CYTOCHEMICAL STUDIES OF MITOCHONDRIA

I. The Separation and Identification of a Membrane Fraction from Isolated Mitochondria

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INTRODUCTION

Mitochondria are now well known to be the site of systems of enzymes facilitating many of the oxidative processes of cells. Some of these enzymes cannot be extracted free of contaminating material and appear to be tenaciously bound to relatively large units within the mitochondria. Thus, it has recently been resuggested by Cleland and Slater (1) that the "insoluble" succinoxidase complex is closely associated with mitochondrial membranes. The present work presents the first direct evidence to support this view.

Prior to now, there has been no unequivocal demonstration that mitochondria even possess membranes. Electron microscope studies have produced evidence that suggests that mitochondria *in situ* possess a complicated, membranous envelope enclosing a more or less homogeneous matrix (2–6). Light microscope examination of mitochondria in intact cells (7) and isolated in suspension (8, 9) has indicated, on the basis of their response to media of various tonicities, that they may be bounded by a semipermeable membrane. It has been pointed out by Harman (10), however, that this behavior can be explained on the basis of a hydratable gel structure without a bounding membrane.

The work to be presented is divided into two parts. The first part deals with the separation of five fractions from liver mitochondria disrupted in sodium deoxycholate (DOC). One of these fractions is shown to consist of material derived from mitochondrial membranes. In the second part (11) biochemical studies of these fractions are presented and, in particular, it is shown that the "membrane fraction" contains the succinoxidase complex in high concentration.

Methods

150 to 200 gm. rats were stunned and decapitated. The quickly excised livers were homogenized (10 per cent weight/volume homogenate) in 0.44 M sucrose (12) solution in the cold (~5°C.). Nuclei, unbroken cells, and debris were removed by centrifugation for 10 minutes at 700 *g* in the Spinco centrifuge at ~4°C. The mitochondrial fraction was isolated from the supernatant by centrifugation at 13,000 *g* (12) for 10 minutes. The resulting pellet was washed

twice by resuspension and homogenization in 0.44 M sucrose and recentrifuged at 13,000 *g* for 10 minutes. At the end of the first wash, the "fluffy layer" was removed by layering the pellet with 0.44 M sucrose, swirling, and decanting. The final pellet was once more resuspended in 0.44 M sucrose. An aliquot of this suspension was rapidly mixed with 1/9 its volume of 3 per cent Na deoxycholate to give a final DOC concentration of 0.3 per cent. On addition of the DOC, the cloudy mitochondrial suspension became clear almost immediately. The DOC solution was prepared by dissolving deoxycholic acid (Wilson Co.) in dilute NaOH solution and back titrating with dilute HCl to pH 7.5 to 7.7.

The DOC–treated mitochondrial suspension was fractionated by differential centrifugation into five fractions. Each fraction (except the first) was obtained from the supernatant of the previous one by sedimenting, respectively, at 13,000 *g* for 10 minutes, 25,000 *g* for 20 minutes, 105,000 *g* for 60 minutes, 150,000 *g* for 60 minutes, and 150,000 *g* for 6 hours. These fractions were prepared for electron microscopy as described below.

All preparations were processed as pellets and fixed in the cold (3 to 5°C.) in 1 per cent OsO_4 in unbuffered 0.44 M sucrose for 16 hours (12). Dehydration in a graded ethyl alcohol series was followed by embedding in butyl methacrylate at 45°C. Sections were cut perpendicular to the broad face of the pellets; that is, in a direction parallel to the radius of the centrifuge. In this way all possible layers in each pellet could be examined. Sections mounted on carbon films (13, 14) were examined in a modified RCA type EMU-1 or Siemens Elmiskop I electron microscopes. In addition to the fractions mentioned the following pellets were treated in the same manner: (1) Pellets of intact, isolated mitochondria. (2) Pellets obtained by differential centrifugation from a suspension of 0.5 per cent DOC–treated mitochondria. (3) Pellets obtained by differential centrifugation from isolated mitochondria after suspension in water. (4) Pellets obtained by differential centrifugation from a water suspension of mitochondria after treatment for 20 minutes in the sonic oscillator (11). Liver slices, treated as described later in the text, were fixed for 4 hours in the cold at 1 per cent OsO_4 in veronal-acetate buffer adjusted to pH 7.3, dehydrated in ethyl alcohol, and embedded in butyl methacrylate.

RESULTS

Mitochondria in Situ.—The appearance in the electron microscope of mitochondria in sections of intact rat liver and other tissues has been described several times (2–6) and will be only briefly reviewed here. In this tissue, the usual appearance of the mitochondrion (Fig. 1) is ovoid with an envelope consisting of two parallel membranes spaced 100 A apart (in sections). Within the mitochondrion are a number of pairs of membranes (cristae mitochondriales, (2)) which in section appear to lie roughly perpendicular to the mitochondrial surface. The thickness of the cristae is 150 to 200 A. An occasional section shows that the membranes of the cristae are sometimes continuous with the inner mitochondrial membrane. In the area within the mitochondrion but outside the paired membranes is the relatively diffuse *mitochondrial matrix.* Embedded in the matrix are a number of dense *mitochondrial granules*, 300 to 350 A in diameter. Although these structures are presumed to exist in the living tissue, it is not known how closely the values given for membrane spacings, for example, reflect such dimensions in the living state.

In this description and in the material which follows the dense lines seen in section are considered to be profiles of membranes. A more complete con-

sideration of the existence of membranes in mitochondria and a definition of "membrane" as we understand it appear in the Discussion.

In addition to mitochondria, the micrograph (Fig. 1) also shows elements of the endoplasmic reticulum (*er* in the figure) (15, 16). These have an appearance in section which shows them to be flattened vesicles bounded by distinct membranes. Adhering to the outer surface of these vesicles are numerous small particles, 100 to 150 A in diameter, which have been found to be rich in ribonucleic acid (RNA) (17). The close spatial association of some of these elements with the mitochondria is especially noticeable in liver.

General Features of the Mitochondrial Fraction.—The pellets of isolated, but otherwise untreated mitochondria, contain in addition to mitochondria a certain amount of contamination with other cellular components. Of these, the most prominent is microsomal and consists of elements of the endoplasmic reticulum together with the RNA-bearing particles adhering to their outer surface (17). Consideration at greater length of microsomal contamination in our preparations appears in the Discussion of this paper. Also present in the pellet are a number of membrane-bounded granules somewhat smaller than mitochondria, which often contain numerous, tiny (*ca.* 50 A), dense particles. These are presumed to be the same as structures described as "peribiliary bodies" by Palade and Siekevitz (17) and as "lysosomes" by Novikoff *et al.* (18).

The propertion of mitochondria showing, in the electron micrographs, serious damage, such as loss of matrix, was very small in the fractions prepared in the present study. This is, of course, no indication of the total amount of mitochondria so damaged during isolation. Such damaged elements would very likely have lower density than intact ones and would be selected out by centrifugation. In the present study, mitochondria were isolated in 0.44 M sucrose to avoid the swelling reported to occur in approximately 15 per cent of mitochondria isolated in 0.25 M sucrose (12).

Appearance of the Isolated Mitochondria. After isolation in 0.44 M sucrose (Fig. 2), mitochondria differ somewhat in appearance from mitochondria *in situ*[1]. The general features, however, are still preserved. The outer mitochondrial membrane surrounds most elements rather loosely and at some points the inner one can be observed as well. The membranes of the cristae are often rather widely spaced while the matrix is homogeneous and dense. The mitochondrial granules are present although often difficult to discern. The high density of the matrix in these preparations masks to some extent the inner mitochondrial membrane, the membranes of the cristae, and the mitochondrial granules and it is for this reason that these structures are seen only with diffi-

[1] The differences in appearance between *in situ* and isolated mitochondria are considered to be substantially due to the differences in optimal fixation conditions. These include absence of buffer, long fixation, and absence of cytoplasm in the preparations of isolated mitochondria

culty in Fig. 2. The high density of the matrix which constitutes the main difference may be due to differences in the fixation conditions and also possibly to alterations in the mitochondria produced by the isolating medium.

