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# The Mechanism of Lead-Induced Mitochondrial $Ca^{2+}$ Efflux

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## Abstract

Addition of $Pb^{2+}$ to rat kidney mitochondria is followed by induction of several reactions: inhibition of $Ca^{2+}$ uptake, collapse of the transmembrane potential, oxidation of pyridine nucleotides, and a fast release of accumulated $Ca^{2+}$. When the incubation media are supplemented with ruthenium red, the effect of $Pb^{2+}$ on NAD(P)H oxidation, membrane $\Delta\Psi$, and $Ca^{2+}$ release are not prevented if malate-glutamate are the oxidizing substrates; however, the latter two lead-induced reactions are prevented by ruthenium red if succinate is the electron donor. It is proposed that in mitochondria oxidizing NAD-dependent substrates, $Pb^{2+}$ induces $Ca^{2+}$ release by promoting NAD(P)H oxidation and a parallel drop in $\Delta\Psi$ due to its binding to thiol groups, located in the cytosol side of the inner membrane. In addition, it is proposed that with succinate as substrate, the $Ca^{2+}$-releasing effect of lead is due to the collapse of the transmembrane potential as a consequence of the uptake of $Pb^{2+}$ through the calcium uniporter, since such effect is ruthenium red sensitive.

**Key Words:** Mitochondria; lead; calcium; NAD(P)H oxidation; calcium transport; mitochondrial calcium; pyridine nucleotide oxidation; kidney mitochondria.

## Introduction

Several reports have been published on the effect of lead on mitochondrial transport functions. Scott *et al.* (1971) have reported that the interaction of lead with the inner mitochondrial membrane results in an increased permeability to $K^+$, as well as in an inhibition of $Ca^{2+}$ uptake; under their conditions, the process depended on the oxidation of ascorbate-$N,N,N',N'$-tetramethylphenylenediamine. These findings were confirmed and extended by Parr and Harris (1976), using pyruvate–malate as energy source for $Ca^{2+}$

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accumulation. Additional data on the effect of $Pb^{2+}$ on mitochondrial $Ca^{2+}$ transport was provided by Kapoor *et al.* (1984), who proposed that in mitochondria oxidizing malate–glutamate, lead stimulates $Ca^{2+}$ release via the calcium uniporter; their proposition is based in the fact that the $Ca^{2+}$-releasing effect of lead is inhibited by ruthenium red (RR). However, it is important to note that concentrations of RR as high as 5 $\mu$M were utilized in their experiments. As a result of their observations they also proposed that the accumulated $Pb^{2+}$ displaces calcium from its intramitochondrial binding sites.

In regard to the ability of mitochondria to retain $Ca^{2+}$, it has been established that a higher value of membrane $\Delta\Psi$ and a parallel higher NAD(P)H/NAD(P) ratio are required (Nicholls and Akerman, 1982; Lehninger *et al.*, 1978). In this respect, Beatrice *et al.* (1980) and Palmer and Pfeiffer (1981) have shown that reagents for thiol groups such as *N*-ethylmaleimide and diamide promote increased permeability to $Ca^{2+}$ efflux by a mechanism in which oxidation or pyridine nucleotides and collapse of membrane potential are central aspects. The role of membrane SH groups in the mechanism of mitochondrial $Ca^{2+}$ release has been further discussed by Vercesi (1984), who reported that $NADP^+$-stimulated $Ca^{2+}$ efflux correlates with changes of the mitochondrial configuration and the oxidation of membrane thiol groups as induced by diamide.

Considering that $Pb^{2+}$ forms stable complexes with sulfhydryl groups (Valle and Ulmer, 1972), the present study was undertaken to establish whether the $Ca^{2+}$-releasing effect shown by $Pb^{2+}$ is related to its capability to react with membrane thiol groups. The results show that addition of $Pb^{2+}$ to mitochondria oxidizing malate–glutamate induces oxidation of pyridine nucleotides, a decay in $\Delta\Psi$, and release of the accumulated $Ca^{2+}$, regardless of the presence of 0.16 $\mu$M ruthenium red. When the effect of $Pb^{2+}$ was analyzed in mitochondria oxidizing succinate, the fall in membrane potential and the concomitant $Ca^{2+}$ release fail to occur in the presence of 0.16 $\mu$M RR. It is proposed that in the presence of NAD-dependent substrates, the $Ca^{2+}$-releasing effect of $Pb^{2+}$, subsequent to the collapse of the transmembrane potential and the parallel diminution of the NAD(P)H/NAD(P) ratio, is due to binding of $Pb^{2+}$ to SH groups located in the cytosol side of the inner membrane. On the other hand, with succinate as substrate, $Ca^{2+}$ efflux must be secondary to membrane depolarization, caused by accumulation of $Pb^{2+}$ through the calcium uniporter.

