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THE EFFECT OF RUTHENIUM RED ON Ca^{2+} TRANSPORT AND RESPIRATION IN RAT LIVER MITOCHONDRIA

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SUMMARY

1. The effect of ruthenium red and K_2RuCl_6 on Ca^{2+} transport and mitochondrial respiration was studied.

2. Low levels of ruthenium red (3–6 nmoles/mg mitochondrial protein) completely inhibited the stimulation of respiration by Ca^{2+} but had no effect on the response of respiration to ADP and 2,4-dinitrophenol.

3. Ruthenium red (3–6 nmoles/mg mitochondrial protein) also inhibited respiration-dependent and ATP-supported Ca^{2+} uptake as well as the metabolism-independent, K^+-driven translocation and the high- and low- affinity binding of Ca^{2+}. However, it had no effect on the release of accumulated Ca^{2+}. Respiration-dependent Sr^{2+} and Mn^{2+} uptake also were inhibited.

4. At slightly higher concentrations (10 or more nmoles/mg mitochondrial protein) than those preventing Ca^{2+} binding and transport, ruthenium red markedly inhibited resting (State 4) respiration and altered, but did not prevent, the stimulation of respiration by ADP and P_i. 2,4-Dinitrophenol also overcame the ruthenium red inhibition of respiration.

5. Latent ATPase was significantly inhibited by 4 nmoles of ruthenium red per mg mitochondrial protein. The Mg^{2+}- and the 2,4-dinitrophenol-stimulated ATPase activities, on the other hand, were slightly, if at all, inhibited by as much as 40 nmoles of ruthenium red per mg protein. Phosphate-induced swelling of mitochondria also was not affected by high levels of ruthenium red.

6. K_2RuCl_6 significantly inhibited State 4 respiration at a concentration of 2.5 μM (1 nmole/mg protein), essentially complete inhibition occurring at 10 to 50 μM (4–20 nmoles/mg protein). The inhibition of respiration by K_2RuCl_6 was overcome by Ca^{2+} just as effectively as by ADP and P_i. 2,4-Dinitrophenol also overcame the inhibition of respiration by K_2RuCl_6. Concentrations of K_2RuCl_6 5–10 times higher than that which markedly inhibited respiration were required to inhibit Ca^{2+} transport.

7. These results indicate that ruthenium red has a dual effect on mitochondrial metabolism; it interferes with the binding and transport of Ca^{2+} and at a slightly higher concentration it markedly inhibits respiration by interacting with the energy coupling pathway.

Abbreviations: TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine; EGTA, ethyleneglycol-bis(aminoethyl)-tetraacetic acid.

INTRODUCTION

Four different Ca^{2+} transport or binding processes in isolated mitochondria have been described (see refs. 1 and 2 for reviews). The first one to be discovered was the energy-linked uptake process which is dependent upon coupled respiration or ATP hydrolysis[3–8] and is inhibited by uncouplers of oxidative phosphorylation and, depending upon the energy source, either by inhibitors of respiration or by oligomycin. The other three processes are metabolism-independent. One is the uptake of Ca^{2+} which is driven by the discharge of a K^+ concentration gradient[9]. The second is the low-affinity binding of Ca^{2+}, which is believed to be due to the interaction of Ca^{2+} with the phospholipids of the mitochondrial membrane and which is inhibited by local anesthetics[10]. The last is the high-affinity binding of Ca^{2+} which has been interpreted to indicate the existence of carrier molecules specific for Ca^{2+} (refs. 11, 12).

At very low concentrations, La^{3+} inhibits the high-affinity binding sites[13], the energy-linked[14] and the K^+-driven Ca^{2+} uptake processes[9]. MELA[14] and CHANCE *et al.*[15] have thus proposed that La^{3+} is a specific inhibitor of the Ca^{2+} carrier in the mitochondrial membrane. However, La^{3+} has very high affinity for phosphate, a phenomenon which explains the transient nature of the inhibition by La^{3+}, and prevents the testing of possible effects of La^{3+} on oxidative phosphorylation.

Very recently, MOORE[16] has briefly reported that a complex ruthenium salt, ruthenium red, which is commonly used in histochemistry as a stain for mucopolysaccharides, is a powerful inhibitor of the energy-linked translocation of Ca^{2+}. Since ruthenium red can be used in the presence of phosphate it was possible to show that concentrations which completely inhibited the uptake of Ca^{2+} had no effect on respiration or ADP phosphorylation. Thus, ruthenium red seems to be a specific inhibitor of the energy-linked translocation of Ca^{2+}, and its effect indicates the possible involvement of a carbohydrate-containing component in the uptake process. We have thus carried out a detailed investigation of the effects of ruthenium red on the different Ca^{2+} binding and transport processes in mitochondria; we have found that ruthenium red not only inhibits the energy-linked translocation of Ca^{2+}, but also the K^+-driven translocation, the low affinity binding, and the high-affinity binding of Ca^{2+}. We have also discovered that, at concentrations slightly higher than those preventing mitochondrial interactions with Ca^{2+}, ruthenium red markedly inhibits resting respiration and alters, but does not prevent, the stimulation of respiration by ADP. The data obtained indicate that in addition to its effects on a Ca^{2+} binding factor ruthenium red also interacts with components of the energy-transfer pathway.

