SOME OBSERVATIONS ON NEGATIVELY STAINED MITOCHONDRIA

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ABSTRACT

A particulate component of mitochondria is described which is observed in negatively stained preparations of unfixed whole mitochondria or their fragments. It consists of a roughly spherical particle, approximately 85 A in diameter, found on the surface of the cristae and probably also on the side of the mitochondrial envelope which faces the matrix. It is attached to the underlying membrane by a narrow stalk 40 to 50 A long. The particle appears to be rather labile and it is destroyed or at least rendered unrecognizable by all conventional fixatives used in electron microscopy, especially OsO_4.

Negative staining of isolated mtiochondria has recently revealed structural details which have not been observed before in fixed and sectioned material (1, 6). Only a short account of these findings has been published (1). Our observations and interpretations differ somewhat from those published and therefore a brief account of the results obtained so far will be given here.

MATERIALS AND TECHNIQUES

Most of the results described in this paper were obtained with isolated mitochondria from *Neurospora crassa*, but a few experiments with rat heart and liver, calf pancreas, guinea pig heart and pancreas, and rabbit brain gave essentially identical results. The material was usually homogenized in 0.44 M sucrose and, after a low speed centrifugation to eliminate nuclei and unbroken cells, the mitochondria were sedimented for 20 minutes at 13,000 *g*. Alternatively, the homogenate was layered over a continuous sucrose density gradient and the mitochondria were collected after equilibrium centrifugation as a broad band with a density of ~1.18. After 8-fold dilution with distilled water the mitochondria were sedimented by 10 minutes' centrifugation at 105,000 *g*.[1] In all cases the resulting pellet was resuspended in distilled water or 4 per cent sucrose to obtain a suitable dilution. A drop of this suspension was applied to the supporting film on an electron microscope grid and the surplus withdrawn with a fine pipette or filter paper. Before the thin layer of fluid remaining on the grid had dried, a drop of 2 per cent phosphotungstic acid, adjusted to pH 6.0-7.2 with KOH, was applied. The surplus was again withdrawn and the grid dried at room temperature. All other operations were carried out at 0-4°C.

In a few experiments 1 per cent uranyl acetate was used as a negative stain. The pH was adjusted to 7.2 in the presence of EDTA. The results were generally less satisfactory but were essentially identical with those obtained with phosphotungstate. In some instances specimens were prepared by spreading whole cells or fresh organ homogenates on the surface of distilled water or salt solutions, against no external pressure or against a constant pressure, following a technique described by Kleinschmidt (3). After transfer to the electron microscope grid, the material was negatively stained as described above. A Siemens electron microscope with double condenser was used and illumination was confined to approximately the area seen on the screen. Electron

[1] We are grateful to Dr. David Luck for discussion and for providing most of the mitochondrial preparations used in this work. A detailed account of the techniques used can be found in his forthcoming paper: *J. Cell Biol.*, 1963, **16**, 483.

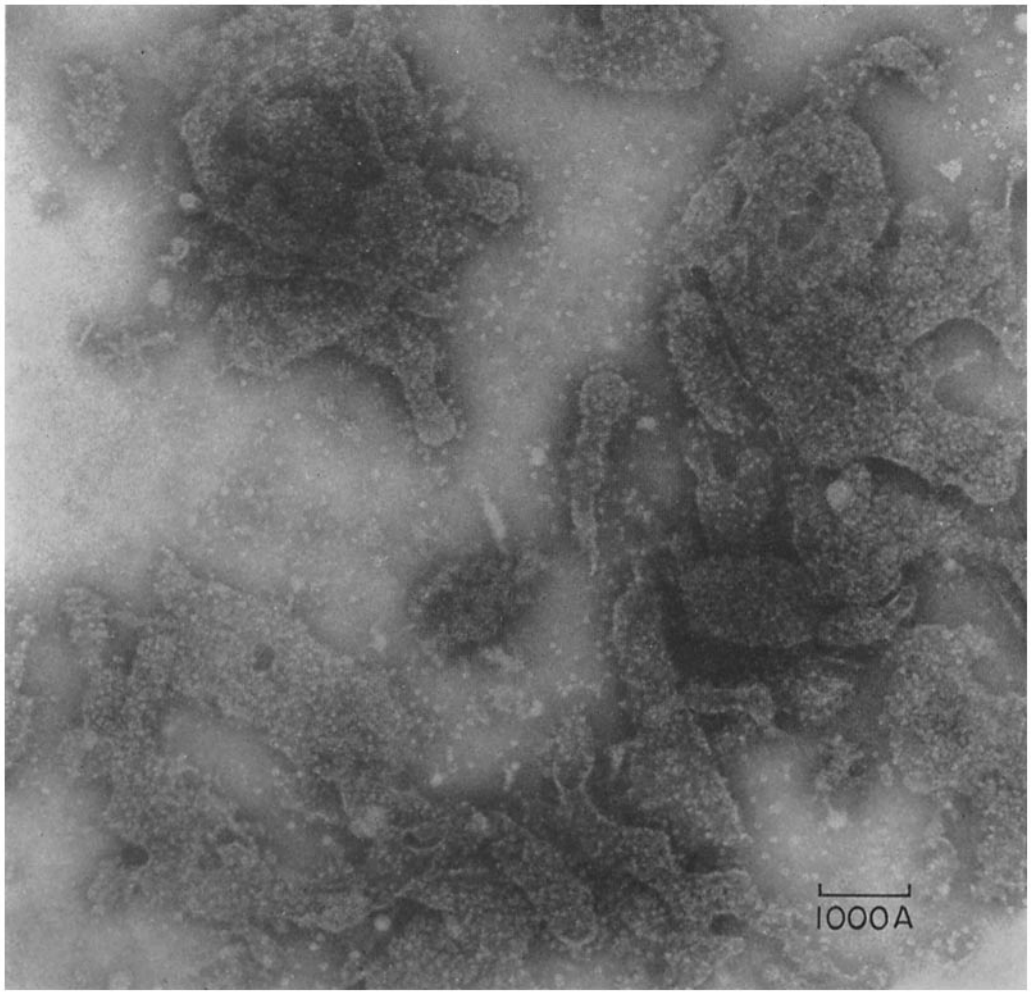


FIGURE 1

Most commonly encountered appearance of mitochondrial fragments. The rather sparse distribution of particles along the edges of some of the membrane fragments may indicate a loss of particles during the preparation, and the isolated particles seen in the background may—with the reservations given in the text—be considered to represent such detached particles. × 120,000.

