THE IMPORTANCE OF STRICT TEMPERATURE CONTROL DURING GRADIENT CENTRIFUGATION OF OSMOTICALLY ACTIVE CELL ORGANELLES.

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SUMMARY

A temporary drop in temperature below $0^{o}C$ in the rotor tube during isopycnic sucrose gradient centrifugation is not usually detectable with the available temperature gauges, but such transient sub-zero temperatures will cause mitochondria to rupture and to redistribute throughout the gradient. Swing-out titanium rotors are particularly prone to such temperature variations as titanium in relation to aluminium is a poor heat conductor.

Equilibrium gradient centrifugation in concentrated sucrose media is frequently used for the separation, isolation and characterization of cell organelles. It has been suggested that centrifugation in concentrated sucrose solutions causes mitochondrial disruption so that several distinct fractions of damaged mitochondria are derived from an originally homogeneous population of mitochondria (1, 2, 3). However in recent years several reports indicated that when conditions are used which are designed to avoid such damage, then at least two fractions of intact mitochondria may be separated by this method (4, 5, 6, 7, 8). Different mitochondrial populations have been separated by means of this technique in our laboratory (9, 10).

During these investigations we became aware of another possible source of mitochondrial damage during gradient centrifugation. Since other osmotically active cell organelles or

particles would be similarly affected, the following findings may be of a more general interest.

MATERIALS AND METHODS

Isopycnic centrifugation of mitochondria isolated from adult female Wistar rats by the method of McMurray and Dawson (11) was carried out in a Spinco Beckman L65 centrifuge at 132,000$g_{av.}$ for 90 min, using a SW 50 rotor in a partial vacuum of 75-200 Torr pressure, obtained by using the rotary pump only, or the SW 41 rotor either with the diffusion pump (5-10 Torr) or the Rotary pump only (75-200 Torr). Protein and phospholipid determinations and electron microscopy and fixation were carried out as described previously (9).

RESULTS AND DISCUSSION

In over 150 experiments using mitochondrial preparations from rat and chick liver the isopycnic sucrose gradient centrifugation method for the separation of mitochondrial populations has given highly reproducible results (7, 9, 10; Darin & Pollak, unpublished). Inconsistent results obtained from 8 consecutive experiments coincided with the use of an unsatisfactory drive unit which caused large temperature variations in the rotor (Morton & Pollak, unpublished).

In subsequent experiments a SW 41 rotor was used for the isopycnic centrifugation of rat liver mitochondria. This rotor is made from titanium (as opposed to the SW 50 rotor which is made from aluminium) and has longer and wider tubes (89x14 mm) than those of the SW 50 rotor (51x13 mm). Gradient centrifugation with the SW 41 rotor at 132,000 $g_{av.}$ for 90 min (using the diffusion pump as recommended by the manufacturer) resulted in an entirely different distribution of mitochondrial bands from that routinely obtained by centrifugation with the SW 50 rotor.

Although the temperature on the indicator showed never less than 2°C, it was found that the sucrose gradients in two of the six tubes in one of these experiments were frozen at the end of the run. Hence the temperature measurements carried out at the centre of the rotor did not give a true indication of tube-temperature. Problems in the use of titanium swing-out rotors for sucrose density gradient centrifugation of mitochondria may arise due to the cumulative effects of the following three factors:

1. the presence of a good vacuum (5-10 Torr);
2. the longer tube length in the SW 41 rotor compared with that in the SW 50 rotor;
3. the relatively thinner metal tube shield, which results in a smaller heat reservoir in the tube region (2mm thickness in the SW 41 rotor, 3mm in the SW 50 rotor).

Thus the chamber temperature may drop temporarily below -1°C causing mitochondria with a sucrose-free space and a freezing-point depression of less than 1°C to freeze. On thawing, these mitochondria would rupture, giving rise to the redistribution of membrane bands and soluble enzymes. Since the gradient used, 0.9-1.9 M sucrose has a considerably greater freezing-point depression, this effect on the mitochondria may not be detected by visual examination.

As in previous investigations (7, 8, 9) the results in Table 1 are interpreted to mean that the B1 fraction consists of mitochondrial membranes, equilibrating at a density of d=1.172 and having a protein/phospholipid ratio of 1.7; B2 are mitochondria with a sucrose-impermeable space equilibrating at a density d=1.190, and having a protein/phospholipid ratio of 3.5; while B3 are "leaky" mitochondria, not possessing a sucrose-impermeable

Table 1.

The effect of the use of the diffusion pump and lowered temperatures on the gradient centrifugation of mitochondria.

Rotor and conditions	Mitochondrial Fraction	density	Protein mg	Protein/ Phospholipid ratio
A.)				
SW50	B1	1.172	<0.01	-
(rotary pump only)	B2	1.190	2.0	3.7
nominal temp. readings 2-4°C	B3	1.222	0.31	3.9
(3 experiments, mean recovery 75% (69-82%) of 3 mg mitochondrial protein applied on to gradient)				
B.)				
SW41	B1	1.173	0.04	2.0
(rotary pump only)	B2	1.199	4.2	3.5
nominal temp. readings 5°C-7°C	B3	1.228	0.14	3.7
(4 experiments, mean recovery 74% (64-83%) of 6 mg mitochondrial protein applied on to gradient)				
C.)				
SW41	B1	1.175	0.55	1.7
(diffusion pump used)	B2	1.193	0.71	2.03
nominal temp. readings 2-4°C	B3	1.225	1.15	2.85
(7 experiments, mean recovery 42% (32-50%) of 6 mg mitochondrial protein applied on to gradient)				

