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PERMEABILITY OF MITOCHONDRIA TO SUCROSE INDUCED BY HYDROSTATIC PRESSURE

SIMONE WATTIAUX-DE CONINCK, FRANZ DUBOIS, JEANNINE MERTENS-STRIJTHAGEN, CHRISTIAN DE SCHRIJVER and ROBERT WATTIAUX

Laboratoire de Chimie Physiologique et Laboratoire de Physiologie, Facultés Universitaires Notre-Dame de la Paix, 61 rue de Bruxelles, 5000 Namur (Belgium)

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Summary

When subjected to increasing pressure at 0°C, rat liver mitochondria become permeable to sucrose, causing them to swell and their outer membrane to rupture. Afterwards they are lysed and their matrix content is released into the medium. This permeation to sucrose may be prevented to some extent by increasing the temperature at which compression is carried out. 0.75 mM imipramine protects mitochondria against lysis caused by hydrostatic pressure, but does not oppose their permeation to sucrose nor the swelling resulting from the compression. At this concentration, the drug does not exhibit a significant effect on the lateral phase separations which take place in the inner mitochondrial membrane under pressure. The mitochondria of rat fetal liver (21 days), kidney and Morris hepatoma 16 become permeable to sucrose when they are subjected to compression; under these conditions, lateral phase separations occur in their inner membrane. Contrary to liver mitochondria, the former do not undergo lysis. Taking into account both present and previous results, events leading to mitochondrial membrane deterioration by hydrostatic pressure may be summarized in the following way. Pressure first leads to a phase transition of the membrane lipids, thus causing a permeation to sucrose; as a result the mitochondria swell because they have absorbed osmotic water. The membrane lipids freeze increasingly as the pressure increases; the inner membrane becomes fragile and finally, in the case of the adult liver organelles, can no longer resist the swelling. All these events can be avoided by increasing the temperature; imipramine only prevents inner membrane lysis.

Introduction

Rat liver mitochondria are damaged when they are subjected to a relatively high hydrostatic pressure [1,2]. To explain this phenomenon, it has been

proposed that pressure makes the inner mitochondrial membrane permeable to sucrose which in turn causes an osmotic swelling of the granules leading to their disruption [3]. On the other hand, freeze-fracture electron microscopy shows that pressure induces lateral phase separations in mitochondrial membrane lipids [4]. This research has been undertaken in order to test the hypothesis that pressure causes a permeability change of the inner membrane and to correlate biochemical and morphological observations concerning pressure effects.

Materials and Methods

Tissue fractionation. All the experiments were performed on mitochondrial fractions corresponding to the sum of the M and L fractions of de Duve et al. [5].

Compression of the granules. As already described, the granules were compressed [4] with the compression unit of Bronfman and Beaufay [2] kindly provided by Professor H. Beaufay.

Sucrose permeability measurements. 1 ml of M + L fraction (0.5 g tissue) was compressed in the presence of 2 μCi of [^{14}C]sucrose in 0.25 M sucrose. After compression, the granules were filtered through a Sepharose 4B column (1.6 X 5.5 cm) equilibrated with 0.25 M sucrose. 1 ml fractions were collected and analyzed for radioactivity and malate dehydrogenase activity. The amount of mitochondria recovered with the excluded material in the first six fractions was estimated by measuring the fraction content in malate dehydrogenase, a mitochondrial matrix enzyme. This enzyme was chosen, rather than a mitochondrial membrane enzyme like cytochrome oxidase, because the distribution of a membrane enzyme could provide a false estimation of the fraction content in the mitochondria. Indeed, in this paper as well as in previous ones [1—4], we have shown that when the pressure increases, a progressive disruption of the mitochondria takes place, thus releasing the matrix content. Under these conditions, mitochondrial membranes are present in the medium together with undisrupted mitochondria (swollen for the most part). After filtration on Sepharose 4B, due to their size, mitochondrial ghosts and undisrupted mitochondria are recovered together in the first fractions. Therefore, the amount of mitochondrial membrane enzymes present in these fractions depends not only on the amount of intact mitochondria but also on the amount of mitochondrial membranes or mitochondrial ghosts. On the other hand, the amount of the mitochondrial matrix enzymes recovered in these fractions corresponds more closely to the undisrupted mitochondria. Indeed, except for some readsorption on membrane after release (minimal in the case of malate dehydrogenase), such an enzyme can only be present in these fractions if it is enclosed in intact mitochondria. The radioactivity associated with the first fractions corresponds to the sucrose which migrated with the mitochondria, indeed, free sucrose is found with the soluble components at the end of the elution.

