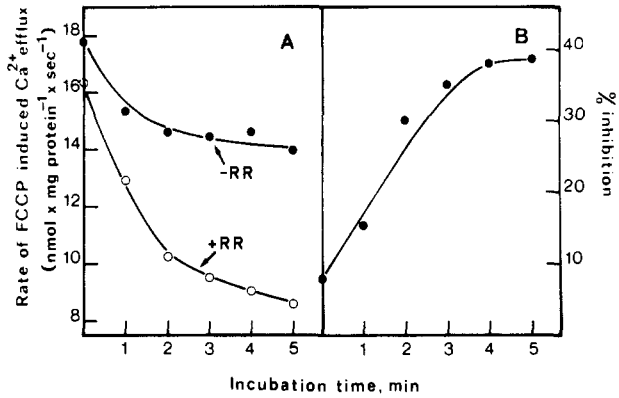

FIGURE 5: Time dependence of Ruthenium Red inhibition of FCCP-induced Ca^{2+} efflux. Experimental conditions are as in Figure 1, with 10 mM acetate and 55 μM $CaCl_2$. The experiments were started by the addition of 1 mg/mL mitochondria, and "zero time" denotes the time required to complete Ca^{2+} uptake with attainment of steady-state Ca^{2+} distribution. (Panel A) (O) 17.5 pmol of Ruthenium Red added at zero time and 0.4 μM FCCP at the time intervals indicated on the abscissa; (●) 0.4 μM FCCP added at the time intervals indicated on the abscissa (chart speed 2 s/in.). (Panel B) Percentage of Ruthenium Red inhibition of FCCP-induced Ca^{2+} efflux as a function of time (data calculated from panel A).

Red supplemented mitochondria resulted in a marked decline of the percent inhibition by Ruthenium Red (Figure 4C). This phenomenon, which is presumably related to the mechanism of the Ruthenium Red inhibition, will be discussed later.

Time Dependence of Ruthenium Red Inhibition. Figure 5A shows that addition of a low amount of Ruthenium Red, after completion of Ca^{2+} uptake, led to a time-dependent inhibition of uncoupler-induced Ca^{2+} efflux. This effect should not be confused with the slight time-dependent decrease of uncoupler-induced Ca^{2+} efflux, occurring also in Ruthenium Red untreated mitochondria. The Ruthenium Red inhibitory effect increased linearly with the time of incubation in the first 4 min, attaining about 40% inhibition. Amounts of Ruthenium Red causing complete inhibition of the uniporter cannot be used in these experiments, since this causes Ca^{2+} efflux per se. However, the titer of Ruthenium Red required for 50% inhibition (I_{50}) of uncoupler-induced Ca^{2+} efflux can be obtained by extrapolation. A Dixon plot for the inhibitory effect of Ruthenium Red on uncoupler-induced Ca^{2+} efflux indicated that a 3-min preincubation with Ruthenium Red resulted in a higher degree of inhibition, with a lowering of the I_{50} from 55 to 8 pmol (mg of protein)$^{-1}$ (not shown). Since the titer of Ruthenium Red may be slightly variable among the various mitochondrial preparations and Ruthenium Red solutions, the appropriate inhibitor concentration causing the time-dependent inhibition without significant Ca^{2+} efflux is to be carefully selected by trial and error in each experiment.

Once ascertained that the degree of Ruthenium Red inhibition on the uncoupler-induced Ca^{2+} efflux is time dependent, it is possible to obtain large inhibitory effects by preincubating directly the mitochondrial stock solution at 0 °C with Ruthenium Red. When these Ruthenium Red treated mitochondria are assessed for Ca^{2+} transport, a pattern such as that shown in Figure 6 is observed, provided that a sufficiently low amount of Ruthenium Red is used [5.4 pmol (mg of protein)$^{-1}$ in this experiment]. The effect of Ruthenium Red on the rate of Ca^{2+} uptake and on the extramitochondrial free Ca^{2+} at steady state was negligible, whereas in the Ruthenium Red treated mitochondria the rate of FCCP-induced Ca^{2+} efflux was clearly inhibited. The experiments of Figures 5 and 6 thus show that, by suitable preincubation, it is possible to obtain a pattern of Ruthenium Red inhibition of Ca^{2+}

