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LATERAL PHASE SEPARATIONS AND STRUCTURAL INTEGRITY OF THE INNER MEMBRANE OF RAT-LIVER MITOCHONDRIA

EFFECT OF COMPRESSION. IMPLICATIONS IN THE CENTRIFUGATION OF THESE ORGANELLES

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Summary

When maintained in the vicinity of the lower transition temperature of their membrane lipids, rat-liver mitochondria undergo lysis as shown by the release of malate dehydrogenase, (an enzyme located within the mitochondrial matrix), in the surrounding medium.

Structural changes take place in the membranes of mitochondria subjected to increasing pressure at 0°C, when the pressure reaches 750 kg/cm^2. Freeze-fracture electron microscopy shows the appearance of smooth areas devoid of particles in fracture faces of mitochondrial membranes, together with zones, where aggregated particles can be seen. Concurrently, a suppression of the malate dehydrogenase structure-linked latency is observed. These structural changes can be prevented by increasing the temperature at which compression is performed. The freeze-etching observations suggest that lateral phase separations occur in mitochondrial membranes subjected to high pressure. This can be explained by supposing that pressure promotes the gel-phase appearance in a lipid system and raises the transition temperature since the transition liquid crystal → gel is accompanied by a decrease in volume. The deterioration of mitochondria subjected to high pressure is interpreted as a result of the lateral phase separation induced by compression in the membranes.

These results are discussed with respect to our interpretation of the damaging effects that hydrostatic pressure, generated by centrifugation, exerts on rat-liver mitochondria.

Introduction

In the course of an investigation on the effects of centrifugation on subcellular structures, we found that rat-liver mitochondrial membranes could be

deeply deteriorated when the hydrostatic pressure, generated during centrifugation, reached a certain level [1—4]. In accordance with our results, Bronfman and Beaufay [5] have shown that mitochondrial membranes are damaged when mitochondria are subjected to compression in a hydraulic press. To explain this phenomenon, we have suggested that the hydrostatic pressure increases the phase transition temperature (liquid crystal → gel) of mitochondrial lipids above the centrifugation temperature; as a result the inner mitochondrial membrane becomes permeable to sucrose, the granule swells and the membranes are disrupted [6].

Our hypothesis is based on two facts: (1) The appearance of the gel phase in a lipid system is accompanied by a volume decrease and is thus promoted by a pressure increase [7]; as a consequence a pressure increase is expected to raise the phase transition temperature. (2) Several authors have shown that the permeability of artificial and natural membranes abruptly increases at the transition liquid crystal → gel of the phospholipids which make up the membrane [8—10]; and that even lysis occurs when the temperature is below the transition temperature [11,12]. Such a hypothesis supposes that lateral phase separations in the mitochondrial membranes can cause a lysis of the mitochondria and that the temperature at which phase separations occur in these membranes is increased by compression. The work reported here was carried out in order to check this hypothesis. We first investigated the structure-linked latency of a mitochondrial-enzyme malate dehydrogenase after inducing lateral phase separations in mitochondrial membranes at atmospheric pressure by keeping the granules below 0°C in the presence of a cryoprotector. Then, using freeze-fracture electron microscopy, we examined mitochondria subjected to hydraulic compression to see whether lateral phase separations really did take place in the mitochondrial membranes at and above 0°C, in a pressure range capable of unmasking latent mitochondrial enzymes [5].

Materials and Methods

Tissue fractionation

All experiments were performed on mitochondrial fractions from rat-liver corresponding to the sum of fractions M and L of the Duve et al. [13]. Density gradient centrifugation was carried out according to the method of Beaufay et al. [14] with the equipment described by de Duve et al. [15] and Beaufay et al. [14]. Granules were layered above the gradient.

Incubation of mitochondria below 0° C

Samples of the mitochondrial fraction containing 40% (v/v) ethylene glycol/1 mM imidazole (pH 7.4)/0.25 M sucrose were pipetted in a test tube and equilibrated for 15 min at the required temperature in a cooling bath. Then, a drop of the granule suspension was transferred to a copper disc at the corresponding temperature and equilibrated for a further 45 min. The samples were frozen with liquid-solid nitrogen and freeze-fractured at —100°C with 1 min etching in a Balzers BAF 300 freeze-etching apparatus. For the enzyme measurements, granules were incubated in the same medium during 30 and 60 min, at the required temperature. After that, the samples were maintained at 0°C, until the enzyme determinations.

Compression of the granules

The experiments were performed according to Bronfman and Beaufay [5] with the compression unit described by these authors and kindly provided by Professeur H. Beaufay.

The pressure vessel, allowing fixation of mitochondria while under pressure, was similar to that described by Landeau and Thibodeau [16] with some modifications. It consisted of two perpex compartments as described by these authors, separated by a parafilm diaphragm. The lower compartment containing a steel cone was filled with 1.5 ml 1.5% (v/v) glutaraldehyde/50 mM phosphate buffer, pH 7.4 (electron microscopy experiments) or with 1.5 ml 0.25 M sucrose/1 mM imidazole pH 7.4 (enzyme latency experiments); the upper compartment was filled with 0.6 ml of the mitochondrial fraction in 0.25 M sucrose/1 mM imidazole, pH 7.4. The two compartments were closed with several thicknesses of parafilm allowing pressure transmission and the vessel introduced in the compression chamber, filled with water, and connected to the hydraulic pump. Pressure was built up within seconds and maintained for 1 h. After that, the compression chamber was valved off, uncoupled from the main line and vigorously shaken. In such conditions, the parafilm diaphragm between the two compartments was perforated by the steel cone and glutaraldehyde or sucrose solutions mixed with the granule preparation. Fixation was performed during 1 h. After restoring atmospheric pressure, the fixed granules were concentrated by centrifuging at 250 000 × *g* per min in the Spinco rotor 40, then resuspended in 1.5% glutaraldehyde, 40% (v/v) glycerol/1 mM imidazole, pH 7.4. They were frozen with liquid-solid nitrogen and freeze-fractured at −100°C with 1 min etching in a Balzers BA 360 freeze-etching apparatus.