Swelling of the mitochondria. The swelling of the mitochondria was estimated by establishing the distribution pattern of the mitochondrial enzymes after isopycnic centrifugation in a glycogen gradient with 0.25 M sucrose as the solvent [6]. The mitochondrial fraction was layered at the top of the gradient before centrifugation. In such a gradient, normal mitochondria are recovered

mainly in the higher-density regions of this gradient, swollen mitochondria are found in the upper fractions, just beside the top fraction where the unsedimentable components are located [7].

Enzyme assays. Sulfite: cytochrome *c* reductase was assayed according to the method of Wattiaux-De Coninck and Wattiaux [8] by following the reduction of cytochrome *c* at 550 nm in a Gilford recording spectrophotometer. The medium contained 0.3 mM Tris buffer (pH 8.5), 0.4 mM NaCN and 0.25 M sucrose, in a volume of 1 ml. Malate dehydrogenase was measured according to the method of Wattiaux-De Coninck et al. [4] by following the oxidation of NADH at 340 nm in a medium containing 25 mM Tris buffer (pH 7.4), 0.15 mM NADH, 0.25 mM oxaloacetate and 0.25 M sucrose in a volume of 1 ml. These enzymes have two distinct activities: (a) free activity, i.e., the activity measured without the addition of the Triton X-100 detergent and (b) total activity, determined in the presence of 0.1% Triton X-100. When the outer mitochondrial membrane is intact, the free activity of sulfite : cytochrome *c* reductase (located in the intermembrane space [8]) is low because the outer mitochondrial membrane is only slightly permeable to cytochrome *c*; when the inner mitochondrial membrane is intact, malate dehydrogenase exhibits a low free activity because the enzyme has no access to external NADH. The determination of the free activity of both enzymes allows an appraisal of the integrity of the inner and outer mitochondrial membranes. Proteins were measured according to the method of Lowry et al. [9].

Electron microscopy. The granules were fixed under pressure and the sample was processed for freeze-fracture electron microscopy as described by Wattiaux-De Coninck et al. [4].

Results

Permeability to sucrose

As illustrated in Fig. 1, the permeability of the mitochondria to sucrose increases as a function of the pressure imposed on the granules. Maximum effects are observed when the pressure rises from 900 to 1200 kg/cm^2. Above this pressure, a slight permeability increase still takes place followed by a definite decrease.

Swelling and disruption of mitochondria

Fig. 2 shows the distribution, after isopycnic centrifugation, of two reference enzymes which exhibit different submitochondrial localizations: sulfite : cytochrome *c* reductase is a component of the intermembrane space [8] and malate dehydrogenase is located in the matrix [10]. The enzymes of uncompressed mitochondria are found mainly at the bottom of the gradient. After compression at 1200 kg/cm^2, sulfite : cytochrome *c* reductase, released because of the disruption of the outer membrane, is largely recovered in the top fraction of the gradient where the granules have been layered. Malate dehydrogenase exhibits a bimodal distribution, one part is present in soluble form in the top fraction, the remainder is mainly associated in the fractions of the gradient close to where the swollen mitochondria equilibrate. After compression at 1700 kg/cm^2, a large part of both enzymes is recovered in the top frac-

