An electron microscopical study of isolated mitochondrial membranes treated with osmium tetroxide, potassium permanganate, and formaldehyde

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With 2 plates (figs. 1 and 2)

Summary

An investigation of isolated mitochondrial membranes, fixed with osmium tetroxide, potassium permanganate, or formaldehyde, shows that a great number of small particles are attached to their surfaces. Sections of specimens fixed in potassium permanganate or osmium tetroxide appear to confirm these findings. An attempt has been made to compare these particles with the 'elementary particles' described by Fernández-Morán and by Green, and also with particles revealed by negative staining of mitochondrial membranes, as reported by Stoeckenius. Specimens fixed with formaldehyde, whether isolated mitochondria or material fixed in bulk, show very poor contrast in electron micrographs.

Introduction

We undertook the investigation of the effect of various methods of specimen preparation on molecules of proteins, lipids, and nucleic acids, and also on comparatively simple biological systems, such as isolated membranes, because we considered that these studies might contribute to the understanding of the effect of these methods on the cells themselves. We have already studied the effect of certain fixatives on protein molecules (Deutsch, Fischer, and Krause, 1964), and we have now extended these investigations to isolated mitochondrial membranes. We thought that a comparison of the effect of various fixatives on these membranes might contribute to knowledge of their structure and of the chemical constitution of their structural elements. We have based our investigations on the information already available about the biochemistry and ultrastructure of mitochondria in sectioned cells, of isolated mitochondria, and of isolated mitochondrial membranes (see especially Watson and Siekevitz (1956); Lehninger and others (1958); Lehninger and Wadkins (1962)), and on the results obtained by Egger and Rapaport (1963) and by Rosenthal and Rapaport (1964) in their study of proteins and enzymes derived from the mitochondria of rat liver.

Materials and methods

The membranes were obtained by the method devised by Schneider and Hogeboom (1951). They were resuspended in a 0.25 M sucrose solution

(pH = 7.2), frozen at a temperature of −20 °C, and stored at −5 °C. After thawing they were homogenized ultrasonically (10 min, 800 kc/s, 5 watts/cm²). They were studied in the electron microscope, either without further treatment or after fixation. They were fixed with osmium tetroxide (2 min in a 1% aqueous solution, buffered with sodium veronal/sodium acetate at pH 7), or with potassium permanganate (2 min in a 0.25% aqueous solution, buffered with sodium veronal/sodium acetate at pH 7), or with formaldehyde (2 min in a 5% aqueous solution of formalin, at pH 7). After fixation the membranes were washed in distilled water. Small droplets of aqueous suspensions of the membranes (untreated, or treated with either osmium tetroxide, potassium permanganate, or formaldehyde) were deposited on specimen grids covered with collodion, and allowed to dry in air. The grids were studied either directly in the electron microscope, or after shadowing with platinum at an angle of 20°. In another series of experiments, membranes that had been fixed and washed as described above were dehydrated in ethanol, embedded in a methacrylate mixture according to standard procedure, and sectioned at about 30 μm on an Ardenne-Westmeyer ultra-microtome. The electron micrographs were taken with a Zeiss Elmi D 2 electron microscope at a voltage of 48 kV.

Results

We would like to emphasize that we are not only presenting micrographs obtained with the 'good' fixatives, but also others, in order to demonstrate the difference between various methods, and because results obtained with 'poor' fixatives may be used to confirm or even invalidate results obtained with 'good' fixatives.

Figs. 1, D to F, and 2, A to E show patches of broken membranes. In some parts the electron density is much higher than in others, indicating that several layers are lying on top of each other. Untreated specimens (fig. 1, D) and specimens treated with osmium tetroxide (fig. 1, F) or potassium permanganate (fig. 2, A) do not show any special features; the contrast of specimens fixed with formaldehyde is very poor (fig. 1, E). By shadowing of untreated specimens a substructure is revealed (fig. 2, B): the membranes are composed of irregularly shaped units (linear dimensions about 20 to 40 μm).

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**FIG. 1** (plate). A, section of membranes fixed with osmium tetroxide, showing a three-layered structure (a). A few particles of the size of ribosomes are attached to the surfaces (r). B, section of membranes fixed with potassium permanganate, showing two parallel rows of small particles (sp). C, section of membranes fixed with formaldehyde, showing poor preservation. D, untreated membranes. The electron dense patches (ed) in this figure and in the following ones indicate that several layers of membranes are lying on top of each other. A few particles (r) of the size of ribosomes are attached to the membranes. The larger particles (lp) are probably aggregations of the smaller ones. E, membrane fixed with formaldehyde. The contrast is very poor. A few particles of the size of ribosomes (r) are attached to the membranes, and many smaller particles are also seen. F, membranes fixed with osmium tetroxide. A few particles of the size of ribosomes (r) are attached to membranes.
FIG. 1