Enzyme assays

Free malate dehydrogenase was assayed spectrophotometrically at 340 nm and 25°C in a medium containing 25 mM Tris buffer (pH 7.4)/0.15 mM NADH/0.25 mM oxalacetate. Total malate dehydrogenase was assayed in the same conditions but in the presence of 0.1% Triton X-100. Cytochrome oxidase was measured according to Appelmans et al. [17] and monoamine oxidase according to Schnaitman et al. [18]. For monoamine oxidase and malate dehydrogenase, units of enzymic activity are defined as the amount of enzyme causing the decomposition of 1 μmol of substrate per min under the conditions of the assay. One cytochrome oxidase unit is defined as the amount of enzyme causing the decadic logarithm of the concentration of reduced cytochrome *c* to decrease by 1 unit/min per 100 ml incubation mixture [13].

Results

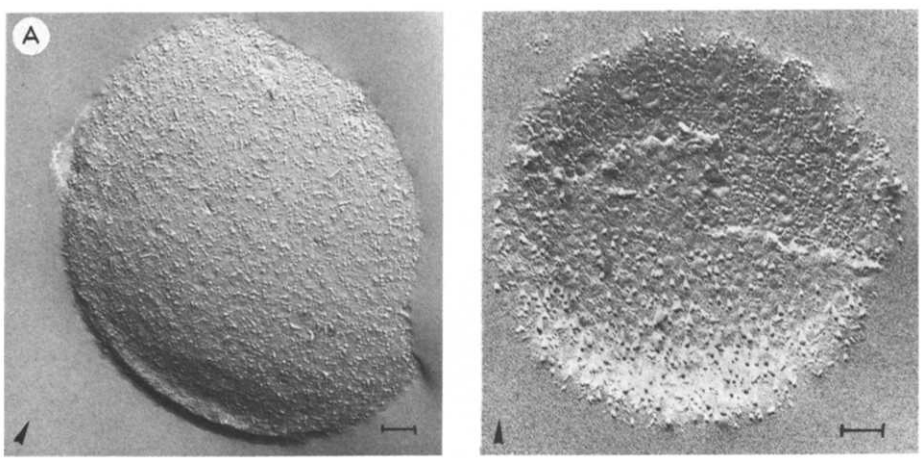
Lateral phase separations at atmospheric pressure and structural integrity of the inner mitochondrial membrane

Freeze-fracture electron microscopy shows that lateral phase separations take place in rat-liver mitochondrial membranes below 0°C [19,20]. According to calorimetric measurements [19], the onset temperatures of the thermotropic transition for the inner membrane are −4°C (transition exotherm) and −15°C (transition endotherm). We monitored the structural integrity of the mitochondria in that temperature range. For this purpose, we measured the free activity

of malate dehydrogenase, an enzyme located within the mitochondrial matrix and established the distribution of this enzyme after isopycnic centrifugation together with those of cytochrome oxidase and monoamine oxidase, the marker enzymes of the inner and the outer mitochondrial membrane respectively. As ascertained by its low free activity, malate dehydrogenase is largely latent in intact mitochondria owing to the impermeability of the inner membrane to external pyridine coenzymes. A deterioration of the inner membrane will increase the dehydrogenase free activity. On the other hand, when mitochondria are intact, malate dehydrogenase, cytochrome oxidase and monoamine oxidase are recovered together in a sucrose gradient after isopycnic centrifugation, if the membranes are disrupted, the matrix and the membrane enzyme distributions no longer coincide.

To demonstrate that thermotropic phase transitions occur in mitochondrial membranes, mitochondria have to be incubated in the presence of a high concentration of cryoptrotector, either glycerol or ethylene glycol [19,20]. We found that in the presence of glycerol, the free activity of malate dehydrogenase is considerably increased at 0°C; we did not observe a similar effect for ethylene glycol. Malate dehydrogenase is largely latent at 0°C, even in the presence of 40% ethylene glycol; therefore, only this cryoprotector was used in the experiments described in this section. Four temperatures were investigated: 0°, −4°, −10° and −15°C. As shown in Fig. 1, and in accordance with the results of Hackenbrock et al. [19], no lateral phase separations were evident at 0°C and −4°C in the inner membrane. In contrast, lateral phase separations are apparent at −10° and −15°C. Free activity of malate dehydrogenase is reported in Fig. 2. It is low when mitochondria are kept at 0° or −4°C and markedly enhanced when the organelles are maintained at −10° or −15°C.

The distribution of malate dehydrogenase, monoamine oxidase and cytochrome oxidase, after isopycnic centrifugation are shown in Fig. 3. Mitochondria were maintained at 0°, −4° or −15°C for 1 h before centrifugation. The distribution patterns are similar when the preparations are kept at 0° and −4°C. In both cases, malate dehydrogenase and cytochrome oxidase exhibit a homogeneous distribution, with a median equilibrium density of 1.197 g/ml.