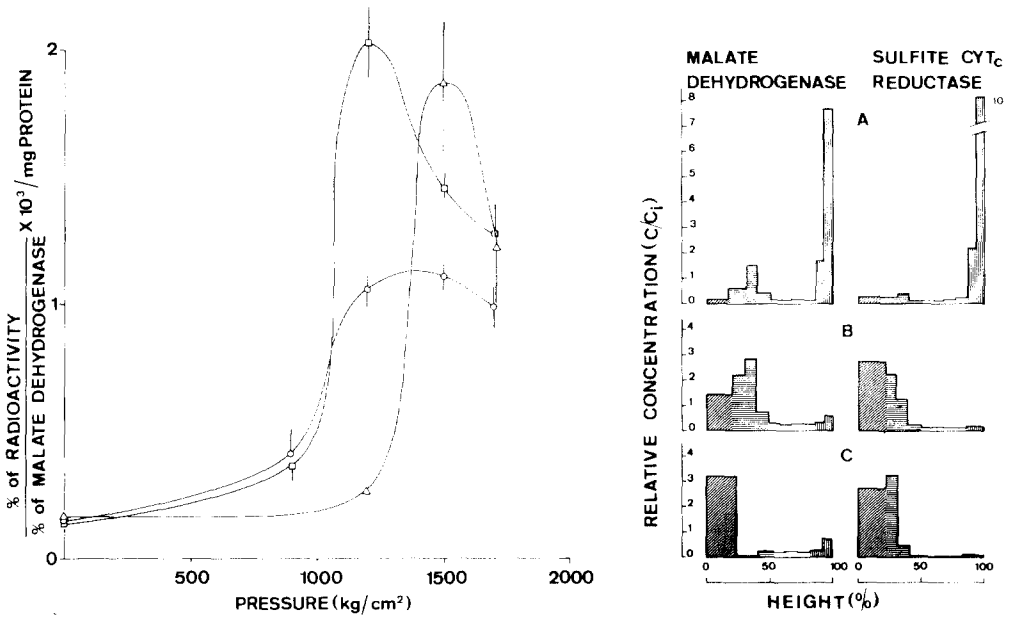


Fig. 1. Penetration of sucrose in rat liver mitochondria submitted to increasing pressure for 1 h. The amount of sucrose associated with mitochondria is expressed as the ratio of the percentage of radioactivity found in the first six fractions after chromatography on Sepharose 4B to the percentage of malate dehydrogenase measured in these fractions. Moreover, taking into account the differences in the yield of the mitochondria recovered in the mitochondrial fraction, this ratio has been divided by the amount of proteins present in the compressed mitochondrial fraction assuming that the proteins present in this fraction essentially originate from the mitochondria [6]. Vertical lines correspond to standard errors. ○———○, compression at 0° C; △———△, compression at 10° C; □———□, compression at 0° C in the presence of 0.75 mM imipramine.

Fig. 2. Distribution of malate dehydrogenase and sulfite : cytochrome *c* reductase after the isopycnic centrifugation of a rat liver mitochondrial fraction in 5—20% (w/w) glycogen gradient using 0.25 M sucrose in water as the solvent. The time integral of the square angular velocity was 144 rad^2/ns. Centrifugations were performed at 39 000 rev./min in an SW 65 Spinco rotor. Particles suspended in 0.25 M sucrose were initially layered 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_i). Abscissa: percentage of the height of the liquid column in the tube. ▨, unsedimentable components; ▤, swollen mitochondria; ▥, normal mitochondria. (A) uncompressed mitochondria, (B) mitochondria compressed for 1 h at 1200 kg/cm^2, (C) mitochondria compressed for 1 h at 1700 kg/cm^2.

tion in unsedimentable form. These observations suggest that under pressure, the mitochondria swell causing the outer membrane to rupture; then they are lysed and release their matrix content into the medium. These results agree with the structure latency modifications of both enzymes when mitochondria are subjected to increasing pressures: sulfite : cytochrome *c* reductase and malate dehydrogenase are unmasked by the pressure, but in a non-simultaneous manner. The intermembrane enzyme is released at a lower pressure than the matrix enzyme (Fig. 3).

Temperature effects

As shown previously when the temperature is increased, lateral phase separation, induced by pressure, is prevented and the mitochondria are protected