Treatment with Deoxycholate.—The addition of DOC to a mitochondrial suspension to give a final concentration of 0.3 per cent results in almost immediate clearing of the preparation. Five fractions were sedimented in the centrifuge from the cleared suspension as described earlier. The pellets of the two heavier fractions (13,000 *g* and 25,000 *g*) resembled grossly pellets of untreated mitochondria except that the pellet of the 13,000 *g* fraction had a dark colored layer of unknown origin as well as a tan layer. The pellet obtained at 105,000 *g*, the membrane fraction, was of a waxy consistency, transparent, and pinkish tan in color. The pellets of the two lighter fractions (150,000 *g*, 60 minutes, and 6 hours, respectively) were the same in gross appearance as the pellet of the membrane fraction (105,000 *g*).

The Appearance of the Five Fractions in the Electron Microscope.—The 13,000 *g* and 25,000 *g* fractions were essentially similar in the electron microscope and will be treated together. The bulk of the two fractions contained mitochondria which were partially disrupted as well as a few elements which for unknown reasons appeared to have escaped alterations by the DOC. In addition to the obvious mitochondrial material there were two other sorts of granules. One of these consisted of membrane-bounded bodies somewhat smaller than mitochondria and containing numerous, tiny (*ca.* 50 A) dense particles previously mentioned as present in the pellets of isolated but untreated mitochondria. In addition there was present another type of granules of about the same size, with no bounding membrane, and having a distinctly laminated structure. The source of these latter granules is completely unknown since they have not been observed *in situ* in liver. A number of lipide droplets were also present.

The most interesting, from our point of view, of the DOC-treated mitochondrial fractions was the one obtained after 60 minutes at 105,000 *g* (Fig. 3). The material of this fraction consists of vesicular elements, both single and compound, ranging in size from 0.2 to 2 microns in diameter. Most of them fall in the range between 0.5 and 1 micron. The vesicles possess a limiting membrane bounding a region whose density is the same as that of the surrounding medium. Many of the larger vesicles are multiple, consisting of a system of several small vesicles enclosed within one or two bounding membranes (arrow, Fig. 3). These elements resemble in structure the membranes of intact mitochondria. There are also others where many closely packed membranes can be seen.

The two 150,000 *g* fractions (60 minutes and 6 hours, respectively) were essentially similar in appearance in the electron microscope and will be discussed together. By and large they resembled the membrane fraction (105,000

g) in containing mainly vesicular material. There were, however, inhomogeneities due to systematic variations in the vesicular elements and due to the presence of other material. With respect to these inhomogeneities we may divide the pellet into three regions. The first of these contained vesicular elements resembling those of the membrane fraction (105,000 *g*, 60 minutes) except that the bounding membranes were more often multiple rather than single (Fig. 4). Also present were numerous smaller single vesicles in the range 100 to 150 A in diameter. A second major part of the pellet contained bundles of closely packed membranes which were not disposed in readily recognizable vesicles (Fig. 5). These were embedded in a heterogeneous mass of poorly resolved material. It is believed that this part consists of a portion of the membranes which have responded to the DOC treatment to a greater extent than the rest. Such a reaction could be explained on the basis of initially incomplete mixing when DOC at high concentration was added to the mitochondrial suspension. This is supported by observations on suspensions treated with 0.5 per cent DOC to be discussed below. The relatively homogeneous material in which these membranes are embedded may represent partially solubilized mitochondrial matrix or patially dissolved membrane or both. The third major portion of the 150,000 *g* fraction (Fig. 6) contained rather disorderly, flattened vesicles and bundles of membranes which were surrounded by numerous dense particles with uniform diameters of about 150 A. It is considered that this represents microsomal contamination present in the original mitochondrial fraction. The resemblance of the small particles to the particles described by Palade as present in many cells (15) and as containing ribonucleic acid (17) is inescapable.

Treatment with 0.5 per cent DOC.—In order to gain more insight into the morphological changes observed particularly in the 150,000 *g* fractions, mitochondria were treated in suspension with a higher final (0.5 per cent) concentration of DOC. This suspension was centrifuged to give three pellets which sedimented at 13,000 *g* in 10 minutes, 25,000 *g* in 20 minutes, and 105,000 *g* in 60 minutes respectively. The two heavier fractions (13,000 *g* and 25,000 *g* respectively) differed little from the corresponding fractions of 0.3 per cent DOC treated mitochondria.

The 105,000 *g* fraction, however, presented a striking picture (Fig. 12). Here are found long (*ca* 5 to 10 μ) arrays of five to ten closely apposed (*ca* 100A) membranes. Such arrays might be formed by fusion of vesicles to form large elements which then come to be parallel to one another by mutual forces of attraction. It is difficult to determine positively whether these are arrays of open-ended membranes or of flattened vesicles. However, the general appearance favors the latter view. In support of this it may be noted that open-ended membranes are rarely if ever encountered within cells *in situ*, in microsomal preparations (17), or in mitochondrial suspensions treated with 0.3 per

cent DOC. These bundles of membranes resemble those found in part of the 150,000 *g* fractions from the 0.3 per cent DOC preparations in their close-packing, great extent, and freedom from small scale vesiculation. This supports our belief that the latter represent a portion of mitochondrial membranes which has been exposed to higher initial concentration of DOC than the average.

Further DOC Experiments.—The experiments thus far presented were carried out on isolated mitochondria in suspension. Two further investigations were made to define more fully the mode of action of DOC.

(*a*) *Effect of DOC on Slices of Intact Liver.*—It was not clear whether the observed reaction of mitochondria with DOC was a general one or whether it occurred only in preparations of isolated mitochondria. To answer this question, free hand slices of rat liver about 14 mm. thick were incubated at 0–5°C. for 1 hour in 0.44 M sucrose either with or without 0.3 per cent DOC. After incubation the slices were prepared for electron microscopy and examined. In many of the cells near the surface of the slices incubated in the presence of DOC, mitochondria were drastically swollen and showed complete loss of matrix (Fig. 11). The pattern of disruption was similar to that observed in mitochondria treated in suspension. Control slices incubated in the absence of DOC showed no such effects. Thus, it is apparent that the disruption of mitochondria in DOC is not a peculiarity of isolated mitochondria, but can be observed *in situ* as well. To eliminate possible effects of sucrose which might influence the mode of mitochondrial disruption a similar pair of experiments was carried out in which veronal-acetate buffer adjusted to pH 7.3 was used in place of sucrose. Essentially the same results were obtained in this procedure as where sucrose was used.

(*b*) *Mode of Disruption of Mitochondria in Pellets Layered with DOC.*—It was our wish to demonstrate that the membranes observed in DOC–treated mitochondrial suspensions were of mitochondrial origin and if possible to find their source in a specific structural element of the mitochondria. To achieve this it was of primary importance to trace in small steps the way in which mitochondria underwent disruption when exposed to DOC. This could not be done in suspensions since the effects were almost instantaneous. Therefore, a pellet of untreated mitochondria was formed at the bottom of a centrifuge tube using the bucket-type rotor of the centrifuge. The use of this head permitted subsequent recentrifugation without smearing of the pellet which would have resulted had the angle head been used. The supernatant was discarded, the top of the pellet washed briefly, and the pellet then covered carefully with a solution of 0.3 per cent DOC in 0.44 M sucrose. The preparation stood at 0–5°C. for 1 hour, and was recentrifuged for 10 minutes at 105,000 *g* to pack the top layers. It was then fixed, embedded, and sectioned parallel to the radius of the centrifuge as described for the other pellets.

In this pellet a population of mitochondria was present which had reacted to a graded concentration of DOC from the maximum (0.3 per cent) at the top to essentially zero concentration at the bottom. Transverse sections through the pellet showed all stages of dissolution in a fairly orderly sequence. These are presented in Figs. 7 to 10.

