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ON THE MECHANISM OF CITRATE AND ISOCITRATE PROTECTIVE ACTION ON RAT LIVER MITOCHONDRIA

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Summary: Both citrate and isocitrate prevent the damage (efflux of endogenous Mg^{2+} and pyridine nucleotides, decay of $\Delta\psi$ and release of accumulated Ca^{2+}) induced in rat liver mitochondria by Ca^{2+} and phosphate fluxes. Addition of fluorocitrate suppresses the action of isocitrate, but not that of citrate. The same results have been obtained with mitochondria isolated from animals treated with fluoroacetate. It is suggested that citrate directly and isocitrate by prior conversion into citrate exert the protective action by chelating and retaining Mg^{2+} within the mitochondria.

Lehninger et al. (1, 2) found that when acetoacetate (or oxaloacetate) is added to respiring mitochondria to bring the pyridine nucleotides (PN) pool into a more oxidized state, accumulated Ca^{2+} is released. The subsequent addition of a reductant, such as 3-hydroxybutyrate or isocitrate is followed by reuptake of this released Ca^{2+}. The inference drawn from these observations was that the redox state of mitochondrial PN somehow controls the efflux pathway of Ca^{2+} (1, 2). This interpretation has however been challenged by Nicholls and Brand (3), Beatrice et al. (4), Wolkowicz and Mc Millin-Wood (5) and Bardsley and Brand (6). These Authors argue that acetoacetate potentiate Ca^{2+}-induced damage to the mitochondrial membrane resulting in a fall of $\Delta\psi$ and Ca^{2+} efflux via a reversal of the uniport.

In order to circumvent the possibility of such damage due to PN oxidation, Roth and Dickstein (7) have studied the reverse situation namely PN reduction and its effect on Ca^{2+} efflux. Since only isocitrate, the oxidation of which in mitochondria is $NADP^{+}$ dependent (8), inhibits the ruthenium red insensitive Ca^{2+} efflux, they have suggested that it is

Abbreviation: $\Delta\psi$, mitochondrial transmembrane electrical potential; ΔE, electrode potential; PN, pyridine nucleotides; Hepes, 4-(2-hydroxyethyl)-1-piperazine ethane sulphonic acid.

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the $NADP^+$/NADPH ratio rather than the NAD^+/NADH ratio which is the primary determinant of the rate of Ca^{2+} release or retention. In support, Moore et al. (9) found that isocitrate causes reuptake of Ca^{2+} released from mitochondria by addition of t-butylhydroperoxide.

We have recently found that the acetoacetate-induced Ca^{2+} efflux is accompanied by a corresponding efflux of endogenous Mg^{2+}. Loss of the latter above a critical value is then followed by the collapse of $\Delta\psi$ (10). The same chain of events also occurs when Ca^{2+} and phosphate are added to mitochondria in the absence of any oxidant. Since isocitrate can both enter mitochondria and chelate Mg^{2+}, one explanation for its action on Ca^{2+} efflux might be that it could aid Mg^{2+} retention.

The results reported in this paper show that in fact isocitrate exerts its effect by prior conversion into citrate, which then chelates and retains Mg^{2+} within the matrix space.

EXPERIMENTAL

Rat liver mitochondria were isolated in 0.25 M sucrose and 5 mM Hepes (pH. 7.4) by the conventional centrifugation method. Mitochondrial protein concentration was assayed by a biuret method with bovine serum albumin as standard.

Membrane potential ($\Delta\psi$) was measured by monitoring the distributions of tetraphenylphosphonium across the mitochondrial membrane with a tetraphenylphosphonium selective electrode prepared in our laboratory according to Kamo et al. (11) using a calomel electrode (Radiometer K401) as the response electrode. The electrode potential is linear with respect to the logarithm of the tetraphenylphosphonium concentration with a slope of 59 mV in agreement with the Nernst equation.

Mg^{2+} efflux and Ca^{2+} movements were estimated by atomic absorption spectroscopy of the supernatant (12).

Oxidized pyridine nucleotides or nicotinamide lost in the supernatant were measured fluorometrically by the method of Ciotti and Kaplan (13).

