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A Mechanism of Latent Cryoinjury and Reparation of Mitochondria

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Changes in the systems of oxidative phosphorylation and ion transport of rat liver mitochondria have been studied during storage or incubation after freeze–thawing. It has been found that two different processes take place in frozen–thawed mitochondria, one of them tends toward resealing membrane defects and is accompanied by a partial reparation of function; and another one leads to a decrease of the membrane potential, release of K^+ and Ca^{2+} from the matrix, accumulation of lipid peroxides, and uncoupling of oxidative phosphorylation (latent cryoinjury). The latent cryoinjury appears as a result of oxidation of endogenous pyridine nucleotides under conditions of high permeability of the inner membrane and low membrane potential, thus causing activation of the membrane lipid peroxidation and enzyme hydrolysis, leakage of cations, and deenergization of mitochondria. Inhibition of the latent cryoinjury favors the restoration of mitochondrial function after freeze–thawing.

Mitochondria are important cellular structures which can transform the energy of substrate oxidation into the energy of ATP and also participate in intracellular ionic distribution. Functional features of mitochondria are related to the structural integrity of the inner membrane which should have low permeability for ions (11, 17).

It has been shown that a freeze–thaw cycle changes the functional activity of isolated mitochondria, the extent of cryoinjury being associated with freezing and cooling rates (2, 18, 25, 26). Freezing injury of the electron transport chain may be avoided to a certain extent by using rapid freeze–thaw protocols (19), but the membrane permeability is impaired (2, 18, 26).

Cryoinjury is known to develop in mitochondria during a certain period after thawing, this is latent injury (23). However, mechanisms of this latent damage remain unclear.

The aim of this report is to study changes in the ionic transport properties of rat liver mitochondria during storage or incubation after rapid freezing and rapid thawing. Two different trends in membranes of frozen–thawed mitochondria have been found. One of these is directed to resealing of membrane defects and the other one leads to "secondary" induction of ionic transport and to uncoupling of oxidative phosphorylation (latent cryoinjury). The latent cryoinjury of mitochondria is conditioned by changes in the metabolic balance leading to the activation of membrane lipid peroxidation and hydrolysis.

MATERIALS AND METHODS

Mitochondria were isolated from the livers of male Wistar rats weighing 150–200 g according to Johnson and Lardy (8) in a medium containing 300 m*M* sucrose, 10 m*M* Tris/HCl, and 0.5 m*M* EDTA, pH 7.4. Mitochondria were washed in an EDTA-free medium and the pellet was resuspended in the latter medium (the mitochondrial protein concentration was 40–60 mg/ml) and frozen in polyethylene ampules to −196°C at a rate of 400–500°C/min by being plunged into liquid nitrogen. Samples were

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thawed in a water bath at 40°C at a rate of 500–600°C/min. Cryoprotectants were not used.

Respiration was measured with a Clark-type oxygen electrode (5) in a 1-ml chamber; further details are indicated in the figure legends. The transport of potassium and calcium ions in mitochondria was estimated by changes in their respective concentrations by means of K^+- and Ca^{2+}-selective electrodes (Radiometer type F2312K and F2112Ca, Copenhagen). Changes in the mitochondrial membrane potential ($\Delta\Psi$) were assigned by the distribution of the lipophilic cation tetraphenylphosphonium (TPP^+) between the mitochondrial matrix and the incubation medium with a TPP^+-selective electrode made according to Kamo (10). Free Ca^{2+}, K^+, and TPP^+ were measured in water-jacketed glass reaction vessels using a colomel reference electrode. The vessel contents were stirred rapidly and smoothly with a magnetic stirrer and circular jollower. The output from the electrodes was relayed from pH/voltmeters (PHM84 Research pH Meter, Radiometer, Copenhagen) to strip-chart recorders. Electrode responses were calibrated over the pCa range 6.0–6.5, pK 3.0–6.0; pTPP 6.0–7.0 in standard medium. Mitochondrial Ca^{2+}, K^+, and TPP^+ transport was measured at 26°C in a final volume of 5.0 ml. The basic medium comprised 250 m*M* sucrose, 30 m*M* Tris/HCl, 10 m*M* KH_2PO_4, 8 m*M* succinate (pH 7.4) to which 1 mg of mitochondrial protein · ml^{-1} was added in the reaction vessel. When studying the potassium transport, KH_2PO_4 in the incubation medium was exchanged for H_3PO_4–Tris. Other additions from concentrated stocks were as noted in the figure legends. The responses of Ca^{2+}, K^+, and TPP^+ electrodes were also calibrated in each system addition of internal standards to the medium.

