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# Prolonged Storage of Mitochondria by Freezing: Retention of Respiratory Control and Energized Swelling

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## *Abstract*

A procedure has been devised for preserving mitochondrial function for prolonged periods of time. Rat liver mitochondria in a solution containing dimethyl sulfoxide and bovine plasma albumin are frozen and stored at liquid nitrogen temperature. Phosphorylation efficiency, respiratory control, and $Mg^{2+}$ control of energized swelling are preserved by this method.

## *Introduction*

In many laboratories mitochondria are prepared daily for study. If a procedure were available for preserving mitochondrial function for prolonged periods of time, investigators would be freed from the daily tedium of isolation. The availability of many small tubes of identical mitochondria from one large-scale preparation would allow direct comparison of results from different experiments. Variables such as condition of animals and inadvertant differences in the isolation procedure would be eliminated. Convenient methods for prolonged storage would make possible greater collaboration between distantly separated laboratories by permitting maintenance of biological integrity during shipment.

ADP control of respiration and $Mg^{2+}$ control of energized swelling are the most sensitive parameters of functional integrity known for mitochondria.[1,2] We present a procedure for prolonged storage of mitochondria by freezing, which preserves oxidative phosphorylation as well as these two labile properties.

## *Methods and Materials*

Rat liver mitochondria were prepared as described previously.[3] Liver was homogenized in 0·21 M mannitol, 0·07 M sucrose, 50 mM Tris.HCl, pH 7·4, and 1 mM EDTA. The mannitol–sucrose solution was treated with Amberlite MB-1 ion-exchange resin to

remove contaminating heavy-metal ions prior to addition of Tris and EDTA. The first resuspension of the mitochondria and the washes thereafter were carried out in the same mannitol-sucrose medium with 10 mM EDTA and 10 mM Tris.HCl.

Mitochondria were finally suspended in 0·21 M mannitol, 0·07 M sucrose, 10 mM Tris.HCl, pH 7·4 (Solution A). Prior to freezing, a part of the mitochondrial suspension was adjusted to a final concentration of 10% dimethyl sulfoxide (DMSO) and 1% bovine plasma albumin (Solution B) by the addition of 100% DMSO and 10% BPA. DMSO (Fisher Certified Reagent) was treated with ion-exchange resin as mentioned above for mannitol–sucrose. Crystallized Bovine Plasma Albumin (BPA) was obtained from Armour Pharmaceutical Co. Five to ten min equilibration in Solution B was allowed before freezing. Samples (0·5–1·0 ml) containing 10–15 mg mitochondrial protein/ml in 5 cc plastic vials (Boston round heavy wall polyethylene bottles, Bel-Art Products, Pequannock, N.J.) were frozen by immersion and gentle swirling in liquid $N_2$. Aliquots were stored at liquid $N_2$ temperature (−196°) in a Linde Liquid Nitrogen Refrigerator, at −70° in a Revco Ultra-Low Temperature Freezer, and at −20° in a Sub-Zero Model 2508 F freezer. Thawing was at 37° in a water bath. As soon as thawing was complete, samples were placed in an ice bucket and promptly assayed for respiratory control and swelling properties.

The thawed samples were used directly for assay. Aliquots (0·02 and 0·10 ml) were pipetted directly into 1·2 or 1·6 ml of swelling assay medium and respiratory control assay medium, respectively. Contents of the assay media are given in the legends to Tables I and II. Further details of the procedures have been presented elsewhere.[3]

The Clark oxygen electrode was used to measure oxygen uptake and the ADP/O ratios were calculated from the enhanced oxygen utilization resulting from addition of known amounts of ADP.[4]

## *Results*

Because the mitochondria used in this study showed some $Mg^{2+}$-stimulated ATPase activity, the basal respiratory rate in State IV (presence of substrates, Pi, $O_2$; no added ADP) is elevated by the ADP produced during breakdown of endogenous ATP. The respiratory rate in presence of oligomycin (which inhibits ATPase and oxidative phosphorylation) may be a more accurate reflection of the basal rate. Thus, for comparison, two respiratory control ratios were calculated, one from the oligomycin-inhibited rate and one from the State IV rate.

The effects of freezing and thawing were studied in mitochondria frozen in two media. In the absence of DMSO and BPA (Solution A), the respiratory control index of mitochondria, assayed immediately after freezing, is decreased by greater than 50% (Table I). In the same solution only 20–30% of the initial ADP-stimulable respiratory activity remains after two months storage at −196°. However, respiratory control activity survives freezing and thawing in the presence of DMSO and BPA (Solution B) to the extent of 66–90% and greater than 60% remains after two month's storage at −196°. Optimum ADP/O ratios (3·0) with glutamate, malate, and malonate as substrate were retained after storage under these conditions (not shown in the table). Storage at −70° gives poor preservation of respiratory control. Respiratory control was rapidly lost at −20° even in the presence of BPA + DMSO, and respiratory activity was almost completely lost after two months storage at this temperature. Respiratory control is clearly best preserved by

storage at −196° in the presence of DMSO and BPA. BPA and DMSO appears to have little effect on respiratory rate at this temperature (Table I).