MATERIALS AND METHODS

Rat liver mitochondria were isolated in 250 mM sucrose from Wistar strain albino rats by a standard procedure and were washed twice. Oxygen consumption by mitochondria was measured polarographically with a Clark oxygen electrode, and the pH changes in the extramitochondrial medium with a sensitive combination electrode and a Beckman Expandomatic pH meter attached to a strip-chart multichannel recorder. The standard incubation medium usually contained 80 mM NaCl, 5 mM Tris–HCl (pH 7.2) and 5 mM sodium succinate in a final volume of 3.8 ml at 25°.

When present, inorganic phosphate was 2.5 mM in most experiments; (other concentrations are specified in the legends to the figures). Additional procedural details are also found in the legends.

The valinomycin-induced efflux of K^+ from mitochondria was measured with a Beckman 39047 K^+ electrode, and a Beckman Expandomatic pH meter. The reaction medium is described in the legend to Fig. 4.

High- and low-affinity Ca^{2+} binding was studied at 0°, in a 2.0-ml assay system containing 250 mM sucrose, 10 mM Tris–HCl (pH 7.4), 1 μM rotenone, 2 μg antimycin A, and 5 mg of mitochondrial protein, essentially as described by REYNAFARJE AND LEHNINGER[11].

Ca^{2+} uptake was measured isotopically, following rapid filtration through millipore membranes (0.45 μm pore size). At specified times 25 μl of the reaction medium containing the mitochondria were filtered in 3 to 5 sec, and the filters were then washed twice with 1 ml of cold reaction medium which did not contain Ca^{2+}. Radioactivity was measured either in a Nuclear Chicago low background gas flow counter, or in a Packard model 2002 scintillation counter. Mitochondrial ATPase was studied at 30°, in a reaction medium containing 75 mM KCl, 25 mM Tris-HCl (pH 7.4), 4 mM $MgCl_2$ (when added), 8 mM Na-ATP and 1 mg of mitochondrial protein, in a final volume of 1.5 ml. After 15 min, 1.5 ml of cold 20 % trichloroacetic acid were added to the tubes. The contents of the tubes were filtered at 0° through Whatman No. 1 filter paper, and phosphate was determined on the filtrate by a standard colorimetric procedure Initial rates of ATPase were determined by measuring the appearance of H^+ in the medium after addition of ATP to a mitochondrial suspension, as described in the legend for Fig. 10.

Large amplitude swelling was followed at 21°, in a Beckman B spectrophotometer at 750 nm, instead of the usual wavelength of 520 nm, to avoid interference by ruthenium red. The medium contained 100 mM ammonium phosphate, 0.1 mM EGTA, 5 mM Tris–HCl (pH 7.4), 0.5 μg antimycin A and 1 μM rotenone.

The protein concentration of the mitochondrial suspension was determined by a biuret reaction.

All the chemical reagents were analytical grade. Ruthenium red was purchased from British Drug House (BDH), and $^{45}CaCl_2$ from New England Nuclear, U.S.A., K_2RuCl_6 was purchased from Johnson Matthey Chemicals Ltd., London, England.

RESULTS

Effect of ruthenium red on ion transport and respiration

In agreement with the findings of MOORE[16], we observed that low levels of ruthenium red (3–6 nmoles/mg mitochondrial protein) completely inhibited the stimulation of respiration by Ca^{2+}, but have no effect on ADP phosphorylation[17]. As expected, ruthenium red also inhibited the respiration-dependent uptake of Ca^{2+} as measured by the Millipore filtration method described in MATERIALS AND METHODS (Fig. 1 A). The inhibition is not restricted to the respiration-dependent uptake process; the ATP-supported uptake also is strongly inhibited (Fig. 1B).

Since ruthenium red inhibits the transport of Ca^{2+} into mitochondria, most likely by interacting with a component of the Ca^{2+} transport system, it was not surprising to find that ruthenium red did not induce the release of accumulated Ca^{2+}

(Fig. 2). By the same reasoning one might expect that ruthenium red would also prevent the release of Ca^{2+} promoted by uncouplers and by respiratory inhibitors[18]. It was found, however, that ruthenium red (3.5 mnoles/mg mitochondrial protein) did not prevent the exit of Ca^{2+} induced by dinitrophenol (Fig. 2) or by antimycin A (Data not shown). This is shown in Fig. 2 by the normal dinitrophenol-induced disappearance of H^+ in the presence of ruthenium red. In fact, even much higher concentrations of ruthenium red (14 nmoles/mg protein) were ineffective (Fig. 2) (it should be noted that these higher concentration of ruthenium red inhibited respiration, an effect that will be discussed below).