This and all following electron micrographs show mitochondrial preparations obtained from *Neurospora crassa* and negatively stained with phosphotungstate as described in the text.

optical magnifications between 40,000 and 160,000 times gave the best results and allowed direct visualization of the structural details on the screen.

RESULTS

A suspension of mitochondria, isolated as described and negatively stained, shows in the electron microscope large numbers of flattened-out membrane fragments of varying size, and occasionally apparently intact or nearly intact mitochondria. The surface of most but not all of the membrane fragments shows irregularly and rather densely distributed light areas ~85 A in diameter (Fig. 1). These areas apparently correspond to roughly spherical particles of that size, which are attached to the membranes. This is borne out by the observation of rows of such particles outside of and parallel to the edges of some membrane fragments where these seem to fold back on themselves and are seen in profile (Fig. 2). Irregular gaps in the

rows may be due to accidental loss of particles. Usually the particles appear to be separated from the edge of the membrane by a narrow empty space. However, under favorable circumstances and at higher magnifications they can be seen to be linked to the membrane by a narrow stalk 40 to 50 A long (Figs. 3 and 4). Where the edge of the membrane fragment is nearly straight the particles seem to be in contact with one another; where the edge is convex they are spread apart slightly. The appearance of particles in contact may, of course, result from an optical overlap of particles lying in different planes.

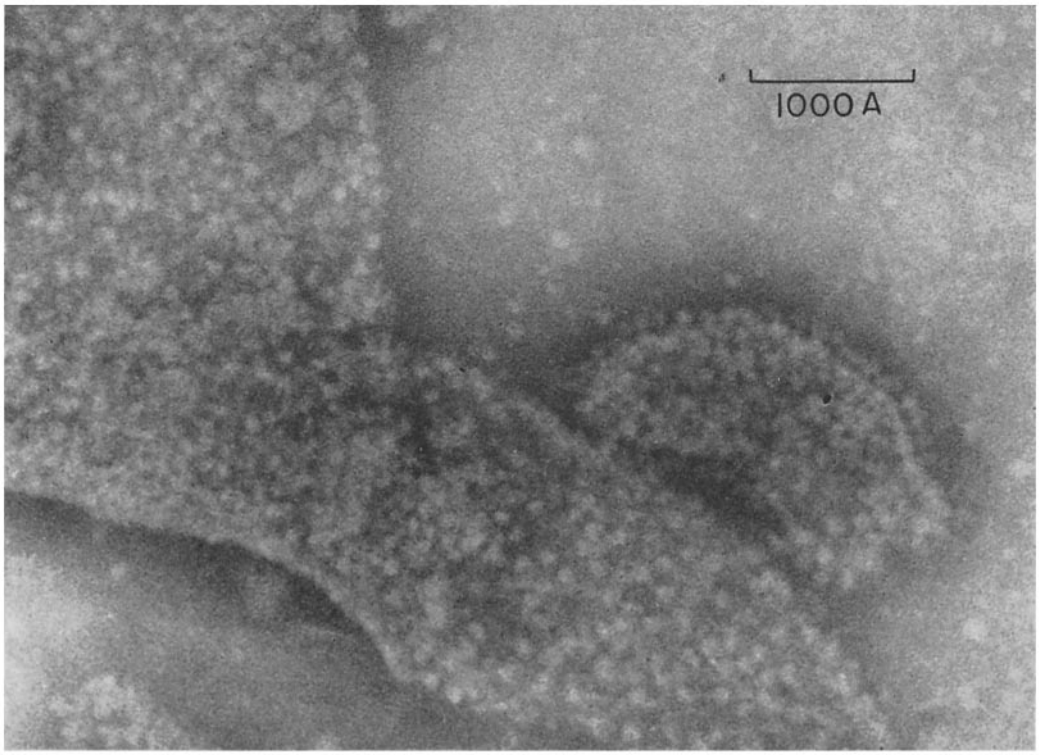


FIGURE 2

Two membrane fragments are shown. One has a smooth edge, whereas the other shows a row of particles along the edge, apparently separated from it by a narrow space. × 240,000.

Another characteristic but much less frequently encountered structure in these preparations can be described as a ribbon with rounded-off ends which is approximately 130 to 140 A wide where it is narrowest. On both sides it is studded with a single row of particles identical in appearance with those on the edges of the membrane fragments described above (Figs. 5 and 6). The composite structure is 380 to 400 A wide and of varying length up to approximately 1 μ. Often the ribbon is split in one or several places along the center over part or over all of its length and the two symmetrical halves are separated by an apparently more or less empty space into which the phosphotungstate has penetrated. This results in a corresponding increase in the total width of the ribbon. These structures probably represent isolated cristae or fragments of cristae seen edge on. This interpretation is supported by observations on more or less intact mitochondria also found in the same preparations, where the same ribbon-like structures can be seen inside the mitochondrion or in the process of extrusion through a rupture in the mitochondrial envelope (Figs. 7 and 8). Moreover, in some instances one or both ends of the ribbon flatten out and are then identical in appearance with the isolated membrane fragments described above which show a single row of particles around the edge.