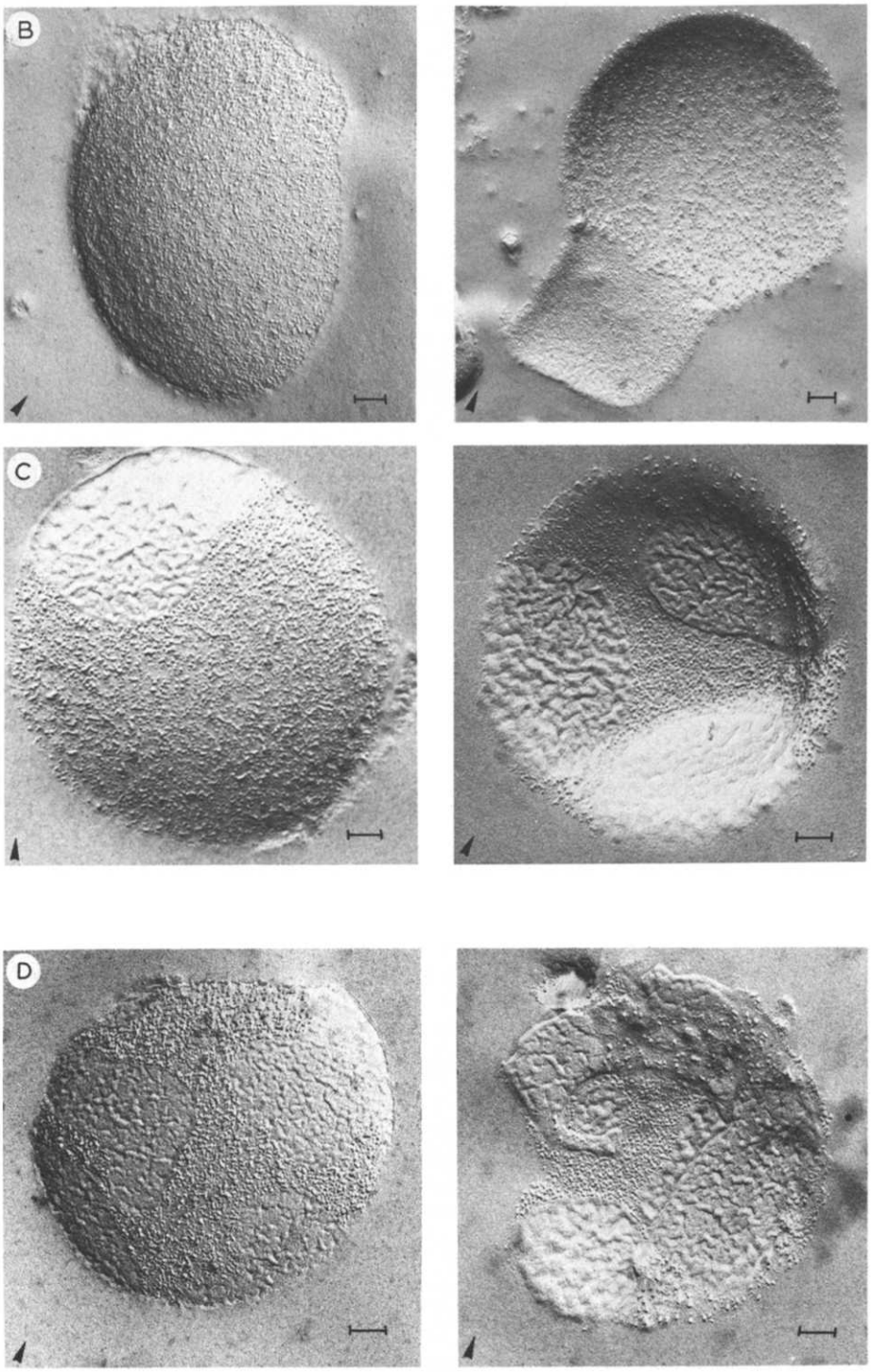


Fig. 1. Convex and concave fracture faces of membranes of mitochondria equilibrated at 0°C (A), −4°C (B), −10°C (C) and −15°C (D) for 1 h and then frozen. Smooth particle-free areas are formed on faces of both membranes when mitochondria are cooled at −10° and −15°C before freezing. Scale bars indicate 0.1 μm.

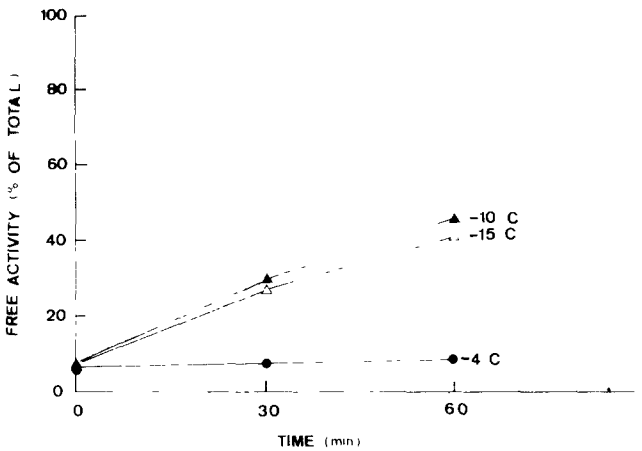


Fig. 2. Free activity of mitochondrial malate dehydrogenase. Aliquots of a total mitochondrial fraction are kept during increasing times at the indicated temperature, in presence of 0.25 M sucrose, 1 mM imidazole pH 7.4 and 40% (v/v) ethylene glycol. After that, free and total activity of malate dehydrogenase were measured. Total enzyme activity was 180 units/g fresh weight of liver.

Thus, in such conditions, the inner mitochondrial membrane was kept intact. Results are quite different when mitochondria are incubated at −15°C. A high amount of malate dehydrogenase was recovered at the top of the gradient, having been released by the treatment at −15°C, in an unsedimentable form. The remainder was spread between 1.198 and 1.214 g/ml mean density fractions. About 50% of cytochrome oxidase and monoamine oxidase and only 5% of malate dehydrogenase are recovered between densities 1.164 g/ml and 1.191 g/ml, with a peak of activity in a fraction of 1.169 g/ml mean density for the membrane enzymes. Therefore, this region of the gradient contains mainly mitochondrial ghosts. The remainder of cytochrome oxidase and monoamine oxidase was found together with malate dehydrogenase between densities 1.198 and 1.214 g/ml. These observations show that maintaining the mitochondria at −15°C causes a true lysis of an important proportion of the organelles.