The intensity of lipid peroxidation was determined by accumulation of malonic dialdehyde (MDA) capable of being stained with thiobarbituric acid (4) using an extinction coefficient of 156 mM^{-1} cm^{-1}. Incubations were for 20 min at 26°C in media containing 120 m*M* KCl, 30 m*M* Tris/HCl, 10 m*M* KH_2PO_4, 8 m*M* succinate (pH 7.4); 1-ml aliquots of mitochondrial suspension (1 mg protein/ml suspension) were transferred to tubes with 0.1 ml of 100% (w/v) trichloroacetic acid and 0.01 ml of 10 m*M* butylated hydroxytoluene (BHT) for 0, 5, 10, 15, and 20 min of incubation. The mitochondrial protein was determined by the method of Lowry *et al.* (15).

RESULTS

Freeze–thawing of mitochondria in media without cryoprotectant is known to lead to uncoupling of oxidative phosphorylation (2, 18, 25, 26). It can be seen from Fig. 1 that the rate of substrate (succinate) oxidation by mitochondria immediately after freeze–thawing is so great that subsequent addition of ADP or the uncoupler carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP) did not increase the oxygen consumption rate. However, after 5 min of storage of dense suspension at room temperature, the addition of ADP increased the respiratory rate (Fig. 1). The data obtained previously on the partial maintenance of mitochondrial function after freeze–thawing without cryoprotectant (2, 18, 26) can be explained by the fact that the time between thawing and measurement was not registered. During further storage, the extent of respiratory stimulation with ADP (the respiratory control index, RCI) increased initially, reaching the maximum in 20 min (RCI = 2.3 ± 0.2), and then decreased. As is seen from Fig. 1, RCI changes are associated with the rate of state 2 substrate oxidation according to Lardy and Welman (13) (in the absence of ADP) because the state 3 respiratory rate (in the presence of ADP) changed but to a lesser extent.

Figure 2 shows the kinetics of state 2 succinate oxidation by mitochondria immediately after freeze–thawing. It can be seen