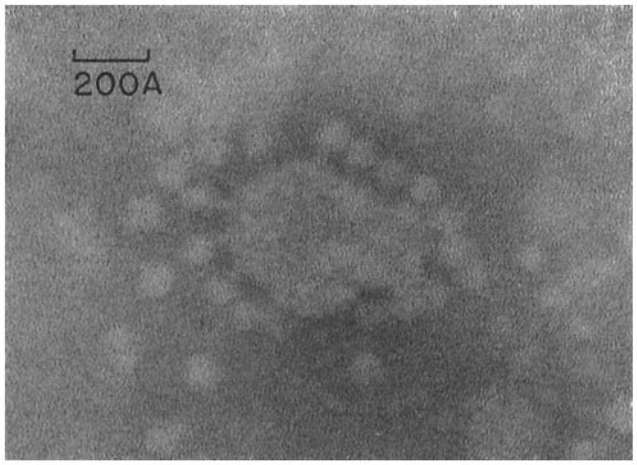


FIGURE 3

Small membrane fragments showing the narrow stalks which connect the particles to the membrane. × 480,000.

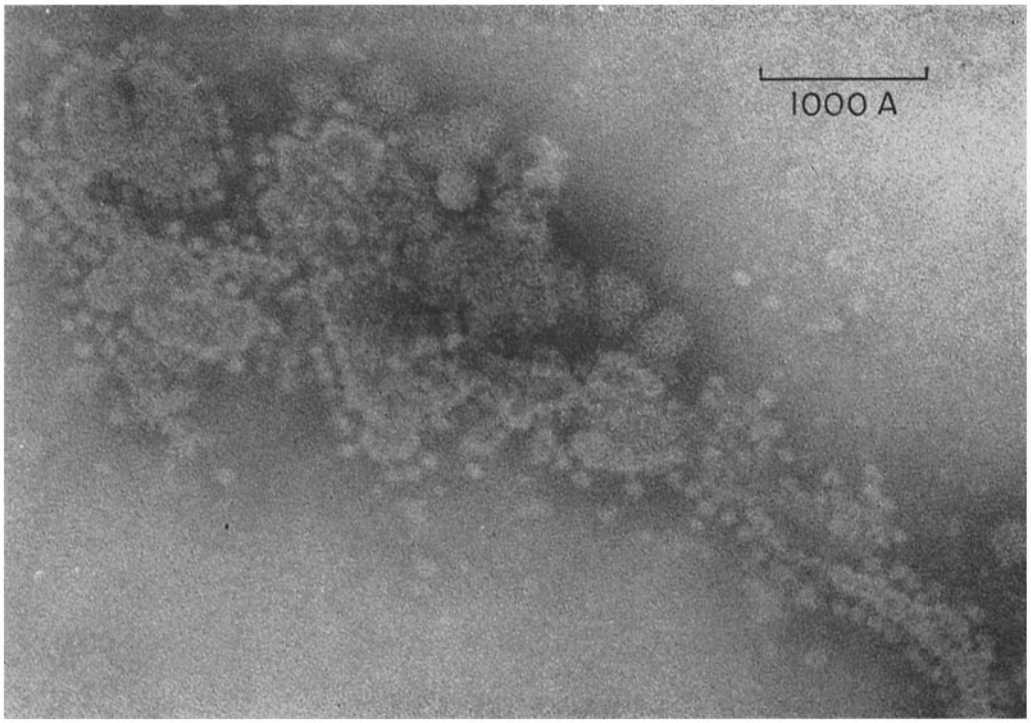
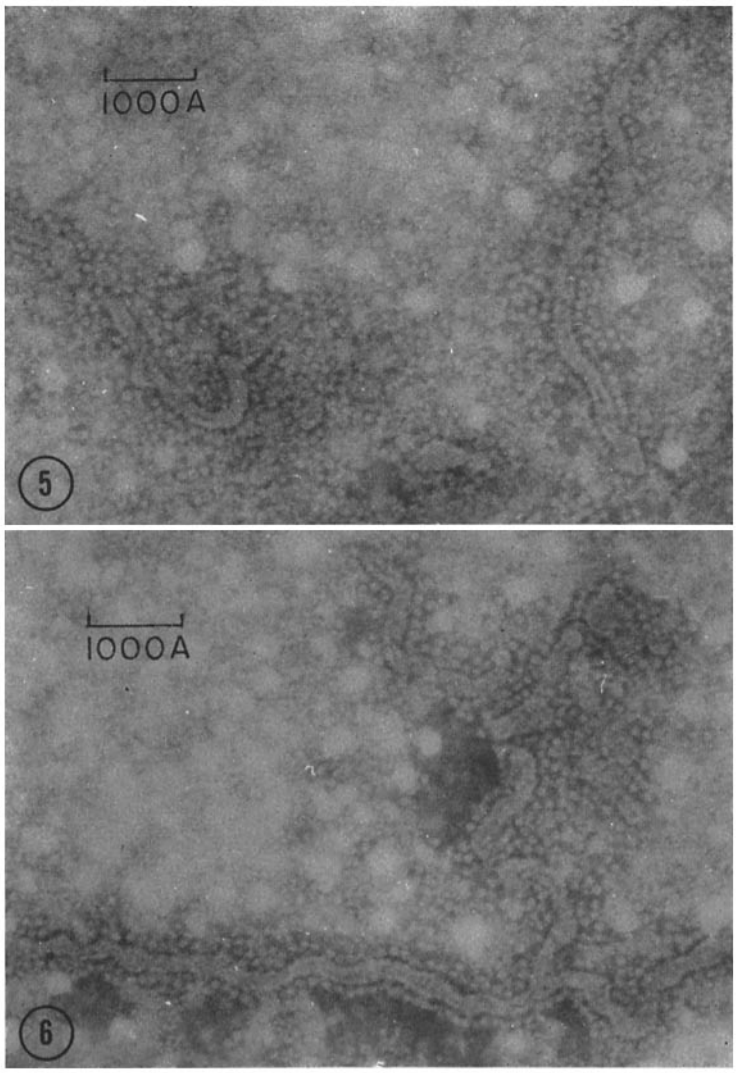


