SPECIFIC INHIBITION OF MITOCHONDRIAL Ca^{++} TRANSPORT BY RUTHENIUM RED

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SUMMARY

The ability of rat liver mitochondria to transport calcium ions has been found to be inhibited specifically by the dye ruthenium red. Since this dye reacts specifically with mucopolysaccharides, and since energy conservation is not inhibited by this dye, it is concluded that mucopolysaccharides (in the form of mucoproteins or muco or glycolipids) are at the active center of the sites of mediation of mitochondrial Ca^{++} transport.

The ability of mitochondria to accumulate mono - and divalent cations has been the subject of investigation in several laboratories for several years and yet the identity (ies) of carriers or binding sites is (are) still speculative, for review see reference 1,2,3.

Recently however, Lehninger (4) has reported on a protein factor isolated and partially purified from mitochondria. This factor demonstrates high and low affinity Ca^{++} binding properties similar to those reported earlier for intact mitochondria (5,6). The insolubility of this factor in low salt (4) makes it quite possible that one is dealing with small fragments of mitochondria which coalesce in low salt solutions.

Data from other laboratories indicate that phospholipids have the capacity to bind mono - and divalent cations, (7,8) and, indeed, when phospholipids are removed from mitochondria the ability to bind or transport Ca^{++} is lost.

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However, readdition of phospholipids to the delipidated mitochondria restores the binding capability (9).

Green and co-workers (10) have indicated that sulfatides also have the capacity to bind cations with the release of H^+ ions. The kinetics of the cation binding reactions of isolated lipids are slower than observed with mitochondria.

In a paper by Bondareff (11) it was observed that mitochondria were stained by the mucopolysaccharide stain (12,13) ruthenium red-$(Ru(NH_3)_4(OH)Cl_2)$. This was also brought to our attention by Nakao (14). Thus, knowing that the sulfate and carboxy groups of mucopolysaccharides are good candidates for Ca^{++} binding, we investigated the ability of ruthenium red to alter the Ca^{++} binding properties of intact mitochondria.

We wish to report on the effect of ruthenium red on Ca^{++} transport, and to indicate from our preliminary studies that mucopolysaccharides (possibly as mucoproteins or glyco-or mucolipids) are at the active center or are intimately involved in the transport of Ca^{++} into mitochondria.

MATERIALS AND METHODS

$^{45}Ca^{++}$ was obtained from the New England Nuclear Co. Trizma base, Sucrose, glutamic and malic acids, and ADP were from the Sigma Chemical Co., St. Louis, Mo. Ruthenium red was a gift from Dr. T. Nakao, Department of Pathology, Albert Einstein College of Medicine, Bronx, N.Y. Oxygen was monitored using the Clark oxygen electrode obtained from the Yellow Spring Instrument Co.

H^+ and K^+ electrodes were 3945 and 3947 Beekman electrodes. The system for monitoring has been described previously (15).

Calcium uptake was studied by rapid centrifugation (after a specified period of incubation) through 0.8 M sucrose containing 5.0 mM each glutamate, malate and tris phosphate pH 7.4, and saturated with oxygen. Aliquots of the supernatant solutions were counted to determine the residual calcium.

Ruthenium red was shown by us to have an absorption maximum at 530 mm. This wavelength was used to quantitate the residual ruthenium red in the supernatant solutions of treated mitochondria.

RESULTS

Calcium added to mitochondria in the presence of adequate substrate (5.0 mM each of glutamate and malate), 1 mM KCl, 10 mM phosphate, and 0.25 mM ADP or ATP, caused a stimulation of respiration as described by several laboratories (cf ref. 1). This stimulation is indicative of an energy linked accumulation of Ca^{++}. Along with the respiratory stimulation, there occurs an increase in the extra-mitochondrial H^+ and K^+ ions.

This is seen in Fig. 1 where the competence of the rat liver mitochondria for oxidative phosphorylation is exemplified by the state 4-3-4 transition upon addition of ADP before and after Ca^{++}.