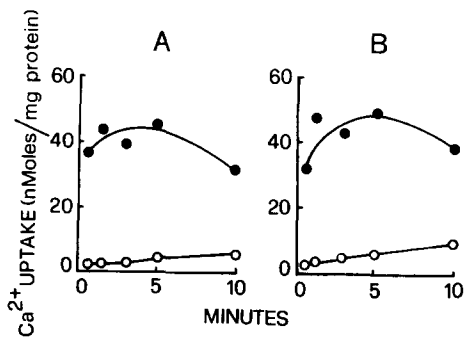

Fig. 1. Effect of ruthenium red on Ca^{2+} transport in rat liver mitochondria. Assay condition: A, the reaction mixture contained 80 mM NaCl, 10 mM sodium succinate, 5 mM Cl_2, 3 mM sodium ATP, 4 mM P_i, 0.1 mM $^{45}CaCl_2$, 5 μM ruthenium red (when added) and 10 mg of mitochondrial protein in a final volume of 10 ml. B, the reaction mixture contained 100 mM sucrose, 75 mM KCl, 33 mM Tris–HCl (pH 7.4), 10 mM Na-ATP, 0.1 mM $^{45}CaCl_2$, 2 μg antimycin A, 2 μM rotenone, 16 μM ruthenium red and 10 mg of mitochondrial protein in a total volume of 3.0 ml. The reaction was carried out at 25° in 50-ml erlenmeyer flasks with continuous stirring. Ca^{2+} uptake was measured by Millipore filtration, as indicated in MATERIALS AND METHODS. ●—●, *minus* ruthenium red; ○—○, *plus* ruthenium red.

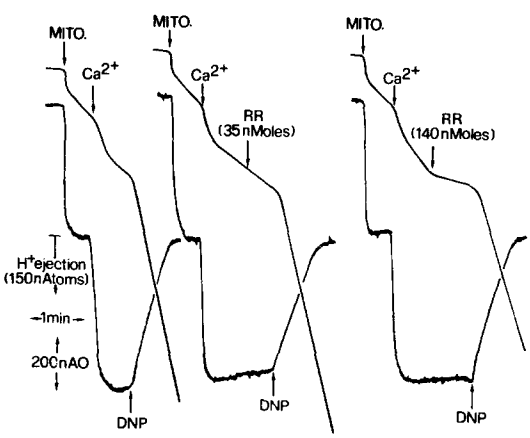

Fig. 2. Effect of ruthenium red (RR) on the release of Ca^{2+}. The reaction medium contained 80 mM NaCl, 2 mM Tris–HCl (pH 7.4), 5 mM sodium succinate and 10 mg of mitochondrial protein in a final volume of 3.8 ml. Where indicated, 600 nmoles of Ca^{2+} and 0.1 mM (final concn.) dinitrophenol (DNP) were added. Oxygen consumption and H^+ movement as indicated in MATERIALS AND METHODS. The incubation temperature was 25°.

Ruthenium red also prevents the stimulation of respiration by Sr^{2+} and Mn^{2+} (Fig. 3). The absence of H^+ ejection from mitochondria upon the addition of Sr^{2+} and Mn^{2+} in the presence of ruthenium red shows that the inhibition of respiratory stimulation is accompanied by the inhibition of the uptake of these bivalent cations. A relatively high amount of Mn^{2+} (4 μmoles) was used, since the affinity of the respiratory chain for Mn^{2+} is much less than for Ca^{2+} and Sr^{2+} (refs. 19, 20).

MOORE[16], has shown that ruthenium red has no effect on the valinomycin induced uptake of K^+, and on the concomitant stimulation of respiration and H^+ release. As can be seen in Fig. 4, however, ruthenium red even inhibits the uptake of Ca^{2+} which normally occurs when the endogenous K^+ is discharged by valinomycin in a K^+-free medium[9]. The valinomycin-induced release of endogenous K^+ from mitochondria is evidently linked mandatorily with the uptake of Ca^{2+} since H^+ or other cations in the medium cannot substitute for Ca^{2+}. Thus when the entrance of Ca^{2+} into mitochondria is inhibited by ruthenium red, the exit of K^+ cannot occur (Fig. 4).