At the bottom of the pellet the mitochondria show essentially no response to DOC. Slightly further up, however, (Fig. 7) some elements are swollen. In these, the diameter has increased and the matrix is decreased in density. In all elements the bounding membranes and membranes of the cristae are visible, as are the mitochondrial granules.

Near the middle of the pellet most mitochondria have become swollen (Fig. 8) to three to five times the average mitochondrial diameter in untreated preparations. The matrix appears as a diffuse background within the mitochondria and contains a number of pale threads and granules. The outer mitochondrial membrane is now stretched thin, but is otherwise intact. In contrast to this, the cristae appear essentially unswollen and lie at the side of the mitochondria. Their membranes are correspondingly denser than the outer limiting membrane. The inner bounding membrane also appears undistended and can be seen in the region of the mitochondrial surface near the cristae.

At the level close to the top of the pellet an abrupt change takes place (Fig. 9). The mitochondria are reduced to about twice the normal diameter and most, though not all, of the matrix has disappeared. The inner bounding membrane and the membranes of the cristae are now stretched and distended presumably because at this level they have reacted with the DOC. Their density is about the same as that of the outer mitochondrial membrane.

On the top of the pellet (Fig. 10), where the mitochondria were exposed directly to the deoxycholate, elements are found which closely resemble the material of the 105,000 *g* fraction from mitochondria treated in suspension with 0.3 per cent DOC. The general mitochondrial outline is preserved as a double envelope consisting of two membranes (now widely spaced) and enclosing a number of single walled vesicles. The matrix has essentially disappeared, as have also the mitochondrial granules. The over-all diameter of these elements is about half as great as that of the swollen elements found in greatest numbers in the middle of the pellet, although it is somewhat greater than the diameter of untreated mitochondria. The difference in appearance found between membranes of the cristae and other membranes of the swollen mitochondria of the middle region of the pellet has disappeared under the more drastic exposure to deoxycholate at the top of the pellet. All parts of the membranes seem now to have responded equally, and the cristae appear as spherical vesicles within the mitochondria. An interpretation of these observations will appear in the Discussion.

As a control for mitochondrial pellets layered with 0.3 per cent DOC, pellets

were layered with 0.44 M sucrose without DOC and incubated for 1 hour at 0–5°C. In this case, the steps of disruption described above were absent and the mitochondria were essentially intact. This indicates that the dissolution of the mitochondria was the result of exposure to DOC and did not arise from other possible effects.

Disruption of Mitochondria by Physical Methods.—The use of a detergent to cause a separation of the mitochondrial matrix from the membranes is a physicochemical approach which might be expected to destroy certain enzymes. It was hoped that a treatment which was more directly physical might produce a preparation enzymatically more active and morphologically more homogeneous. Three methods were tried: treatment with water alone, blending at high speed, and exposure to sonic energy.

Water-Treated Mitochondria.—A pellet of mitochondria isolated in 0.44 M sucrose was resuspended by homogenization in water. This treatment results in considerable swelling of most mitochondria and disruption of a few. Fractions obtained by centrifugation of the suspension at 5,000 *g* for 10 minutes and 105,000 *g* for 30 minutes were examined in the electron microscope. The heavier fraction (5,000 *g*) was composed almost entirely of mitochondria swollen about five times in diameter (Fig. 13). Few if any elements were broken, and all appeared to contain a substantial amount of matrix. The cristae were unswollen and appeared as a cluster of small, elongated vesicles near the margins of the sectioned mitochondria. The outer mitochondrial membranes rarely appeared double. However, a large bubble or bleb of irregular outline was often apparent proceeding from the outer surface. A cluster of cristae is often present at the site of one of these blebs. It is probable that the "crescents" described by Harman (10) in light microscopic observations of water-treated mitochondria correspond to the clustered cristae. The accompanying bleb described here is apparently a new finding.

The lighter fraction (105,000 *g*, 30 minutes) (Fig. 14) was small in amount and was, in part, similar to the heavier fraction with the exception that less matrix was present. In addition were present many small, vesiculated elements. Some of the larger elements, obviously swollen mitochondria, appeared to be ruptured.

Treatment with water alone, therefore, resulted in substantial swelling of the mitochondria, but failed to remove the matrix adequately. Following or preceding the water treatment with high-speed blending or sonic energy failed to improve matters. Both procedures resulted in considerable alterations of the mitochondrial structure, in which blending (Fig. 15) simply broke the membranes but left matrix adhering, while sonics (Fig. 16) produced a uniform mass of small vesicles which appeared still to contain matrix in the electron micrographs. The fact that succinoxidase was concentrated to only a small extent in these fractions (about twice, (11)) supported the view that matrix was still present. The work of Hogeboom and Schneider (19) on sonic-treated mitochondria will be discussed in greater detail in the subsequent paper (11), but their results were in essential agreement with those reported here.

The Isolation and Treatment of Mitochondria from Rat Kidney Cortex.—It seemed desirable to attempt the isolation of mitochondria from rat kidney cortex which

contains a lesser amount of endoplasmic reticulum than does liver. It was thought that a mitochondrial fraction free of membranes of the endoplasmic reticulum and RNA-containing particles might be obtained.

Cells of the tubules in the rat kidney cortex are characterized by the presence at their basal ends of large numbers of rod-shaped mitochondria. These mitochondria are intimately associated with deep infoldings of the cell membrane (6, 20). In the preparations obtained, there was present with the mitochondria a considerable amount of membranous material (probably from the cell membranes) as contaminant. While, in general, the picture with DOC treatment of kidney mitochondria was similar to that obtained with liver mitochondria, the large amount of membranous contamination discouraged further investigation.

DISCUSSION

Much of the data presented in this paper arose from experiments designed to clarify two primary observations. A suspension of mitochondria isolated from rat liver and treated with 0.3 per cent DOC yielded in the centrifuge a fraction which in the electron microscope appeared to be composed of membranes and which in addition was of biochemical interest since, as will be presented (11), it contained the succinoxidase complex in high concentration. It seemed likely that this material was a product of the disruption of mitochondria by DOC. It was not clear, however, whether this product represented a specific structural element of the original mitochondria which survived exposure to DOC, and, if so, whether this part was present in mitochondria not subjected to isolation. Finally, in the case of affirmative answers to these points, we wished to know from where in the intact mitochondria this material came.

Mitochondrial suspensions treated with 0.3 per cent DOC undergo a prompt and drastic change. Differential centrifugation of this suspension yields at 105,000 *g* a transparent, pinkish tan pellet which on fixation, embedding, and sectioning appears in the electron microscope to consist of vesicular elements containing material of no greater density than the surrounding medium. That a similar material could be obtained from mitochondria treated *in situ* with DOC was demonstrated by examination of liver slices incubated in 0.44 M sucrose containing 0.3 per cent DOC. Mitochondria in cells near the surface of these slices showed typical disruption in the presence of DOC which was absent in slices incubated in 0.44 M sucrose alone. A similar pair of experiments carried out in veronal-acetate buffer, pH 7.3, gave essentially the same results. Thus it was shown that the isolation procedures were not necessary for the production of membranous material by the action of DOC. The source of the membranous elements must, however, be known before the observation of them is of much value. These elements could be derived directly from membranes present in mitochondria or in the contaminating endoplasmic reticulum or they could be artifactual. Such an artifact might be formed from interaction of the deoxycholate with non-membranous material such as mitochondrial matrix,

or with previously dissolved membranes, or it could be formed in the subsequent stages of preparation for electron microscopy.

These questions proved to be interdependent and were answered together by a detailed study of the way in which mitochondria were disrupted by DOC. The examination of transverse sections of pellets of mitochondria layered over with 0.3 per cent DOC yielded a picture consistent with the idea that much of the membrane material of the suspension-treated mitochondria was indeed derived from mitochondrial membranes. It was found that the degree of disruption of mitochondria in such pellets was an inverse function of their distance from the exposed surface of the pellet. The stages of interaction were as follows: (1) swelling without appreciable loss of matrix to three to five times in diameter, (2) shrinkage to about twice the diameter of untreated mitochondria, which was accompanied by loss of matrix. During the swelling period, the cristae showed relatively little change in appearance, while during shrinkage, they became vesiculated and appeared in many cases to separate from the inner mitochondrial membrane. The structure of membranous elements observed at the top of the layered pellet represents a distorted image of certain essential features of membranes of intact mitochondria. Such elements were usually bounded by two concentric membranes enclosing a number of smaller spherical vesicles.