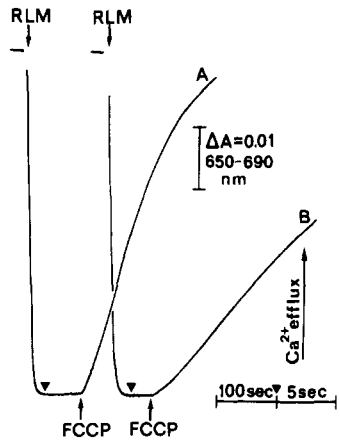

FIGURE 6: Effect of Ruthenium Red pretreatment on FCCP-induced Ca^{2+} efflux. Experimental conditions are as in Figure 1, with 10 mM acetate, 55 μM $CaCl_2$, and 20 μM Arsenazo III instead of Antipyrilazo III. Where indicated, 2 mg of mitochondria (RLM) and 0.4 μM FCCP were added. (Trace B) The mitochondrial stock solution was incubated with 5.4 pmol of Ruthenium Red (mg of protein)$^{-1}$ for 40 min at 0 °C. (Trace A) Untreated mitochondria (stock solution supplemented with an equivalent volume of buffer). Note the time scale change at (▼).

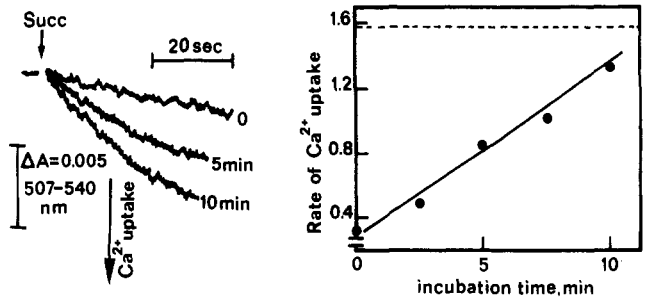

FIGURE 7: Time dependent release of Ruthenium Red inhibition of Ca^{2+} uptake. Experimental conditions are as in Figure 1, with 10 mM acetate, 5 mM $MgCl_2$, 0.1 mM $CaCl_2$, and 0.1 mM Murexide instead of Antipyrilazo III. Succinate was omitted. The experiments were started by the addition of 1 mg/mL mitochondria. A total of 22 pmol of Ruthenium Red (mg of protein)$^{-1}$ was added 1 min after mitochondria (not shown), followed by the addition of 2 mM succinate (Succ) after the time indicated on each trace. (Right) Dependence of the rate of Ca^{2+} uptake on the incubation time. The rate of Ca^{2+} uptake is expressed in nmol (mg of protein)$^{-1}$ s^{-1}, and the dotted line represents the rate of Ca^{2+} uptake of mitochondria untreated with Ruthenium Red.

transport opposite to that generally observed, namely, Ruthenium Red acting as inhibitor of Ca^{2+} efflux rather than of Ca^{2+} influx. Higher amounts of Ruthenium Red could not be used in preincubation experiments, since this results in a significant residual inhibition of the Ca^{2+} uniporter, which causes a lowering of the steady-state Ca^{2+} accumulation ratio.