Effect of ruthenium red on high- and low-affinity binding of Ca^{2+}

REYNAFARJE AND LEHNINGER[11] have shown that liver mitochondria contain both high- and low-affinity Ca^{2+} binding sites. Whereas the low-affinity binding sites apparently represent non-specific binding of Ca^{2+} to mitochondrial phospholipids[10,21], the high-affiinity sites are thought to be Ca^{2+}-specific carrier molecules in the mitochondrial membrane[11,12]. Since ruthenium red inhibits energy-dependent Ca^{2+} transport and since the high-affinity Ca^{2+} binding sites seem to be implicated in this

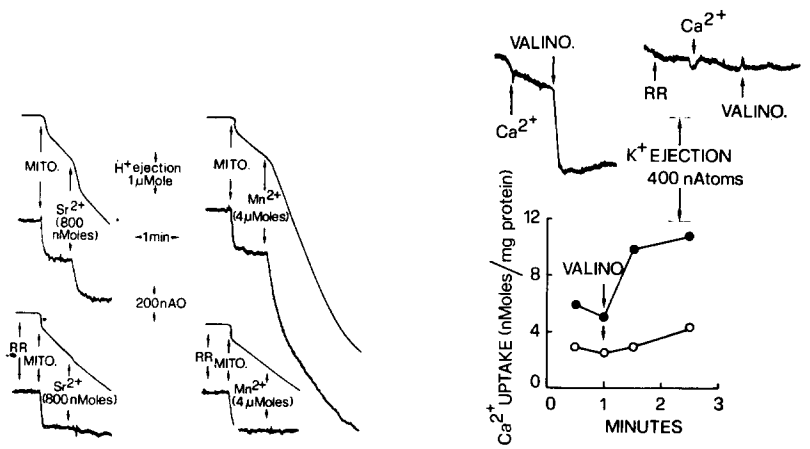

Fig. 3. Effect of ruthenium red on Sr^{2+} and Mn^{2+} uptake by mitochondria. The reaction mixture and experimental conditions were the same as described in Fig. 2 except that 2.5 mM P_i and 6.05 mg of mitochondrial protein were added to the test system. Where indicated 10 μM (final concn.) ruthenium red (RR) was added.

Fig. 4. Effect of ruthenium red (RR) on the K^+-driven uptake of Ca^{2+}. The reaction mixture contained 180 mM sucrose, 2 mM NaCl, 5 mM Tris–HCl (pH 7.4), 5 μg antimycin A, 2 μM rotenone, and 10 mg of mitochondrial protein in a final volume of 3 ml. In the experiment shown at the top of the figure K^+ release was measured. Where indicated, 800 natoms of Ca^{2+}, 16 μM (final concn.) ruthenium red and 10 nM valinomycin were added. In the experiment shown at the bottom of the figure, Ca^{2+} uptake was measured by the millipore filtration method as described in MATERIALS AND METHODS. The amount of Ca^{2+} (labeled with $^{45}Ca^{2+}$) present in the reaction mixture was 800 natoms. ●—●, *minus* ruthenium red; ○—○, *plus* 16 μM ruthenium red.

transport process, the effect of ruthenium red on the high- and low-affinity binding sites was tested. As can be seen in Table I, ruthenium red inhibited the binding of Ca^{2+} to the high affinity sites approximately 90 % but it also inhibited the low-affinity sites about 60 %. Thus the effect of ruthenium red on Ca^{2+} binding is less specific than that of La^{3+} and the local anesthetics[2,22].

TABLE I

EFFECT OF RUTHENIUM RED ON HIGH- AND LOW-AFFINITY BINDING OF Ca^{2+} BY RAT LIVER MITOCHONDRIA

The experiment was carried out essentially as described by REYNAFARJE AND LEHNINGER[11], in a total volume of 2 ml containing 5 mg of mitochondrial protein, at 0°. The concentration of ruthenium red was 5 nmoles/mg of mitochondrial protein.

	High-affinity binding (nmoles/mg protein)		*Inhibition (%)*	*Low-affinity binding (nmoles/mg protein)*		*Inhibition (%)*
	Ca^{2+} added	*Ca^{2+} bound*		*Ca^{2+} added*	*Ca^{2+} bound*	
No addition	2.00	1.94		160	21.0	
+ Ruthenium red	2.00	0.27	87	160	8.2	60

Effect of ruthenium red on mitochondrial respiration

At a level of 4–6 nmoles of ruthenium red per mg of mitochondrial protein the major effects of this compound on mitochondrial metabolism is an inhibition of bivalent cation transport and the concomitant stimulation of respiration. However, it was noted that even at this level of ruthenium red there also was a significant inhibition of resting (State 4) respiration when succinate was the oxidizable substrate. At higher concentrations (10–50 nmoles/mg of protein), ruthenium red also inhibited State 4 respiration when β-hydroxybutyrate, or ascorbate *plus* TMPD were oxidized but the effect with ascorbate was much less pronounced than with the other two substrates. The extent of the inhibition was dependent upon the amount of ruthenium red added per mg of mitochondrial protein (Fig. 5). When succinate was the respiratory substrate, there was approx. 80 % inhibition of resting respiration in a system containing 40 nmoles of ruthenium red per mg mitochondrial protein but less than 50 % inhibition at a ratio of 10 nmoles of ruthenium red per mg of mitochondrial protein. At the levels of ruthenium red tested, dinitrophenol rapidly overcame its inhibition but the uncoupled rate was 20–30 % slower than that observed in mitochondria which have not been treated with ruthenium red.