The mitochondria at the top of the layered pellet were exposed directly to the 0.3 per cent DOC solution and thus the final reaction product at this place is essentially the same as that in mitochondria treated in suspension. We are able, therefore, to trace backward in the layered pellet all stages of dissolution from material like that in the membrane fractions to intact mitochondria. Since at no point in this process did it become difficult to identify membranes, there was no evidence to support the idea that the original mitochondrial membranes dissolved and new ones were formed. Loss of matrix and mitochondrial granules, which was apparently complete at the top of the pellet, began at the stage of maximum swelling. On the basis of these observations we assert that the vesicular material isolated from disrupted mitochondria is not artifactual, but consists of membranes of mitochondria which are substantially free of matrix.

It is of some interest to attempt an explanation of the mechanism of dissolution of the mitochondria. Toward this end a model for the mitochondrial structure is proposed. We will consider that the mitochondrial matrix consists in part of material organized to form a loosely linked network which readily becomes hydrated when exposed to 0.44 M sucrose solution and tends to swell as a result. We further propose that the outer mitochondrial membrane acts as an elastic net to prevent swelling of the matrix. The presence of DOC causes the outer mitochondrial membrane to lose some of its "tone" thus allowing the matrix to swell. What happens to the inner membrane is not clear, though from the micrographs of the layered pellet it appears

to break. The matrix can now swell until the mitochondrial diameter has increased three- to fivefold. At this stage DOC penetrates the outer membrane and affects a change in the matrix, the mitochondrial granules, and the inner membrane and membranes of the cristae, which up to now have been protected from the DOC. The matrix and mitochondrial granules lose their organization and the products leak out while the inner membranes become vesiculated. As the matrix leaks out, residual 'tone' of the outer membrane causes the mitochondrion to shrink. Finally, the picture seen at the top of the layered pellet and in the membrane fraction develops. The model of the mitochondrion indicated above thus proposes the existence of a gel-like matrix whose tendency to swell under hydration forces is resisted by the presence of a bounding, semipermeable membrane under tension. This model includes both the concept of a semipermeable membrane so often proposed to explain certain properties of mitochondria (7, 9), and the concept of a hydratable gel structure suggested by Harman (10).

The Purity of the Membrane Fraction.—In the preceding discussion emphasis has been laid on the identification of the vesicular elements of the membrane fraction with the membranes of mitochondria. The morphological evidence presented indicates that all or nearly all the mitochondrial matrix and granules were rendered non-sedimentable by the DOC at the centrifugal forces used, and that therefore the membrane preparations were essentially free of this material. It has been pointed out that microsomal contamination was present in all the mitochondrial isolates, this contamination consisting of membranes of the endoplasmic reticulum plus RNA-containing particles. Morphological examination revealed the presence of the small particles in the membrane fraction, but such observations are difficult to evaluate quantitatively. In addition, the membranes of the endoplasmic reticulum which are presumably also present are indistinguishable morphologically after DOC treatment from those of mitochondria, especially in cases in which the small particles are no longer adherent. An independent estimate of the amount of microsomal contamination present in the membrane fraction is therefore of interest.

According to Palade and Siekevitz (17) about 10 per cent of the total solids of the microsomal fraction of rat liver is RNA, this being responsible for about 30 per cent of the mass of the small particles of the microsomes. It can be calculated from these data that the total mass of the membranes of the endoplasmic reticulum is about seven times the mass of the RNA present in the microsomes. Now, about 3 per cent of the membrane fraction obtained from mitochondria treated with 0.3 per cent DOC was RNA (11). Seven times this or 21 per cent of the pellet could thus be derived from membranes of the endoplasmic reticulum while about 9 per cent of the pellet could consist of the small particles. However, Palade and Siekevitz (17) showed that approximately 60 per cent of the membranes of the endoplasmic reticulum are rendered nonsedimentable at 105,000 *g* by treatment with 0.3 per cent DOC. Thus, the

membrane fraction from mitochondria contains only about 8 per cent (21 per cent × 0.4) material derived from the membranes of the endoplasmic reticulum and 9 per cent derived from small particles, or 17 per cent from the whole microsome fraction. This calculation assumes: (1) all the RNA in the pellet is derived from the microsomes and none from the mitochondria, (2) the microsomal RNA is not solubilized by treatment with 0.3 per cent DOC, (3) the mitochondrial membranes are not solubilized by treatment with 0.3 per cent DOC, and (4) the small particles are not dissolved or selected against after treatment. If assumption (1) and (4) are wrong, the figure of 17 per cent will be decreased, and if (2) and (3) are wrong, it will be increased. In any case, we conclude that very likely less than one quarter of the membrane pellet is derived from the microsomes.

It should be pointed out that even if as much as 25 per cent of the membrane fraction is due to microsomal contamination, this does not invalidate the conclusions presented here or in the subsequent paper (11). Our principal interest in the membrane fraction lies in demonstrating its major source in the mitochondria and in its content of enzymes, namely the cytochrome oxidase and succinoxidase complex (11), which are not present in the microsomes (9). Thus the principal effect of microsomal contamination on our results is only to lower the specific activities of these enzymes in the membrane fraction.

Repeatedly throughout this paper the term "membrane" has been applied to certain structures. It is pertinent to define what we mean by this term and to examine the implications of our findings. A membrane, in our view, is essentially a flexible sheet, much smaller in one dimension than in two others at right angles to it. It is continuous morphologically in the sense that above a certain resolution at which the observation is made, a substantial part of the membrane exhibits no breaks or holes. In the case of mitochondrial membranes, we can assert that no holes in single membranes can be demonstrated confidently at resolutions greater than 20 to 50 A. Up to this point in our description, a membrane does not differ substantially from an interface between two immiscible liquids, which also is a continuous sheet possessing tensile strength. A membrane, however, possesses a higher degree of organization than does an interface, since within certain limits, its integrity is independent of the medium which surrounds it. Clearly, we cannot ordinarily isolate an interface from its two bounding liquids. The membrane depends for its existence on its own structure which is essentially independent of a variety of bathing media while the interface is the site and product of continuing interaction between two different specific substances. Thus, we visualize a membrane as a continuous sheet possessing, in general, non-rigid structural integrity which resists certain manipulations and exposure to a variety of media.

The electron microscopy of sections of tissue blocks has consistently revealed the presence of pairs of thin, dense lines within sectioned mitochondria. These have been interpreted as profiles of essentially two dimensional structures and accordingly have been designated as "membranes." It is clear, however, that the only general

properties of these structures which could be determined by such techniques are those of "two dimensionality" and of continuity. Palade (2) concluded on the basis of the response to solutions of various tonicities of mitochondria *in situ* and after isolation that the structures he observed in section in the electron microscope were indeed membranes. Sjöstrand and collaborators (4–6), agreeing with Palade on the existence of membranes, inferred that the double appearance of the limiting and internal membranes reflected a three-layered structure of single membranes in which two osmiophilic protein layers were separated by a non-osmiophilic lipide layer. Reversible swelling and shrinkage on exposure to hypo- and hypertonic solutions have been observed in intact cells (7), in saline isolates (8), and in sucrose suspensions (9). These observations can be interpreted to indicate that mitochondria behave as osmometers and are consequently enclosed within a semipermeable membrane. However, as indicated earlier, Harman (10) from similar observations proposed a hydratable gel structure for mitochondria with no limiting membrane. Thus, it is difficult to differentiate on the basis of apparent osmometry alone between the semipermeable membrane structure and the hydratable gel structure or a combination of these. What is missing from these observations (7–9) is the demonstration that the "two dimensional" structures seen in section are not simply interfaces, but have a structure more or less independent of the cell cytoplasm and of the rest of the mitochondrion.