The experiments of Figures 4–6 further demonstrate that uncoupler-induced Ca^{2+} efflux is highly sensitive to Ruthenium Red and that a relevant increase of the degree of inhibition can be obtained with a suitable preincubation. This may be interpreted as due to a penetration of Ruthenium Red into the inner membrane, until inhibitory sites more deeply embedded in the membrane or situated at the matrix side are reached. Figure 7 shows an experiment that supports this hypothesis. Mitochondria were pretreated with a low concentration of Ruthenium Red, and Ca^{2+} uptake was initiated by the addition of succinate at various time intervals after the addition of the inhibitor. It is seen that in the sample where succinate was added immediately after Ruthenium Red, the rate of Ca^{2+} uptake was strongly inhibited. On the other hand, in the samples where Ca^{2+} uptake was initiated after 5–10 min of preincubation the inhibitory effect of Ruthenium Red was markedly reduced. In the inset, it is shown that the degree of Ruthenium Red inhibition on the rate of Ca^{2+} uptake decreased linearly with the time of incubation. A total of 10 min of aerobic preincubation was necessary to abolish about 90% of the inhibitory effect of 22 pmol of Ruthenium Red (mg of protein)$^{-1}$. A trivial explanation such as binding to the glass cuvette is unlikely: while the inhibitory effect of Ruthenium Red on Ca^{2+} influx decreases during the time course of incubation, the inhibition of Ca^{2+} efflux increases. In this type of experiment, low amounts of Ruthenium Red were used, in order to minimize the binding of the dye to unspecific sites of lower affinity. This bound Ruthenium Red would act as a reservoir for the high-affinity sites, thus precluding the time-dependent decrease of inhibition to be observed. Furthermore, Mg^{2+} was included, in order to slow down the rate of the uniporter to levels compatible with the maximal rate of H^+ extrusion by the redox H^+ pumps.

The evidence for an asymmetry in the inhibitory effects of Ruthenium Red over the processes of Ca^{2+} influx and efflux raises the problem of the titer of the inhibition, i.e., the number of inhibitory sites. In experiments where FCCP was added after 3 min of preincubation with Ruthenium Red, the concentration necessary for 50% inhibition of the process of Ca^{2+} efflux was 8 pmol (mg of protein)$^{-1}$. In experiments utilizing the K^+ diffusion potential as the driving force for Ca^{2+} influx (Scarpa & Azzone, 1970), in the presence of a large excess of valinomycin (Bragadin et al., 1979), we found that the inhibition of Ca^{2+} influx by Ruthenium Red is noncompetitive and that the amount of Ruthenium Red required for 50% inhibition is 28 pmol (mg of protein)$^{-1}$ (not shown). This amount is close to that found, after suitable preincubation, to cause 50% inhibition of the rate of uncoupler-induced Ca^{2+} efflux. It appears therefore that when Ca^{2+} influx and efflux are assayed for the inhibitory effect of Ruthenium Red under suitable conditions (no incubation for the influx and prolonged incubation for the efflux), the titer tends to be similar. This is in accord with the view that the uncoupler-induced Ca^{2+} efflux proceeds through the uniport Ca^{2+} carrier.

Discussion

The process of Ca^{2+} transport through the uniport carrier is an electrophoretic process. Hence, the driving force is given by

$$\Delta\tilde{\mu}_{Ca^{2+}} = RT \ln \frac{\gamma^{in}|Ca^{2+}|^{in}}{\gamma^{out}|Ca^{2+}|^{out}} + zF\Delta\psi \qquad (1)$$

where γ^{in} and γ^{out} are the activity coefficients for Ca^{2+}, $|Ca^{2+}|^{in}$ and $|Ca^{2+}|^{out}$ are the concentrations of Ca^{2+} in the matrix and cytosolic space, z is the valence of Ca^{2+}, and $\Delta\psi$ is the transmembrane potential. Equation 1 predicts that Ca^{2+} influx will take place as long as the electrical term (negative inside) exceeds the chemical term. Equation 1 also predicts that the dimension of the chemical Ca^{2+} gradient depends on the γ^{in}/γ^{out} ratio and that any change on this ratio will affect the rate of Ca^{2+} transport. If Ca^{2+} moved across the inner mitochondrial membrane only through the uniport Ca^{2+} carrier, its distribution should reach electrochemical equilibrium. Since this is not the case at high $\Delta\psi$, it has been postulated that an independent pathway for Ca^{2+} efflux also exists (Puskin et al., 1976; Pozzan et al., 1977; Nicholls, 1978; Carafoli, 1979; Bernardi & Azzone, 1982, 1983), which prevents the attainment of electrochemical equilibrium. The concept of an independent pathway for Ca^{2+} efflux is supported by the observation that addition of Ruthenium Red at steady state

promotes Ca^{2+} efflux [reviewed by Nicholls & Akerman (1982)].