Thus, cooling mitochondria to temperatures situated in the region of the transition endotherm of their inner membrane causes a suppression of the malate dehydrogenase structure-linked latency resulting from a release of the enzyme in the medium.

Lateral phase separations induced by compression

Lateral phase separations observed by freeze fracture electron microscopy in mitochondrial membranes are reversible. Mitochondria maintained at low temperature but warmed above 0°C before freezing do not exhibit lateral phase separations in their membranes [19]. We wondered whether lateral separations could be made irreversible by glutaraldehyde treatment. It was essential to answer this question in order to carry out the experiments where the mitochondria had to be subjected to hydrostatic pressure. Indeed, it was expected that if lateral phase separations do occur under pressure in mitochondrial membranes, it would probably be reversible and therefore unobservable when normal pressure would be restored before freezing. We compared mitochondria

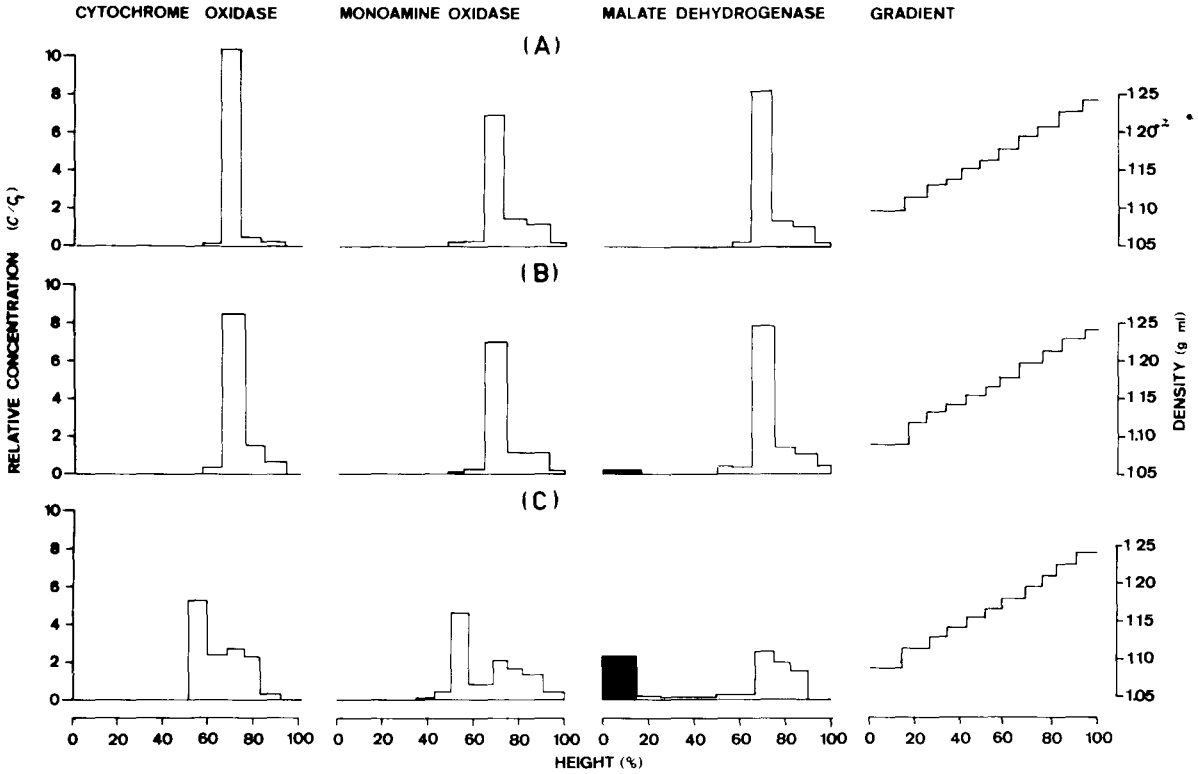


Fig. 3. Distribution of mitochondrial enzymes after centrifugation (for 2.5 h at 39 000 rev./min in SW 65 head of the Spinco model L 65 B preparative ultracentrifuge) of a mitochondrial fraction of rat-liver through a 0.776—2.969 molal sucrose gradient. Mitochondrial fraction in 0.25 M sucrose, 40% (v/v) ethylene glycol, 1 mM imidazole pH 7.4 was kept at 0°C (A), at —4°C (B) or at —15°C for 1 h (C) before layering at the top of the gradient. Ordinate: relative concentration, i.e., ratio of the observed activity (C) to that which would have been found if the enzyme had been homogeneously distributed throughout the whole gradient (C_1). Abscissa Percentage of the height of the liquid column in tube. Filled blocks are used for the top subfraction to indicate that it includes unsedimentable material. Mean Recovery was 96.6% for cytochrome oxidase, 98.0% for monoamine oxidase and 100.1% for malate dehydrogenase. Absolute values of enzyme activity in the mitochondrial fraction were 22.0, 0.75 and 176 units/g fresh weight of liver, respectively, for cytochrome oxidase, monoamine oxidase and malate dehydrogenase.

