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ORGANIZATION OF MITOCHONDRIAL STRUCTURE AS REVEALED BY FREEZE-ETCHING

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SUMMARY

1. A freeze-etch study of rat-liver and rabbit-heart mitochondria reveals detailed structure not recognized by the usual procedures of electron microscopy.

2. These include a fibrous network in the matrix of contracted mitochondria, particulate components associated with the inner membrane, and smooth patches covering the particles of the inner membrane.

3. Chemical fixation is found to prevent maximum resolution of fine detail but glutaraldehyde-fixed mitochondria still maintain the general morphological features observed by other techniques.

INTRODUCTION

Since the early work of PALADE[1] on the general morphology of the mitochondrion, electron microscopy has been a major technique for structural investigations of this cellular organelle. Although it has been apparent that changes in mitochondrial structure accompany alterations in functional state[2-6], these observations have so far been confined to describing changes in membrane arrangement rather than changes in membrane ultrastructure. However, recent work using positive staining[7-9], negative staining[10,11] and freeze-etch[12-14] techniques suggests the presence of organized substructures in the mitochondrial membrane and offers new possibilities for using electron microscopy to investigate the relationship between structure and function. The technique of freeze-etching in particular has the advantage of yielding high information content on events occurring in the plane of the membrane.

Several investigators have described the general appearance of mitochondria in freeze-etch preparations[15-17], but, because most of these descriptions were part of a more general *in vivo* study of cellular tissue, an integrated description of mitochondrial freeze-etch morphology is still lacking. In addition, freeze-etch studies on isolated mitochondria[13,18] show that the metabolic and structural state of the mitochondrion and the conditions of preparation play a crucial role in determining the amount of fine structure ultimately seen. The purpose of the present study was to obtain a detailed freeze-etch description of the mitochondrion and to define the conditions under which the technique can be used to follow structural changes which accompany alterations in functional state.

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MATERIALS AND METHODS

Rat-liver mitochondria were prepared as described previously[19]. Submitochondrial vesicles were obtained by sonication and centrifugation at 104000 × *g* for 20 min. Energy-dependent uptake of sodium phosphate was induced at pH 7.8 by adding permeant ion to a mitochondrial suspension with sodium succinate as an energy source. Details have been described previously[20]. Before freezing, the samples were pretreated in one of the following two ways: (a) chemically fixed (0.5 % glutaraldehyde for 15 min), washed by centrifugation in either water, 20 or 50 % glycerol and centrifuged to a pellet; (b) unfixed, washed by successive centrifugation in distilled water or in 20 % glycerol containing either 70 or 300 mM sucrose. A drop of the pelleted sample was rapidly frozen in Freon 22 and replicas obtained according to MOOR AND MÜHLETHALER[15] on a Balzers freeze–etching apparatus. For certain replicas, a home-made stage which accepts four different samples at a time was used. Unless otherwise indicated, specimens were fractured and etched at —100°. Time of etching was 2 min except in experiments with no etching where specimens were fractured and immediately replicated. Replicas were examined using an Elmiscope Ia (at the University of California, Berkeley) and a RCA (at the Veterans Administration Hospital, Martinez, Calif.) electron microscope. All photographs presented are positives, *i.e.* were processed so as to show shadows in white. Shadow direction is indicated by an encircled arrow on each figure.

Submitochondrial vesicles were negatively stained by applying a drop of the dilute suspension to an electron microscope grid, blotting, applying a drop of 2 % phosphotungstic acid in 0.01 M phosphate buffer, blotting again and drying.

RESULTS

Chemically-fixed mitochondria

Replicas of mitochondria fixed with glutaraldehyde in 70 mM and 300 mM sucrose are compared in Fig. 1. Both cross section and face views can be seen. In cross section, a difference in matrix organization between contracted (fixed in 300 mM sucrose) and expanded (fixed in 70 mM sucrose) can be distinguished but fine detail is lacking. Two different kinds of faces are present, one relatively smooth and a second, underlying layer, covered with particles ranging in diameter from 50 to 150 Å. The relationship between these two faces is strikingly illustrated in Fig. 2 where an inner "bag" appears to extrude through a ruptured covering membrane. There were no observable differences between mitochondria osmotically expanded in sucrose solutions and those expanded by means of metabolically-induced ion uptake.