FIGURE 4

Other membrane fragments probably originating from cristae. The particles in the rows along the edge appear rather densely packed and the stalks are visible in several places. × 240,000.

In some of the relatively well preserved mitochondria the two peripheral membranes which constitute the mitochondrial envelope can be distinguished. No particles have been observed on the outer one,[2] whereas the inner one shows the same particles seen on the membrane fragments and cristae (Fig. 9). These could, of course, actually

[2] In a few instances characteristic patterns different from those seen on the cristae have been observed on what appears to be the outer of the two membranes constituting the mitochondrial envelope. But they do not appear regularly enough to warrant a description here.



FIGURES 5 AND 6

Ribbon-like structures bearing parallel rows of particles on both sides. They are assumed to represent cristae or fragments of cristae seen in profile. In several places, especially in Fig. 5, the cristae appear split along the center line so that the two constituent membranes can be individually observed separated by a dense band. × 140,000.

belong to underlying cristae, but, in view of the often observed rather uniform distribution over the whole visible area of the membrane, this explanation appears less likely. It seems more likely that the particles cover the whole inner mitochondrial surface which is in contact with the matrix.

In many instances only one peripheral membrane is visible in the partly intact mitochondria. This may be due either to loss of the outer one, or to failure of the phosphotungstate to penetrate between the two. The micrographs obtained so far usually do not allow a decision between these alternatives.

Unless the particles described are very firmly bound to the membranes, one would also expect to find isolated particles in these preparations. There are usually many small bodies seen between the membrane fragments, which, according to their shape and size, could represent detached particles (Fig. 1). Unfortunately a variety of larger and

smaller bodies of unknown origin is also present, which makes positive identification of a specific type of particle very difficult. One hardly ever finds an isolated particle with an appendage which could correspond to the "stalk" seen in membrane-bound particles, nor does one find membranes with "stalks" bearing no particles. The "stalks," however, are close to the limit of visibility in the micrographs and therefore they are not a good criterion for identification of the particles. The question as to how many of the isolated bodies represent detached particles remains open. Conditions which cause a loss of particles from the membranes, for instance prolonged storage of the mitochondrial suspension in dilute sucrose, do not seem to increase the number of isolated particles. It is possible that the particles disintegrate while still attached to the membrane or shortly after having been detached. The isolated particles often appear slightly larger than those attached to membranes; some show a hollow center of variable diameter and indications of substructure. These could represent stages in the disintegration, but no definite conclusions can be drawn so far (Fig. 10).

No particles identical with or similar to the ones described here have been found on other biological membranes, such as the erythrocyte ghost or the endoplasmic reticulum. A few membrane fragments bearing particles which have been observed in the microsomal fraction of mammalian tissue have been taken as an indication of contamination from the mitochondrial fraction.

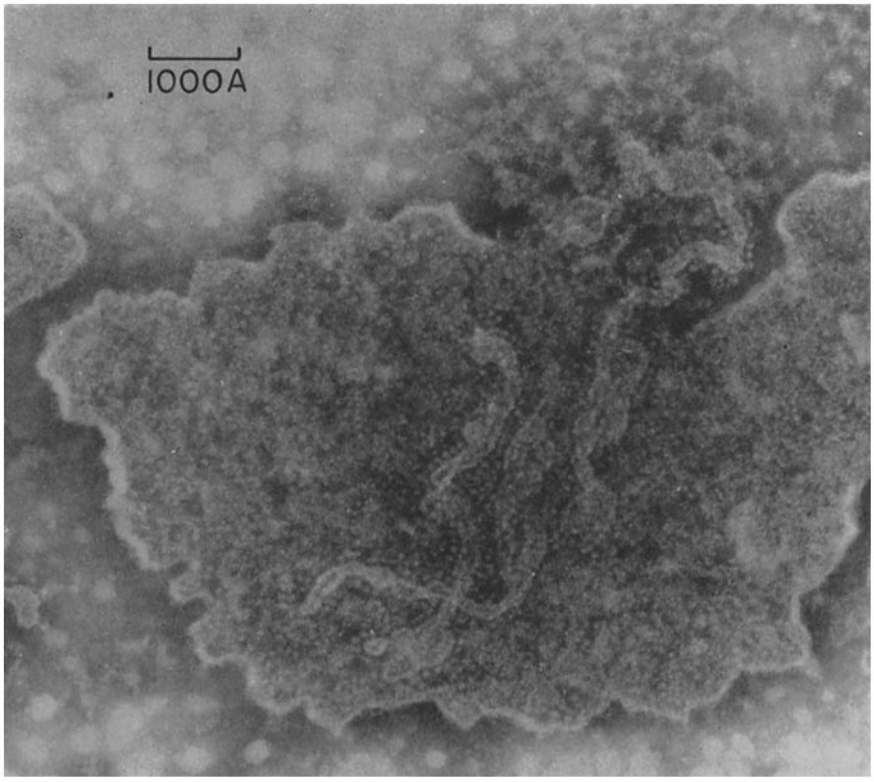


FIGURE 7

Partly intact mitochondrion with cristae protruding through what appears to be a rupture in the mitochondrial envelope. Notice the tendency of the cristae to split along the center line and to swell, a phenomenon also encountered regularly in fixed and sectional material. × 140,000.

