ening are demonstrated by the infrared spectrum of the mercuric mercaptide of 3-mercapto-1,2-propanediol. This is in agreement with the expected reduction of osmium tetroxide by the sulfhydryl groups in forming the polymeric mercaptide. The 60-Mc nuclear magnetic resonance proton spectrum of the tetramer or pentamer shows a sharp peak at $\delta = 4.67$ parts per million (ppm), which is indirectly attributable to the OH protons of the polymers; protons from SH were ruled out by the infrared spectra of the tetramer and pentamer mentioned above, and protons from the solvent (D_2O) accounted for only one-fifth of the peak integral. In addition, there is an extremely broad peak extending from $\delta = 1$ ppm to $\delta = 7$ ppm, with its summit at $\delta = 3.7$ ppm, which is attributable to OCH and OCH_2 protons. The broadness of this peak is probably due to the paramagnetism of the polymers, which was confirmed by electron spin resonance spectra. The nuclear magnetic resonance spectra indicate that these polymers are in solution.

One milliliter of an aqueous solution containing 40 mg of the tetramer and 40 mg of sucrose was injected intravenously into each of several albino mice. The dark brown solution colored the blood, skin areas rich in blood vessels, and the eyes. Within 45 minutes the blood cleared, the eyes returned to normal color, and the dark material was found in the urine. This is biological evidence that the polymer is in true solution. No acute toxicity was noted.

Although the formation of polymers of osmium have frequently been postulated (*11*, *12*), this is the first example of their characterization. The synthesis of other soluble coordination compounds of osmium, including some with multiple ionic charges, and their use as stains for light and electron microscopy is the subject of a separate communication (*13*).

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14. Supported by research grant (CA-02478) from the National Cancer Institute, Bethesda, Maryland.

7 February 1967; revised 26 May 1967

Dimethyl Sulfoxide Protects Tightly Coupled Mitochondria from Freezing Damage

Abstract. *Dimethyl sulfoxide prevented loss of respiratory control and decrease in efficiency of oxidative phosphorylation when plant mitochondria were stored in liquid nitrogen. Respiration was severely inhibited and was not stimulated by adenosine diphosphate when mitochondria were frozen in liquid nitrogen without dimethyl sulfoxide. Thus, isolated mitochondria provide a model system for the study of the effects of freezing on biological membranes and of the prevention, by dimethyl sulfoxide, of freezing damage.*

Freezing causes extensive disorganization of mitochondrial and other membranes within living cells, and cryoprotective agents such as dimethyl sulfoxide (DMSO) prevent this damage (*1*). According to Trump *et al.* (*1*), there is no satisfactory theory to explain such protective treatment. Damage to isolated mitochondria by freezing includes rupture of membranes, loss of soluble enzymes, and increase in the activity of adenosine triphosphatase (*2*). Freezing abolishes respiration and phosphorylation of isolated spinach mitochondria, but sucrose provides partial protection (*3*). Glycerol and DMSO prevent decreases in respiration and oxidative phosphorylation when rat-liver mitochondria are frozen (*4*). There are no reports on whether freezing affects respiratory control of tightly coupled mitochondria. We have tried to develop a method for storing tightly coupled mitochondria and to learn whether isolated plant mitochondria can be used for studies on the mode of action of cryoprotective agents.

Mitochondria were isolated from wall tissue of mature green tomato fruits (variety Kc146) with the methods of Drury and Garrison (*5*). Isolated mitochondria were suspended in a solution containing 0.5 mole of mannitol, 1.5 g of bovine serum albumin, and 5 mmole of sodium barbital per liter at *p*H 7.5. Oxygen uptake was measured polarographically (*6*) with a Clark platinum electrode. The ratio of adenosine diphosphate to oxygen consumed (ADP/O) and the respiratory control ratios were calculated according to Chance and Williams (*6*). The latter ratio is equal to the rate of oxygen uptake stimulated by ADP divided by the subsequent rate limited by ADP.

The reaction mixture contained a solution with 0.5 mole of mannitol, 5 mmole of $MgCl_2$, 10 mmole of tris(hydroxymethyl)aminomethane, 10 mmole of KH_2PO_4, and 0.5 mmole of ethylenediaminetetraacetate per liter plus mitochondria, substrate, and ADP in a final volume of 2.95 ml at *p*H 7.5 and 23°C. For the freezing experiments, 0.5-ml samples of mitochondrial suspension were placed in 50-ml polycarbonate tubes or 1-dram glass vials. To obtain various cooling rates and storage temperatures we placed the samples in a deep freeze (−18°C), partially immersed them in liquid nitro-

gen, or suspended them over liquid nitrogen (30 ml of liquid nitrogen in a 300-ml Dewar flask). A sample to be thawed was placed in crushed ice for 10 minutes and then briefly held at about 30°C. The temperatures of the samples did not exceed 3°C. They were measured to −40°C with a telethermometer (Yellow Springs Instrument Co.) which was equipped with a stainless steel probe.