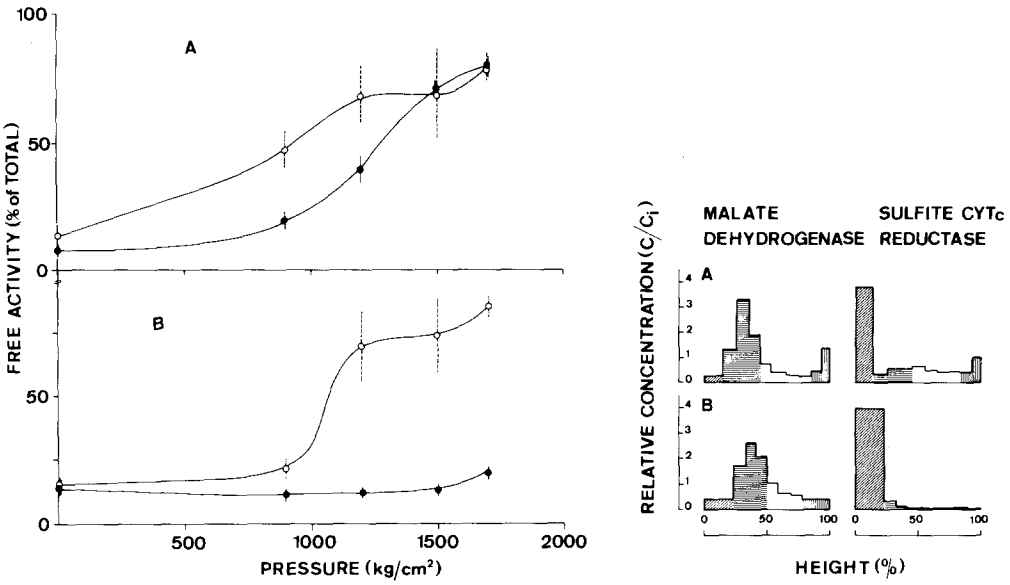


Fig. 3. Sulfite : cytochrome *c* reductase (○———○) and malate dehydrogenase (●———●) free activity of rat liver mitochondria submitted to increasing pressures for 1 h at 0°C. Vertical lines correspond to standard errors. A, in the absence of imipramine; B, in the presence of 0.75 mM imipramine.

Fig. 4. Distribution of malate dehydrogenase and sulfite : cytochrome *c* reductase after the isopycnic centrifugation of a rat liver mitochondrial fraction in 5—20% (w/w) glycogen gradient using 0.25 M sucrose in water as the solvent. Mitochondria were compressed at 1200 kg/cm^2 (A) and at 1700 kg/cm^2 (B) for 1 h at 0°C, in the presence of 0.75 mM imipramine. For an explanation of the graph, see legend to Fig. 2.

against lysis [4]. Fig. 1 shows that to some extent, an increase in temperature opposes the permeation of the inner membrane to sucrose. The curve illustrating the permeability change as a function of the pressure is shifted toward a higher pressure when compression takes place at 10°C.

Effect of imipramine

Imipramine is an antidepressant which protects the mitochondria from lysis caused by the hydrostatic pressure generated during centrifugation [11] and by a hydraulic press [12]. The drug does not oppose sucrose permeation resulting from compression (Fig. 1). On the contrary, in the presence of 0.75 mM imipramine, the phenomenon is even more apparent; a sharp permeability increase occurs followed by a net decrease. Accordingly, the drug does not prevent the mitochondria from swelling, as indicated by the distribution curves of sulfite : cytochrome *c* reductase and malate dehydrogenase after isopycnic centrifugation (Fig. 4). When mitochondria have been compressed in the presence of imipramine, sulfite : cytochrome *c* reductase is released during compression and is found in the top fraction; on the other hand, malate dehydrogenase is quasi-totally recovered in the fractions containing the swollen mitochondria. Therefore, when imipramine is present, mitochondria swell but do not undergo lysis. This confirms the latency experiments which, in accordance with Bronfman's results [12], show that imipramine does not prevent the