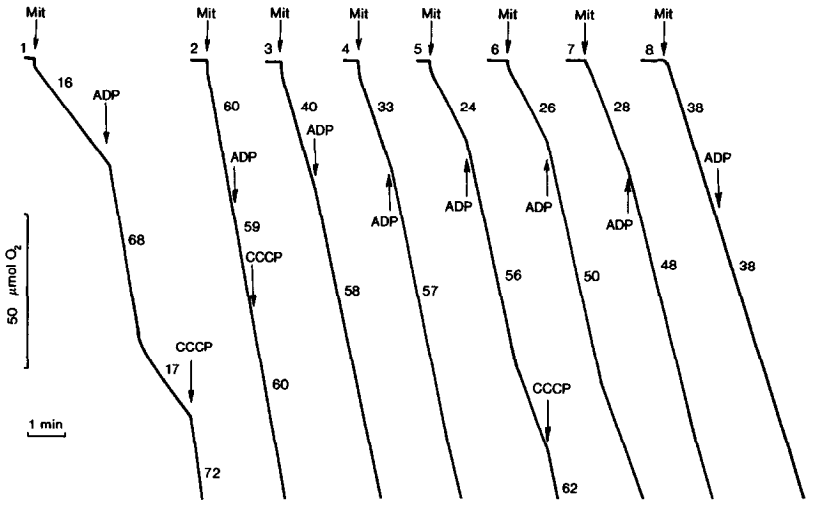

FIG. 1. The effect of the time of storage of frozen–thawed mitochondria at 20°C on oxidative phosphorylation. Trace 1 shows the oxygen uptake for fresh mitochondria, with an increase in oxygen uptake after ADP was added. Trace 2 shows the response seen with frozen–thawed mitochondria—in this case there was a large oxygen uptake even before ADP addition, showing that these mitochondria were uncoupled. Traces 3–8 show changes in oxygen uptake during storage of frozen–thawed mitochondria at 20°C for 5 min (trace 3), 10 min (trace 4), 20 min (trace 5), 30 min (trace 6), 40 min (trace 7), and 60 min (trace 8). It can be seen that a large oxygen uptake before ADP decreased during storage up to 20 min, was accompanied by coupling of oxidative phosphorylation and then increased again showing uncoupled mitochondria after 60 min of storage. Incubation medium: 250 m*M* sucrose, 30 m*M* Tris–HCl, 10 m*M* KH_2PO_4, 8 m*M* succinate (pH 7.4). Mitochondria were stored in the freezing medium. Oxygen consumption rates are indicated on curves (nmol O_2 min^{-1} mg $protein^{-1}$). Additives: mitochondria (Mit) (3–5 mg protein), 250 μ*M* ADP, 1 μ*M* CCCP. Cell volume was 1 ml. The incubation temperature was 26°C. The results of an experiment characteristic of eight trials are presented.

that during incubation the high oxygen consumption rate decreases initially and then increases; i.e., changes are similar to those described in Fig. 1 for the stored, closely packed suspension, which, however, developed faster. The rate of state 2 succinate oxidation by native mitochondria did not change in the course of the experiment, and subsequent addition of the uncoupler induced its stimulation (Fig. 2).

When frozen–thawed mitochondria were transferred from the closely packed suspension to the incubation medium containing succinate and phosphate, the potential was generated on their inner membranes, whose magnitude could be judged by changes in the content of lipophilic cation TPP^+ accumulated in mitochondria in proportion to the membrane potential (10). Figure 3 shows that the membrane potential of frozen–thawed mitochondria is much lower than that of native organelles; it is unstable and begins to decrease after 4–6 min of incubation. In the presence of exogenous cytochrome c, the membrane potential reaches greater values; however, it is still unstable and dissipates rapidly.

Figure 4 shows that the content of K^+ ions in frozen–thawed mitochondria is somewhat lower than that in the control. This is indicated by the data on the comparison between the initial level of K^+ after addition of mitochondria and the final level in the presence of the uncoupler plus valinomycine (inducing complete release of K^+ into the medium). The decrease in the content of K^+ after thawing can be explained by the partial loss of the cation during freezing. It has been shown previously (18) that after freeze–thawing under similar condi-

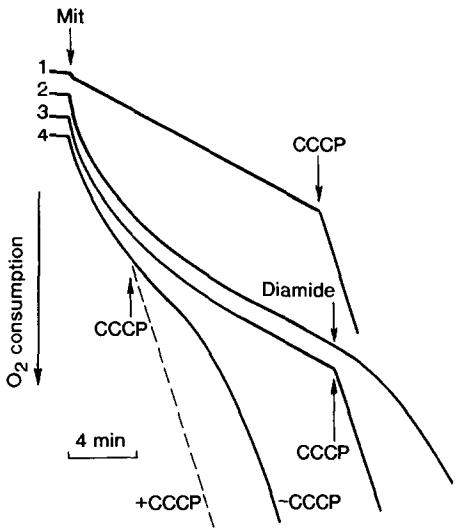

FIG. 2. Changes in the rate of oxygen consumption by state 2 fresh (trace 1) and frozen–thawed (traces 2–4) mitochondria. Trace 1 shows the oxygen uptake for fresh mitochondria, with a stable low rate of oxygen uptake during incubation. Trace 4 shows a change in the oxygen uptake in frozen–thawed mitochondria when the high oxygen rate initially decreases and then increases. Trace 2 shows a change in the oxygen uptake for frozen–thawed mitochondria in the reference medium where succinate is replaced for 5 m*M* glutamate plus malate. Trace 3 shows a change in the oxygen uptake for frozen–thawed mitochondria in the reference medium containing 50 μ*M* BHT, 200 μ*M* nupercaine, 1 m*M* EDTA, 5 m*M* citrate, or 1 μ*M* rotenone. Traces 2 and 3 show that the additives prevent a "secondary" increase in oxygen uptake and the effect of diamide (50 μ*M*) is reversible. The incubation medium composition and conditions are the same as those in Fig. 1. The content of mitochondrial protein was 1 mg.