## Material and Methods

Mitochondria from rat kidney cortex were prepared as described elsewhere (Chávez *et al.*, 1985). Mitochondrial membrane energization was

assayed as described by Akerman and Wikström (1976) in a dual-wavelength spectrophotometer at 533–511 nm by using the dye safranine. Calcium uptake was followed by incubation of mitochondria in $^{45}CaCl_2$ (specific activity $3 \times 10^6$ counts/min/$\mu$mol) and, at the indicated times, an aliquot of 0.2 ml was filtered through a Millipore filter of 0.45 $\mu$m pore size; the radioactivity retained by the washed, dried filters was measured in a liquid scintillation spectrometer. Changes in the oxidation–reduction state of mitochondrial pyridine nucleotides were estimated spectrophotometrically at 370 minus 340 nm. Protein was determined according to the method of Lowry *et al.* (1951). The nitrate salt of lead was used in all experiments.

## Results

Data shown in Fig. 1 indicate that the addition of increasing concentrations of lead progressively inhibits calcium uptake into mitochondria regardless of whether succinate or malate–glutamate were employed. It is important to note that $Ca^{2+}$ transport is particularly sensitive to $Pb^{2+}$ when malate and glutamate are the oxidizing substrates; at a concentration of 2 $\mu$M $Pb^{2+}$, the inhibition attains values as high as 65%, whereas $Ca^{2+}$ transport remains unaffected when succinate is utilized as the substrate. A significant difference is also observed with 4 $\mu$M $Pb^{2+}$.

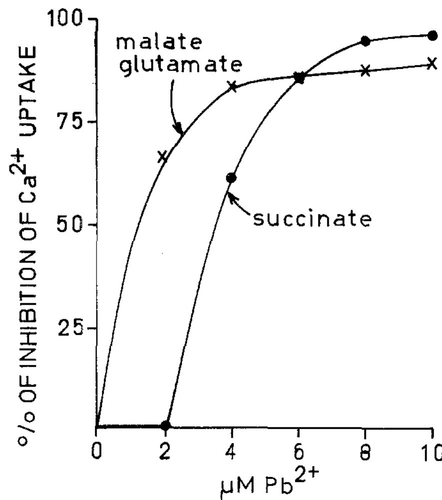


**Fig. 1.** Inhibition of $Ca^{2+}$ uptake by lead depending on the substrate used. Mitochondrial protein (1 mg) was incubated in a mixture (1 ml) containing 125 mM KCl, 10 mM Tris-HCl, pH 7.3, 10 mM acetate-Tris, pH 7.3, 50 $\mu$M $^{45}CaCl_2$, and the indicated concentrations of $Pb(NO_3)_2$. Where indicated, 5 mM succinate plus 10 $\mu$g rotenone or 3 mM malate–1 mM glutamate was added. Incubation time 1 min. Temperature 22°C.

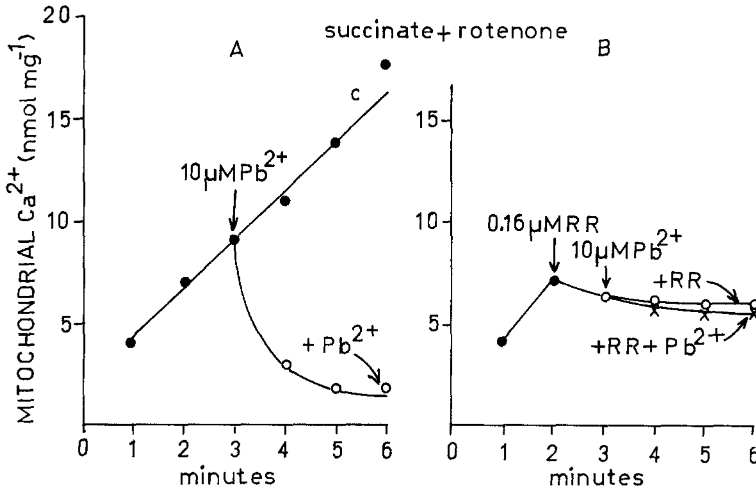


**Fig. 2.** Mitochondrial $Ca^{2+}$ release induced by lead in the presence of succinate as substrate. Mitochondria (2 mg protein) were incubated in a medium (2 ml) as described in Fig. 1. At the indicated times, the indicated concentrations of $Pb^{2+}$ (A) and RR (B) were added, and aliquots of 0.2 ml were withdrawn and filtered as described in Materials and Methods. Temperature 4°C.