In the present work the "membrane" profiles seen in sections of tissue blocks have been found to be present in sections of isolated mitochondria in sucrose, in mitochondria during all stages of dissolution with DOC, and finally in suspensions of mitochondria treated with DOC. Furthermore, it has been possible to isolate from from these suspensions relatively pure preparations of this material. The sections of all these preparations show that the material possesses the property of being much thinner in one dimension than in two others at right angles to it (*i.e.*, the property of "two dimensionality") and also the property of continuity. The fact that this material persists after isolation in sucrose and after treatment with DOC fulfills our third important condition for a membrane: the structure does not depend for its integrity and existence on the presence of a highly specific bathing media, but is to an extent independent of the environment. Thus, we have demonstrated the existence of membranes in mitochondria by isolating them in a relatively pure state and finding that they fulfill certain conditions considered essential for membranes.

SUMMARY

Mitochondria isolated from rat liver and suspended in 0.44 M sucrose were disrupted by treatment with 0.3 per cent Na deoxycholate. The treated suspension was fractionated by differential centrifugation into a number of fractions and the respective pellets were examined in sections in the electron microscope.

One of these fractions was found to consist of apparently membrane-bound (vesicular) elements.

The difference between interfaces and membranes was discussed and the material of this fraction was found to meet stated requirements identifying it as membranous.

A detailed study of the disruption process undergone by mitochondria in the presence of Na deoxycholate showed that the elements of this fraction were derived from structural elements assumed to be mitochondrial membranes.

The findings thus demonstrate that mitochondria do possess membranes as defined and that these membranes can be isolated in a relatively pure form.

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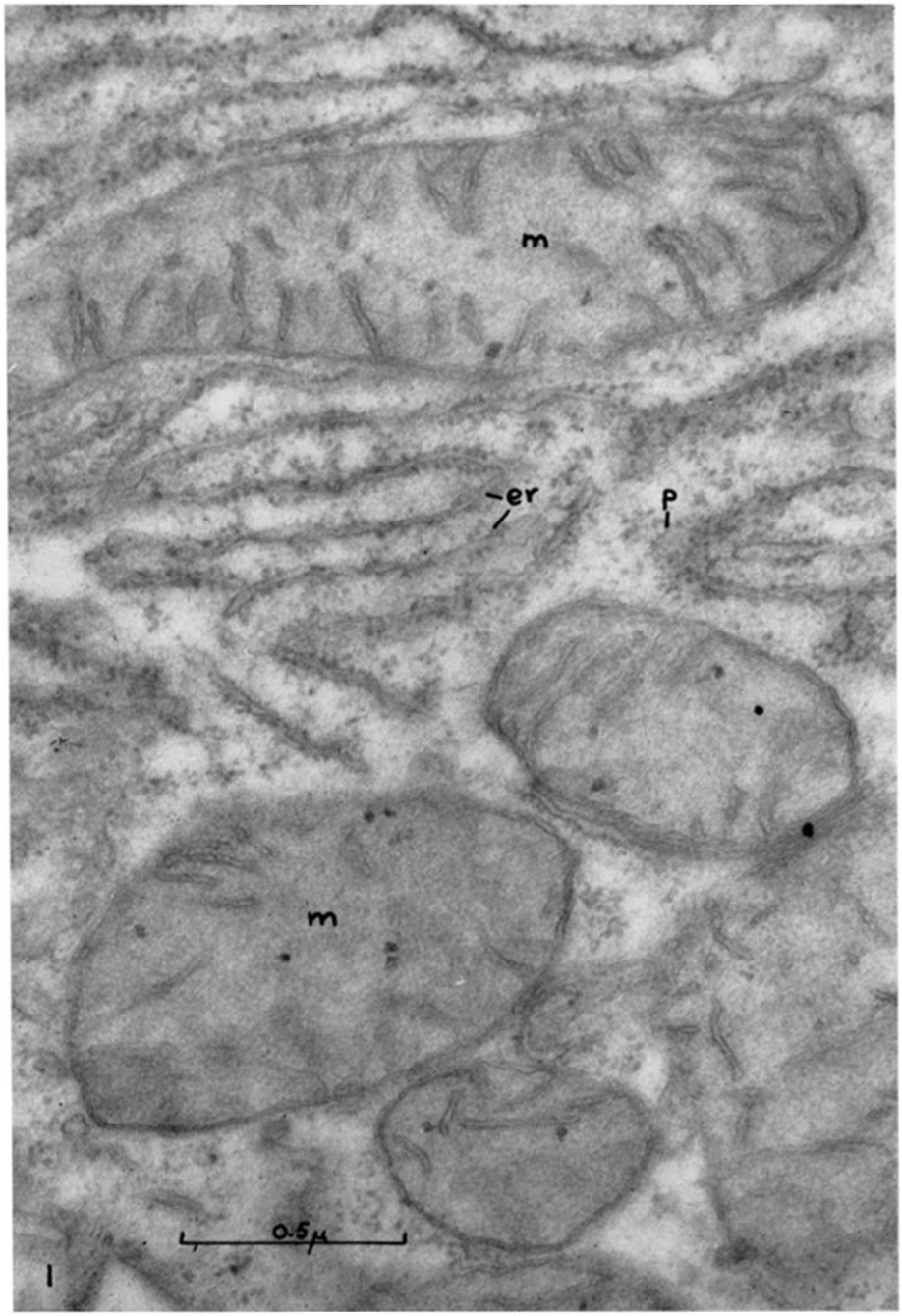
EXPLANATION OF PLATES

PLATE 163

FIG. 1. Section of rat liver showing mitochondria (*m*) and elements of the endoplasmic reticulum (*er*) with adhering small particles (*p*). The endoplasmic reticulum and small particles together form the microsome fraction of liver homogenates (17). The mitochondria are characterized by a pair of closely apposed, enveloping membranes, enclosing a relatively homogeneous matrix in which lie the paired membranes of the cristae mitochondriales and the mitochondrial granules. Magnification 60,000.



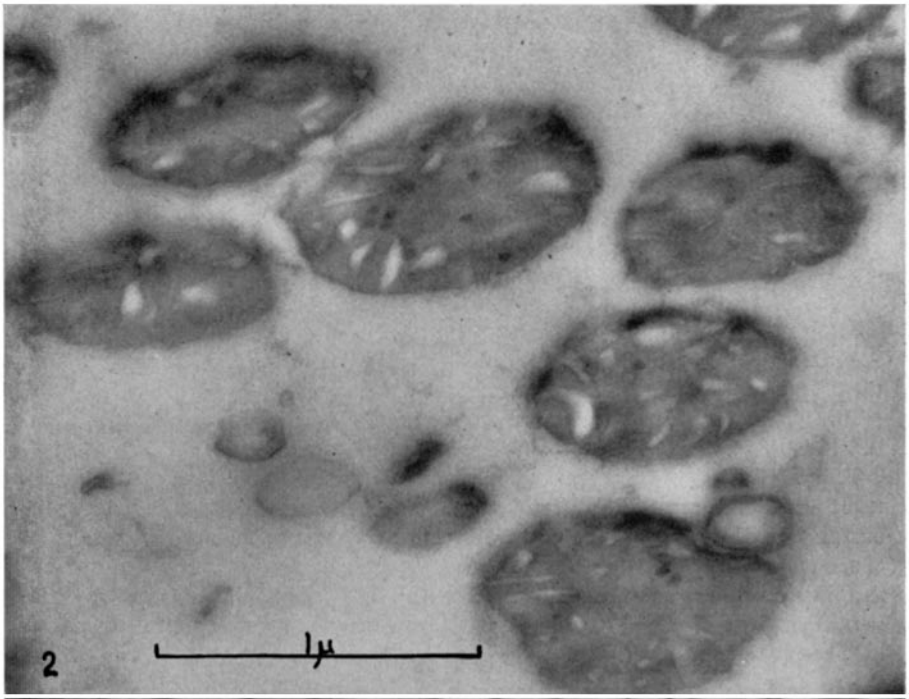
(Watson and Siekevitz: Cytochemical studies of mitochondria. I)