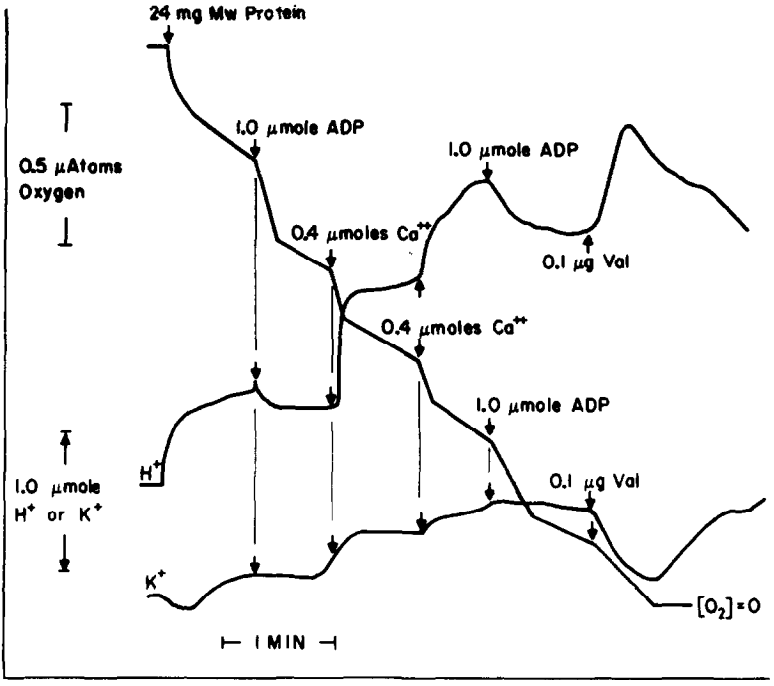

FIGURE 1. Oxygen, K^+ and H^+ tracings of rat liver mitochondria (Mw), and the effect of ADP and Ca^{++}: The reaction is carried out at 23°C and the changes are measured as described in the methods. An upward deflection of the H^+ and K^+ traces indicates an increase of these ions in the medium. The medium (5 ml) contains 20 mM Choline chloride, 2 mM Tris HCl 220 mM Sucrose, 2 mM Tris Phosphate, 10 mM Glutamate and 10 mM Malate, 1 mM KCl, pH 7.4.

The Ca^{++} to oxygen ratio was 2.4 ± 0.4 when 0.2 - 1.0 µmoles of Ca^{++} were added to 26 ± 8 mg mitochondrial protein. The Ca^{++} to H^+ ratio was

0.4 - 0.7 and the Ca^{++} to K^+ ratio was 0.7 - 1.0.

The exactness of these numbers relates only to the present experimental conditions and may be altered under others.

In Fig. 2, one sees that the addition of 0.2 μmoles ruthenium red after the first Ca^{++} addition inhibited further stimulation of respiration by Ca^{++}, but was without effect on the state 3 rate or the valinomycin induced uptake of K^+, release of H^+ and respiratory stimulation.

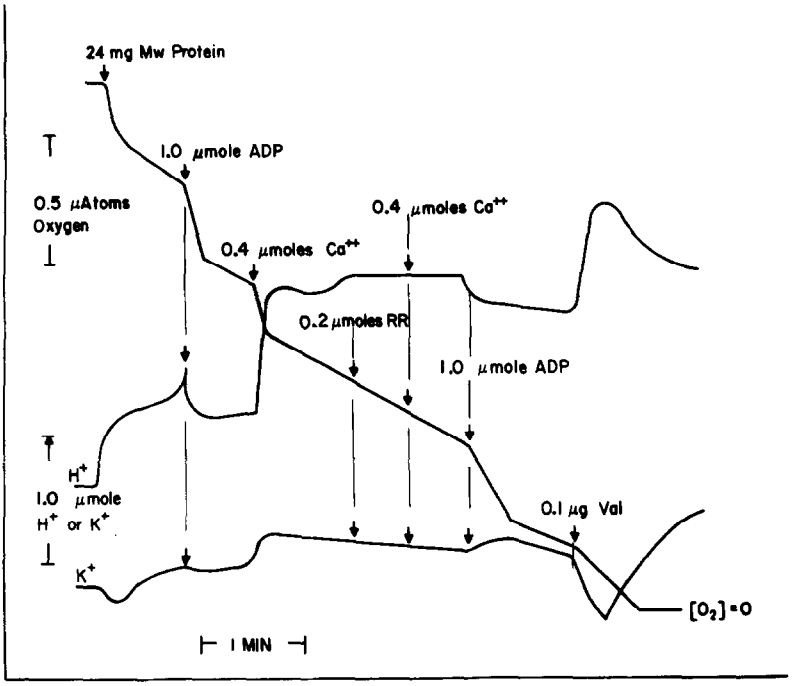

FIGURE 2. The effect of ruthenium red (RR) on the state 3 rate of rat liver mitochondria (Mw), the Ca^{++} induced changes in respiration, and K^+ and H^+ movements. Note also the lack of effect on the valinomycin (Val) induced K/H exchange and respiratory stimulation. The medium and volume are as described for Fig. 1.