Mitochondrial incubation were carried out at 20°C with 1 mg mitochondrial protein/ml in the following standard medium: 200 mM sucrose, 10 mM Hepes (pH 7), 1.25 μM rotenone, 2 mM sodium phosphate, 1 μM tetraphenylphosphonium chloride. Final Ca^{2+} concentration was brought to 20 μM after having determined Ca^{2+} content of the medium by atomic spectroscopy.

RESULTS

Fig. 1 shows the changes of $\Delta\psi$ and the movements of Ca^{2+} and Mg^{2+} which occur when rat liver mitochondria are incubated at pH 7.0 in the presence of Ca^{2+} and phosphate. If succinate is also present $\Delta\psi$ quickly attains a value of 200 mV and mitochondria accumulate external Ca^{2+}. As

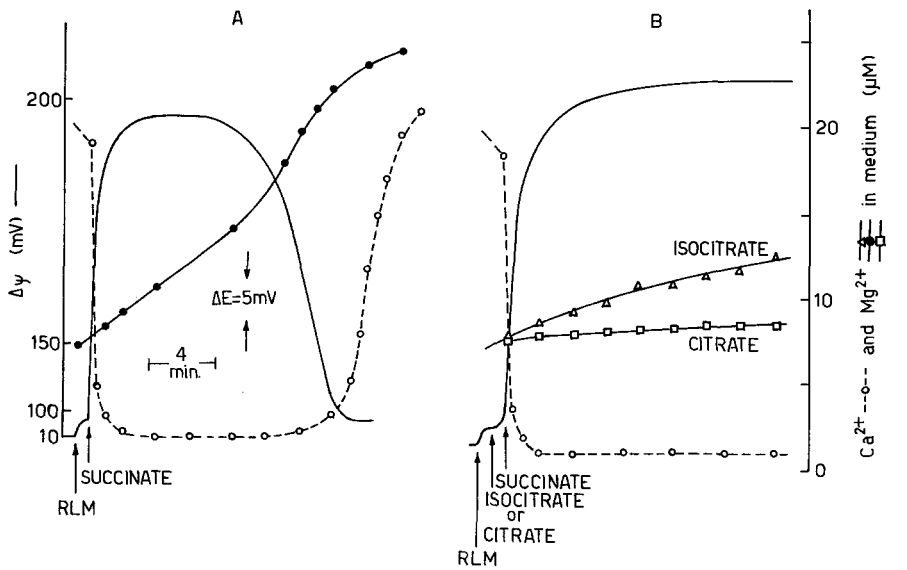

Fig. 1: Effect of Ca^{2+} and phosphate movements on $\Delta\Psi$ and on Ca^{2+} and Mg^{2+} retention in rat liver mitochondria (A); protective action of citrate and isocitrate (B).
Rat liver mitochondria (RLM) were incubated in the standard medium described in Experimental. At the arrows 5 mM succinate, 1 mM citrate and 1 mM isocitrate were added.

soon as the concentration of endogenous Mg^{2+} decreases below approximately 50%, $\Delta\Psi$ begins to drop and soon afterwards the accumulated Ca^{2+} escapes (Fig. 1A). Addition of either isocitrate or citrate before succinate, however, results in prevention of all these functional changes (Fig. 1B). In particular citrate inhibited Mg^{2+} efflux more efficiently than isocitrate. Both isocitrate and citrate also prevent the release of mitochondrial PN caused by Ca^{2+} and phosphate movements (Fig. 2). Furthermore, as shown in Fig. 3, the ruthenium red insensitive Ca^{2+} efflux is inhibited by isocitrate and still more by citrate.

Assuming that isocitrate acts, as it is generally believed, by reducing mitochondrial $NADP^{+}$, the problem arises as to whether citrate acts after its conversion into isocitrate. To test this hypothesis we have repeated the above described experiments in the presence of fluorocitrate, which prevents the citrate-isocitrate interconversion by inhibiting aconitase activity (14). Unexpectally citrate but not isocitrate was effective in the presence of fluorocitrate. For instance, as shown in Fig. 4A, in the presence of fluorocitrate, isocitrate no longer protected energy linked processes (preservation of $\Delta\Psi$ and retention of Ca^{2+} and Mg^{2+}) from