The inhibition of respiration by the higher amounts of ruthenium red (10–50 nmoles per mg of mitochondrial protein) was relieved also by the addition of ADP *plus* P_i but not by ADP or P_i alone (Fig. 6). However, the rate of the ADP-stimulated respiration was significantly influenced by the concentration of P_i in the incubation medium. The ADP-induced cycles of respiration took place with essentially optimal rates and stoichiometries in the presence of 2.5 mM P_i but with ruthenium red also present, the ADP- stimulated respiration corresponded to the State 4 rather than to the State 3 rate (Fig. 6). Yet the pH trace shown in Fig. 6 shows clearly that ATP was synthesized during the ADP-stimulated respiration in the presence of ruthenium red. When the P_i concentration in the medium was raised to 25 mM, ruthenium red inhib-

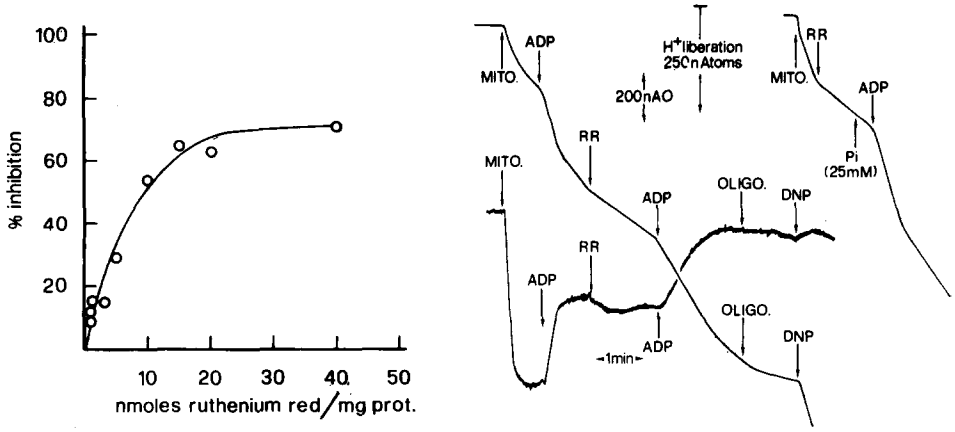

Fig. 5. The effect of ruthenium red concentration on mitochondria respiration. The reaction mixture contained 80 nM NaCl, 5 mM Tris–HCl (pH 7.4), 10 mM sodium succinate and 10 mg of mitochondrial protein in a final volume of 3.8 ml. The incubation temperature was 25°. Ruthenium red was added in the amounts indicated.

Fig. 6. The ADP release of ruthenium red inhibition of respiration and the concomitant synthesis of ATP. The reaction mixture contained 80 mM NaCl, 5 mM Tris–HCl (pH 7.4), 5 mM sodium succinate and 5 mg of mitochondrial protein in a final volume of 3.8 ml. The incubation temperature was 25°. In the experiment shown on the left portion of the figure, 2.5 mM (final concn.) P_i was added to the reaction mixture prior to the addition of mitochondria. In the experiment shown on the right side of the figure, 25 mM (final concn.) P_i was added at the point shown. Where indicated, 500 nmoles ADP, 2.5 μg oligomycin, 50 μM (final concentration) ruthenium red (RR) and 0.1 mM (final concentration) dinitrophenol (DNP) were added.

ited resting respiration as much as it did in the presence of 2.5 mM P_i (Fig. 6). However, at 25 mM P_i, ADP induced a more normal cyclic respiratory response. Moreover, after ADP had been phosphorylated, respiration returned to a rate which was considerably faster than the ruthenium red inhibited rate. This phenomenon occurred even in the presence of 2.5 mM P_i (Fig. 6).

Effect of ruthenium red on ATPase and mitochondrial swelling

The finding that the rate of respiration following ADP stimulation did not return to the ruthenium red-inhibited rate suggested the possibility that ruthenium red stimulated mitochondrial ATPase activity, thereby providing a constant supply of ADP in the medium to sustain a stimulated respiration. Therefore, the effect of ruthenium red on ATPase activity was measured. Surprisingly, ruthenium red has no stimulatory effect on ATPase, either in the absence or presence of activators; rather it inhibited the latent ATPase quite substantially, it had a negligible effect on the Mg^{2+} stimulated ATPase, and it decreased the dinitrophenol-induced ATPase very modestly (Fig. 7 B). Almost maximal inhibition occured in the presence of 10 μM (38 nmoles/mg protein) ruthenium red; increasing the concentration to 100 μM had little additional effect. The initial rate of ATPase activity was evaluated under conditions more similar to those in which the inhibition of respiration by ruthenium red was routinely studied by measuring the H^+ liberation into the extramitochondrial medium following the addition of ATP. Also under these conditions, the latent ATPase associated with State 4 respiration was inhibited, while the dinitrophenol-stimulated ATPase was not affected (Fig. 7A).