To establish the differences in the effect of $Pb^{2+}$ on mitochondrial $Ca^{2+}$ release, depending on the substrate, the experiments presented in Figs. 2 and 3 were performed. Figure 2A shows that the addition of 10 $\mu$M $Pb^{2+}$ to mitochondria, accumulating $Ca^{2+}$ by oxidation of succinate as substrate, induces a prompt release of the total cation content at a rate of 6.3 nmol/mg during the first minute. If 0.16 $\mu$M ruthenium red is added, the $Ca^{2+}$-releasing effect of $Pb^{2+}$ is prevented (Fig. 2B). These results were also obtained by Kapoor and van Rossum (1984) who used malate–glutamate as substrates and 5 $\mu$M RR.

Figure 3A shows that when mitochondria accumulate $Ca^{2+}$ in the presence of malate and glutamate, addition of 10 $\mu$M $Pb^{2+}$ causes a fast efflux of stored $Ca^{2+}$ (6.5 nmol/mg in the first minute), as in mitochondria oxidizing succinate. However, it should be noted that with malate–glutamate as the substrates, RR fails to protect against the calcium-releasing effect of $Pb^{2+}$ (Fig. 3B), although the efflux rate is lower than without RR (2.5 nmol/mg in 1 min).

The ability of lead to promote mitochondrial $Ca^{2+}$ efflux could be related to its efficiency to collapse the membrane $\Delta\Psi$, as has been proposed for other thiol reagents (Jung and Brierley, 1982). As shown in trace A of Fig. 4, addition of 10 $\mu$M $Pb^{2+}$ induces a drop in $\Delta\Psi$ built up by oxidation of 5 mM succinate. As reported (Scott *et al.*, 1971; Parr and Harris, 1976), the effect of $Pb^{2+}$ on mitochondrial functions can be exerted by the uptake

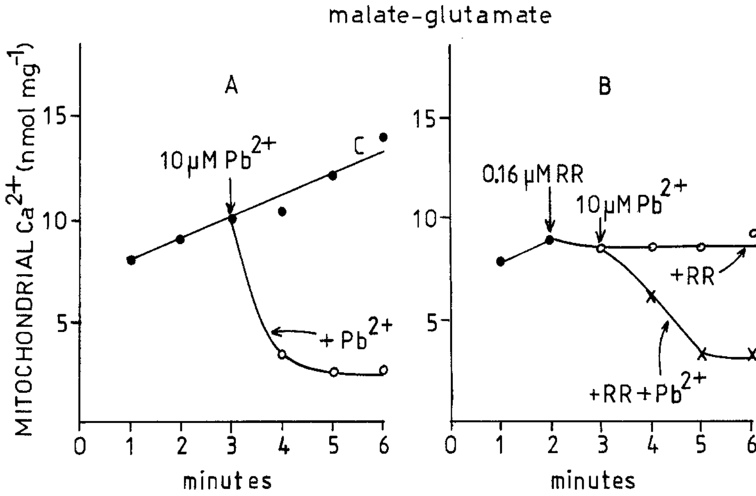


**Fig. 3.** Mitochondrial $Ca^{2+}$ release induced by lead in the presence of NAD-dependent substrates. Protein from mitochondria (2 mg) was added to an incubation medium (2 ml) similar to that described in Fig. 1. At the times indicated, $Pb^{2+}$ (A) and RR (B) were added, and aliquots of 0.2 ml were withdrawn and filtered as described in Materials and Methods. Temperature 4°C.