space and hence equilibrating in the denser region of the gradient d=1.222, but having a protein/phospholipid ratio similar to that of the B2 mitochondria. If freezing occurs, the B2 mitochondria would be expected to be damaged first, due to the small freezing-point depression of their sucrose-free space. Any subsequent increase in temperature may then cause the mitochondria to thaw and rupture; the membrane fragments will then

equilibrate in the B1 region, while the soluble proteins will be distributed throughout part of the gradient, but would not be visually detectable as a band. B3 values would be increased significantly, if the damage is slight as only the inner membrane of the B2 mitochondria is ruptured by the freezing and thawing, resulting in artefactually produced "leaky" mitochondria (see Table 1). The B3 mitochondria being sucrose-permeable will only rupture under more extreme temperature conditions, hence exposure of the gradient to temperatures between $-1^{o}C$ to $-2^{o}C$ would result in increased B1 values, low B2 values and further increased B3 values, together with significant losses in the total mitochondrial protein recovered from the three bands. More extreme temperature conditions would totally disrupt B2 mitochondria resulting mainly in membrane fragments (B1) and soluble enzymes. Under extreme temperature conditions, B3 mitochondria would also contribute to the B1 fraction.

Table 1 shows clearly that when the SW 41 rotor is used under conditions such that freezing is likely to occur, the results are typical when compared with results obtained in the SW 50 rotor; when the diffusion pump was not used and the temperature was manually adjusted several times during the run to ensure that sub-zero temperatures were avoided, the results obtained with the SW 41 rotor are comparable with those obtained in the SW 41 rotor are comparable with those obtained in the SW 50 rotor.

Monitoring gradient runs by electron microscopy confirmed the above observations, as demonstrated in Fig. 1a and 1b. The figure shows B2 fractions isolated under conditions B and C (Fig. 1). The lower recovery in protein (Table 1) and the morphological damage (Fig. 1b) resulting from the freezing and

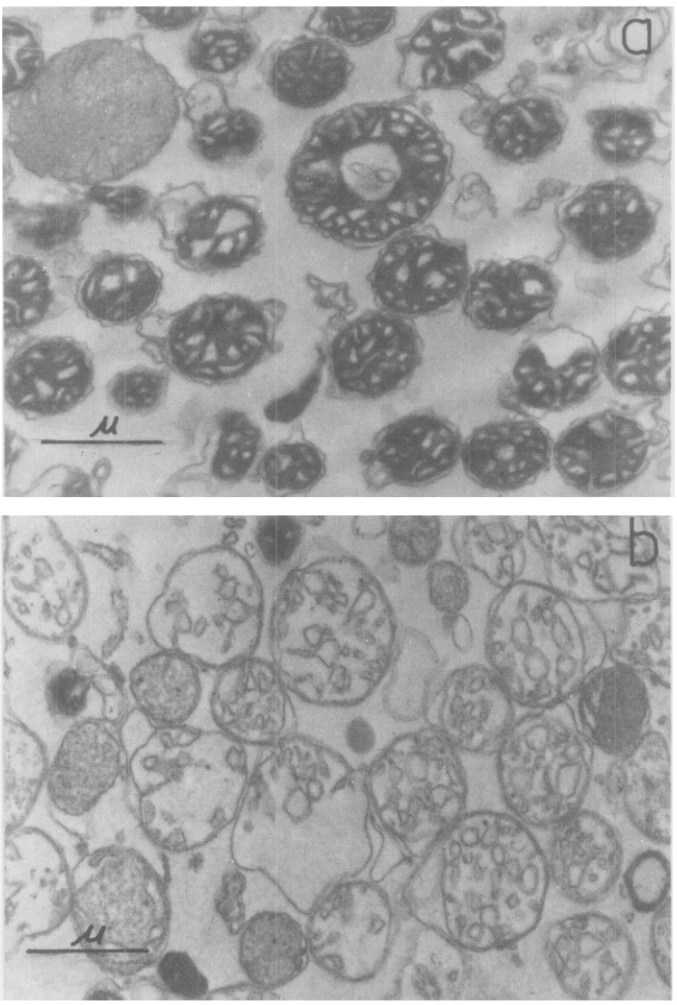

Figure 1a. B2 mitochondria isolated under condition B (Table 1). Note that most of the mitochondria are well preserved and in the "condensed state", the dense matrix-space being a prominent feature.

Figure 1b. B2 mitochondria isolated under condition C (Table 1). Mitochondria are swollen, inner membrane is still present, but in most instances the dense matrix material is completely lost.

(bars represent 1 µm)

thawing of mitochondria shows certain similarities to the results obtained by Wattiaux and Wattiaux-De Coninck (3), though these authors reached different conclusions.

Recent reports indicate that osmotically undamaged mitochondria may be isolated by sucrose density centrifugation under conditions used in the present investigation (7, 8, 9). A more detailed study demonstrating the use of sucrose gradients to separate heterogeneous populations of mitochondria into at least two separate populations has been completed and will be reported shortly (Pollak, in preparation).

The present study shows that a temporary drop in the tube temperature to $-1^{\circ}C$ or lower is a potential source of artefactual segregation and separation of mitochondria due to damage and rupture caused by freezing and thawing; the use of swing-out titanium rotors accentuates this danger.

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