Unfixed mitochondria

The matrix material in chemically-unfixed mitochondria is different in contracted and expanded mitochondria (Fig. 3). In the former, strands of material up to 1000 Å in length and approx. 100 Å in width fill the matrix space in a seemingly random manner. When the inner compartment has expanded to the limit of the outer membrane, the matrix material assumes a more diffuse granular appearance.

The main characteristics of exposed mitochondrial faces are shown in Fig. 4. The two kinds of faces which are seen in glutaraldehyde-fixed samples are also distinguishable in these unfixed samples but more fine detail is apparent. The smooth outer face

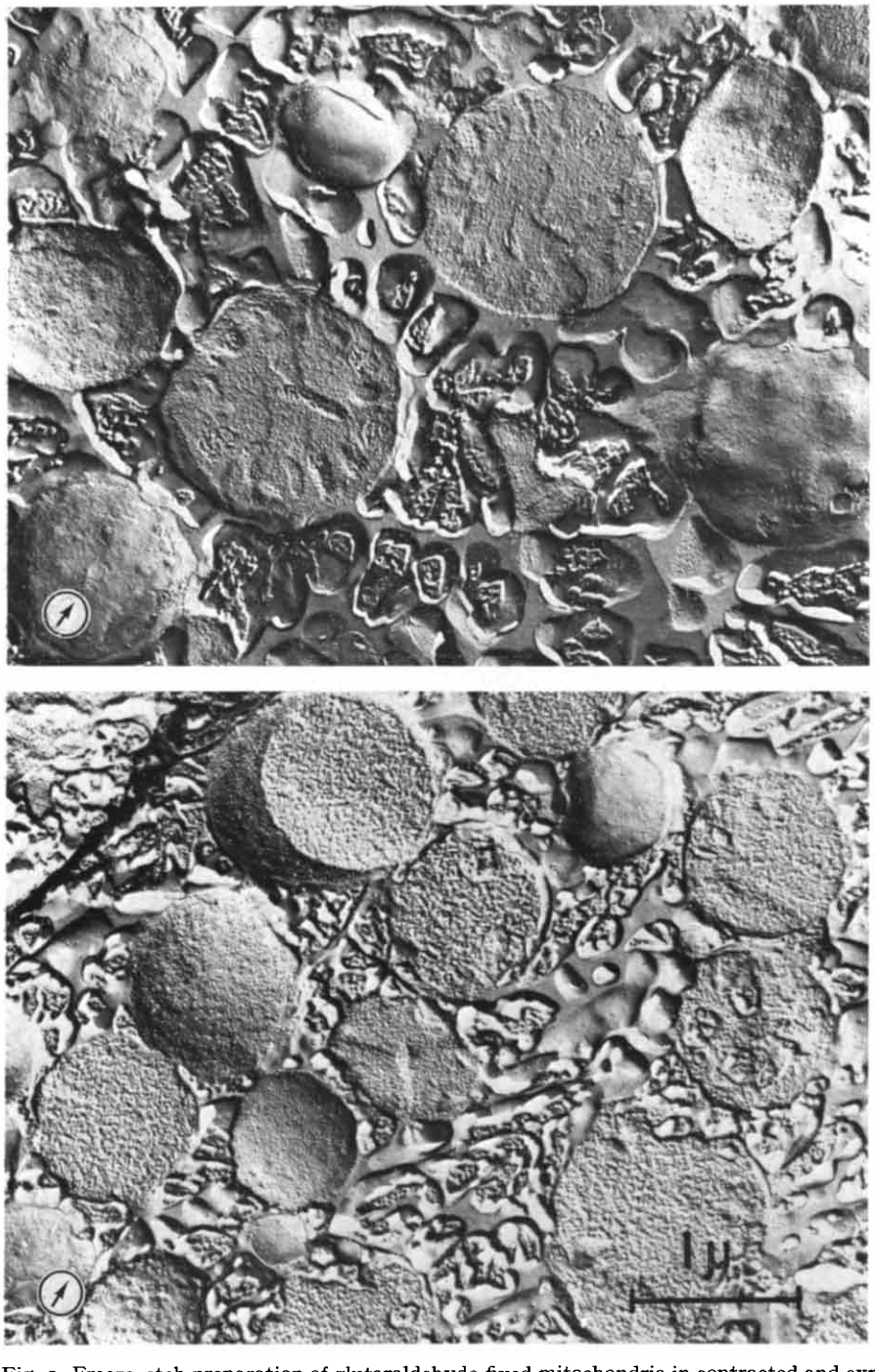

Fig. 1. Freeze–etch preparation of glutaraldehyde-fixed mitochondria in contracted and expanded states. Upper: fixed in 300 mM sucrose; lower: fixed in 70 mM sucrose. Conditions of fixation were 0.5% glutaraldehyde for 15 min at 0°. After fixation, the mitochondria were washed by centrifugation in distilled water and suspended in 20% glycerol for freezing.

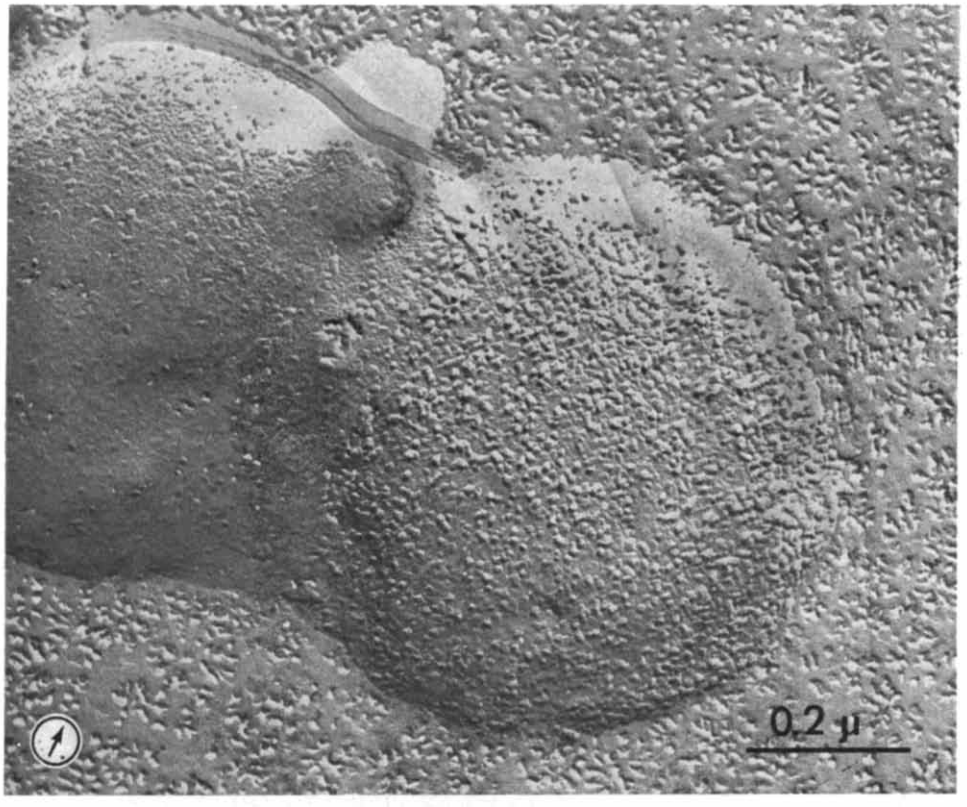

Fig. 2. Freeze–etch preparation of a glutaraldehyde-fixed mitochondrion showing the two membrane faces exposed by fracture. The mitochondrial sample was fixed in 70 mM sucrose as described in Fig. 1, and suspended in 50% glycerol for freezing.