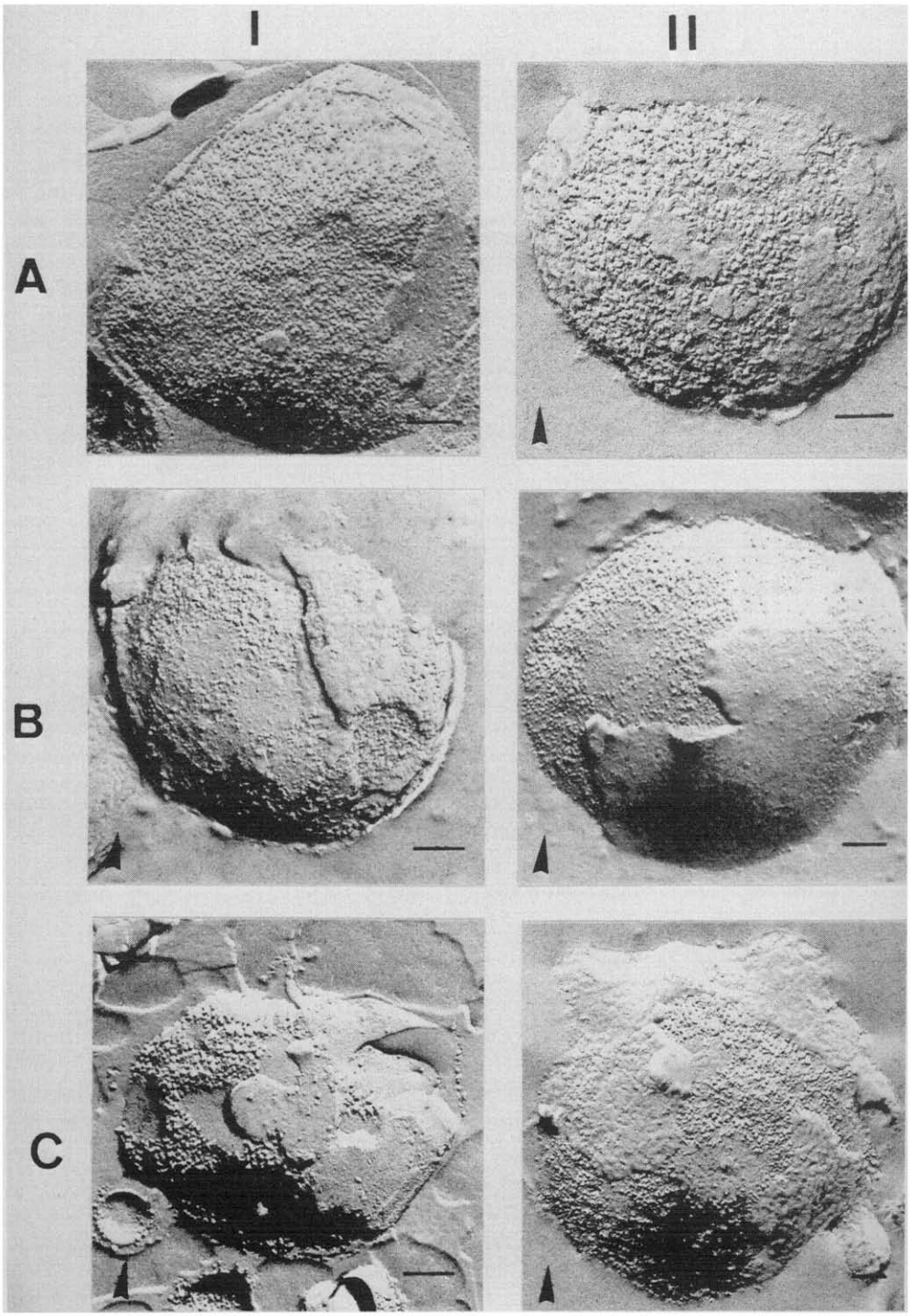


Fig. 5. Convex fracture faces of membranes of rat liver mitochondria compressed in the absence (I) or in the presence of 0.75 mM imipramine (II) for 1 h at 0°C, fixed and frozen. (A) 1 kg/cm^2, (B) 1200 kg/cm^2, (C) 1700 kg/cm^2. Bars represent 0.1 μm.

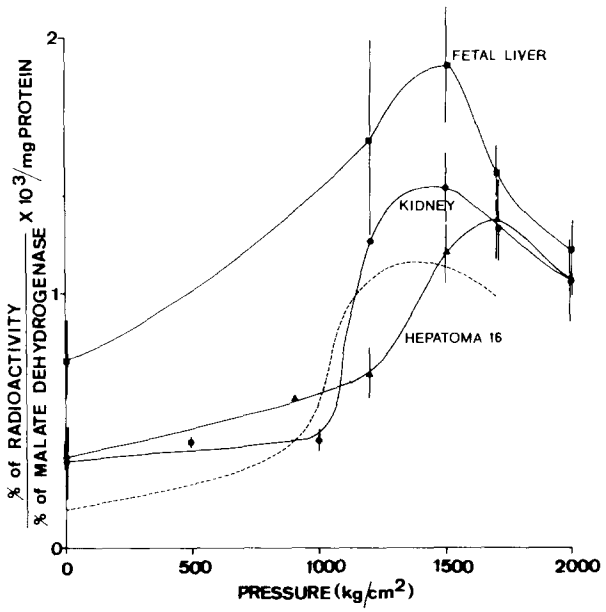


Fig. 6. Penetration of sucrose in fetal liver (21 days), kidney and Morris hepatoma 16 mitochondria submitted to increasing pressure for 1 h at 0°C. For an explanation of the graph see legend to Fig. 1. To facilitate comparison, the curve corresponding to adult liver mitochondria (Fig. 1) is represented by a dashed line.

increase of free sulfite : cytochrome *c* reductase caused by compression, but inhibits the increase of malate dehydrogenase free activity (Fig. 3).