The polarity of Ca^{2+} fluxes and the Ca^{2+} distribution in steady state can be analyzed by a nonequilibrium thermodynamic approach (Azzone et al., 1983) where the process of Ca^{2+} flow, $J_{Ca^{2+}}$, through two independent pathways, one electrical, $J_{Ca^{2+}}^{e}$, and one electroneutral, $2H^+/Ca^{2+}$, $J_{Ca^{2+}}^{n}$, is described as follows:

$$J_{Ca^{2+}} = J_{Ca^{2+}}^{e} + J_{Ca^{2+}}^{n} = L_{Ca^{2+}}^{e}\Delta\tilde{\mu}_{Ca^{2+}} + L_{Ca^{2+}}^{n}(\Delta\tilde{\mu}_{Ca^{2+}} - 2\Delta\tilde{\mu}_{H}) \quad (2)$$

where $L_{Ca^{2+}}^{e}$ and $L_{Ca^{2+}}^{n}$ are the conductances through the electrical and electroneutral pathway, respectively. In steady state, where $J_{Ca^{2+}} = 0$, the distribution ratio is then given by

$$\frac{RT}{zF}\ln\frac{\gamma_{Ca^{2+}}^{in}|Ca^{2+}|^{in}}{\gamma_{Ca^{2+}}^{out}|Ca^{2+}|^{out}} = -\left(\frac{1}{1 + L_{Ca^{2+}}^{n}/L_{Ca^{2+}}^{e}}\Delta\psi + \frac{1}{1 + L_{Ca^{2+}}^{e}/L_{Ca^{2+}}^{n}}\frac{RT}{F}2.303\Delta pH\right) \quad (3)$$

Equation 3 predicts that

$$\frac{RT}{zF}\ln\frac{\gamma_{Ca^{2+}}^{in}|Ca^{2+}|^{in}}{\gamma_{Ca^{2+}}^{out}|Ca^{2+}|^{out}} = -\Delta\psi$$

when $L_{Ca^{2+}}^{n} \ll L_{Ca^{2+}}^{e}$, i.e., when the rate of operation of the electroneutral pathway is negligible with respect to the rate of operation of the uniport electrical pathway. Thus, the observation that in resting mitochondria Ca^{2+} distribution is shifted from electrochemical equilibrium means, in terms of eq 3, that $L_{Ca^{2+}}^{n}$ is very low but not negligible, with respect to $L_{Ca^{2+}}^{e}$. The low rate of respiration in Ca^{2+}-supplemented resting mitochondria also indicates a low rate of Ca^{2+} cycling. According to eq 3, the accumulation ratio of Ca^{2+} deviates from $\Delta\psi$ (variable extents of disequilibrium in the steady-state Ca^{2+} distribution) proportionally to the increase of the $L_{Ca^{2+}}^{n}/L_{Ca^{2+}}^{e}$ ratio, i.e., consequent to activation of the electroneutral pathway or to inhibition of the electrical pathway.

This explains why the Ca^{2+} accumulation ratio is depressed proportionally to the extent of inhibition of the uniport carrier by Ruthenium Red. The present analysis is not modified in principle in case the independent pathway is a $2Na^+/Ca^{2+}$ rather than a $2H^+/Ca^{2+}$ exchange.