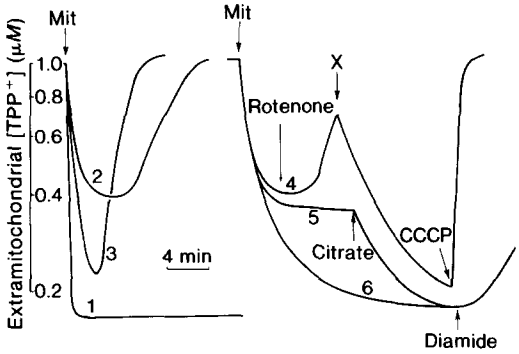

FIG. 3. Distribution of TPP^+ in intact (trace 1) and frozen–thawed (traces 2–6) mitochondria. Trace 1 shows that fresh mitochondria take up TPP^+ rapidly and keep it during the experiment. Traces 2 and 4 show that frozen–thawed mitochondria take up a lesser quantity of TPP^+ which is released subsequently to the medium. Trace 3 shows that cytochrome c (30 μ*M*) increases the TPP^+ uptake by means of frozen–thawed mitochondria and accelerates its release to the medium. Trace 5 shows the TPP^+ uptake by frozen–thawed mitochondria in the presence of rotenone (1 μ*M*). Trace 6 shows that in the presence of BHT (50 μ*M*), nupercaine (200 μ*M*), citrate (5 m*M*), or 5 m*M* glutamate plus 5 m*M* malate instead of succinate, frozen–thawed mitochondria take up the same quantity of TPP^+ as fresh mitochondria but at a lower rate. The additives were introduced simultaneously with frozen–thawed mitochondria. (X) The compounds are added as compounds indicated in curve 6. Mit, mitochondria, 1 mg of protein/ml. Cell volume was 5 ml. The incubation medium contained 1 μ*M* TPP^+.

tions, the mitochondrial permeability for K^+ ions increases. These studies were performed on deenergized mitochondria, i.e., without additional substrate but in the presence of respiratory inhibitor. In this case, the maximum permeability for K^+ was observed in the first minutes of incubation. In our study, it was shown that during incubation of frozen–thawed mitochondria in a medium with substrate (succinate), the content of K^+ did not change for the first 4–6 min, but then began to decrease. On the other hand, the control mitochondria were capable of maintaining endogenous K^+ during the experiment (20–25 min).

After freeze–thawing, mitochondria are capable of taking up a small amount of Ca^{2+} from the medium in which it is present as a contaminant, but cannot retain it during incubation. Figure 5 shows a release of all mitochondrial Ca^{2+}. Rapid release of Ca^{2+} from the matrix of both intact and frozen–thawed mitochondria can be induced by the added uncoupler.

Addition of the antioxidant BHT or the inhibitor of phospholipase A_2, the local anesthetic agent nupercaine (27), prevented the decrease in the membrane potential, the increase of the oxygen consumption velocity, and the liberation of cations from mito-

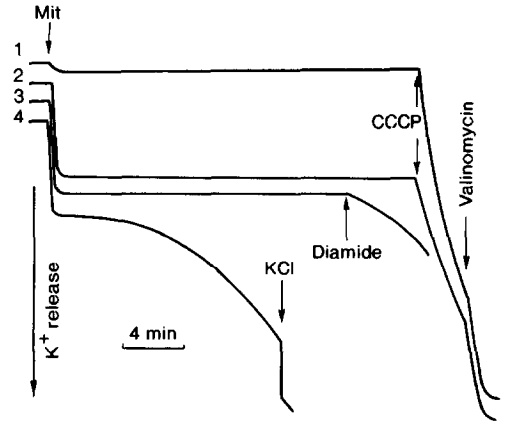

FIG. 4. Release of K^+ from native (trace 1) and frozen–thawed (traces 2–4) mitochondria. Trace 1 shows that fresh mitochondria do not lose endogenous K^+ during incubation. Trace 4 shows that frozen–thawed mitochondria lose K^+ during incubation. Traces 2 and 3 show that K^+ is not released to the medium when succinate is replaced with 5 m*M* glutamate and 5 m*M* malate (trace 2), 50 μ*M* BHT, 200 μ*M* nupercaine, 1 m*M* EDTA, 5 m*M* citrate, or 1 μ*M* rotenone (Trace 3) is added simultaneously to frozen–thawed mitochondria. In the reference medium KH_2PO_4 in the incubation medium is exchanged with H_3PO_4–Tris. Changes in pK were measured in the range of 3.5 to 5.0. Concentration of mitochondrial protein was 1 mg/ml. Cell volume was 5 ml. Additives: 1 μ*M* CCCP, 1 μ*M* valinomycine, 50 μ*M* diamide, 100 nmol/ml KCl.