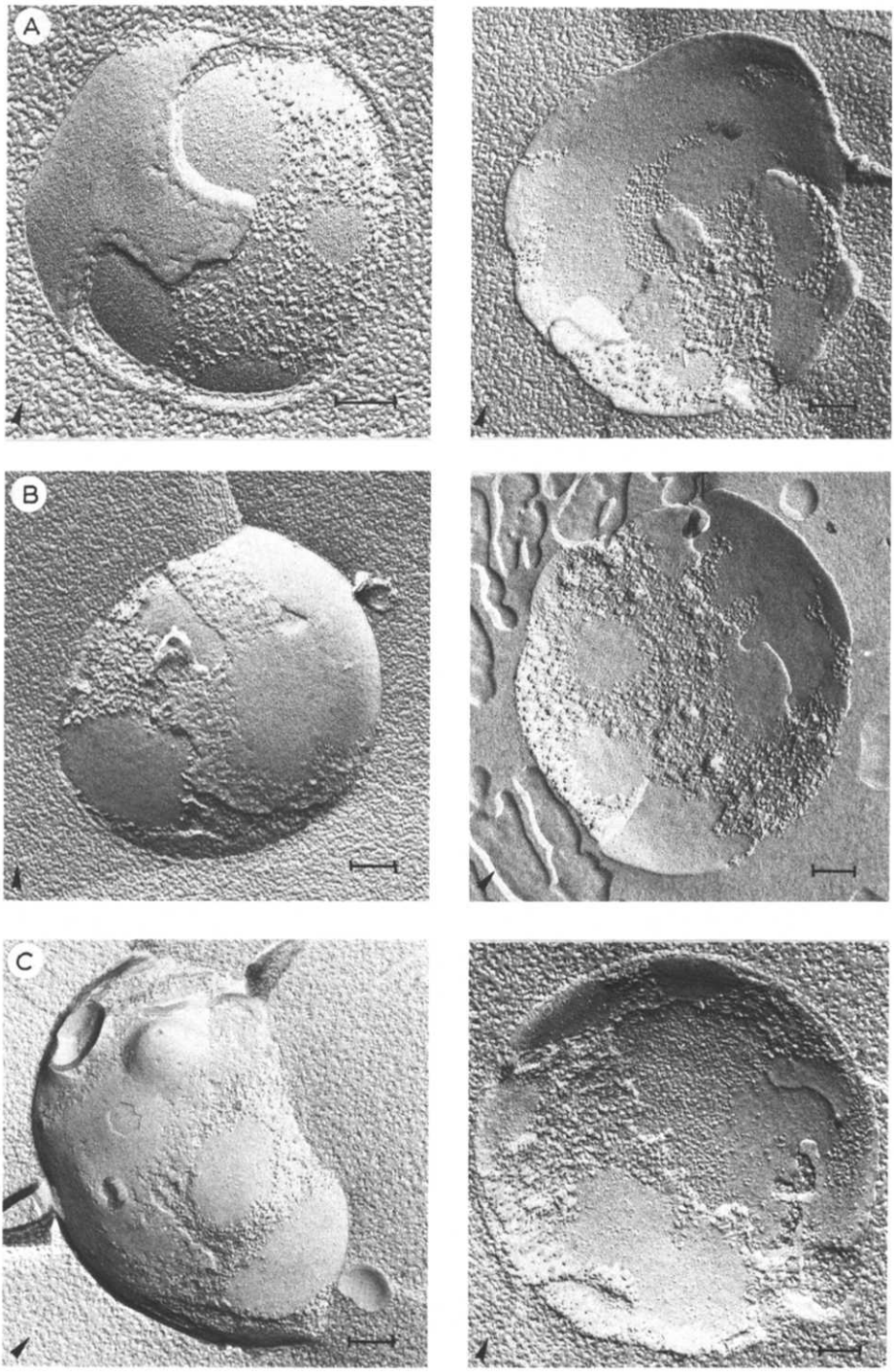


Fig. 4. Convex and concave fracture faces of membranes of mitochondria maintained at −15°C for 1 h before freezing. A. Mitochondria frozen without fixation; B, mitochondria fixed with 1.5% glutaraldehyde for 2 h and then frozen, C, mitochondria fixed with 1.5% glutaraldehyde for 2 h, warmed to 0°C and frozen. Scale bars indicate 0.1 μm