Freshly isolated mitochondria exhibited respiratory control, and the value of ADP/O was about 1.2 for succinate oxidation (Fig. 1, curve A). This ratio and that of respiratory control were not decreased when the mitochondrial suspension was made 5 percent (volume/volume) in DMSO 10 minutes before assay (curve B). There was little change when mitochondria with DMSO were frozen by suspension over liquid nitrogen for 15 minutes and then immersed in liquid nitrogen for 24 hours (curve C). The rate of cooling over liquid nitrogen was 20° to 25°C per minute, and the duration of the freezing plateau was about 0.5 minute. The same freezing treatment without DMSO (curve D) caused 88 percent inhibition of the ADP-stimulated rate of respiration; the low rate of respiration was not increased by several additions of ADP or uncoupling concentrations of 2,4-dinitrophenol. Dimethyl sulfoxide failed to prevent damage to mitochondria stored at −18°C (curve E).

In other experiments mitochondria were exposed to DMSO (at 5 and 15 percent) for 2 hours before being frozen and 2 hours after thawing, and no adverse effects were observed. Dimethyl sulfoxide (5 percent) also prevented freezing damage when mitochondria were transferred directly from crushed ice to liquid nitrogen or were cooled slowly to −40°C over liquid nitrogen before immersion in the liquid nitrogen (the duration of the freezing plateau was about 4 minutes, and an additional 20 minutes elapsed before −40°C was reached). In other studies, mitochondria were stored with 5 percent DMSO in liquid nitrogen several days, and in one case for 4 weeks, without a decrease of ADP/O or loss of respiratory control.

The data indicate that, after isolation, tightly coupled mitochondria can be stored successfully in liquid nitrogen. It is likely that DMSO protects enzymes of oxidative phosphorylation and the electron transport chain of mitochondria within intact cells when the cells are frozen. Such enzymes are associated with membranes (*7*), and the effect of DMSO is probably related to its protection of mitochondrial membranes, as observed by Trump *et al.* (see *1*).

The marked difference in function between mitochondria frozen with and without DMSO (Fig. 1, curves C and D) provides an approach to finding exactly what constitutes freezing damage and how DMSO protects biological membranes during freezing. Freezing damage has been ascribed to the presence of a high concentration of solute (*2*) and to denaturation of lipoprotein following withdrawal of water (*3*). Denaturation of β-lipoprotein from human plasma coincides with removal of the last traces of water from the medium (*8*). Karow and Webb (*9*) suggested that DMSO may induce the formation of water lattices; such an effect might stabilize mitochondrial membranes during freezing and thawing by preventing withdrawal of water. Dimethyl sulfoxide breaks hydrogen bonds between base pairs of RNA molecules (*10*). Hence, if water is withdrawn from mitochondrial lipoproteins during freezing and thawing, DMSO may prevent interaction of polar groups that were formerly attached to water molecules by hydrogen bonds.

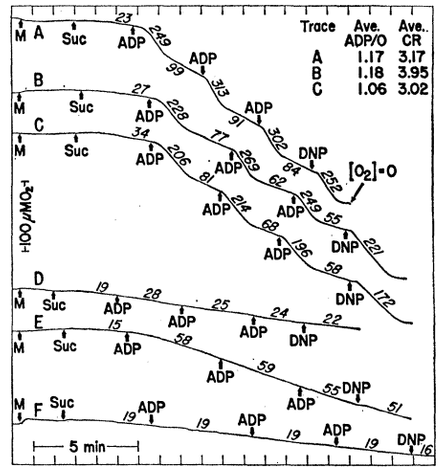

Fig. 1. Polarograph tracing of oxygen uptake by isolated tomato mitochondria as affected by dimethyl sulfoxide and freezing. The reaction mixture and method of freezing are described in the text. Additions to the reaction mixture and their final concentrations were: *M*, mitochondria (126 μg of nitrogen without DMSO or 120 μg of nitrogen if DMSO was present); *suc*, potassium succinate (20 mmole/liter); *ADP*, adenosine diphosphate (0.1 mmole/liter at each addition); *DNP*, dinitrophenol (0.1 mmole/liter). The numbers on each curve are rates of oxygen uptake in millimicroatoms of oxygen per minute. Curve A, freshly isolated mitochondria, no DMSO. Curve B, freshly isolated mitochondria incubated with 5 percent DMSO in ice 10 minutes before assay. Curve C, 5 percent DMSO was added; mitochondria were placed over liquid nitrogen for 15 minutes and then immersed in liquid nitrogen. Mitochondria were thawed after 24 hours and assayed immediately. Curve D, same as C except that no DMSO was added. Curve E, 5 percent DMSO was added, mitochondria were placed over liquid nitrogen for 15 minutes (as in C), and the frozen samples were transferred to −18°C. Mitochondria were thawed after 24 hours and assayed immediately. Curve F, same as E except that no DMSO was added. The average *ADP/O* and respiratory control (*CR*) ratios were calculated as described in the text.

It is interesting that DMSO did not prevent freezing damage when mitochondria were stored at −18°C. Our results are similar to those of Lusena (*2*), who found that DMSO reduced, but did not prevent, changes in permeability of rat-liver mitochondria stored at −15°C. Grant and Alburn (*11*) found that certain enzymatic and nonenzymatic reactions proceed more rapidly at −18°C than at 1°C, so it is likely that extensive changes occurred in mitochondria stored at that temperature. Whether the reduced respiration associated with freezing damage is caused by disruption of the electron transport chain at one or at many points remains to be seen.

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23 March 1967