DISCUSSION

The observations described suggest the existence of a new structural component of mitochondria. It appears as a roughly spherical particle 85 A in

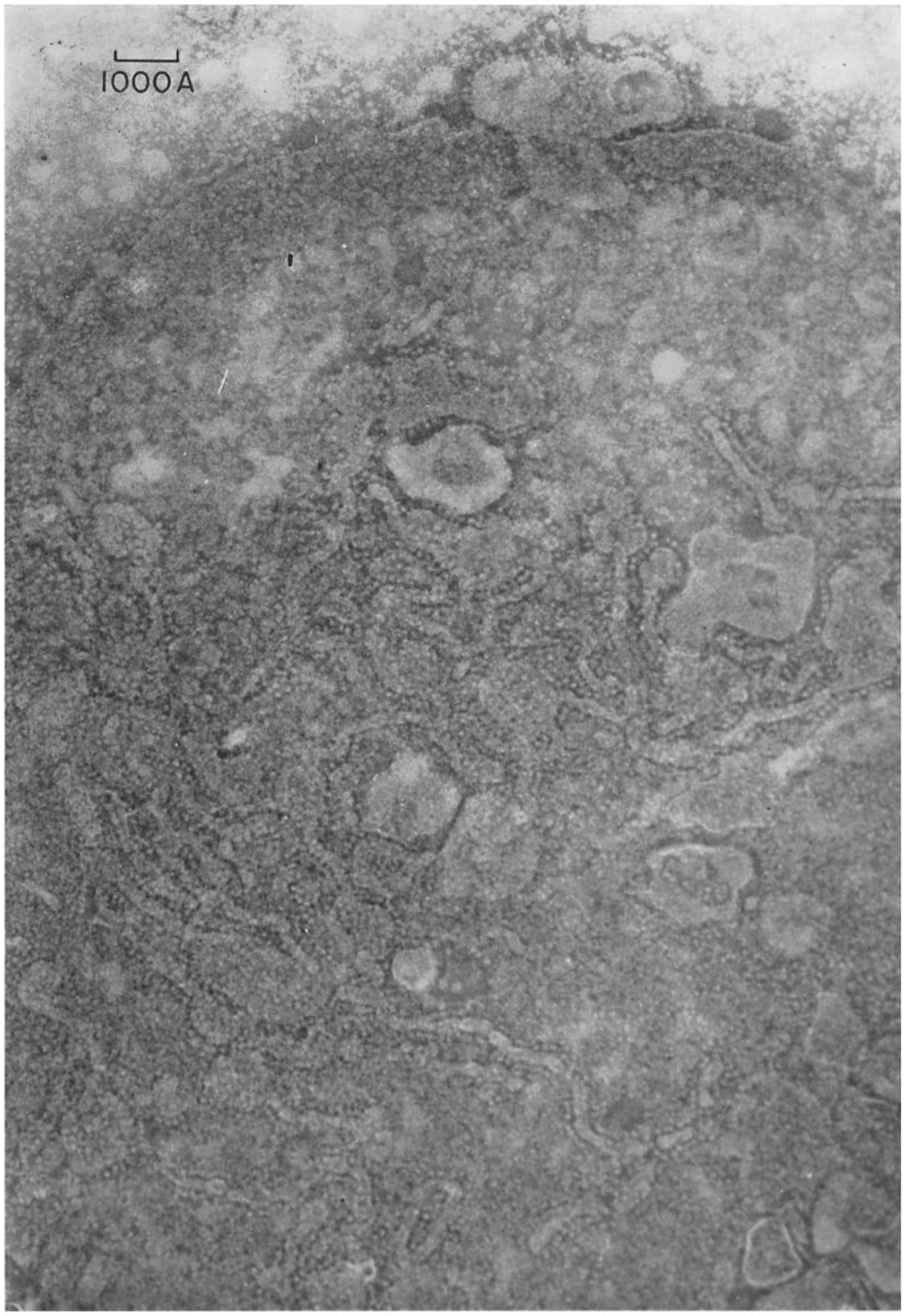


FIGURE 8

Part of a relatively well preserved large mitochondrion, showing the cristae in the interior partly in profile and partly in face view. × 108,000.