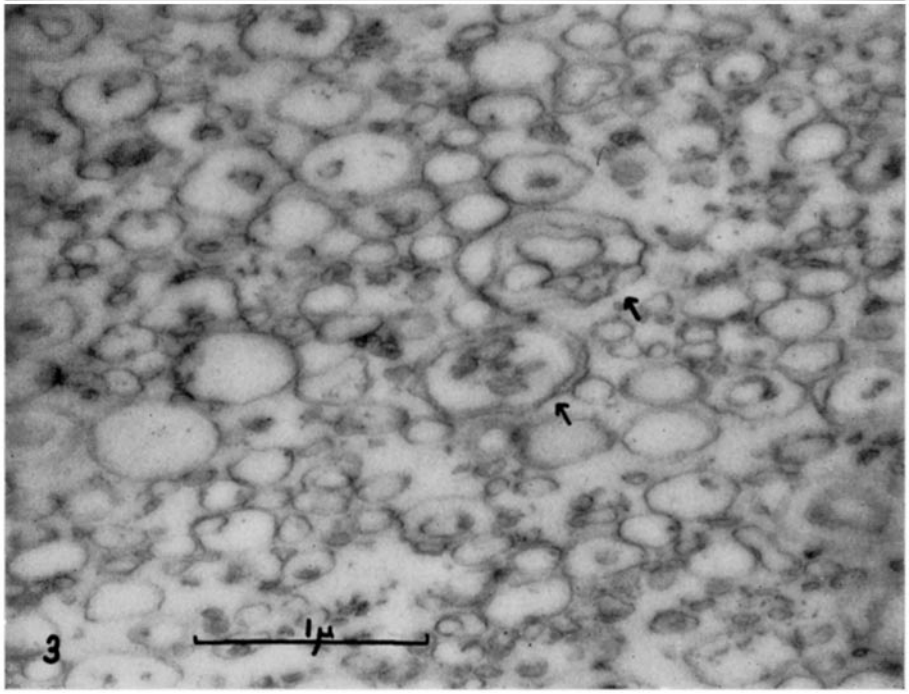
PLATE 164

FIG. 2. Rat liver mitochondria after isolation in 0.44 M sucrose. The relatively high density of the matrix obscures the bounding membranes, the membranes of the cristae, and the mitochondrial granules. Nevertheless these structures can be made out. The outer mitochondrial membrane is partially detached at many points around the mitochondria. Magnification 43,000.

The high apparent density of the matrix as compared with that seen in mitochondria *in situ* is due to differences in fixation conditions and possibly, also, to effects of the isolating medium. A distortion is evident in the separation of the membranes of the cristae.

FIG. 3. Membrane fraction obtained by centrifugation at 105,000 *g* for 60 minutes from a suspension of isolated rat liver mitochondria treated with 0.3 per cent DOC. The pellet consists of vesicular elements ranging in diameter from 0.2 to about 2.0 μ, most of them being in the range of 0.2 to 1.0 μ. Many of the larger elements are compound and a few (arrow) possess a distorted resemblance to the disposition of membranes in intact mitochondria. The smaller elements are usually single. The contents of the vesicles have the same density as the surrounding medium. No DOC appears in the micrograph, since any present in the original pellet is dissolved out by the dehydrating alcohol. Magnification 30,000.





(Watson and Siekevitz: Cytochemical studies of mitochondria. I)

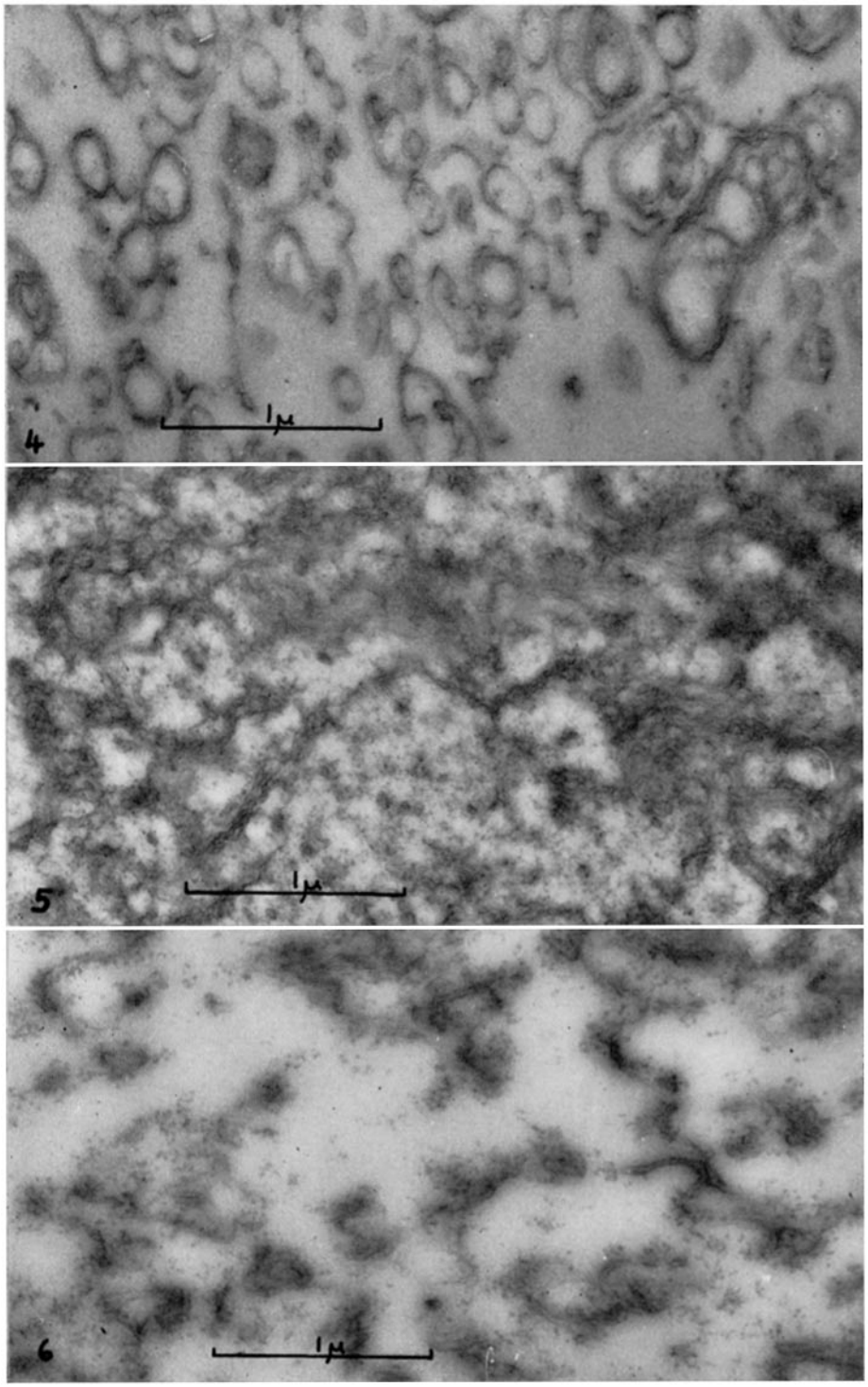
PLATE 165

FIGS. 4 to 6 show the appearance of different parts of the 150,000 *g* pellets from the 0.3 per cent DOC–treated mitochrondrial suspensions.

FIG. 4. The elements shown here are essentially indistinguishable from the material of the membrane fraction (*cf.* Fig. 3). Magnification 30,000.

FIG. 5. Multilayered stacks of membranes, interpreted to be closely packed, flattened vesicles. Surrounding these elements is a poorly resolved, rather heterogeneous, background material. The packs of membranes are considered to be those parts of the original suspension which came into contact with high concentrations of DOC as that reagent was added initially (*cf.* Fig. 12). The background material may consist of partially dissolved membranes, matrix, etc., which were sedimented at this high speed. Magnification 30,000.