Since mitochondria responded normally to ADP addition in the presence of inhibitory levels of ruthenium red when the P_i concentration was increased to 25 mM (Fig. 6), the other possibility considered at this point was that ruthenium red may competively inhibit the penetration of P_i into mitochondria, thereby slowing down the rate of ADP supported respiration. However, when the penetration of phosphate was measured by the swelling technique of CHAPPELL AND CROFTS[23], using ammonium as the permeant counter cation for P_i, 50 μM ruthenium red (71 nmoles/mg protein) had little effect on phosphate induced swelling, suggesting that the effect of ruthenium red on respiration was not due to an inhibition of P_i transport.

Effect of K_2RuCl_6 on Ca^{2+} transport and respiration

To determine whether the effect of ruthenium red on Ca^{2+} transport and respiration was a unique property of this compound, or only of ruthenium, the effect of another ruthenium compound, K_2RuCl_6, on mitochondrial metabolism was studied. At 2.5 μM (1 nmole/mg of mitochondrial protein), K_2RuCl_6 inhibited State 4 respiration in a measurable manner, and the inhibition became essentially complete at 10–50 μM (Fig. 8). As in the case of the inhibition by ruthenium red, ADP *plus* P_i

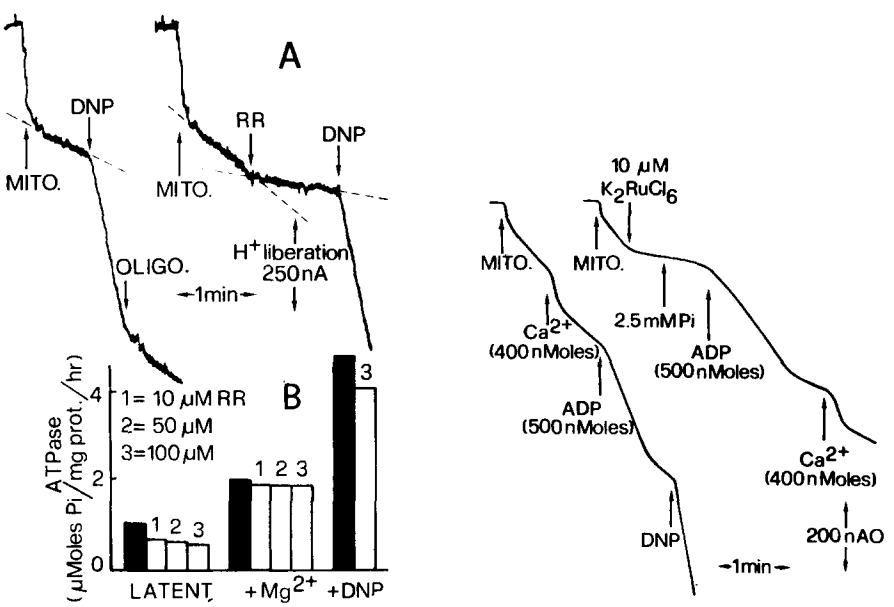

Fig. 7. The effect of ruthenium red (RR) on mitochondrial ATPase activity. Assay conditions: (A) The reaction mixture and experimental conditions were the same as described in Fig. 6 except that P_i was omitted, and 5 mM ATP was added. ATPase activity was determinated by measuring H^+ production. (B) ATPase activity was measured by the release of P_i as described in MATERIALS AND METHODS. When present, the dinitrophenol (DNP) concentration was 0.1 mM and the $MgCl_2$ concentration was 4 mM.

Fig. 8. Effect of K_2RuCl_6 on mitchondrial respiration and energy-linked reactions. The reaction mixture and the experimental conditions were the same as described in Fig. 6 except that the pH was 7.0 and 10 mg of mitochondrial protein was used. In the experiment shown on the extreme left side of the figure. 2.5 mM P_i was added prior to the addition of mitochondria. In the other experiment a final concentration of 2.5 mM P_i and 10 μM K_2RuCl_6 were added at the points indicated.

overcame the inhibition of respiration by K_2RuCl_6. However, Ca^{2+} overcame the inhibition of respiration just as effectively as did ADP *plus* P_i, yielding a State 3 to State 4 response. Interestingly, after Ca^{2+} was taken up by the mitochondria, the respiratory rate returns to the State 4 rather than the inhibited rate. A similar effect was observed when ruthenium red inhibition of respiration was overcome by ADP and 25 mM P_i (Fig. 6).