Question of the Independent Pathway for Uncoupler-Induced Ca^{2+} Efflux. Equations 2 and 3 predict that polarity of Ca^{2+} flux and Ca^{2+} distribution depend on the relative conductances of the electrical and electroneutral pathways and on the dimension of the two electrochemical gradients, $\Delta\tilde{\mu}_H$ and $\Delta\tilde{\mu}_{Ca^{2+}}$. Since addition of uncouplers to Ca^{2+}-loaded mitochondria results in a complete Ca^{2+} release, eq 2 and 3 predict that the effect of uncoupler can be due either to an increase of the electroneutral conductance $L_{Ca^{2+}}^{n}$ or to a decrease of the H^+ and Ca^{2+} electrochemical gradients, $\Delta\tilde{\mu}_H$ and $\Delta\tilde{\mu}_{Ca^{2+}}$. The rate of uncoupler-induced Ca^{2+} efflux varies within a wide range, depending on the Ca^{2+} load and on the presence of P_i or acetate (Figure 1). Nevertheless, the V_{max} of Ca^{2+} efflux is 3 orders of magnitude higher than the rate of operation of the Ruthenium Red insensitive Ca^{2+} efflux pathway. The hypothesis that the uncoupler-induced Ca^{2+} efflux occurs through the independent pathway implies an enormous increase of the electroneutral conductance, so that $L_{Ca^{2+}}^{n} \gg L_{Ca^{2+}}^{e}$. There is no experimental evidence that the electroneutral pathway may be activated to such an extent by uncouplers or respiratory inhibitors, while the independent pathway is inhibited parallel to the depression of $\Delta\psi$ (Bernardi & Azzone, 1982, 1983). Furthermore, in deenergized, uncoupler-treated mitochondria the rate of Ruthenium Red insensitive, ΔpH-driven Ca^{2+} uptake is negligible (Bernardi & Azzone, 1979). It is therefore likely that $L_{Ca^{2+}}^{n}$ and $L_{Ca^{2+}}^{e}$ are relatively unchanged, while the inversion of polarity reflects a modification of $\Delta\tilde{\mu}_H$ and $\Delta\tilde{\mu}_{Ca^{2+}}$ induced by the uncouplers; i.e., uncouplers modify the Ca^{2+} electrochemical gradient across the uniport Ca^{2+} carrier by inducing a depression of $\Delta\psi$. With respect to the reported discrepancies as to the Ruthenium Red sensitivity of uncoupler-induced Ca^{2+} efflux, our data indicate the following. (i) Under suitable conditions, the amount of Ruthenium Red required for 50% inhibition of Ca^{2+} efflux is only 8 pmol (mg of protein)$^{-1}$ which is even lower than the amount required for 50% inhibition of Ca^{2+} influx; this suggests that $\Delta\psi$-driven Ca^{2+} influx and uncoupler-induced Ca^{2+} efflux go through the same pathway. (ii) The uncoupler concentrations required to obtain maximal rates of Ca^{2+} efflux are much higher than those required to stimulate respiration, presumably due to the necessity of collapsing both the H^+ pump driven and the Ca^{2+} diffusion linked electrical potentials. (iii) The apparent sensitivity to Ruthenium Red depends on whether the rate-limiting step for Ca^{2+} efflux is the H^+ conductance (low sensitivity) or the Ca^{2+} conductance (high sensitivity).

The dependence of the Ruthenium Red sensitivity on the uncoupler concentration at high Ca^{2+} load may explain many of the reported discrepancies. Luthra & Olson (1977) and Rigoni et al. (1980) have found that FCCP-induced Ca^{2+} efflux is highly sensitive to Ruthenium Red at low Ca^{2+} [Ca^{2+} load ~10 nmol (mg of protein)$^{-1}$], i.e., under conditions where the H^+ permeability is not rate limiting even at very low uncoupler concentration (Figure 4A). Zoccarato & Nicholls (1982) have observed a high sensitivity to Ruthenium Red of FCCP-induced Ca^{2+} efflux under conditions of high Ca^{2+} load [70 nmol (mg of protein)$^{-1}$], provided that P_i was added. The Ruthenium Red sensitivity was lost when P_i was omitted. This has led to the suggestion that the independent pathway is activated by uncoupler and depressed parallel to the P_i-induced lowering of matrix free Ca^{2+} (Zoccarato & Nicholls, 1982). However, the present data suggest that the high Ruthenium Red sensitivity in the presence of P_i was due to the fact that uncoupler-induced H^+ permeability was not rate limiting, whereas it was so in the absence of added P_i. Indeed, Zoccarato & Nicholls used low uncoupler concentrations, i.e., 0.2 μM FCCP in the presence of 16 μM bovine serum albumin, which binds 80–90% of the added FCCP (Bernardi & Azzone, 1982; Toninello & Siliprandi, 1982). This leads to an underestimation of the rate of Ca^{2+} efflux in the absence of P_i (i.e., with rate-limiting H^+ permeability), which explains the apparent lack of effect of 3.3 mM P_i on the rate of uncoupler-induced Ca^{2+} efflux (Zoccarato & Nicholls, 1982).