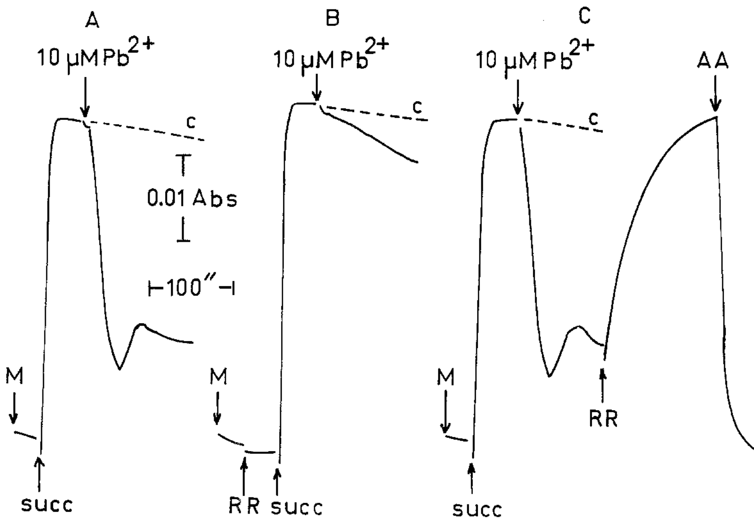


**Fig. 4.** The effect of lead on succinate-induced membrane potential. Mitochondria (M) (2 mg protein) were added to a medium similar to that described in Fig. 1, lacking $CaCl_2$. In addition, the medium contained 10 µM safranine and 10 µg rotenone. Where indicated, 5 mM succinate (A), 0.16 µM ruthenium red (RR) (B), and 0.1 µg antimycin A (AA) (C) were added. Final volume 3 ml. Temperature 22°C.

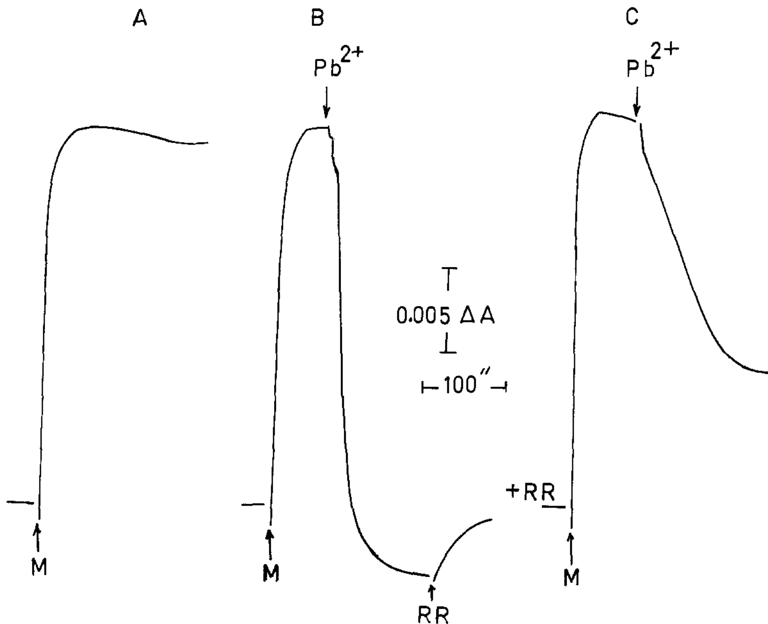


**Fig. 5.** The effect of $Pb^{2+}$ on mitochondrial energization by oxidizing malate–glutamate. Mitochondria (M) (2 mg protein) were added to a medium containing 125 mM KCl, 3 mM malate, 1 mM glutamate, 10 mM acetate-Tris, and 10 μM safranine. Where indicated, 0.16 μM ruthenium red (RR) and 10 μM $Pb^{2+}$ were added. Final volume 3 ml. Temperature 22°C.

of the heavy metal through the $Ca^{2+}$ carrier system. In agreement with the above, trace B of Fig. 4 shows that the addition of 0.16 μM ruthenium red inhibits almost completely the effect of $Pb^{2+}$ on membrane $\Delta\Psi$. Trace C shows that addition of RR promotes recovery of the membrane potential supported by succinate oxidation. The fact that ruthenium red restores $\Delta\Psi$ could indicate that the depolarization of the membrane is produced by the heavy metal transport rather than by an inhibitory action on the succinic dehydrogenase by lead.