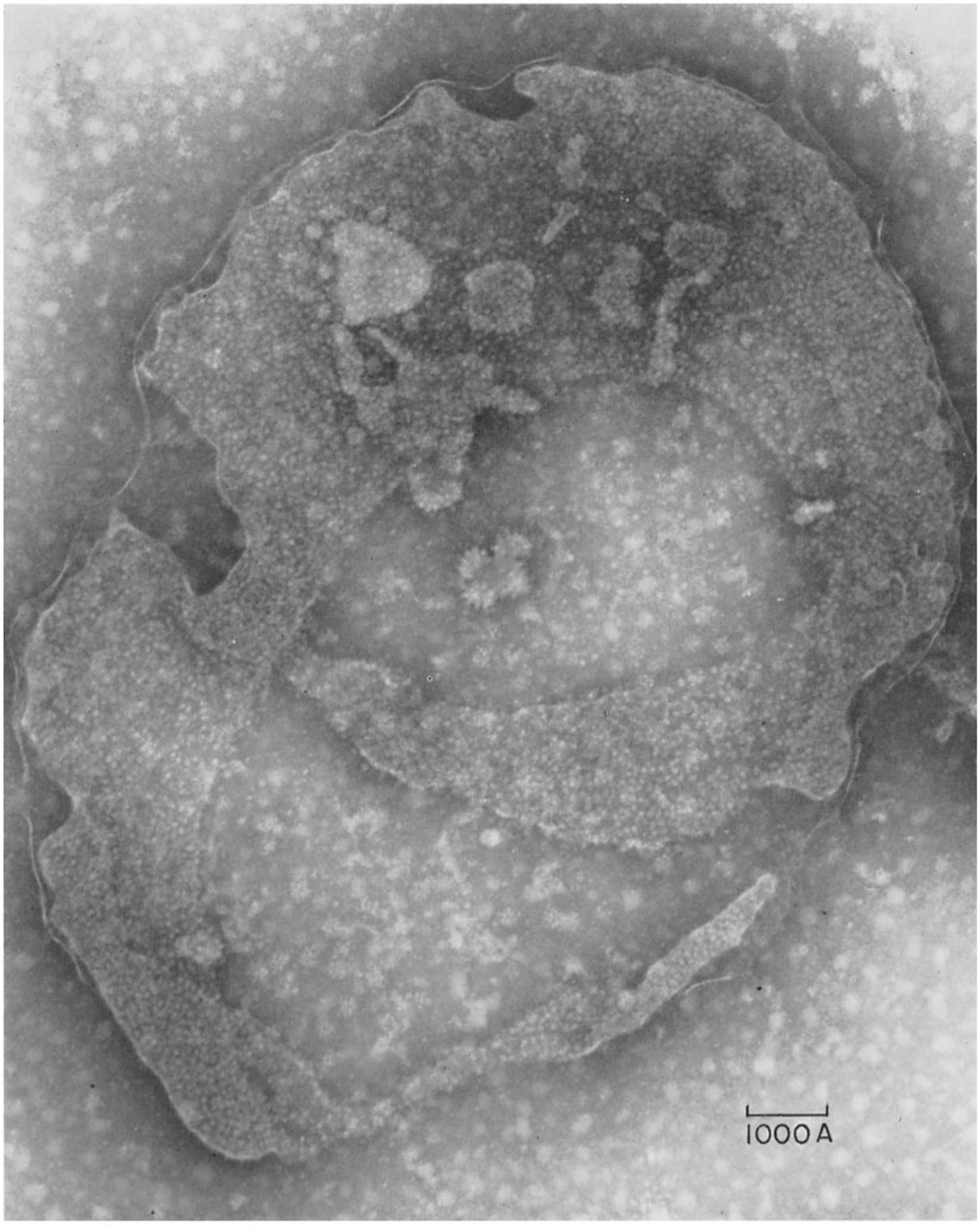


FIGURE 9

Partly intact mitochondrion where the outer membrane of the envelope can be distinguished. Its structure apparently differs from that of the inner membrane, and it does not show the characteristic 85 A particles. × 120,000.

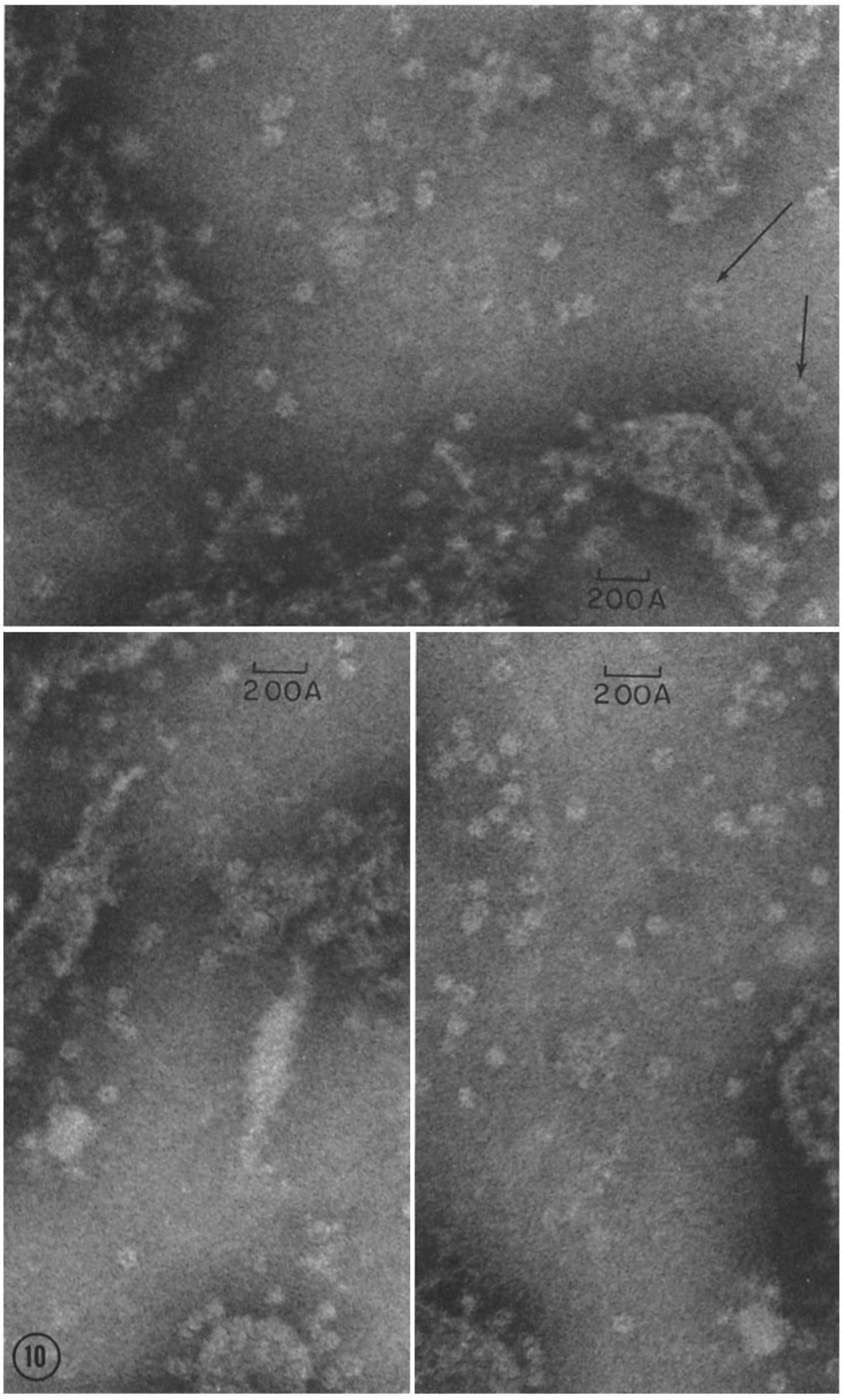
diameter linked to the mitochondrial membrane by a narrow stalk 40 to 50 A long. It is found on the surface of the cristae and probably also on the inner surface of the mitochodrial envelope. This is consistent with the now generally accepted view that the membrane of the cristae, at least in some places, is continuous with the inner membrane of the mitochondrial envelope. The distribution of these particles on the membranes appears irregular. In one preparation, 3000 particles per 1 μ^2 have been counted. They occupy in this case one-fifth to one-sixth of the total available area, but it is doubtful whether this figure is very meaningful. On the one hand, a considerable loss of particles may occur during the preparative procedures, in which case the particle count would be too low. On the other hand, it is known from sectioned material that mitochondrial membrane fragments usually form closed vesicles, and we assume that they are present mainly as flattened-out vesicles in our preparations. We cannot be sure that we are actually observing only one and not both surfaces of these vesicles, and if the latter were the case the figure given above could be too high by a factor of two. The same reasoning applies to whole or partly intact mitochondria, with the added complication that these should include cristae, which, in many cases, cannot be clearly distinguished from the over- or underlying parts of the envelope. In some small regions a much closer spacing of the particles has been observed, and it is possible that smooth-appearing membranes actually represent areas where the particles are too densely packed to be resolved.