Similarly, in Fig. 3, while 6 mμmoles/ml of ruthenium red decreased the extent of Ca^{++} induced respiratory stimulation, 12 mμmoles/ml was totally inhibitory.

Fig. 4 shows the effect of ruthenium red on the uptake of Ca^{++} by mitochondria. At low concentrations of ruthenium red, below 2.6 mμmole/mg,

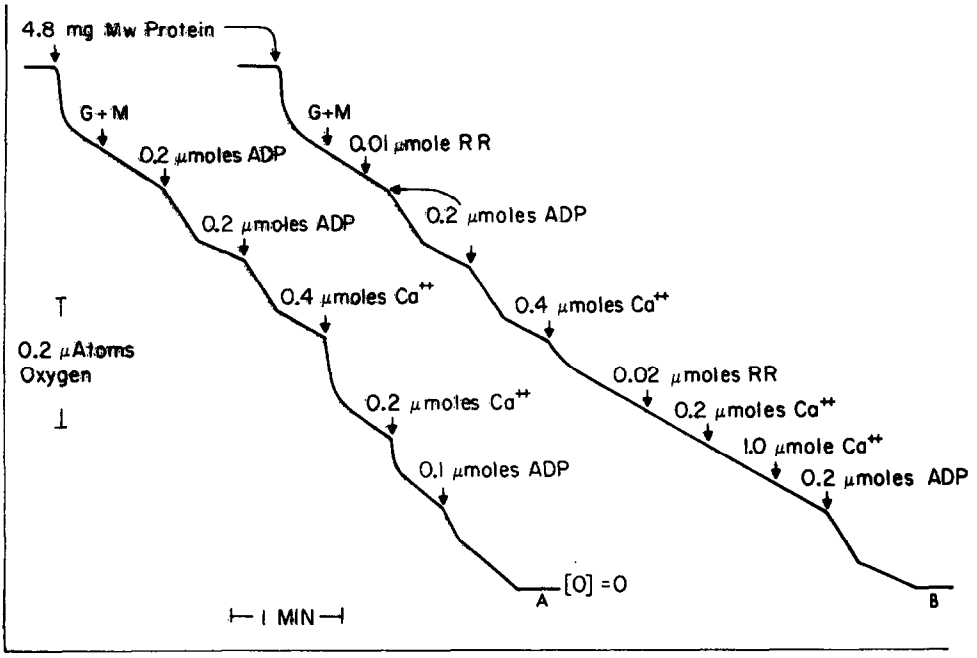

FIGURE 3. The effect of lower ruthenium red (RR) concentrations on Ca^{++} induced respiration: Trace A shows the effect of added ADP and Ca^{++} on 4.8 mg mitochondrial protein (Mw) in 1.5 ml respiration medium containing 250 mM Sucrose, 2 mM KCl, 10 mM Tris Phosphate and 5 mM Tris HCl, pH 7.4. Trace B demonstrates the effect of 0.01 μmoles ruthenium red (RR) on the extent and rate of calcium induced respiration, and the complete inhibition by 0.03 μmoles RR. Note the lack of any inhibition of the state 3 rate either before or after addition of calcium.

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the curve has one slope, but above this concentration the effectiveness of ruthenium red is much greater, as indicated by the steepness of the slope. This effect is still under investigation with respect to the high and low affinities described previously (5,6).

When mitochondrial samples were treated with 0.42 to 4.62 μmoles ruthenium red/mg protein, the data obtained in Fig. 4 were obtained.