Analysis of the effect of $Pb^{2+}$ on mitochondria energized by malate–glutamate consumption shows a large depolarization after $Pb^{2+}$ addition, such depolarization not being reversed by RR (Fig. 5B). In addition, and in contrast to what occurs with succinate, RR is now unable to prevent lead-induced membrane deenergization (Fig. 5C) since, as observed, $Pb^{2+}$ produces a fall of 70% in $\Delta\Psi$ in the presence of ruthenium red.

Besides mitochondrial depolarization, pyridine nucleotide oxidation has also been invoked to explain the induction of mitochondrial $Ca^{2+}$ release by thiol reagents. To point out the effect of $Pb^{2+}$ on this mitochondrial parameter, the experiment shown in Fig. 6 was performed. Trace A shows

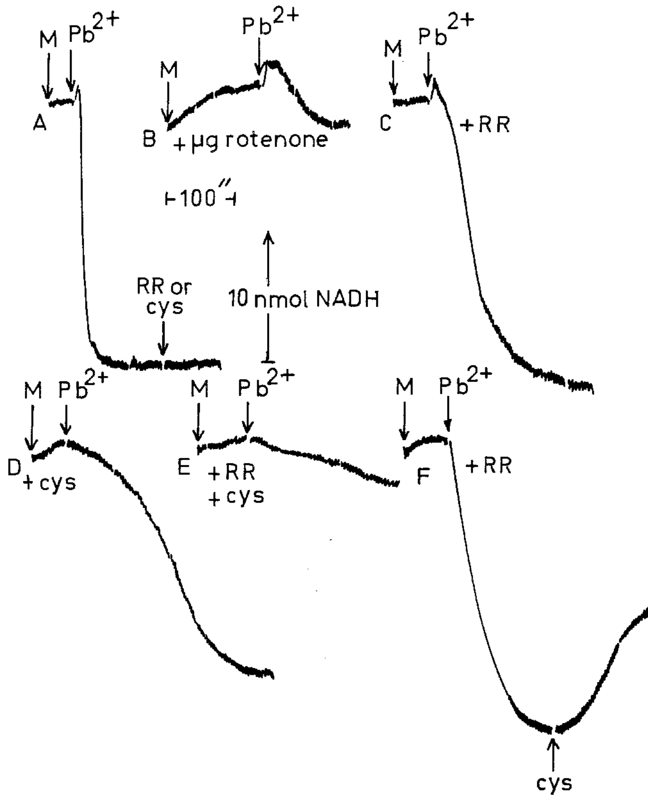


**Fig. 6.** The effect of lead on the oxidation of pyridine nucleotides. Mitochondrial protein (M) (2 mg) was added to a basic medium similar to that described in Fig. 1. The substrates used were 3 mM malate–1 mM glutamate. Where indicated, 10 $\mu$M $Pb(NO_3)_2$, 0.16 $\mu$M ruthenium red (RR), or 200 $\mu$M cysteine (cys) was added. Final volume 3 ml. Temperature 22°C.

that 10 $\mu$M $Pb^{2+}$ induces a burst in NAD(P)H oxidation (10 nmol/mg protein). This effect is hindered by the addition of 10 $\mu$g rotenone (trace B). The addition of 0.16 $\mu$M RR inhibits the initial rate of pyridine nucleotide oxidation; however, the redox steady state is attained at similar levels (trace C). The same concentration of ruthenium red inhibits almost completely the effect of $Pb^{2+}$ on $Ca^{2+}$ release (Fig. 2) and the collapse of membrane $\Delta\Psi$ when succinate is the substrate (Fig. 4), but fails to do so when mitochondria oxidize NAD-dependent substrates (Figs. 3 and 5). Considering that the effect of the heavy metal could be related to its binding to external SH groups, 200 $\mu$M cysteine was added to the incubation mixture and, as observed (trace D), a considerable inhibition in the NAD(P)H oxidation was attained. However, the experimental trace E would indicate that the effect of lead can be produced by its transport to the mitochondrial matrix, as well as