The fact that most of the membrane fragments seen in these preparations are in all probability flattened-out vesicles would readily account for the two different types encountered. Vesicles that bear the particles on the outside would appear to be surrounded by a single row of particles which are seen to be connected to the edge of the membranes by the stalk. If a vesicle bears the particles on the inside, they would be seen only on the face of the membrane and the stalks would not be visible. The appearance of the partly intact mitochondria suggests that the cristae bear the particles only on the surface facing the matrix and that the mitochondrial envelope bears them only on the inner surface also facing the matrix. It seems reasonable to assume that the closing up of membrane fragments to form vesicles will usually follow the preexisting curvature of the membrane, and the different appearance of the fragments seen may therefore give an indication of their origin from envelope or cristae.

That the ribbon-like structures with attached particles are cristae seen in profile is mainly deduced from their occurrence in the interior of intact or partly intact mitochondria. The light ribbon 135 A wide should then correspond to the two membranes of a crista which are closely apposed and possibly include some unidentified material. The same width for a crista is measured in fixed and embedded material in places where the two membranes of the crista appear to be in contact and are seen in cross-section. The particles are therefore assumed to be attached on the outside of the cristal membranes and to protrude into the matrix. This is further borne out by the observation that the membrane pairs of the cristae show a tendency to separate in the negatively stained preparations, which results in an increase in the total width of the cristae, a phenomenon also regularly encountered in sectioned material. In the case of the negatively stained preparations PTA will penetrate between the membranes and appear as either a central dense band or a wider, roughly oval dense area. In the case of the OsO_4-fixed sections the central space appears lighter than the membranes.

It is of course possible that the width of the cristae *in vivo* is greater than 135 A and that they contain some material enclosed between membranes. After partial or total loss of this material, the cristae either would collapse to the width of 135 A with the membranes now closely apposed, or might swell to a variable greater width. This will not be discussed here, but it should be emphasized that in our interpretation the particles are located outside the membranes of the cristae.