At 0.75 mM, imipramine does not prevent the lateral phase separation which takes place under pressure. As illustrated in Fig. 5, smooth areas devoid of particles can be seen in the fracture faces of the inner mitochondrial membrane at 1200 and 1700 kg/cm^2 whether imipramine is present or absent.

Fetal liver, Morris hepatoma 16 and kidney mitochondria

Contrary to liver mitochondria, late fetal liver [13] and Morris hepatoma 16 [14] mitochondria do not seem to be affected by hydrostatic pressure generated by centrifugation. Under the same conditions, rat kidney mitochondria distributions are modified but less drastically than liver mitochondria (Goffin-Tasiaux, personal communication). It was interesting to investigate the behavior of these granules when they were compressed in a hydraulic press. The three kinds of mitochondria become permeable to sucrose when they are subjected to pressure (Fig. 6). For hepatoma 16 mitochondria, the permeation curve is shifted towards a higher pressure when compared to the permeation curve of adult liver mitochondria. Lateral phase separation occurs in the inner membrane of the compressed organelles whatever their origin, as shown by Fig. 7. On the other hand, malate dehydrogenase latency is not affected by pressure (Fig. 8).

Discussion

When subjected to pressure, the inner mitochondrial membrane becomes permeable to sucrose. This phenomenon is apparent for the four kinds of mito-