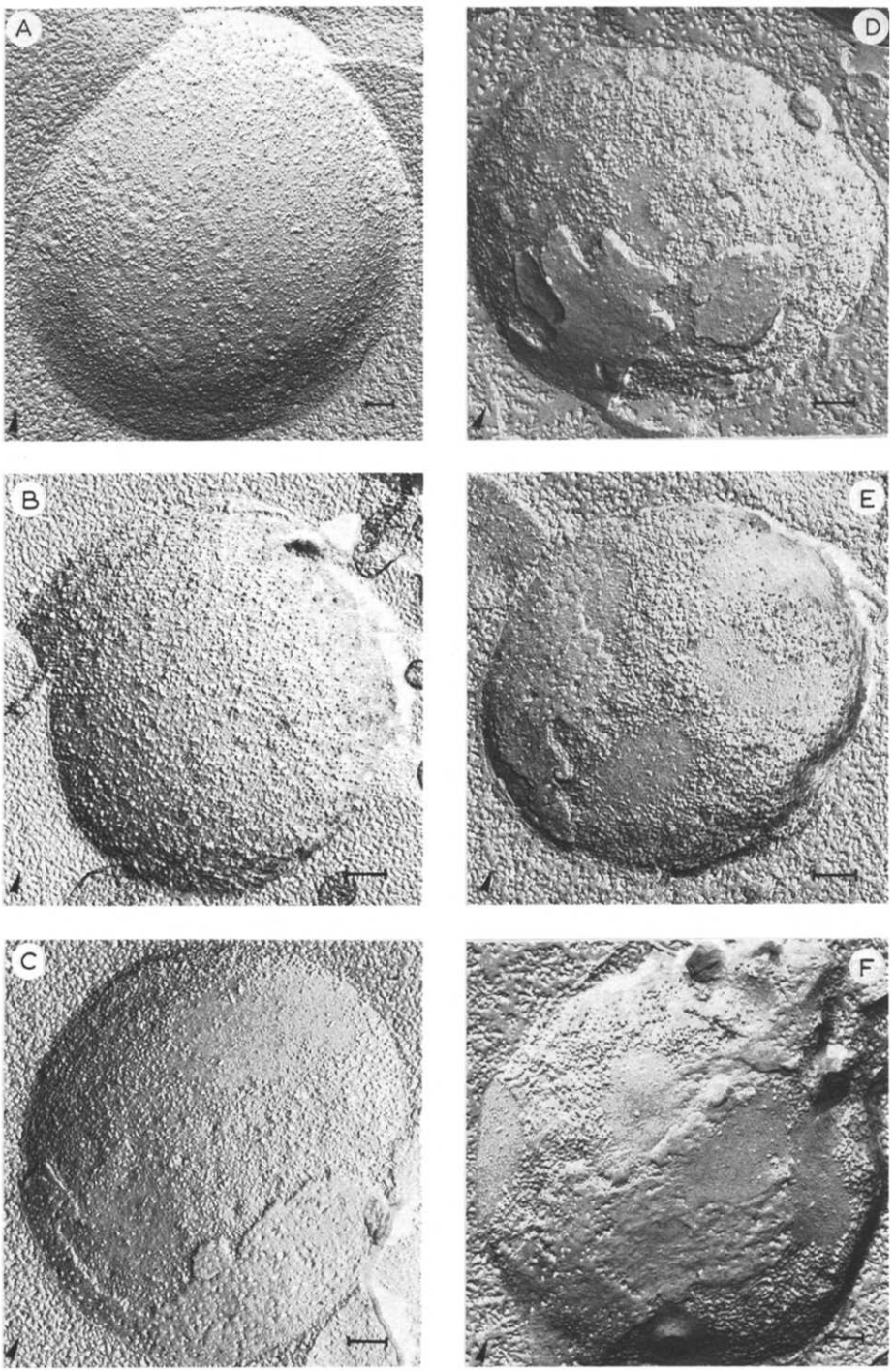


Fig. 5. Convex fracture faces of membranes of mitochondria subjected to different pressures at 0°C during 1 h fixed and frozen. A, Atmospheric pressure; B, 500 kg/cm^2; C, 750 kg/cm^2; D, 1000 kg/cm^2, E, 1250 kg/cm^2; F, 1500 kg/cm^2. Smooth areas devoid of particles begin to be seen when pressure reaches 750 kg/cm^2. Scale bars indicate 0.1 μm.

equilibrated at −15°C and then frozen in liquid solid nitrogen and mitochondria equilibrated at the same temperature, but fixed with 1.5% (v/v) glutaraldehyde during 2 h before freezing. No significant differences were observed. After glutaraldehyde fixation, mitochondria were warmed at 0°C before freezing; results were similar, large smooth areas devoid of particles were seen in the fracture faces of both membranes (Fig. 4). These results show that the glutaraldehyde treatment prevents lateral phase separations in mitochondrial membranes from being reversible. Consequently, in the following experiments, the fixation of mitochondria with glutaraldehyde was performed under pressure, before restoring atmospheric pressure and freezing for electron microscopy.

Fig. 5 shows mitochondrial membrane fracture faces observed when mitochondria have been exposed at a pressure ranging from 500 kg/cm^2 to 1500 kg/cm^2 at 0°C. At 500 kg/cm^2, there is no sign of structural changes in the membranes. In particular, the convex fracture face of the inner membrane is covered with a great number of regularly disseminated particles. Structural changes become obvious when mitochondria are subjected to a pressure of 750 kg/cm^2, smooth areas devoid of particles begin to be seen in the fracture faces. This phenomenon is particularly evident when the pressure is higher; at 1500 kg/cm^2, the smooth areas are extensive, they are contiguous with zones where aggregated particles can be seen.

An appreciation of the structure-linked latency of malate dehydrogenase, when mitochondria are compressed is shown in Fig. 6. The results are in agreement with the observations of Bronfman and Beaufay [5]. Free malate dehydrogenase activity begins to increase when the pressure is at 750 kg/cm^2 and reaches a maximum when compression is performed under 1250—1500 kg/cm^2. Suppression of the structure-linked latency of the mitochondrial matrix enzyme parallels lateral phase separation.

The structural changes caused by compression can be prevented by increasing the temperature at which compression is performed. This is illustrated in

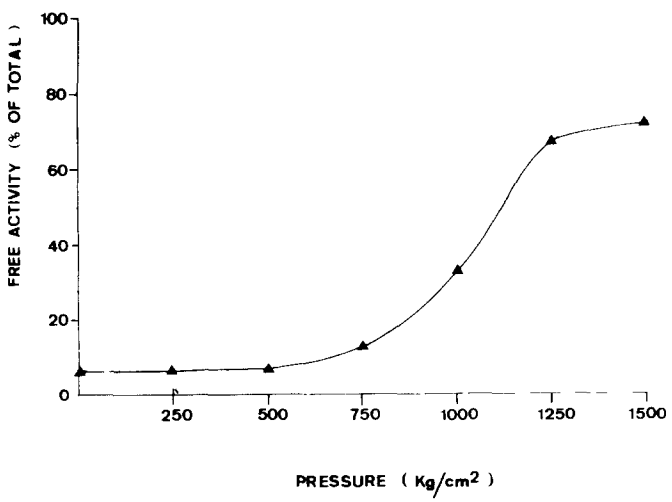


Fig. 6. Malate dehydrogenase free activity of mitochondria submitted to increasing pressures during 1 h. Total enzyme activity was 170.0 units/g fresh weight of liver.