TABLE I. Effects of freezing and prolonged storage on respiratory control ratio and ADP-stimulated oxidation rate

| | Before freezing | Immediately after freezing | Stored at −196° | Stored at −70° |
|---|---|---|---|---|
| A. Respiratory control index based on oligomycin-inhibited rate | | | | |
| Mitochondria in Solution A | | | | |
| Prep 1 | 10·1 | 4·1 | 2·8 | 1·7 |
| Prep 2 | 8·1 | 3·6 | 2·9 | 1·6 |
| Mitochondria in Solution B | | | | |
| Prep 1 | 9·2 | 6·3 | 5·9 | 2·1 |
| Prep 2 | 8·4 | 6·7 | 5·4 | 1·8 |
| B. Respiratory control index based on State IV rate | | | | |
| Mitochondria in Solution A | | | | |
| Prep 1 | 5·2 | 1·9 | 1·0 | 0·68 |
| Prep 2 | 7·0 | 1·8 | 1·8 | 0·68 |
| Mitochondria in Solution B | | | | |
| Prep 1 | 4·5 | 3·9 | 3·2 | 1·1 |
| Prep 2 | 4·8 | 4·7 | 3·9 | 1·4 |
| C. ADP-stimulated respiratory rate ($\mu$g atoms 0/min/mg protein) | | | | |
| Mitochondria in Solution A | | | | |
| Prep 1 | 0·115 | 0·102 | 0·098 | 0·043 |
| Prep 2 | 0·243 | 0·203 | 0·208 | 0·062 |
| Mitochondria in Solution B | | | | |
| Prep 1 | 0·093 | 0·088 | 0·099 | 0·062 |
| Prep 2 | 0·243 | 0·246 | 0·168 | 0·084 |

Solution A contained 0·21 M mannitol, 0·07 M sucrose, 10 mM Tris.HCl, pH 7·4. Solution B is Solution A + 10% DMSO and 1% BPA. Storage at −196° and −70° was for two months. Respiratory rates were measured with a Clark oxygen electrode at 37° in the following medium: 0·14 M mannitol, 0·05 M sucrose, 10 mM each of sodium glutamate, sodium malate, and sodium malonate, 5 $\mu$M cytochrome *c*, 5 mM $MgCl_2$, 0·5 mM EDTA, 10 mM $Na_3PO_4$ buffer, pH 7·4, 2 mM Tris.HCl buffer, pH 7·4, and 0·2% bovine serum albumin. The reaction was started by addition of 0·6–1·0 mg of mitochondria protein (contained in 0·05–0·1 ml) to 1·6 ml of the above medium. After the initial (State IV) rate had been determined, 0·4 $\mu$mole of ADP was added (State III). Following measurement of the ADP-stimulated rate, 10 $\mu$g oligomycin was added after depletion of added ADP for determination of the oligomycin-inhibited basal rate. Respiratory control indices were calculated by dividing the ADP-stimulated rates by either the State IV initial basal rate or the oligomycin-inhibited basal rate.

Mitochondria as isolated here show unique swelling properties.[2,3] ATP energizes swelling in a sodium acetate medium. The presence of $Mg^{2+}$ prevents this swelling or, if added after swelling has taken place, it reverses the process. Absorbance change at 520 m$\mu$ can be used to measure such major volume changes in mitochondria.[2] Absorbance was

measured here under conditions which promote swelling (absence of $Mg^{2+}$) and under conditions which prevent or reverse swelling (presence of $Mg^{2+}$ before or after swelling, respectively). The initial absorbance was also measured.

Table II shows that initial absorbance varied markedly under the different conditions. Under the poorest conditions for preservation (storage at −20° in absence of DMSO and BPA) the initial absorbance was only 22% of that for the unfrozen control mitochondria. Storage in presence of DMSO and BPA at −196° (the best conditions for preservation) allowed retention of 88% of the absorbance of the unfrozen mitochondria. Conditions

TABLE II. Effect of freezing and storage on initial absorbance and $Mg^{2+}$ control of swelling

| Sample and conditions | Initial absorbance ($0D_{520}$/mg protein/ml) | % decrease in absorbance in 5 min: Energized swelling (no $Mg^{2+}$) | % decrease in absorbance in 5 min: Prevention of swelling (+$Mg^{2+}$) | Reversal of swelling ($Mg^{2+}$ added after swelling) |
|---|---|---|---|---|
| Control mitochondria in Solution A | 3·2 | 45 | 0 | ++ |
| Mitochondria stored at −196° | | | | |
| in Solution A | 2·0 | 30 | 6 | + |
| in Solution B | 2·8 | 26 | 2 | ++ |
| Mitochondria stored at −70° | | | | |
| in Solution A | 1·5 | 36 | 30 | − |
| in Solution B | 1·9 | 20 | 4 | + |
| Mitochondria stored at −20° | | | | |
| in Solution A | 0·7 | 8 | 4 | − |
| in Solution B | 1·2 | 12 | 5 | − |