FIG. 6. Flattened, vesicular elements together with many small dense particles. The size and density of the particles indicate that they are probably the same as the RNA-containing particles of intact cells. The flattened vesicles resemble the characteristically flattened elements of the endoplasmic reticulum present in liver. This material is thought to be principally microsomal. Magnification 30,000.



(Watson and Siekevitz: Cytochemical studies of mitochondria. I)

PLATE 166

FIGS. 7 to 10. Transverse sections through the pellet of intact, isolated mitochondria layered with 0.3 per cent DOC, showing the appearance of different layers in the pellet.

FIG. 7. Near the bottom of the pellet (remote from the DOC) are many essentially intact mitochondria (m_i) as well as a number which have begun to swell (m_s). In all these elements one can discern the limiting membranes, membranes of the cristae, the matrix, and the mitochondrial granules. Microsomal contamination (μ) is also present. Magnification 30,000.

FIG. 8. In the middle of the pellet most of the mitochondria have greatly swollen to three to five times normal diameter. This is the stage of maximum swelling. The greatly distended outer limiting membrane appears intact. The inner limiting membrane does not completely enclose the swollen mitochondria, but appears somewhat thicker than the outer membrane and occupies a relatively short segment at the margin of the mitochondria. The membranes of the cristae do not appear swollen, but share the higher thickness and density of the inner membrane. The cristae are found in a patch at one side of the mitochondria and are often associated with the segments of the mitochondrial margin where both outer and inner membranes can be seen. The mitochondrial matrix is present in substantial amounts, and the mitochondrial granules can be discerned. Outside of the mitochondria, elements of the endoplasmic reticulum are present as contaminant and can be recognized by the small particles adhering to their outer surface. Magnification 30,000.

FIG. 9. Near the top of the layered pellet a considerable change has taken place. The mitochondria have shrunk to about twice normal diameter and the mitochondrial matrix is much reduced in amount. There are now no systematic differences in the thickness and density of the membranes. Presumably, all membranes including the membranes of the cristae have reacted equally to the DOC. Magnification 30,000.

FIG. 10. At the surface of the pellet, the material present has been exposed to essentially the same treatment as were the suspension-treated mitochondria. The situation described in the previous figure has developed somewhat further so that essentially all the matrix is gone, and the general disposition of the membranes, while resembling that in intact mitochondria, is now somewhat more disorderly. Such elements are like those indicated in Fig. 3 (arrow). Magnification 30,000.

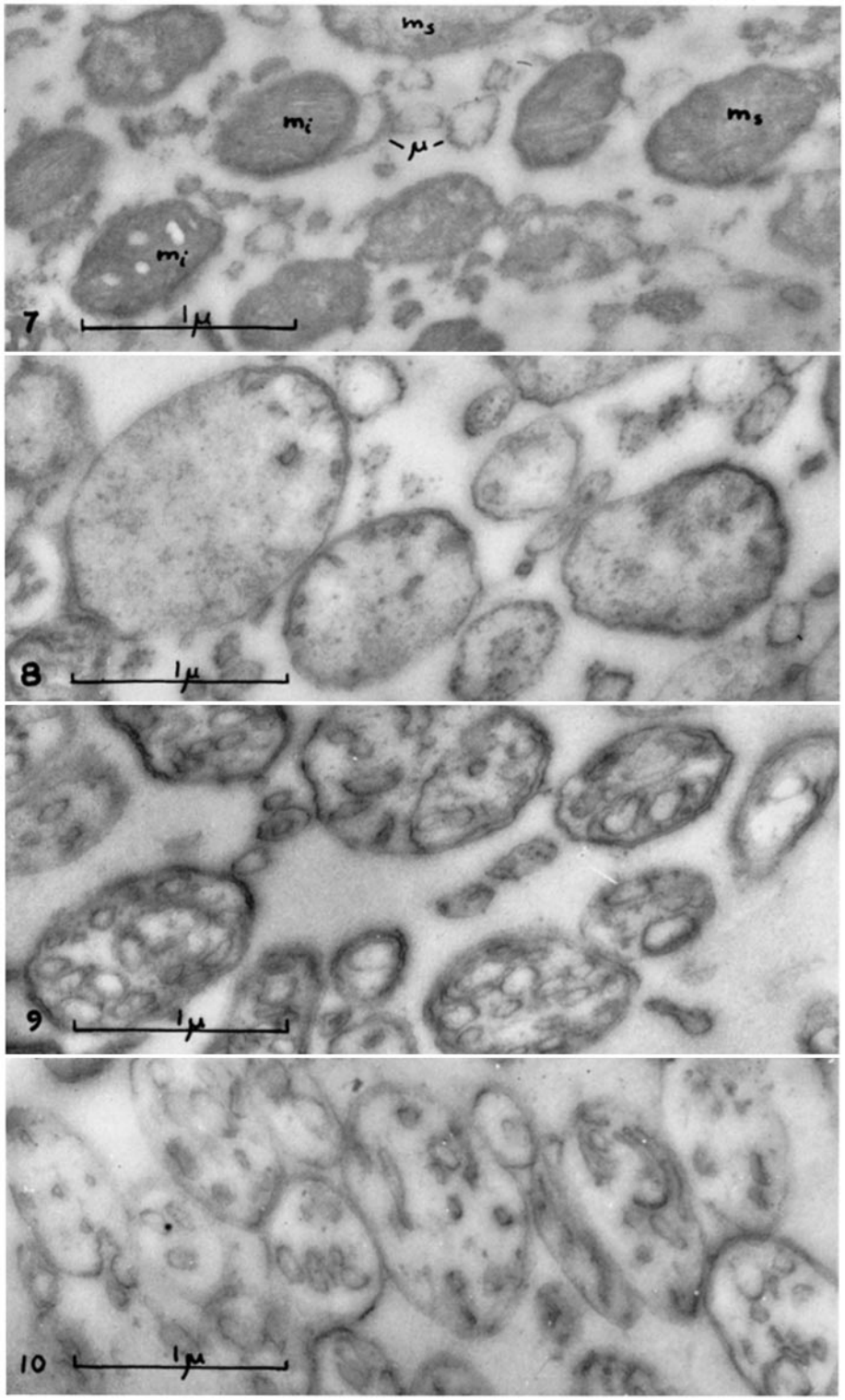


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PLATE 167

FIG. 11. The appearance of mitochondria in slices of rat liver incubated at 0°C. in 0.44 M sucrose containing 0.3 per cent DOC. The swelling, loss of matrix, and vesiculation of cristae are apparent here as in the mitochondria treated in suspension with DOC. Magnification 30,000.

FIG. 12. The appearance of much of the fraction isolated at 105,000 *g* from mitochondria treated in suspension with 0.5 per cent DOC. Here, the changes have proceeded further, resulting in the appearance of extensive stacks of many layers of closely parallel membranes. It is considered likely that the membranes are disposed in much flattened vesicles, although this cannot be convincingly demonstrated in the micrograph. Magnification 50,000.