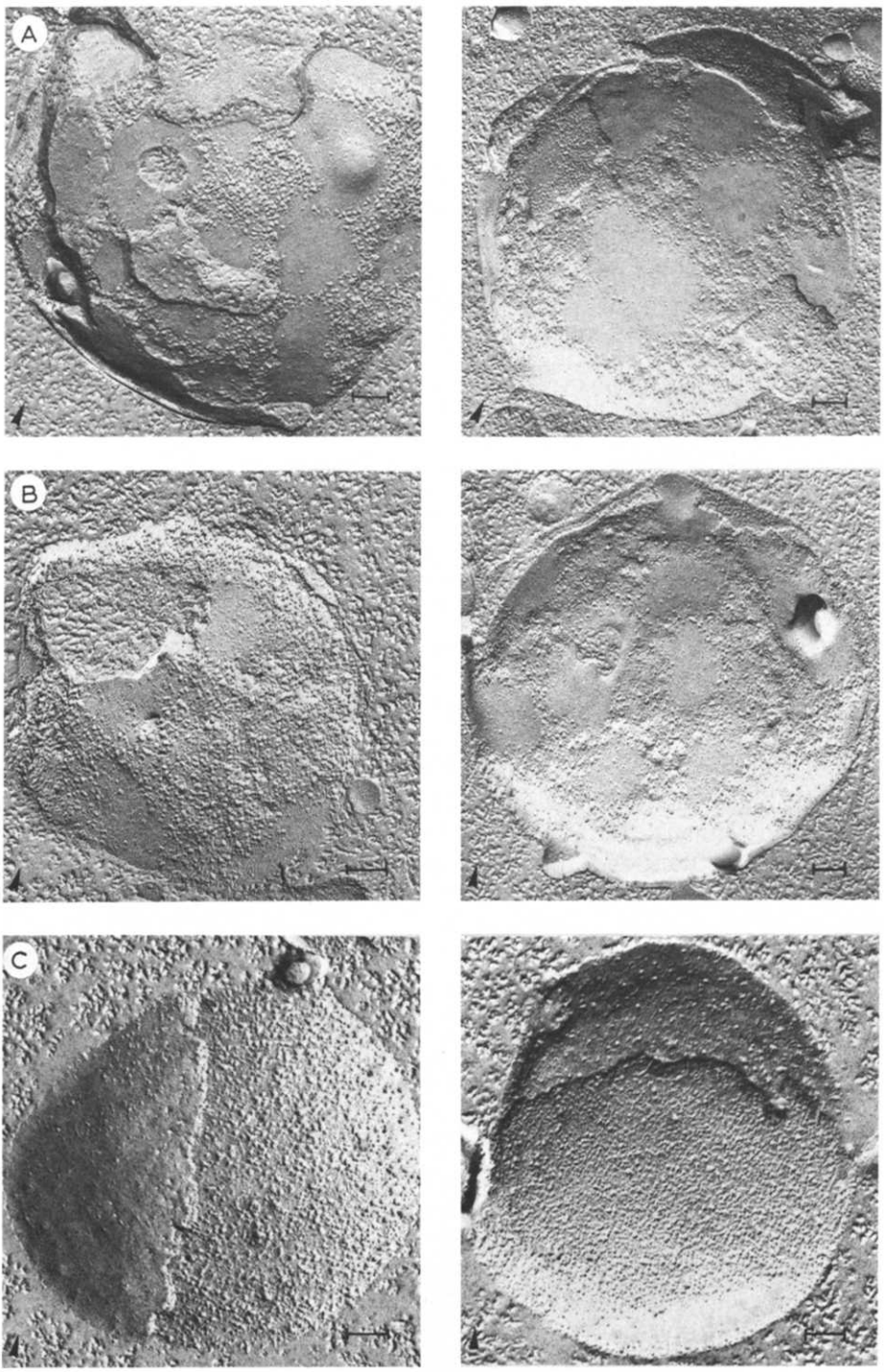


Fig. 7. Convex and concave fracture faces of membranes of mitochondria exposed at a pressure of 1500 kg/cm^2 during 1 h at different temperatures, fixed and frozen. A, 0°C; B, 5°C, C, 10°C. Smooth areas devoid of particles are seen when compression is performed at 0° or 5°C, but absent at 10°C. Scale bars indicate 0.1 μm.

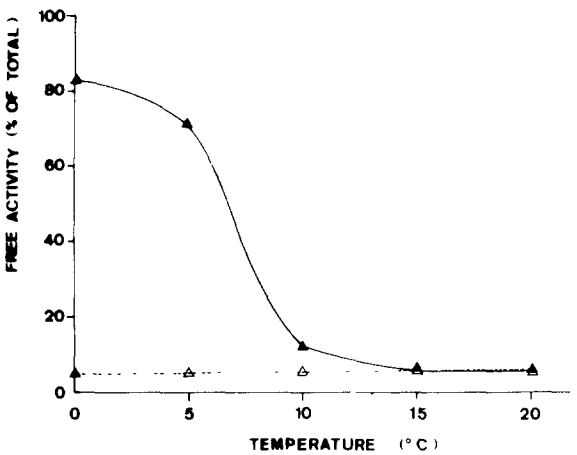


Fig. 8. Malate dehydrogenase free activity of mitochondria exposed at atmospheric pressure (· - - - - -) or at a pressure of 1500 kg/cm^2 during 1 h (▲———▲) at different temperatures. Total enzyme activity was 164.0 units/g fresh weight of liver.

Fig. 7; it shows the fracture faces of mitochondrial membranes after compression at 1500 kg/cm^2 and at different temperatures. At 10°C, particles in the fracture faces exhibit a distribution similar to that seen in the membranes of mitochondria equilibrated at 0°C under atmospheric pressure. In the same way, increasing the temperature prevents loss of the malate dehydrogenase latency (Fig. 8).