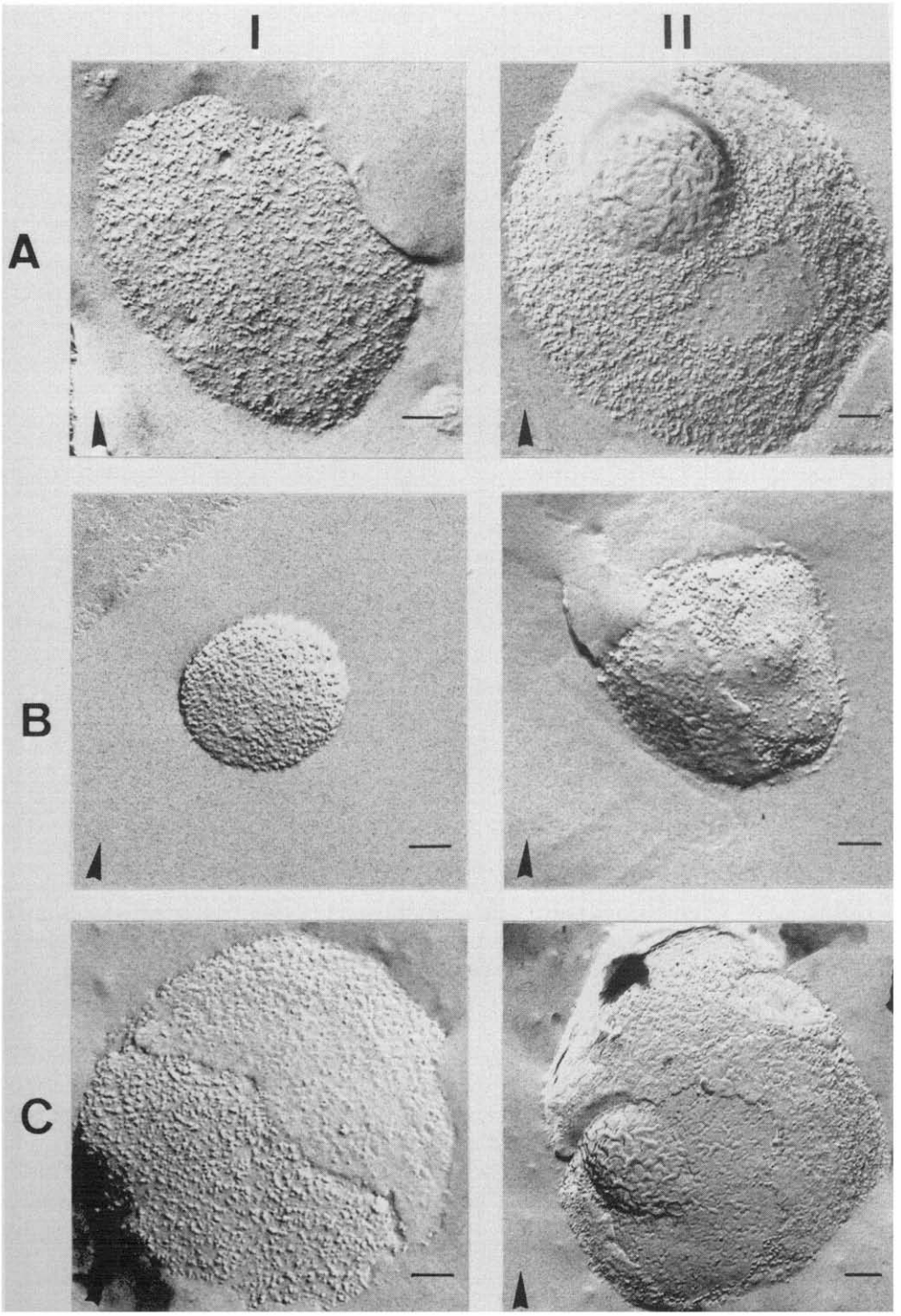


Fig. 7. Convex fracture faces of membrane of fetal liver (A), Morris hepatoma 16 (B) and kidney (C) mitochondria; maintained at atmospheric pressure (I), or compressed for 1 h at 0°C (II) at 1200 kg/cm^2 (kidney and fetal liver mitochondria) or 1500 kg/cm^2 (hepatoma mitochondria). Bars represent 0.1 μM.

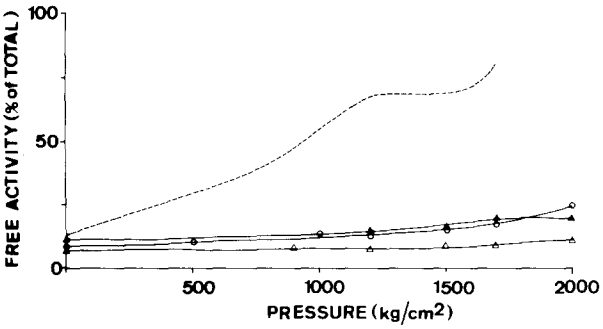


Fig. 8. Malate dehydrogenase free activity of fetal liver (▲———▲), kidney (○———○) and Morris hepatoma 16 (△———△) mitochondria submitted to increasing pressure for 1 h at 0°C. Dashed line, free activity of adult liver mitochondria as shown in Fig. 3.

chondria we have studied. The curve illustrating this process passes through a maximum. There are two plausible explanations. The fact that labelled sucrose (which penetrated into the mitochondria under pressure) remains inside the organelles supposes that a certain resealing of the inner membrane occurs after atmospheric pressure is re-established. If not, labelled sucrose would be considerably diluted during its filtration on Sepharose in the presence of 0.25 M unlabelled sucrose. In fact, the amount of sucrose associated with the mitochondria in our experiments does not strictly correspond to the true amount which penetrated under pressure. It is probable that a certain proportion of radioactive sucrose was redistributed in the cold sucrose medium after returning to atmospheric pressure. Therefore, a possible explanation as to why the accumulation of sucrose in mitochondria passes through a maximum could be that the resealing of the inner membrane is less adequate when the granules have been subjected to a high pressure. There is, however, another possibility. As shown by several authors [15—17], the permeability of some artificial and natural membranes reaches a maximum at the transition from liquid crystal to gel crystal of the phospholipids of which they are composed. If, as discussed below, permeation induced by pressure results from the freezing of the membrane lipids, it is possible that a maximum of permeability could take place in the pressure range where phase transition occurs in the inner mitochondrial membrane.

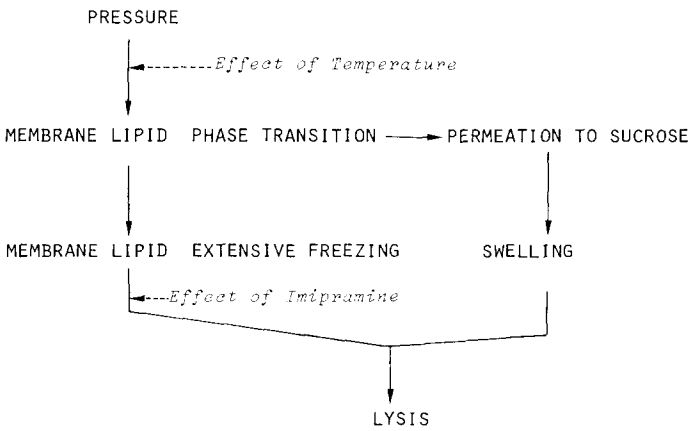
In parallel with the permeation to sucrose, freeze-fracture electron microscopy shows that smooth zones devoid of particles spread out in the inner membrane of adult liver [4], kidney, Morris hepatoma 16 and fetal liver mitochondria. As already discussed [4], these pictures can be interpreted as morphological illustration of lateral phase separations caused by thermotropic phase transition induced by pressure. It is tempting to hold the phase transition process responsible for this permeation and to draw a parallel between our observations and those of the above-mentioned authors [15—17]. Differential scanning calorimetry [18] shows that thermotropic transition occurs in the inner mitochondrial membrane between —4 and —15°C under atmospheric pressure. Pressures like those used in our experiments must shift the transition temperature domain above 0°C [4]. For example, referring to De Smedt et al. [19] and Trudell et al. [20], if we suppose that a change of pressure of 100 kg/