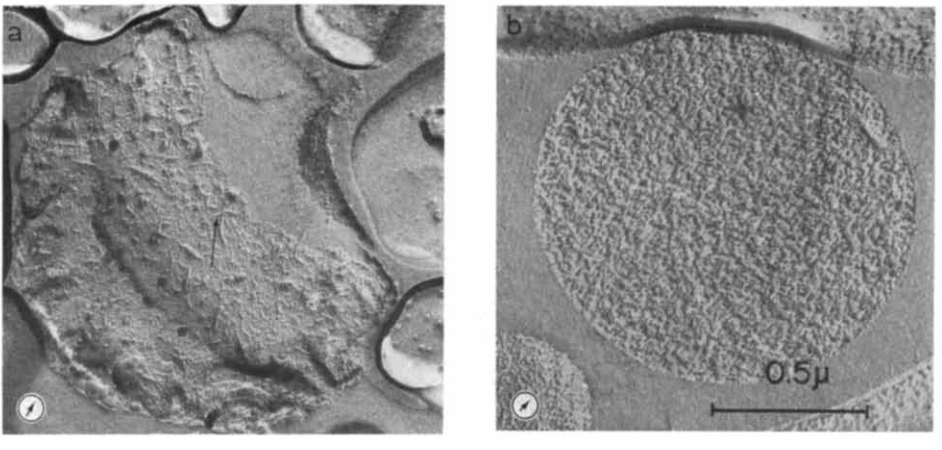

Fig. 3. Freeze–etch preparation of unfixed mitochondria showing cross-sectional views in the contracted (a) and expanded (b) state. The mitochondrial sample was suspended in 20% glycerol containing 300 (contracted) and 70 mM (expanded) sucrose, and centrifuged to a pellet for immediate freezing. Note the fibrous nature of the matrix in the contracted state (arrow).

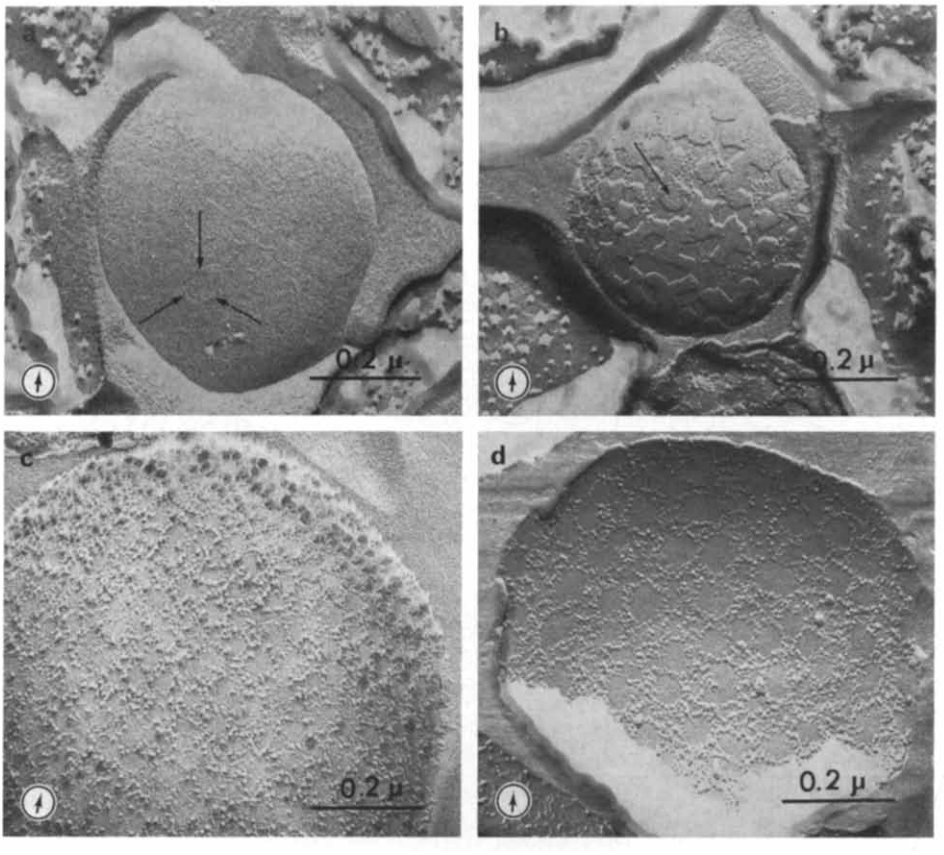

Fig. 4. Freeze–etch preparations of unfixed mitochondria showing the various faces exposed during fracture. Conditions of preparation as in Fig. 3 (contracted). a. Smooth fracture face of outer membrane showing a faint circular pattern (arrows). b. Fracture face showing smooth patches (arrow) overlying the particle-covered inner membrane face. c. Convex fracture face of inner membrane. d. Concave fracture face of inner membrane.