At concentrations of K_2RuCl_6 higher than 50 μM (20 nmoles/mg protein) the response to Ca^{2+} and to ADP *plus* P_i becomes more and more sluggish. The transport of Ca^{2+} begins to be significantly inhibited between 50 and 250 μM K_2RuCl_6 (Fig. 9), and is completely suppressed at 500 μM. As seen in Fig. 9, in the presence of phosphate the inhibition becomes evident at lower concentrations of K_2RuCl_6. In addition, phosphate enhances the discharge of the Ca^{2+} that had been accumulated. Thus both ruthenium red and K_2RuCl_6 inhibit State 4 respiration; however, an essential difference between the two compounds lies in the response of mitochondrial respiration to Ca^{2+}, which is abolished by ruthenium red well before State 4 respiration is inhibited, and well before the response to ADP becomes disturbed, whereas in the case of K_2RuCl_6 it is affected by concentrations approximately 5–10 times higher than required to markedly inhibit State 4 respiration. The effect of K_2RuCl_6 obviously differs from that of $RuCl_3$, which was found by MOORE[16] to inhibit electron transport and other energy linked functions, at 1 mM, but not in the range of 10–100 μM, where the effect of K_2RuCl_6 is maximal.

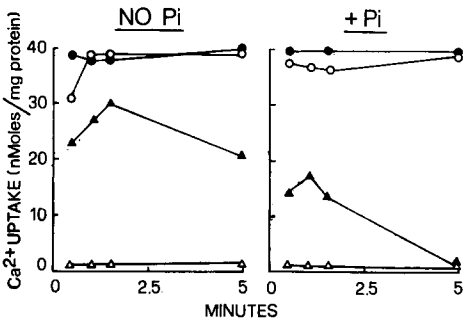

Fig. 9. Effect of K_2RuCl_6 on Ca^{2+} uptake. The reaction mixture and experimental conditions were the same as described in Fig. 8. ●—●, *minus* K_2RuCl_6; ○—○, *plus* 50 μM K_2RuCl_6; ▲—▲, *plus* 250 μM K_2RuCl_6; △—△, *plus* 500 μM K_2RuCl_6.

It should be mentioned at this point that the inhibition of respiration by K_2RuCl_6 was found to be some what variable, *i.e.* in some experiments it did not inhibit as effectively as in others. The reason for this variability is not completely understood at this time but we have observed that solutions of K_2RuCl_6 that were 12–24 h old were more effective than freshly prepared solution. Moreover, it is a more effective and reproducible inhibitor of respiration at slightly acidic pH's (approx. pH 6.6). This is probably because K_2RuCl_6 is quite insoluble in neutral or basic solutions. Because of its insolubility properties, K_2RuCl_6 was dissolved in distilled water for use in the experiments reported here, yielding an acidic solution. Thus, one possible explanation for the change in activity of the K_2RuCl_6 solution upon standing is that it undergoes hydrolysis at the acid pH to form an hydroxide complex which may be the active form of the compound. We are investigating this problem further.

DISCUSSION

Our results show that ruthenium red, at very low concentrations, inhibits all types of Ca^{2+} reactions with mitochondria. It inhibits Ca^{2+} binding to both the high-affinity[11] and the low-affinity[10] sites, a unique property among the inhibitors that have been described so far. Indeed, at concentrations that inhibit energy-linked Ca^{2+} transport, La^{3+}, the classical inhibitor of high-affinity binding has no effect on the activity of the low-affinity sites[13], whereas local anesthetics like butacaine inhibit only the low-affinity binding reaction[2,22]. Complete inhibition of high-affinity binding, and very severe inhibition of low-affinity binding, were obtained at concentrations of ruthenium red between 5 and 20 nmoles/mg of mitochondrial protein; since the average number of nmoles of Ca^{2+} bound to the high-affinity sites in rat liver mitochondria is between 1 and 10 nmoles per mg of mitochondria protein, ruthenium red may exert its effect by titrating the high affinity sites. On the other hand, the number of nmoles of Ca^{2+} bound to the low affinity sites in rat liver mitochondria is between 50 and 70 nmoles per mg of mitochondrial protein, and hence, the inhibition by ruthenium red cannot be due to a stoichiometric titration of these binding sites. Low-affinity sites have so far been considered to be represented by phospholipids of the mitochondrial membranes[21]. However, the effect of ruthenium red, a specific polysaccharide reagent[24–25], on the low-affinity sites indicates the participation of a carbohydrate in this reaction. It is also clear from the effects of ruthenium red that the low- and high-affinity sites, so far considered different in nature, must have a common denominator in their reaction with Ca^{2+}. The high-affinity sites have been proposed to represent carrier molecules specific for Ca^{2+} in the mitochondrial membrane which are involved in a necessary, preliminary step in the process of active Ca^{2+} translocation[11,12]. However, high-affinity binding of Ca^{2+} cannot be demonstrated in some mitochondrial preparations such as water-washed mitochondrial "ghosts"[11,12], which are still capable of active Ca^{2+} transport. It has been postulated that these preparations have lost the specific, high-affinity Ca^{2+} carrier and that Ca^{2+} transport takes place by way of a carrier-independent process[11,12]. However, we have found that ruthenium red effectively inhibited the energy-linked uptake of Ca^{2+} in mitochondrial ghosts[17]. Hence, whatever their nature, the ruthenium red sensitive components are also present in mitochondrial preparations which are not capable of high-affinity binding. Moreover, these findings indicate that the effect of ruthenium red on the active uptake of Ca^{2+} in intact mitochondria cannot be ascribed solely, or necessarily, to its inhibition of the high-affinity Ca^{2+} binding sites.