The particles described here have been clearly observed only in fresh material stained negatively with PTA, or with uranyl-acetate complexed with EDTA and used as a negative stain. The question then arises whether they could be an artifact, for instance matrix material adsorbed on the cristae during the isolation or negative staining of the preparation. This does not seem very likely, since they always appear to be attached to the membrane by the stalk and usually no isolated particles with stalks are seen in the preparations. Moreover, spreading a suspension of intact tissue culture cells on a large, clear water surface, which results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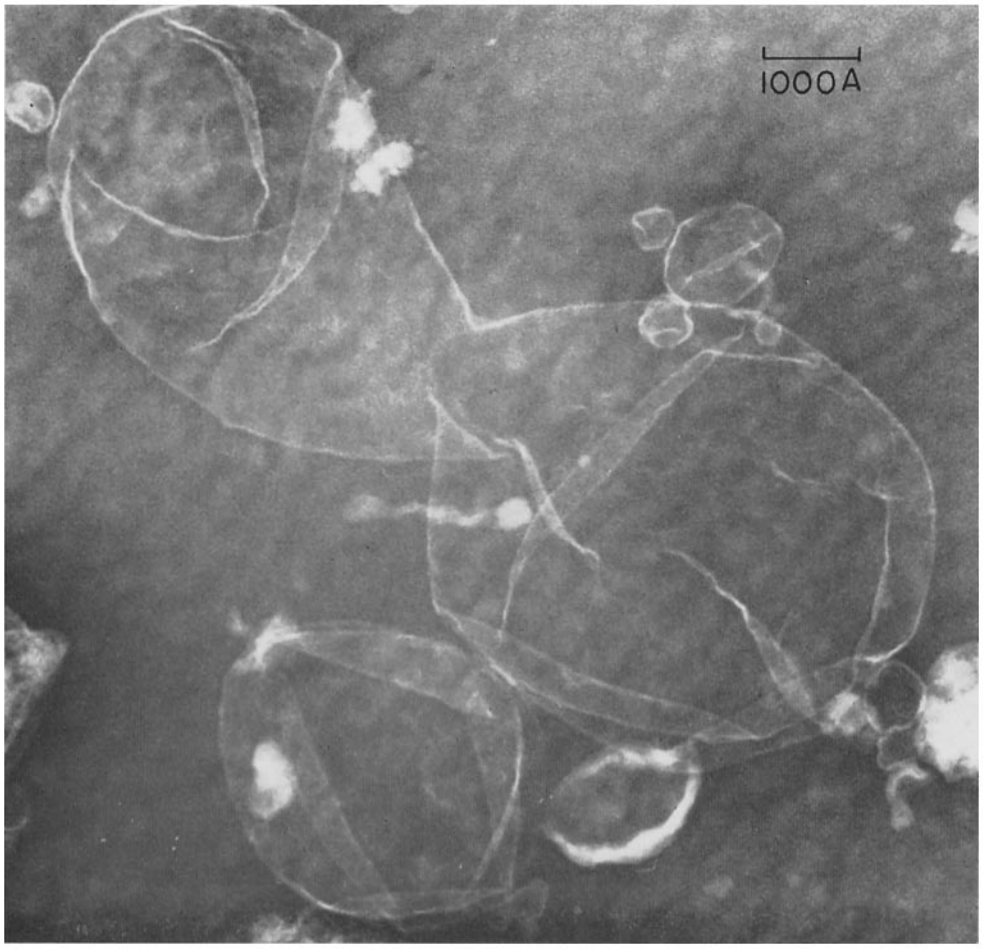

FIGURE 11

Preparation of mitochondria identical in all respects with those in Figs. 1 to 8 except for an exposure to OsO_4 vapor before negative staining. Practically only naked membranes are found in this material. × 140,000.

in instant disruption of the cells and their mitochondria, should minimize adsorption effects, because all small particulate material is caught in a rapidly expanding surface film. Nevertheless the mitochondrial fragments in such a preparation show the same concentration of bound particles that is found in mitochondrial material prepared by adsorption to the supporting film from a suspension. Finally, if the particles are not found on the mitochondrial membranes, because of changes in the preparative technique used, neither are they found anywhere else in the preparation. It seems therefore more likely that they are very labile particles which are easily destroyed by

FIGURE 10

Isolated particles, sometimes found in the preparations. They may represent particles detached from the membrane and seen in various stages of disintegration. The form with a central hole and a roughly pentagonal shape is the most frequently encountered. The two structures marked by arrows could represent very advanced stages in the disintegration. × 400,000.

conventional preparative techniques or are transformed to such an extent that they become unrecognizable. This assumption is borne out by the observation that an exposure of the mitochondrial suspension to OsO_4 vapor or solution at 0°C or to a $KMnO_4$ solution before negative staining causes a complete disappearance of the particles (Fig. 11). This, of course, explains why they have not been observed in tissue OsO_4-fixed, embedded, and sectioned in the conventional way. Other fixatives, such as dichromate, formaldehyde, acrolein, and glutaraldehyde, have similar but less drastic effects or at least do not stabilize the particles to any useful extent. Crotonaldehyde and methacrolein preserve the particles slightly better in negatively stained preparations but they do not stabilize them sufficiently against conventional dehydration and embedding procedures to yield satisfactory pictures in sectioned material. Moreover, it is difficult to introduce contrast in sections which is comparable to the contrast found in negatively stained material.

It has long been realized that the mitochondrial enzymes of oxidative phosphorylation must have a definite spatial relationship (2, 4, 5, 7) and it is therefore tempting to assume that the particles described represent the morphological substrate for this postulate, *i.e.* that they are fixed enzyme complexes. Direct evidence for this assumption has not been presented so far, and because of the apparent lability of the particles will probably be difficult to obtain. However, once these particles have been established as a definite morphological component of the mitochondrion, their isolation and biochemical identification should be of paramount interest. Attempts in this direction have been made, but so far apparently without conclusive results (8). Because of their size, the particles cannot contain constituents with a combined molecular weight much higher than 500,000 and therefore cannot be identical with Green's "electron transport particles" unless the latter are assumed to be aggregates of these particles, which remains to be shown.[3] In the meanwhile the particles have proved useful as a sensitive morphological criterion for detecting mitochondrial fragments in microsomal tissue fractions by electron microscopy. The simple and fast technique of negative staining should also be of value in the further characterization of other biological membranes.

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Note Added in Proof:

While this paper was in press, Dr. D. F. Parsons has reported on negatively stained mitochondria in Abstracts of the 7th Annual Meeting of the Biophysical Society, February 18–20th, 1963, New York City, and *J. Cell Biol.*, 1963, **16,** 620. His observations are in good agreement with the findings reported here.

[3] Dr. Fernández-Morán (1) reports "successful isolation in Dr. D. E. Green's laboratory of uniform submitochondrial particles, 70 to 90 A in diameter, which exhibit fine structure of 10 to 20 A and are otherwise similar to the elementary particles of the intact mitochondrial membranes." As reference is given: Green, D. E., A climactic point in the study of the mitochondrion, *Arch. Biochem. and Biophysics*, 1961, in press. The paper seems not to have appeared yet.

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