(Fig. 4a) can now be seen to bear a faint patchwork-like pattern. When fracture occurs, approx. 500-Å-wide patches appear attached to the exposed inner membrane (Fig. 4b). The exposed fracture face of the inner membrane (Fig. 4c) is covered with numerous different size particles, ranging from 50 to 150 Å in diameter (Fig. 5). The size distribution of the particles is given in Fig. 6. Most particles fall in the size range 60–100 Å but there is a significant number of larger, 100–150 Å, sized particles. The distribution could indicate either different size particles or one particle in different orientations. In many cases, three or more particles are arranged in a row and their arrangement over the face is uneven. Smooth areas of approx. 500 Å in width are scattered over the face forming a network-like pattern. This is more clearly seen in a concave fracture face as shown in Fig. 4d. The presence and arrangement of these particles is not altered by raising the fracture temperature to $-85°$.

At least two faces are exposed in preparations of submitochondrial vesicles in distilled water (Fig. 7). One is a fracture face (Fig. 7a) containing membrane particles ranging in size from 50 to 150 Å in diameter. Such fracture faces are seen in samples

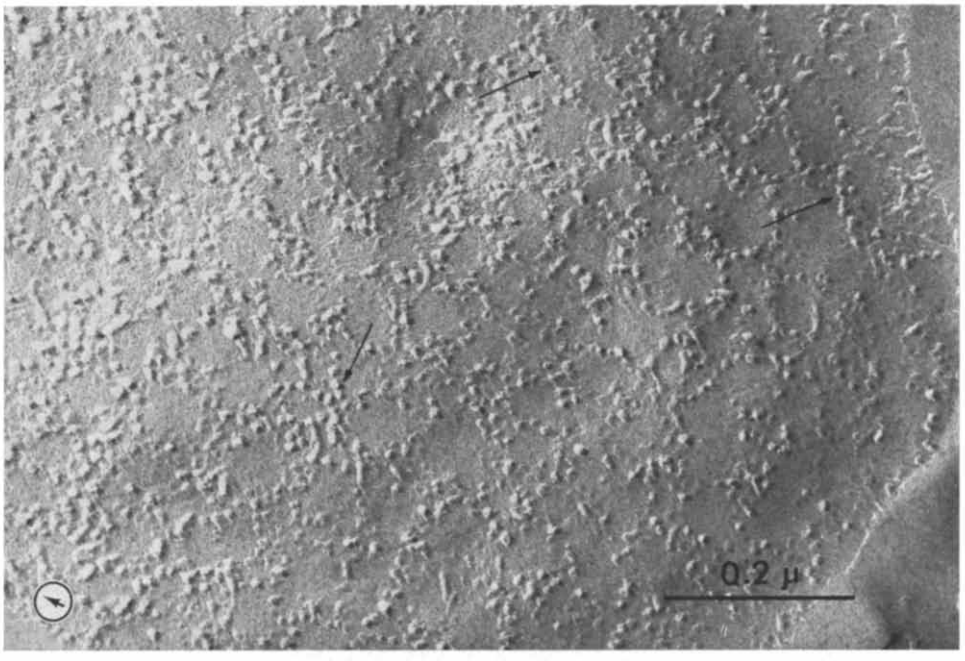

Fig. 5. Fracture face of an inner mitochondrial membrane, similar to Fig. 4c, but at higher magnification to show particle size and organization. Conditions of preparation as in Fig. 3. The particles on the face are often arranged in a linear array (arrows).

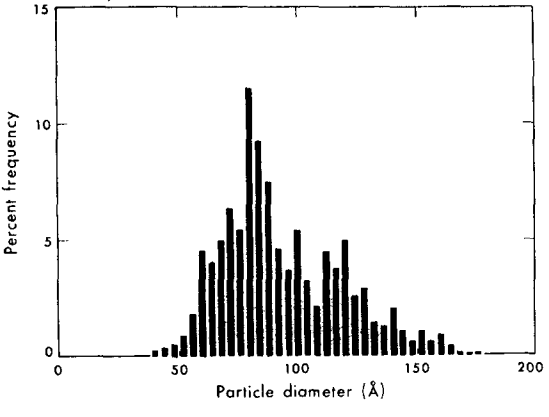

Fig. 6. Frequency distribution of particle sizes on the inner membrane fracture face. Over 1000 particles were counted on different mitochondrial faces.

which receive no etching. When the specimens were both fractured and etched, an additional membrane face is exposed (Fig. 7b). This etched face is smooth and does not show the particulate arrays seen on the fracture face. Negatively-stained samples of the same submitochondrial vesicles (Fig. 8) show the dumbbell-shaped protrusions usually found on mitochondrial inner membranes[21]. Protrusions of this shape were never seen on either the fracture face or the etch face of mitochondrial membranes.