The particle aggregation caused by compression in the mitochondrial membranes is reversible. Membrane fracture faces of mitochondria subjected to a pressure of 1500 kg/cm^2, during 1 h, then reequilibrated at atmospheric pressure are similar to those found in mitochondria that have not been compressed.

Discussion

Several authors have found that the permeability of liposomes and cells may be deeply increased when thermotropic liquid to gel crystalline transition occurs [8—10]. Moreover, as shown by McEhlaney et al. [11], and Van Zoelen et al. [12], in their works on *Acholeplasma laidlawii*, the osmotic behaviour of the cell depends on the physical state of the membrane lipids. In particular, when the membrane lipids are in gel state, the cell can no longer swell and complete lysis occurs in hypotonic sucrose solutions. Our results show that rat-liver mitochondria kept in the vicinity of the lower thermal transition temperature of their membrane lipids undergo lysis. By analogy with what has been found for liposomes and cells, it may be supposed that thermotropic phase transition causes an increase of the inner mitochondrial membrane permeability to sucrose, an entry of osmotic water and a disruption of the membrane owing to the lowering of the organelle swelling capacity.

The outer mitochondrial membrane probably does not play a key role in this phenomenon. It is freely permeable to relatively large molecular weight solutes and it is not involved in the osmotic behavior of mitochondria [21]. It is to be pointed out that lateral phase separations occur in the outer membrane at

several degrees above 0°C [19] without apparently damaging mitochondria. It is, however, not excluded that in the region of the lower thermal transition temperature of their lipids, which is the same as that of the inner membrane lipids [19], the rigidity of the outer membrane contributes to the lowering of the mitochondrial swelling capacity by counteracting the inner membrane expansion.

When rat-liver mitochondria are subjected to compression at approx. 750 kg/cm^2, particle-free zones contiguous to zones with a high density of particles begin to be observed in mitochondrial membranes. The phenomenon is more and more apparent when the pressure increases and is impressive at 1500 kg/cm^2. Similar pictures are seen in mitochondrial [19,20] and various biological membranes [22] when they are equilibrated at a temperature which is able to cause a thermotropic phase transition of their lipids, as ascertained by physical measurements: EPR, differential calorimetry. They are interpreted as morphological illutrations of lateral phase separations which take place in these membranes during thermotropic phase transitions. In these conditions, lipid areas in liquid crystalline state coexist with lipid areas in gel crystalline state; intramembrane particles (integral proteins) are squeezed out of the gel state zones and are sequestered in the liquid state zones [22]. Thus our freeze etching observations strongly suggest that lateral phase separations can take place in the mitochondrial membranes when they are subjected to a high pressure. Such an interpretation is strengthened by the fact that this can be prevented by increasing the temperature at which the compression is performed.

Freeze-fracture electron microscopy shows that, under atmospheric pressure, lipid phase separations occur in mitochondrial membranes at subzero temperatures [19,20]. Lateral phase separations are particularly impressive when the temperature is in the vicinity of the lower thermal transition temperature (−15°C according to calorimetric measurements, [19]). At 0°C, phase separations begin to appear when the pressure reaches 750 kg/cm^2; pictures at 1500 kg/cm^2 are comparable to those seen at −15°C under atmospheric pressure. Obviously, according to our morphological results, it is not possible to determine exactly to what extent the transition temperature of mitochondrial lipids is increased by compression. By refering to experiments where a pressure of 1500 kg/cm^2 was applied at different temperatures, one has to admit that such a pressure does shift the transition temperature by several degrees, above 0°C. Indeed, we have shown that lateral phase separation induced by that pressure is prevented if compression is performed at 10°C. This could be explained if the lower transition temperature (−15°C) could rise to approx. 1.5°C by 100 kg/cm^2 elevation of pressure. Such a value is not improbable. ESR measurements of phase transitions in dipalmitoyl phosphatidylcholine bilayers show that the application of 100 atm of helium pressure increases the primary phase transition temperature by approx. 2.2°C and the lower transition temperature of the lipids by approx 1.2°C and also that the increase is linear with pressure [23]. De Smedt et al. [24] have studied by light-scattering phase transitions in dimyristoyl phosphatidylcholine subjected to high pressure. They have found that phase transition temperature increases linearly with pressure up to 1500 kg/cm^2. A change in pressure of 100 kg/cm^2 causes a 2°C increase of the transition temperature.

The experiments reported in this paper were mainly performed to check our hypothesis concerning the effect of the hydrostatic pressure on subcellular structures submitted to centrifugation [1—4,6]. We suppose: (1) That in certain cases, pressure generated by centrifugation could be sufficient to increase the transition temperature of mitochondrial lipids above the centrifugation temperature with, as a result, lipid phase separations in the membranes: (2) That the phase separation causes an increase of the inner mitochondrial membrane permeability to sucrose and an osmotic lysis of the particles.

Our results strongly support the first part of the hypothesis. Pressures of several hundred and, even more, to one thousand kg/cm^2 are readily developed during centrifugation in conventional rotors [6]. Thus, it is very probable that in such conditions lateral phase separations take place in mitochondrial membranes.

With respect to the second part of our hypothesis, it is to be noted that lateral phase separations induced by compression in mitochondrial membranes go hand in hand with a loss of mitochondrial enzyme structure-linked latency and a lysis of the particles. One could ask whether the two phenomena are independently brought about by the pressure or whether they are linked, lateral phase separations being the cause of the membrane deterioration. Our observations are in favor of such a causal relationship in that, like other membranes systems, mitochondria undergo spontaneous lysis when thermotropic liquid to gel crystalline transition occurs in their membranes. It is therefore very probably that thermotropic transition induced by compression in the mitochondrial membranes has the same effect.

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