Solutions A and B are described in the legend for Table I. Absorbance at 520 mμ was measured as detailed by Dow, *et al.*[8] Initial absorbance was determined by extrapolating the absorbance curve to zero time (the first reading was taken at ~5 sec). Swelling was initiated by addition of 0·2–0·4 mg mitochondrial protein to 1·2 ml of the following medium at 23°: 20 mM sodium acetate, 110 mM NaCl, 5 mM ATP, pH 7·0, 2·5 mM Tris.HCl, pH 7·4, and 0·2% bovine serum albumin. To test for $Mg^{2+}$ prevention of swelling, 5 mM $MgCl_2$ was added to a separate cuvette containing swelling medium. To study reversal of swelling, mitochondria were first allowed to swell for 5 min in the above swelling medium. $MgCl_2$, 5 mM, was then added to initiate reversal. Absolute quantitation of rates of swelling reversal was not possible, but three comparative categories could be easily distinguished; rapid swelling reversal, ++; slow swelling reversal, +; and absence of swelling reversal, −.

which gave intermediate preservation of respiratory control and $Mg^{2+}$ control of swelling also gave intermediate values of initial absorbance.

Though some capacity for swelling was maintained under all conditions tested, $Mg^{2+}$ control of swelling appeared to parallel respiratory control in its stability. Mitochondria stored two months at −196° in presence of DMSO and BPA maintained their ability for rapid reversal of swelling when $Mg^{2+}$ was added. All other conditions tested showed either a slower reversal or complete loss of the ability for reversal of swelling. $Mg^{2+}$ was effective in preventing swelling in all cases where some ability for reversal of swelling was preserved. However, where this ability had been totally lost, $Mg^{2+}$ was ineffective in preventing swelling (e.g. after storage at −70° in absence of DMSO and BPA).

## *Discussion*

Freezing and storage of rat liver mitochondria at −196° preserved oxidative phosphorylation as well as the two labile properties, ADP control of respiration and $Mg^{2+}$ control of energized swelling. Best preservation was obtained when the mitochondrial suspension was fortified with 10% DMSO and 1% BPA prior to freezing. The temperature of storage was important, since −70° was much less effective than −196°, and −20° was totally ineffective in preserving these properties for prolonged periods of time.

The initial absorbance of mitochondria was found to decrease with increasing damage sustained during freezing and storage. Three sensitive properties, respiratory control, $Mg^{2+}$ control of energized swelling, and mitochondrial absorbance at 520 m$\mu$, appear to show parallel changes under various conditions of storage.

It may be noted that DMSO itself lowered the respiratory control ratio. This effect could be minimized by treating the DMSO with ion exchanger and by inclusion of 1% BPA in the medium.

Glycerol was also found to have a protective effect during freezing, but, like DMSO, showed inhibition due to heavy-metal-ion contamination. DMSO was more convenient to use because the viscosity is much lower than for glycerol.

Heart mitochondria are relatively more amenable to storage than mitochondria from liver or brain. Oxidative phosphorylation has previously been preserved by freezing beef heart mitochondria, suspended in a sucrose solution, at −20°.[5] Addanki *et al.* have shown that preservation of $Ca^{2+}$-transport properties of heart mitochondria is improved by the addition of DMSO to a suspension of mitochondria in sucrose solution before freezing.[8] Oxidative phosphorylation of brain mitochondria does not survive ordinary freezing and storage at −20°.[7] Preservation of oxidative phosphorylation in rat liver mitochondria can be achieved with freezing at −192°.[6]

Respiratory control and control of energized swelling by $Mg^{2+}$ in rat liver mitochondria can be preserved by the method described in this communication. Thus, mitochondria can now be added to the list of biological materials successfully preserved for long periods. Spermatozoa, erythrocytes, lymphocytes, chloroplasts and cultured myocardial cells are some of the other materials that have been preserved by freezing.[9, 12] In most cases, addition of substances such as glycerol, dimethyl sulfoxide, sugars (especially disaccharides), weak electrolytes or even large polymers such as polyvinylpyrrolidone has been necessary to preserve high activity.[10] The protective effect of these substances probably results from their ability to prevent formation of localized high salt concentrations and to decrease crystal formation. Glycerol and dimethyl sulfoxide penetrate most membranes quite rapidly, which may partially explain their superiority as protective agents against freezing damage.[10, 11]

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