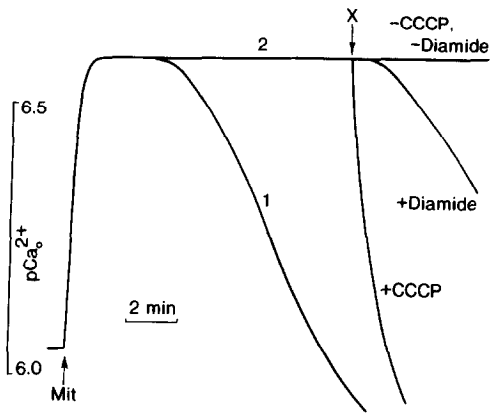

FIG. 5. Transport of Ca^{2+} by native and frozen–thawed mitochondria. Trace 1 shows release of Ca^{2+} from frozen–thawed mitochondria without additive; trace 2 shows that fresh mitochondria without additive or mitochondria frozen–thawed in the presence of 1 μ*M* rotenone, 50 μ*M* BHT, 200 μ*M* nupercaine, 5 m*M* citrate, or succinate exchanged for 5 m*M* glutamate plus 5 m*M* malate do not lose Ca^{2+} during incubation in the reference medium. Cell volume was 5 ml. The concentration of mitochondrial protein was 1 mg/ml. (X) Addition of 1 μ*M* CCCP or 50 μ*M* diamide.

chondria subjected to freeze–thawing (Figs. 2–5). A similar effect can be achieved by the addition of Ca^{2+} chelators (EDTA) or citrate as well as by replacing succinate in the incubation medium with malate plus glutamate. Exogenous NADH or NADPH did not affect the processes studied (not shown). When inhibitors of lipid peroxidation and phospholipase A_2 or NAD(P)-dependent substrates were added simultaneously with frozen–thawed mitochondria, the magnitude of membrane potential increased slowly to the values observed for native mitochondria (Fig. 3).

Inhibition of the oxidation of endogenous pyridine nucleotides with rotenone following the addition of mitochondria immediately after thawing resulted in stabilization of the membrane potential. However, the addition of rotenone 2–3 min after the onset of incubation of mitochondria exerted no effect (Fig. 3). The effect of NAD(P)-dependent substrates and rotenone on the formation of membrane potential and on the cation transport could be reversed by added diamide (Figs. 3–5).

Figure 6 shows that the contents of MDA before and after freeze–thawing do not differ. The content of MDA remained unchanged in the control mitochondria incubated in the medium containing succinate and phosphate. However, the incubation of frozen–thawed mitochondria under similar conditions was accompanied by the accumulation of TBA-reactive substances. The MDA concentration increased with time but insignificantly, when succinate was substituted for glutamate plus malate.

DISCUSSION

The changes of mitochondrial parameters described in this report appeared during a certain period after freeze–thawing follow-

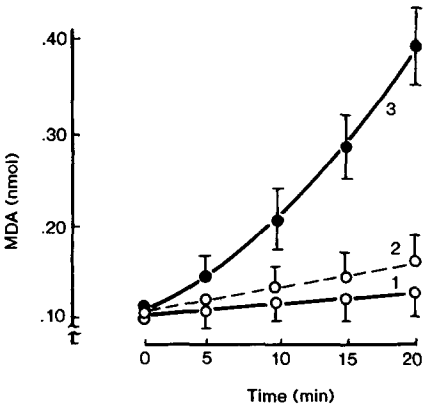

FIG. 6. The effect of NAD(P)-dependent substrates on the production of MDA in mitochondria before (trace 1) and after (traces 2 and 3) freeze–thawing. Incubation was carried out in the reference medium with sucrose exchanged for 120 m*M* KCl. Trace 1 shows no accumulation of MDA during incubation of fresh mitochondria. Trace 3 shows accumulation of MDA during incubation of frozen–thawed mitochondria. Trace 2 shows that MDA production is inhibited in frozen–thawed mitochondria when succinate is exchanged for 5 m*M* glutamate plus 5 m*M* malate. The medium volume is 5 ml. Each 5-min 1-ml suspension sample was added to tubes containing 0.1 ml of 100% (w/v) trichloroacetic acid and 0.01 ml of 10 m*M* BHT. The content of MDA was determined as indicated under Materials and Methods. The incubation temperature was 26°C. The content of mitochondrial protein was 1 mg/ml.