Puskin et al. (1976) have reported that Ca^{2+} efflux induced by 18 μM chlorocarbonyl cyanide phenylhydrazone (CCCP) is insensitive to Ruthenium Red [Ca^{2+} load ~90 nmol of Ca^{2+} (mg of protein)$^{-1}$ in the presence of 10 mM acetate]. However, sampling at 3-min intervals, there employed, may not be adequate to detect processes that occur at rates at 10–20 nmol of Ca^{2+} (mg of protein)$^{-1}$ s^{-1} with $t_{1/2}$ of a few seconds. Indeed, Sottocasa et al. (1977) reported a high Ruthenium Red sensitivity of uncoupler-induced Ca^{2+} efflux at high Ca^{2+} load by using a spectrophotometric method.

Mechanism of Ruthenium Red Inhibition. Once established that the degree of sensitivity of uncoupler-induced Ca^{2+} efflux to Ruthenium Red depends on whether Ca^{2+} or H^+ transport is rate-limiting in the overall process of Ca^{2+} release, the question arises as to why part of uncoupler-induced Ca^{2+} efflux is insensitive to Ruthenium Red even at low Ca^{2+} load, i.e.,

under conditions where Ca^{2+} efflux is not limited by the H^+ permeability. A novel feature of the inhibitory effect is its time dependence, in that the degree of inhibition of Ca^{2+} efflux increases with the incubation time while, vice versa, the degree of inhibition of Ca^{2+} influx decreases. This is circumstantial evidence that Ruthenium Red is transferred from the cytoplasmic to the matrix side of the inner membrane. Note that the Nernst equation predicts that the accumulation ratio for a hexavalent cation, although certainly not readily permeable as Ruthenium Red, increases by 1 order of magnitude every 10-mV increase of the membrane potential.

The partial insensitivity of Ca^{2+} efflux to Ruthenium Red and the time dependence of the Ruthenium Red inhibition are compatible with an asymmetric inhibitory effect, suggesting the following. (i) There are two recognition sites, at the C and at the M side; the Ca^{2+} carrier is inhibited only when Ruthenium Red is bound to the proper recognition site. (ii) Under $\Delta\psi$, Ruthenium Red is transported from the recognition site(s) at the C side to that at the M side. Thus, a minor part of Ca^{2+} efflux is insensitive to Ruthenium Red because Ca^{2+} efflux is initiated soon after the addition of the inhibitor, so that the Ruthenium Red concentration at the M side of the membrane is not sufficient for complete inhibition.

The rate of Ruthenium Red transport is presumably faster at high $\Delta\psi$. Indeed, the process of Ca^{2+} efflux induced by EGTA in state 4 mitochondria loaded with high Ca^{2+} in the presence of acetate is highly sensitive to Ruthenium Red, in spite of the fact that under these conditions the rate of Ca^{2+} efflux is limited by the rate of H^+ back-flow (Pozzan et al., 1977). The concept of Ruthenium Red transport would explain the low sensitivity to the inhibitor at very high uncoupler concentrations, although induction of unspecific Ca^{2+} pathways cannot be excluded.

After this work was submitted for publication, a paper by Jurkowitz et al. (1983), also dealing with the Ruthenium Red sensitivity of uncoupler-induced Ca^{2+} efflux, came to our attention. The conclusion that under conditions of mitochondrial swelling, NADH oxidation, and loss of Mg^{2+} and adenine nucleotides there is activation of a Ruthenium Red insensitive Ca^{2+} efflux, which adds to the Ruthenium Red sensitive Ca^{2+} efflux, is not in contrast with this work, where Ca^{2+} transport has been analyzed only in native mitochondria.

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Registry No. FCCP, 370-86-5; Ca, 7440-70-2; H^+, 12408-02-5; acetic acid, 64-19-7; phosphate, 14265-44-2; Ruthenium Red, 11103-72-3.

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