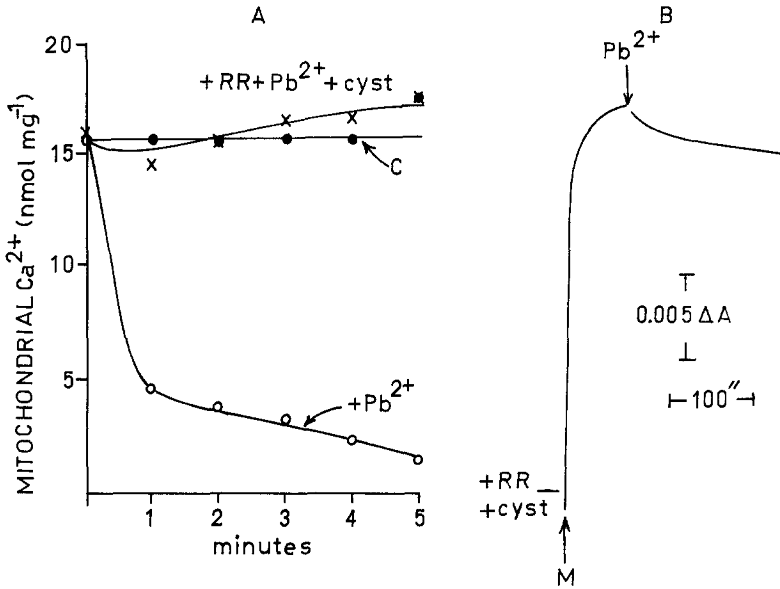


**Fig. 7.** Combined effect of ruthenium red and cysteine on the $Ca^{2+}$-releasing effect of $Pb^{2+}$ (A) and on mitochondrial deenergization as induced by $Pb^{2+}$ (B). In A, 2 mg of protein from mitochondria was preincubated during 10 min in a similar medium as described in Fig. 3; at this time an aliquot of 0.2 ml was withdrawn to determine mitochondrial $Ca^{2+}$ content, and immediately 10 $\mu$M $Pb^{2+}$ or 0.16 $\mu$M RR plus 10 $\mu$M $Pb^{2+}$ and 200 $\mu$M cysteine was added, and at the indicated times aliquots were taken. Final volume 2 ml. Temperature 4°C. In B, 2 mg of mitochondrial protein was added to a medium similar to that described in Fig. 5; where indicated, 0.16 $\mu$M ruthenium red (RR) and 200 $\mu$M cysteine (cyst) were added. Volume 3 ml. Temperature 22°C.

by its reaction with external thiols, since the addition of RR combined with cysteine totally inhibits the oxidation of pyridine nucleotides as induced by $Pb^{2+}$. Trace F shows that the addition of cysteine leads to the reduction of NAD(P)+ in mitochondria treated with RR and $Pb^{2+}$.

The protective effect of ruthenium red plus cysteine was also reproduced in the $Pb^{2+}$-stimulated $Ca^{2+}$ efflux reaction, as shown in Fig. 7A. Regardless of the addition of 10 $\mu$M $Pb^{2+}$, $Ca^{2+}$ remains in mitochondrial matrix when the medium is supplied with 0.16 $\mu$M ruthenium red plus 200 $\mu$M cysteine. This is in line with the protection given by these two latter reagents against the deleterious effect of $Pb^{2+}$ on membrane $\Delta\Psi$ (Fig. 7B).

Apparently, this protective effect of cysteine is not due to a reduction in the ionic concentration of $Pb^{2+}$, or a mass action effect, since its addition in the absence of RR does not provide any protection (Fig. 6A and D).

## Discussion

Kapoor and van Rossum (1984) reported that, in kidney mitochondria, $Pb^{2+}$ is able to inhibit the energy-linked $Ca^{2+}$ uptake as well as to induce efflux of the accumulated calcium. The studies presented here confirm the above statement; in addition, they establish differences in the effect of lead on mitochondrial $Ca^{2+}$ fluxes, depending on the substrates that support $Ca^{2+}$ uptake. Figure 1 reveals that $Pb^{2+}$ at very low concentration (2 $\mu$M) greatly inhibits the uptake of $Ca^{2+}$ (65%) when NAD-dependent substrates are used, as compared to the concentration required when succinate is the electron donor. This marked difference in the sensitivity of $Pb^{2+}$ is unlikely to be caused by a greater inhibition of the energy production when malate–glutamate are used, because higher concentrations of $Pb^{2+}$, such as 10 $\mu$M, slightly inhibited oxygen consumption, using either malate–glutamate or succinate (not shown). Moreover, Scott *et al.* (1971) showed that, in submitochondrial particles, succinate oxidation is more sensitive to $Pb^{2+}$ than NADH oxidation. The high sensitivity to $Pb^{2+}$ could be due to the effect of lead on pyridine nucleotide oxidation as shown in this work, an important factor that controls membrane permeability to $Ca^{2+}$ (Palmer and Pfeiffer, 1981).