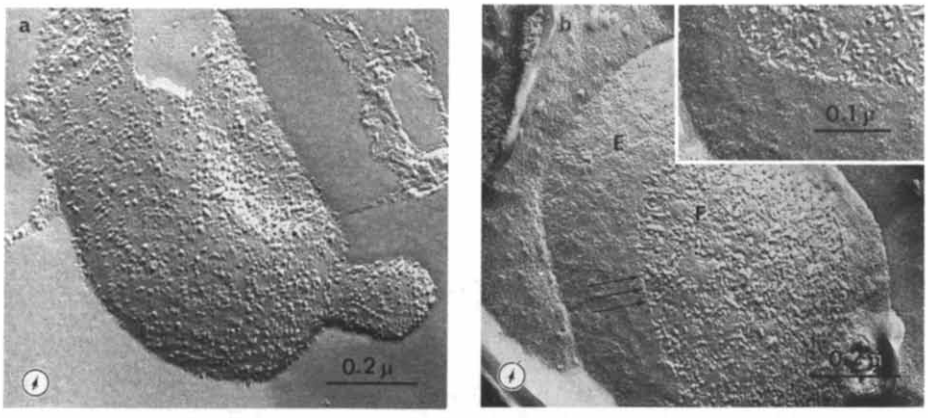

Fig. 7. Freeze–etch preparation of unfixed submitochondrial vesicles. The samples were suspended in distilled water for freezing. a. No etching, showing particle-covered fracture face. b. 2 min etching, showing smooth etch face (E) and particle-studded fracture face (F). A ridge (triple arrow) separates the fracture face from the etched face. Inset: Higher magnification view of the etch and fracture faces.

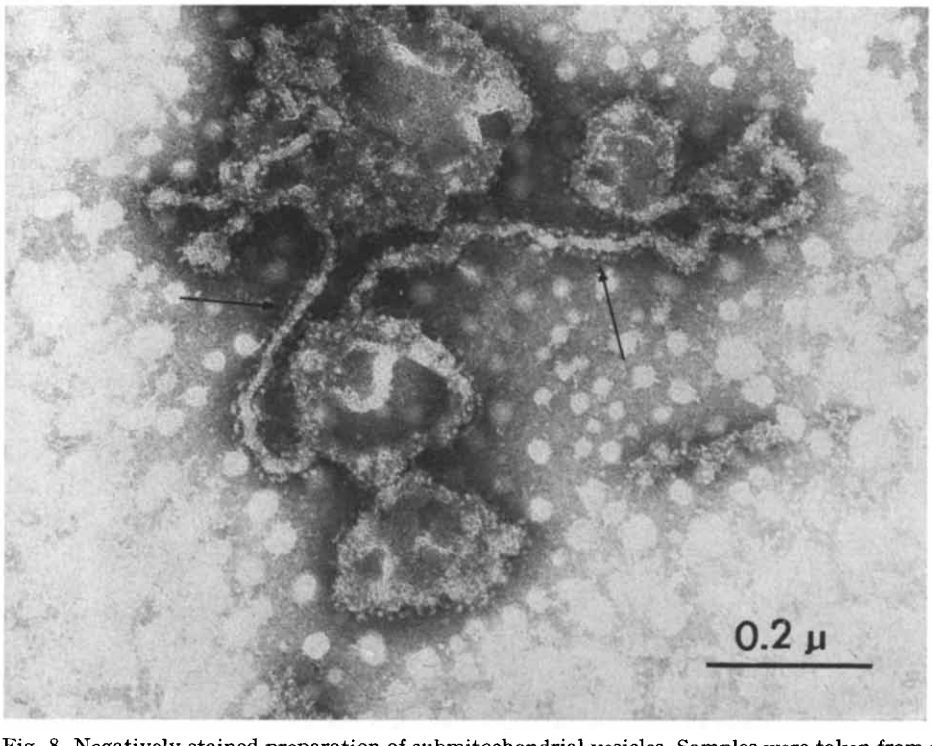

Fig. 8. Negatively-stained preparation of submitochondrial vesicles. Samples were taken from the same preparation used to prepare the freeze–etch replica shown in Fig. 7. The protruding knob structures on the membranes can clearly be seen (arrows).