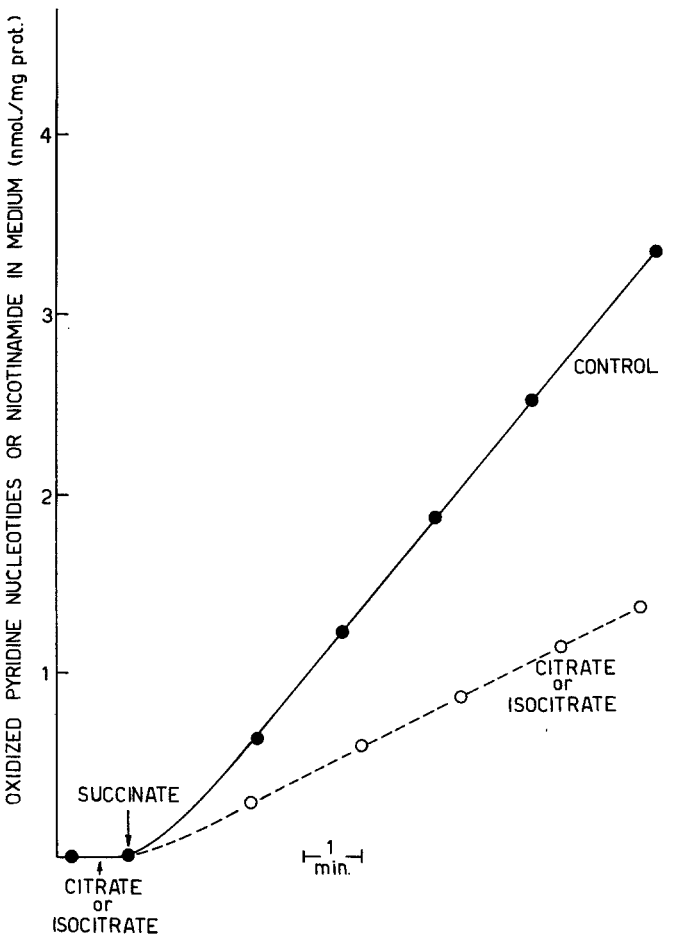

Fig. 2: Prevention of mitochondrial pyridine nucleotides loss by citrate and isocitrate.
Experimental conditions as in Fig. 1.

the deleterious action of Ca^{2+} and phosphate whereas citrate was equally effective in the presence, as well as in the absence, of fluorocitrate (Fig. 4B). Likewise, unlike isocitrate, citrate prevented the efflux of PN and inhibited the ruthenium red insensitive Ca^{2+} efflux (Fig. 3) even in the presence of fluorocitrate. In other words fluorocitrate suppresses the protective action of isocitrate but not that of citrate.

The same results (not reported) have also been obtained using mitochondria prepared from rats treated with fluoroacetate one hour before their death (15). In vivo fluoroacetate is incorporated into fluorocitrate and the latter is present in isolated mitochondria (14), which are consequently unable to convert citrate into isocitrate and viceversa.

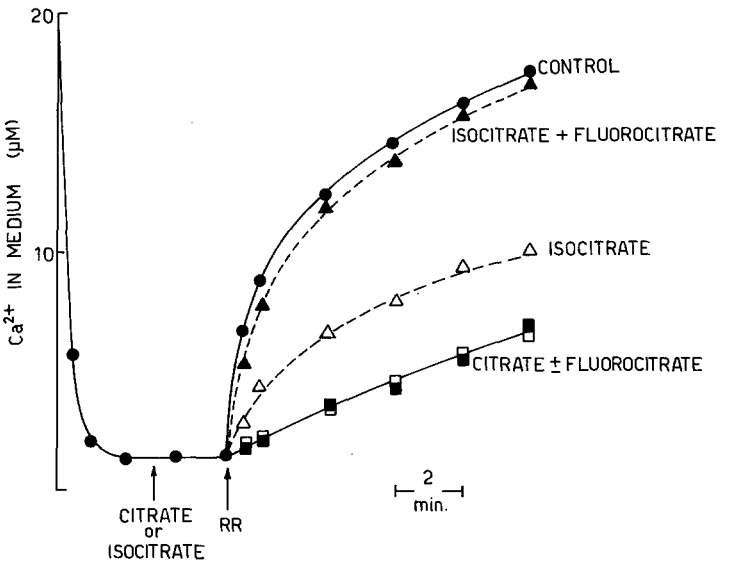

Fig. 3: Inhibition of ruthenium red-insensitive Ca^{2+} efflux by citrate and isocitrate and effects of fluorocitrate.
Experimental conditions as in Fig. 1 with 5 mM succinate in the medium. When present 20 μM fluorocitrate. Added ruthenium red (RR) was 0.5 μM.