The ability of lead to inhibit $Ca^{2+}$ uptake, together with the sensitivity of the respiration-dependent $Pb^{2+}$ uptake to lanthanum, suggests that $Pb^{2+}$ uses the $Ca^{2+}$ carrier system for its effect on mitochondria (Mela, 1969; Lehninger, 1971). By the same token, Kapoor and van Rossum (1984) showed that addition of 5 $\mu$M ruthenium red inhibits $Pb^{2+}$-induced release of calcium accumulated by oxidation of malate–glutamate. The present study shows that RR at a concentration of 0.16 $\mu$M hinders the $Ca^{2+}$-releasing effect of $Pb^{2+}$ when succinate is used as substrate (Fig. 2B); in contrast, RR does not protect against the effect of $Pb^{2+}$ when malate–glutamate are the substrates (Fig. 3B). The discrepancy between these results and those reported by Kapoor and van Rossum (1984) seems to be related to the concentration of ruthenium red used in the experiments here (0.16 $\mu$M) since we found that the effect of lead was blocked when using 5 $\mu$M of ruthenium red with malate–glutamate as substrates (not shown). It could be possible that ruthenium red, added at a concentration which far exceeds the required one to block the $Ca^{2+}$ carrer system (as 5 $\mu$M), binds to sites other than the $Ca^{2+}$ uniport; these nonspecific sites could be the binding sites for lead. Certainly, RR binds to other sites different from the $Ca^{2+}$ uniporter as shown by Jung and Brierly (1984), who reported that the dye is able to open a pathway for $K^+$ by a mechanism in which the $Ca^{2+}$ uniport is not involved.

Membrane depolarization caused by $Pb^{2+}$ with succinate as substrate must be due to the intramitochondrial accumulation of $Pb^{2+}$ through the $Ca^{2+}$ carrier, since it is inhibited at the same ruthenium red titer (Fig. 4) as reported for the $Ca^{2+}$ uniport (Nicholls and Akerman, 1982; Jurkowitz *et al.*, 1983). However, the effect of lead on membrane potential when it was built up by NAD-dependent substrates must be caused by the binding of lead to external functional groups, as under these conditions the reaction was found to be insensitive to RR.

As to mitochondrial deenergization, the requirements for a permeability increase to $Ca^{2+}$ efflux include oxidation of pyridine nucleotides. In this respect, it has been proposed that a higher intramitochondrial NAD(P)H/NAD(P) ratio is necessary to maintain a higher level of reduced glutathion, which presumably maintains a reduced state of membrane SH groups (Beatrice *et al.*, 1984) which in turn controls membrane permeability to cations (Brierley, 1978; Chávez *et al.*, 1977; Jung *et al.*, 1977). The results indicate that NAD(P)H oxidation was stimulated by $Pb^{2+}$ and was not inhibited by ruthenium red.

Considering that $Pb^{2+}$ acts as a thiol modifier reagent (Valle and Ulmer, 1972) we prepose that the mechanism by which it depletes mitochondrial $Ca^{2+}$, collapses membrane $\Delta\Psi$, and induces pyridine nucleotide oxidation is produced through its binding to SH groups. Other authors (Pfeiffer *et al.*, 1979; Siliprandi *et al.*, 1979; Palmer and Pfeiffer, 1981) have shown that a variety of sulfhydryl reagents, such as diamide and *N*-ethylmaleimide, cause similar effects in mitochondria. Based on this latter finding and the fact that ruthenium red does not inhibit the effects of lead when NAD-dependent substrates are used, we propose that, under these circumstances, such $Pb^{2+}$-induced mitochondrial modification must be produced by the interaction of the heavy metal with SH groups located in the cytosol side of the inner membrane. This suggestion is in agreement with the proposal of Vercesi (1984) who reported that $Ca^{2+}$ efflux dependent on pyridine nucleotides oxidation is related to the oxidation of external SH groups by diamide. In addition, we suggest that when succinate is the oxidizable substrate, $Pb^{2+}$-induced $Ca^{2+}$ release must be produced by the uptake of lead through the calcium uniporter which results in membrane depolarization and $Ca^{2+}$ efflux.

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