Freeze–etch preparations of rabbit-heart mitochondria show similar face details to those of rat-liver mitochondria (Fig. 9).

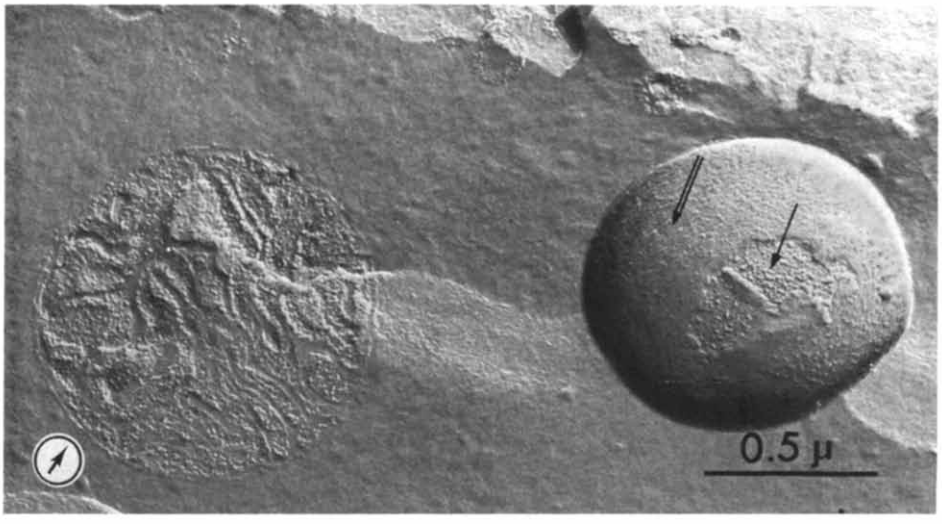

Fig. 9. Freeze–etch preparation of unfixed rabbit-heart mitochondria. Conditions of preparation as in Fig. 3. Note the presence of numerous cristae in cross section compared to rat-liver mitochondria (Fig. 3). In relief, the smooth fracture face of the outer membrane (double arrow) can be seen to overlie the particle-covered fracture face of the inner membrane (arrow).

DISCUSSION

The freeze–etch morphology of the mitochondrion, as revealed in this study, contains features which have been observed by other techniques as well as structural detail not recognized by the usual procedures of electron microscopy. Easily recognizable in both conventional and freeze–etch preparations are cross-sectional views showing outer and inner membranes, and an inner matrix compartment which responds osmotically to the external osmolarity. Additional structural organization revealed by freeze–etching includes: (a) a fibrous network in the matrix of contracted mitochondria; (b) particles associated with the inner membrane; and (c) smooth patches covering these particles. Since the manifestation and interpretation of this detail depends strongly on the preparation of the sample, a close consideration of the pretreatment conditions is of considerable importance.

Preparation of sample

The general features of mitochondrial morphology are well preserved by glutaraldehyde fixation prior to freezing. This is in contrast to osmium or permanganate treatment in which no fracture faces are seen (unpublished observations). On the other hand, there is a loss of fine detail after glutaraldehyde fixation. The fixative also appears to increase the extent of the fracture plane. The patchwork appearance often seen in untreated samples is not present.

Glycerol, generally at a concentration of 20 %, was used in the present study as a cryoprotective agent. It was found to prevent ice-crystal damage especially in chemically-unfixed mitochondria where high internal concentrations of inorganic salts are present. As reported previously[22], a concentration of 20 % was sufficient to prevent most ice-crystal damage when rapid freezing was employed. In submitochondrial

vesicles, washed and suspended in distilled water, glycerol was not needed to prevent ice-crystal damage. In these samples, the presence or absence of glycerol had no apparent effect on morphology.