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Inspection of specimens fixed with osmium tetroxide, potassium permanganate, or formaldehyde and subsequently shadowed shows that a great number of small particles are attached to their surfaces; their diameter is about 8 \( \mu \)m (fig. 2, c to E). In specimens fixed with formaldehyde or potassium permanganate the particles are not so clearly separated as in those fixed with osmium tetroxide. Sections of specimens fixed with potassium permanganate show two parallel rows of small electron-dense particles, of diameter about 8 \( \mu \)m (fig. 1, B). The space between the particles has about the width of a mitochondrial membrane, and about the same electron density as the embedding medium. One has thus the impression that fixation by potassium permanganate reveals the particles that are attached to the surface of the membrane, but not the membrane itself. Sections of membranes fixed with osmium tetroxide show the well-known three-layered structure (fig. 1, A). In sections of specimens fixed with formaldehyde the outlines of the membranes are not clearly visible (fig. 1, c). The thickness of the membranes, as estimated by a study of sectioned isolated mitochondria that had been fixed with osmium tetroxide, is about the same as that of mitochondrial membranes in sections of whole cells.

A comparatively small number of larger particles (about the size of ribosomes) are attached to untreated specimens and to those fixed with formaldehyde or osmium tetroxide, and also to sectioned specimens fixed with osmium (fig. 1, A, D to F); but they are not found in specimens treated with potassium permanganate specimens (fig. 2, A).

**Discussion**

The large particles attached to untreated specimens and to those fixed with osmium tetroxide or formaldehyde (fig. 1, A, D to F) are of the same size as ribosomes. They probably are in fact ribosomes. Their preservation by osmium tetroxide but not by potassium permanganate (fig. 2, A) suggests this (Bradbury and Meek, 1960). Thus they actually constitute a contamination.

Sections of isolated membranes fixed with osmium tetroxide show the same structure as the same membranes in sections of cells (Watson and Siekevitz, 1956), and their thickness is about the same (fig. 1, A). Formaldehyde fixation and presumably also the subsequent treatment preparatory to sectioning has a very adverse effect on the preservation of isolated membranes (and also on membranes in cells) so far as electron microscopical work is concerned (fig. 1, C, E). In classical histology formaldehyde is considered to be a good fixative, and, as our previous investigations on the influence of fixatives on...
proteins have shown (Deutsch, Fischer, and Krause), it also increases the contrast of proteins (as osmium tetroxide does). But the membranes also contain phospholipids, which are fixed by osmium tetroxide but not by formaldehyde (Mercer and Birbeck, 1961). This difference may explain why the membranes are very poorly preserved after treatment with formalin. The adverse effect of formaldehyde fixation, however, can be counteracted by 'staining' (Magnan, 1961). Thus the 'stain', besides increasing the contrast, also acts as a complementary fixative. The appearance of isolated membranes, whether sectioned or not, is very different from the appearance of membranes in sections of whole cells, when potassium permanganate is used as fixative (fig. 1, B). It is very likely that the methods used for preparing isolated membranes bring about structural changes. In view of the investigation by Bradbury and Meek (1960) it is not surprising that membranes treated with potassium permanganate are not so well preserved as those treated with osmium tetroxide. According to these authors potassium permanganate is not a true fixative.

The substructures of the membranes are revealed by the shadowing of untreated membranes (fig. 2, B). The units observed in untreated shadowed specimens have about the same size as the electron transport particles ('ETP') (Green and Oda, 1961; Ziegler, Linnane, Green, Dass, and Ris, 1958). Their shape is somewhat different, but this may be the result of difference in preparation, if, indeed, the units observed by us are identical with the ETP.

The particles attached to the membranes, as observed in specimens fixed with osmium tetroxide, potassium permanganate, or formaldehyde and subsequently shadowed (fig. 2, c to e), are of special interest. These particles are also seen in specimens fixed with potassium permanganate and then sectioned (fig. 1, B). They have roughly the same size as the EPs (elementary particles) described by Fernández-Morán (1962, 1963) and by Green, Blair, and Oda (1963), and it is quite possible that they are identical with them. Particles of about the same size attached to the mitochondrial membrane have also been described by Stoekenius (1963). But we have not found any evidence for the existence of the stalks, by which, according to Stoekenius, they are attached to the membranes. The resolution of the instrument that we have used is presumably not adequate to show the stalks. Stoekenius, who studied mitochondrial membranes after negative staining, found about 3,000 particles per $\mu^2$; we counted about 4,000 particles per $\mu^2$, though, as Stoekenius points out, the figures may not be very significant. He claims that fixation destroys these particles. It should, however, be pointed out that, so far as we know, he only investigated unshadowed specimens, and with unshadowed specimens our findings agree with his. But in view of the evidence provided by shadowed specimens and by those fixed with potassium permanganate and then sectioned, we think it more likely that the contrast in unshadowed specimens is not sufficient to reveal the particles.

Our results support the findings of Fernández-Morán, Green, and Stoekenius, since we have also found that a great number of small particles are
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attached to the surfaces of the membranes. We think it very likely that the particles described by the authors named are essentially identical with ours. The proof, however, rests only on morphological evidence. We hope, by further investigations, to contribute to the understanding of the biological significance of these particles.

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References