cm^2 causes a 1.2—2.2°C increase in the transition temperature, at 1200 kg/cm^2 the transition temperature domain of the inner mitochondrial membrane lipids must definitely be located above 0°C. Thus, the permeation to sucrose we observed under pressure occurs when the mitochondrial lipids are subjected to phase transition. The fact that phase transition causes permeability to sucrose is strengthened by the inhibition effect of a temperature increase on both phenomena.

Imipramine does not oppose the permeation of mitochondria to sucrose caused by pressure. On the contrary, the accumulation of sucrose in the mitochondria is apparently higher in the presence of the drug. A possible explanation is that the resealing of the inner membrane is more effective in the presence of the drug, thus allowing more labelled sucrose to remain in the granule. The fact that sucrose enters into the mitochondria could explain why they swell under pressure, even with imipramine in the medium. It is interesting to note that imipramine has only a slight effect on the lateral phase separation in the inner membrane. This observation offers an additional illustration of the linkage existing between the permeation to sucrose and the phase transition induced by pressure.

Imipramine prevents the disruption of the inner membrane. As discussed earlier [4], the disruption of the inner membrane by pressure probably results from the fact that, when the membrane lipids are in a gel state, mitochondria can no longer swell and lysis occurs. This situation is similar to the situations described by McElhaney et al. [21] and van Zoelen et al. [22] concerning *Acholeplasma laidlawii* cells maintained in a hypotonic solution at different temperatures. The lysis of these cells takes place when the temperature is low enough to freeze all the membrane lipids. How imipramine prevents the disruption of the mitochondrial membrane is not known. Perhaps this effect of the drug is related to its antihemolytic property [23]. However, we found that at the concentrations used in our experiments, imipramine does not inhibit the mitochondria lysis arising from hypotonicity (data not shown).

Considering both temperature and imipramine effects, the deterioration of mitochondria by hydrostatic pressure may be considered as taking place according to the scheme presented in Scheme 1. The pressure first leads to phase transition which causes permeation to sucrose and, because they absorb osmotic water, the mitochondria swell. The membrane lipids freeze extensively as the pressure increases, the inner membrane becomes fragile and finally can no longer resist the swelling, at least in the case of the adult liver granules. By increasing the temperature, all these events can be prevented; imipramine only opposes the disruption of the membrane. The distinction between permeation to sucrose and lysis becomes apparent when mitochondria other than the liver mitochondria are subjected to pressure. Pressure induces lateral phase separation in the inner membrane of these mitochondria as well as making it permeable to sucrose, but does not cause a significant lysis of the organelle. The reason for this particular resistance of the inner membrane is not clear. We must point out that these results agree with centrifugation results indicating that, contrary to adult liver mitochondria, fetal liver, kidney and hepatoma 16 mitochondria are not disrupted during high-speed centrifugation [13,14]. On the other hand, these observations illustrate that the membranes of an organelle



Scheme 1. Sequence of events leading to the deterioration of the mitochondrial membrane by hydrostatic pressure.

could be subjected to modifications by hydrostatic pressure generated by centrifugation without altering the distribution of their enzymes.

Finally, we would like to draw attention to a practical application of our observations. They show that it is possible to give rise to phase transition in a membrane by submitting it to pressure and to study the modification of the properties resulting from the physical change. Such a procedure could be interesting when the lipids under investigation exhibit a transition temperature below 0°C. Indeed, to bring about a thermotropic transition in these lipids by lowering the temperature, it would first be necessary to add a high concentration of cryoprotector to the medium. But these substances can deeply affect the biological membranes, as illustrated by the effects of glycerol on the plasma membrane ultrastructure [24].

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