Matrix organization

The present work clearly reveals an organization of matrix material in freeze-etch preparations of mitochondria (Fig. 3). The linear arrays in the matrix of contracted mitochondria disappear when the inner compartment is expanded either by passive osmotic means or by energy-dependent ion transport. The functional significance of this organization has yet to be revealed, but HACKENBROCK[18] has postulated, on the basis of electron microscopic evidence, that a protein network which is physically continuous with the inner membrane could play a controlling role in determining the volume of the inner membrane compartment during mitochondrial energy metabolism. Recent work on conformational changes in mitochondrial proteins[23] suggests that such a control could be mediated *via* the energy-dependent movement of H^+. Biochemical studies also provide evidence for some form of matrix organization and structure[24]. The varying extractability of different mitochondrial enzymes suggests that the matrix is not a simple solution of enzymes but an organized series of components which differ in binding and association. The present results may be a visualization of such organization.

Membrane structure

Any interpretation of the various views of mitochondrial membranes requires an understanding of how the faces are exposed by fracture and by etching. Examination of the views presented in Figs. 5 and 7a, showing the inner membrane from the external (Figs. 4c and 5) and internal (matrix) side (Figs. 4d and 7a), reveals in both cases a fracture face covered with particles. Examination of submitochondrial vesicles shows that this particle-covered fracture face lies beneath a smoother etch surface (Fig. 7b). The depth of the edge between fracture and etch face is no deeper than 80 Å. These facts can be explained by assuming that the fracture plane follows the hydrophobic interior of the inner membrane (Fig. 10). The particles would then represent components embedded within the membrane. The smooth patches on the inner membrane may be areas where the fracture plane is deflected from the membrane interior to the membrane surface. Alternatively, the patches may be localized adhesions of the outer membrane[18]. Further work is required to clarify this problem as well as the nature of fracture planes in the outer membrane. It is, however, clear that the etch face must *a priori* represent either the true membrane surface or that surface with a thin overlay of nonsublimable water[25].

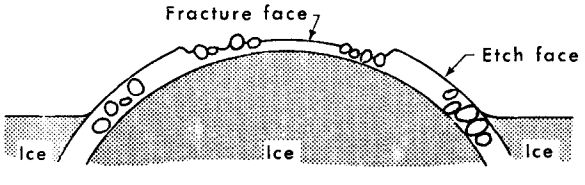

Fig. 10. Schematic representation of the inner mitochondrial membrane as found in a submitochondrial vesicle based on the present freeze–etching study. Two exposed faces are shown, a smooth, etched face and a particle-studded fracture face.

Particulate components in the inner mitochondrial membrane have been described in freeze–etch preparations by BRANTON AND MOOR[16], BAUER AND TANAKA[12], KEYHANI AND KRIZ[13], and RUSKA AND RUSKA[14]. Functional identification has necessarily been tentative. In this study, the replicas of submitochondrial vesicles show the surface (etch face) of the inner membrane to be relatively smooth. There are no particles which could be identified with those seen by the negative-staining technique. We therefore conclude that the stalked knob appearance of ATPase seen in negative staining may be the result of either a penetration of the stain into the membrane, or a modification of the membrane during negative staining.

Both biochemical[11,26–28] and theoretical[29] considerations now support the idea that a discrete spatial arrangement of electron transport components is necessary for energy coupling in mitochondria. A theoretical frequency distribution of the components of the respiratory chain can be calculated from a knowledge of the approximate molecular weights and the molar composition of the respiratory chain[30]. In such a distribution, the cytochromes would comprise the majority of the protein components and fall in the particle size range 40–60 Å with a peak at 60 Å due to cytochromes *a* and a_3. ATPase and the various dehydrogenases would give the distribution a low frequency tail between 70–130 Å. Assuming a platinum deposition thickness of 10–15 Å, this distribution would closely correspond to the particle size distribution shown in Fig. 6. The present results also suggest a definite spatial organization of the particles throughout the membrane. A more positive identification of individual components may be possible by illuminating at different absorption bands to cause selective etching. Such techniques are the object of current investigations.

ACKNOWLEDGMENTS

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