The translocation of Ca^{2+} in the reverse direction, *i.e.* from the intramitochondrial milieu to the extramitochondrial medium, is not inhibited by ruthenium red. All the inhibitors of membrane carriers so far described (*e.g.* atractyloside[26] for the ADP–ATP exchange across the mitochondrial membrane, SH-reagents[27–29] for the translocation of inorganic phosphate, butylmalonate[30] for some of the Krebs cycle intermediates in mitochondria) inhibit the transport in either direction, thus the lack of inhibition of Ca^{2+} release by ruthenium red suggests that the paths for Ca^{2+} uptake and discharge may be different.

As mentioned above, the effect of ruthenium red strongly suggests the involvement of a carbohydrate in the chain of the events that lead to the influx of Ca^{2+} into mitochondria. Glycoproteins are present in the inner mitochondrial membrane in

rather large amounts[31,32]; upon degradation of the membrane, one or more of these glycoproteins are invariably found in the fractions where the Ca^{2+} binding activity is strongest (unpublished results). Very recently, in collaboration with Dr. G. Sottocasa and his co-workers, we have been able to isolate and to purify to a very considerable extent from the inner membrane of beef liver mitochondria a glycoprotein capable of binding Ca^{2+} with a high affinity in a reaction which is strongly inhibited by ruthenium red[33].

Apart from its ability to prevent the interaction of Ca^{2+} with mitochondria, ruthenium red has interesting effects on mitochondrial respiration. The main effect is a very evident inhibition of State 4 respiration, which is noticable even at 3–5 nmoles of ruthenium red per mg of mitochondrial protein when succinate is the respiratory substrate; the inhibition is essentially complete at about 50 nmoles of ruthenium red per mg of mitochondrial protein. In considering the mechanism of the inhibition, a block of the type induced by antimycin A or rotenone can be excluded, since the inhibition is promptly relieved by ADP *plus* P_i and by uncouplers of oxidative phosphorylation. The fact that both succinate and β-hydroxybutyrate supported respiration are inhibited and that uncouplers yield near maximal rates of respiration in the presence of the inhibitor, rules out an effect of ruthenium red at the level of the membrane permeases for the respiratory substrates. This leaves the energy-coupling chain as the only alternative for the site of action of ruthenium red and places it in the same category as oligomycin, the classical inhibitor of the energy-coupling chain. The site of action of ruthenium red must, however, be closer to the respiratory chain than the site of action of oligomycin, since the former inhibits State 4 respiration, whereas the latter does not.

The suggestion is presented here that ruthenium red forms a stable complex with one of the hypothetical components of the energy-coupling sequence, possibly the same component with which uncouplers interact and certainly a component that is closer to the respiratory chain than the one with which oligomycin interacts. The observed removal of the ruthenium red inhibition by added uncouplers, or by adequate amounts of P_i (25 mM) and ADP, is in line with this suggestion, since uncouplers and ADP *plus* P_i successfully compete with ruthenium red for the component it has complexed with. It is also in line with the observation that only an imperfect removal of the inhibition is obtained when ADP is added together with much smaller amounts of phosphate (2.5 mM) which apparently compete inadequately with ruthenium red.

The possibility that ruthenium red limited the access of inorganic phosphate to the region by inhibiting the phosphate permease was considered unlikely, following the swelling experiments in ammonium phosphate. These experiments cannot, however, be considered conclusive, since they were carried out using a very high concentration of inorganic phosphate.

The fact that Ca^{2+} does not overcome the ruthenium red inhibition of respiration is most likely due to its inaccessibility to the region because ruthenium red also blocks Ca^{2+} interaction with the Ca^{2+} carrier. This view is supported by the observation that the inhibition of State 4 respiration by another ruthenium compound, K_2RuCl_6 was overcome just as effectively by Ca^{2+} as by ADP *plus* P_i. Since Ca^{2+} uptake is not prevented by the low amounts of K_2RuCl_6 which strongly inhibit respiration, Ca^{2+} can reach the region where energy coupling takes place and successfully competes with K_2RuCl_6.

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