ing storage or incubation. Therefore, they can be referred to as "latent" cryoinjury (23). The latent cryoinjury involves the appearance of transmembrane fluxes of K^+ and Ca^{2+}, an increase in the rate of state 2 succinate oxidation according to Lardy and Welman (13), a decrease in the membrane potential, and an accumulation of lipid peroxides. Compounds of diverse natures such as antioxidants, local anesthetics, Ca^{2+} chelators, and NAD(P)-linked substrates act unidirectionally by inhibiting the development of this injury. These data indicate the interrelationship between the systems of oxidative phosphorylation, ionic transport, and lipid peroxidation and hydrolysis by phospholipase A_2 in mitochondria. Similar changes in these systems were found previously under the effects of different factors, such as high concentrations of Ca^{2+} and phosphate (1, 20), low pH of the incubation medium (24), organic hydroperoxides (3, 9), and oxidation of pyridine nucleotides (14). As a result, the main biochemical reactions which lead to the deenergization of mitochondria have been discovered. Of great interest was the question of the trigger mechanism of this process. In this connection, rapid freeze–thawing protocols are rather attractive because they allow us to exclude essentially the involvement of the biochemical reactions discussed above. In this case, membrane structural changes can be explained by such physical events as the formation of intramitochondrial ice, intense water flow across the membrane, and sharp changes in pressure.

When mitochondria were returned from the low temperature zone to nearly physiological conditions, structural changes in the membrane were reflected by the impairment of the inner membrane permeability (18) and by the high rates of state 2 substrate oxidation determined by the permeability for ions (6). This may be the main cause of the uncoupling of mitochondrial oxidative phosphorylation after freeze–thawing.

Immediately after thawing, the rate of substrate oxidation was stimulated by neither ADP nor the uncoupler. However, a small $\Delta\Psi$ was observed on their membranes. This indicates that after freeze–thawing, mitochondrial respiration enzymes working to maximum activity could compensate partially for a passive leakage of hydrogen ions at the expense of substrate oxidation energy. The membrane potential was stabilized for some time, the state 2 respiration rate decreased and could be increased by the addition of the uncoupler (Fig. 2). At this stage, frozen–thawed mitochondria could retain endogenous K^+ and Ca^{2+} as well as effect respiratory control when stored in a closely packed suspension. It can be suggested that the resto-

ration of mitochondrial function after freeze–thawing (increasing RCI) was due to "resealing" of membrane defects caused by low temperatures which was achieved by decreasing the state 2 respiratory rate, i.e., by decreasing the inner membrane permeability for ions (6). The mechanisms of this event require further investigation.

The membrane potential decreased with further incubation of frozen–thawed mitochondria, along with a loss of endogenous cations and a "secondary" increase in state 2 respiratory rate. A partial loss of cytochrome c during freeze–thawing may be the critical factor (19) of formation of low and unsteady membrane potential under our experimental conditions of maximum activity of the respiratory system. It has been shown (19) that the succinate oxidase activity was restored entirely by the addition of exogenous cytochrome c. In our experiments, added cytochrome c increased $\Delta\Psi$, but at the same time accelerated its dissipation (Fig. 3). This might be caused by the activation of lipid peroxidation in the presence of cytochrome c heme iron. The data on the accumulation of TBA-reactive substances due to deenergization of mitochondria, and under the effect of antioxidant BHT, indicate that the lipid peroxidation contributed to the development of latent cryoinjury.

However, in the present experiments, the development of latent cryoinjury was prevented by introducing both the antioxidant and the inhibitor of phospholipase A_2 nupercaine. In this case, the EDTA may be reduced along with the phospholipase A_2 activity, as with lipid peroxidation (22, 27). These findings indicate the activation of both lipid peroxidation and hydrolysis with phospholipase A_2 and the interrelation between the two processes. Indeed, as is shown elsewhere (22, 28), the activation of lipid peroxidation leads to an increase in the activity of phospholipase A_2 and, alternatively, the activation of phospholipase A_2 induces lipid peroxidation.