When the samples containing 4.6 mμmoles ruthenium red/mg protein were centrifuged, the original supernatant solution, plus that obtained after passage through 0.5 M sucrose, contained 80 - 90% of the ruthenium red. Under these conditions the mitochondria failed to accumulate Ca^{++}, although the control samples still did. The treated and control samples

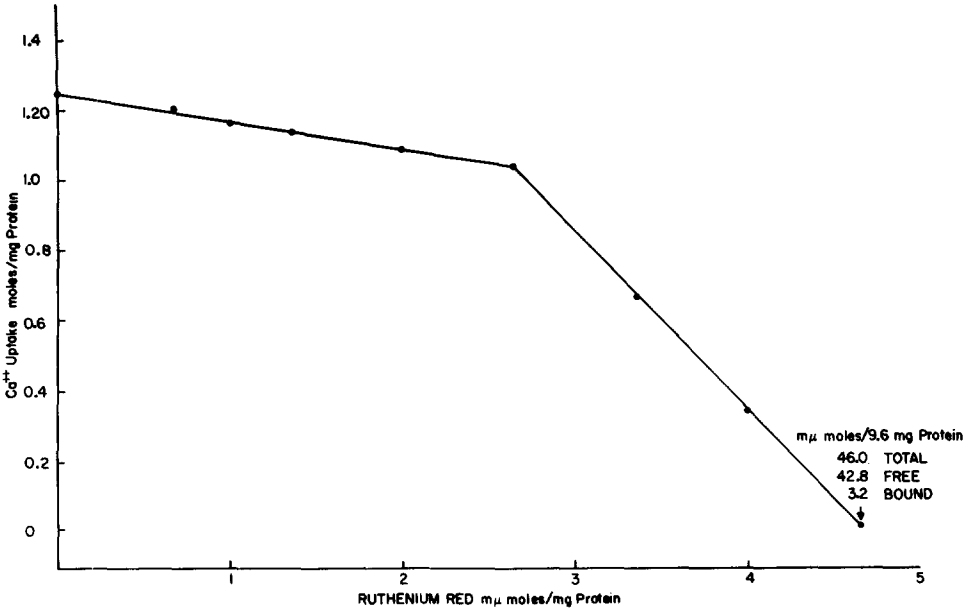

FIGURE 4. The inhibition of Ca^{++} uptake by increasing concentrations of ruthenium red (RR). Ruthenium red was added to 9.6 mg mitochondrial protein/ml. $^{45}Ca^{++}$ was added and after 2.5 mins. the sample was centrifuged through 0.8 M Sucrose containing 5.0 mM each of the Glutamate, Malate and Tris Phosphate, pH 7.4, and saturated with oxygen. Aliquots of the supernatant solution after centrifugation at 32,000 g were counted and the amount of Ca^{++} accumulated, calculated from the specific activity of the calcium added. The numbers on the right side of the figure represent the total amount of ruthenium red added to 9.6 mg mitochondrial protein in a parallel experiment. The amount bound is calculated from the difference between that added and that recoverable in the supernatant solutions after two washings.

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still exhibited decreased but substantial respiratory control.

DISCUSSION

These findings indicate that approximately 0.35 mμmoles of ruthenium red is bound per mg of rat liver mitochondrial protein. If one were to equate this binding with the number of Ca^{++} binding sites (muco-sites), then the number of Ca^{++} binding sites related to active transport of Ca^{++} could be set in the vicinity of 0.35 mμ equivalent sites/mg protein. This is 25 to 30% of the value extimated by Lehninger for high affinity binding (1.2 mμ equivalent) and must be considered to be essentially the same order of magnitude.

As to the binding characteristic of ruthenium red, the possibility that Ru^{+++} in the preparation was responsible for the effects seen was eliminated by using $RuCl_3$ instead of ruthenium red. It was found that 10^{-3} M $RuCl_3$ was inhibitory to electron transport and with it all energy linked functions, but 10^{-4} or 10^{-5} was without effect.

Gustafson and Pihl have shown that ruthenium red reacts selectively with the extra-cellular matrix of cartilage (12) a tissue shown to be abundant in mucopolysaccharide. Luft has also shown ruthenium red to react with the mucopolysaccharide (heparin) of mast cell granules (13). Bondareff has also found that ruthenium red stains or reacts with mitochondria in intact tissue (11), while Nakao has extended this to isolated mitochondria (14).

These facts and the present findings lead us to propose that the mitochondrial binding site involved in calcium transport contains mucopolysaccharide at its active center. The mucopolysaccharide could be in the form of mucoproteins or muco-or glycolipids, and could be identical with the high affinity binding sites reported earlier(5).

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