DISCUSSION

Our results show that citrate, either added to isolated mitochondria or generated from isocitrate, protects energy linked processes such as preservation of $\Delta\psi$, retention of endogenous pyridine nucleotides and

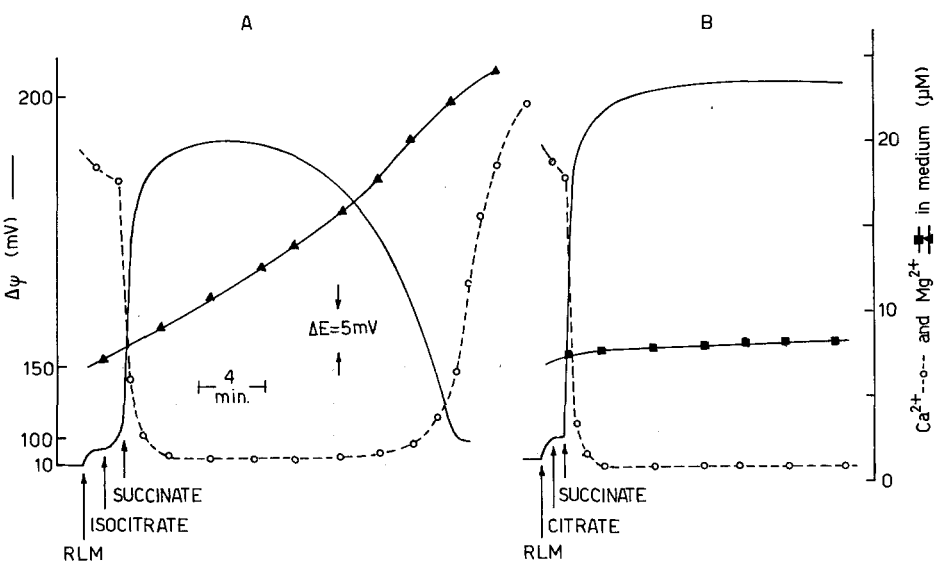

Fig. 4: Fluorocitrate suppresses the protective action of isocitrate (A) but not that of citrate (B). (Compare with Fig. 1B).
Experimental conditions as in Fig. 1. When present 20 μM fluorocitrate.

retention of accumulated Ca^{2+} and Mg^{2+} from the damaging effects of externally added Ca^{2+} and phosphate. Isocitrate shares this protective action provided it is converted into citrate. In fact when this conversion is prevented by fluorocitrate, a potent inhibitor of the aconitase reaction, the action of isocitrate disappears. In the light of these findings the assumption that isocitrate action is only dependent upon the capability of this substrate to keep mitochondrial $NADP^{+}$ and the dependent glutathione system in a reduced state (16) must be reconsidered. An additional, or superimposed, factor emerging from the present results is the capability of citrate, either added or generated from isocitrate, to retain endogenous Mg^{2+} within mitochondria (Fig. 2), thus preventing the impairment of inner membrane permeability properties, which follow Mg^{2+} loss.

It is known that the binding constant between citrate and Mg^{2+} is more than 10 times higher than that between isocitrate and Mg^{2+} (17, 18); this may explain why isocitrate exhibits its action only after its conversion into citrate. The retention of Mg^{2+} within the matrix not only inhibits Ca^{2+} efflux (Fig. 1), probably by the same mechanism as that by which external Mg^{2+} slows down Ca^{2+} uptake (19), but may also be a sufficient condition for the protection of other mitochondrial functions, irrespective of the status of the cation. For instance in the present experimental conditions Mg^{2+} is presumably retained as a citrate chelate. The release of Mg^{2+} induced by Ca^{2+} and phosphate is accompanied by a closely parallel release of adenine nucleotides (20) and pyridine nucleotides (Fig. 2) so that it is likely that Mg^{2+} retention is important not in itself but in that it implies a retention of other factors fundamental for the preservation of energy linked processes.

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