It should be pointed out that the content of TBA-reactive substances is virtually the same before and after freezing. This indicates that the lipid changes are activated during subsequent incubation under nearly physiological conditions rather than in the region of low temperatures. The induced lipid peroxidation and hydrolysis can lead to changes in the nonspecific membrane permeability (12), release of cations from the matrix, and deenergization of mitochondria (3, 24). Substitution of succinate in the incubation medium for NAD(P)-dependent substrates (glutamate plus malate) suppressed the accumulation of lipid peroxidation substances (Fig. 5). On the other hand, NAD(P)-dependent substrates could restore the membrane potential and prevent the acceleration of state respiration and release of cations from the mitochondrial matrix. The data indicate that activation of the lipid peroxidation is related to the state of endogenous pyridine nucleotides which can be lost during freeze–thawing. It has been found that added NADH or NADPH could not reconstitute the membrane potential, perhaps because the inner membrane is impermeable for pyridine nucleotides. However, the inhibition of oxidation of endogenous pyridine nucleotides in the mitochondrial respiratory chain with rotenone prevented the decrease in the membrane potential. Added rotenone acted immediately after mitochondrial freeze–thawing, while 2–3 min later it was already ineffective. The results obtained in the experiments on the rotenone action indicated rapid oxidation of pyridine nucleotides.

The intensity of lipid autoxidation is known to be regulated by the glutathione-dependent enzymes (7). Involving these enzymes in the development of latent cryoinjury is supported by the fact that added diamide (which oxides glutathione (16)) to the medium leads to a decrease in the membrane potential and cation release. The restoration of reduced glutathione is brought

about by glutathione reductase. The activity of this enzyme is determined by the redox state of pyridine nucleotides in the matrix (7, 21). The reduction state of pyridine nucleotides is much higher in the presence of glutamate plus malate or citrate than with succinate, thus providing for the effective inhibition of lipid peroxidation and phospholipase A_2 activity. The inhibition of lipid degradation using both specific inhibitors and increasing the content of reduced pyridine nucleotides prevents the cation release from the mitochondrial matrix and the decrease of the membrane potential.

Introducing these compounds both to the incubation and to the freezing media prevented latent cryoinjury and created conditions for reparation of function after thawing. As is shown in Fig. 7, the rate of state 2 substrate oxidation decreased to the control value, and RCI reached 70% during post-thaw storage of closely packed suspension of frozen–thawed mitochondria in the presence of EDTA.

The consequences in mitochondria after rapid freezing and rapid thawing can be revealed from the present data. When thawed mitochondria were transferred to the medium, rapid substrate oxidation occurred due to changes in the membrane barrier properties. In this case, two different processes were observed: one of them tended toward resealing membrane defects and to reparation of the functional activity, and the other one tended toward increasing the permeability for ions and uncoupling of oxidative phosphorylation. The latter (latent cryoinjury) appeared as a result of pyridine nucleotide oxidation under conditions of high permeability of the inner membrane and low membrane potential, which would inhibit the glutathione-dependent enzymes and activate membrane lipid peroxidation and hydrolysis. The activation of these processes leads to a leakage of endogenous cations (K^+, Ca^{2+}), a decrease in the membrane potential, and acceleration of the state 2 respiration, i.e., to the uncoupling of oxidative phosphorylation of frozen–thawed mitochondria. Our findings show that latent cryoinjury can develop as a result of the impairment of the metabolic balance in mitochondria rather than as a result of any one specific action of freeze–thawing.

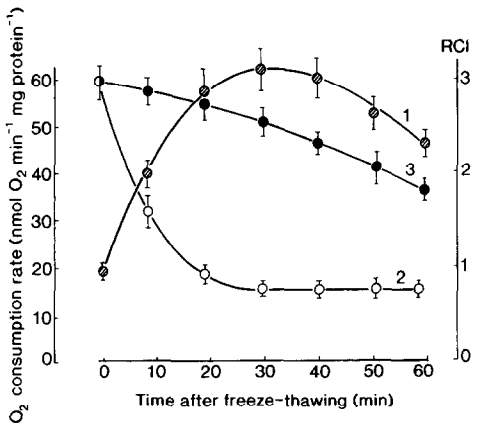

FIG. 7. Changes in RCI (trace 1) and respiration rate of state 2 (trace 2) and state 3 (trace 3) mitochondria (frozen–thawed in the medium containing 1 m*M* EDTA) during storage at 20°C.

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