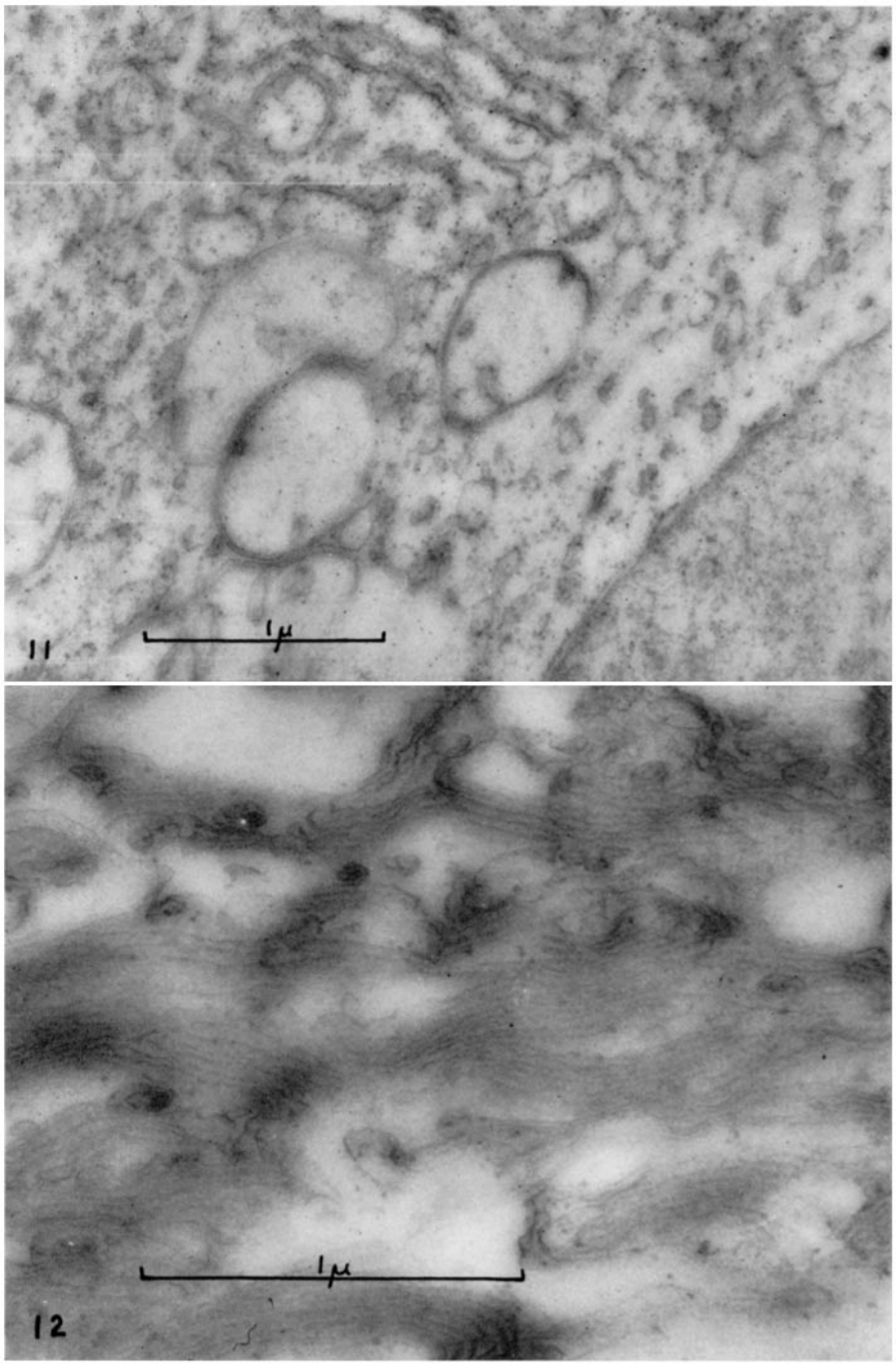

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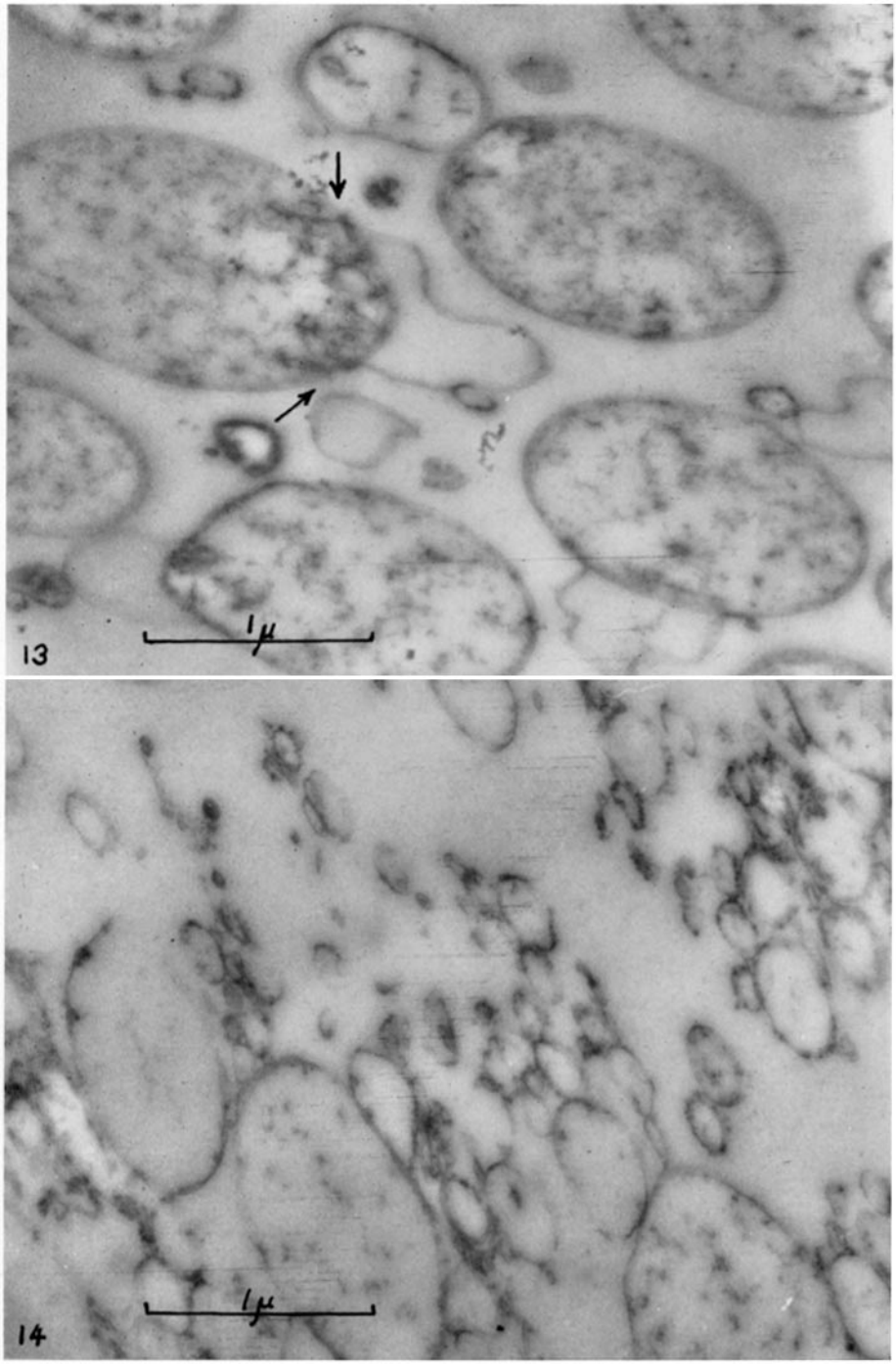
PLATE 168

FIG. 13. Appearance of mitochondria isolated from rat liver after resuspension in water and centrifugation at 5,000 *g*. Swelling to three to five times normal diameter has taken place, but much of the matrix remains within. The cristae, found in a patch at one side of the mitochondria (arrows) are not swollen. At segments of the margin of the mitochondria occupied by the cristae it is usual to find a membranous bleb or bubble extending away from the mitochondria. The identification of this with the inner or outer mitochondrial membrane cannot be made at present. Magnification 30,000.

FIG. 14. Pellet obtained after centrifuging at 105,000 *g* of supernatant from fraction described in Fig. 13. A number of large elements recognizable as swollen mitochondria are present together with many smaller vesicles. Nearly all elements, large or small, contain material of higher density than the surrounding medium and this is thought to be matrix. Most mitochondria are not disrupted by treatment with water alone, but simply swell up without loss of matrix. This fraction was considerably smaller than the 5,000 *g* fraction (see Fig. 13). Magnification 30,000.

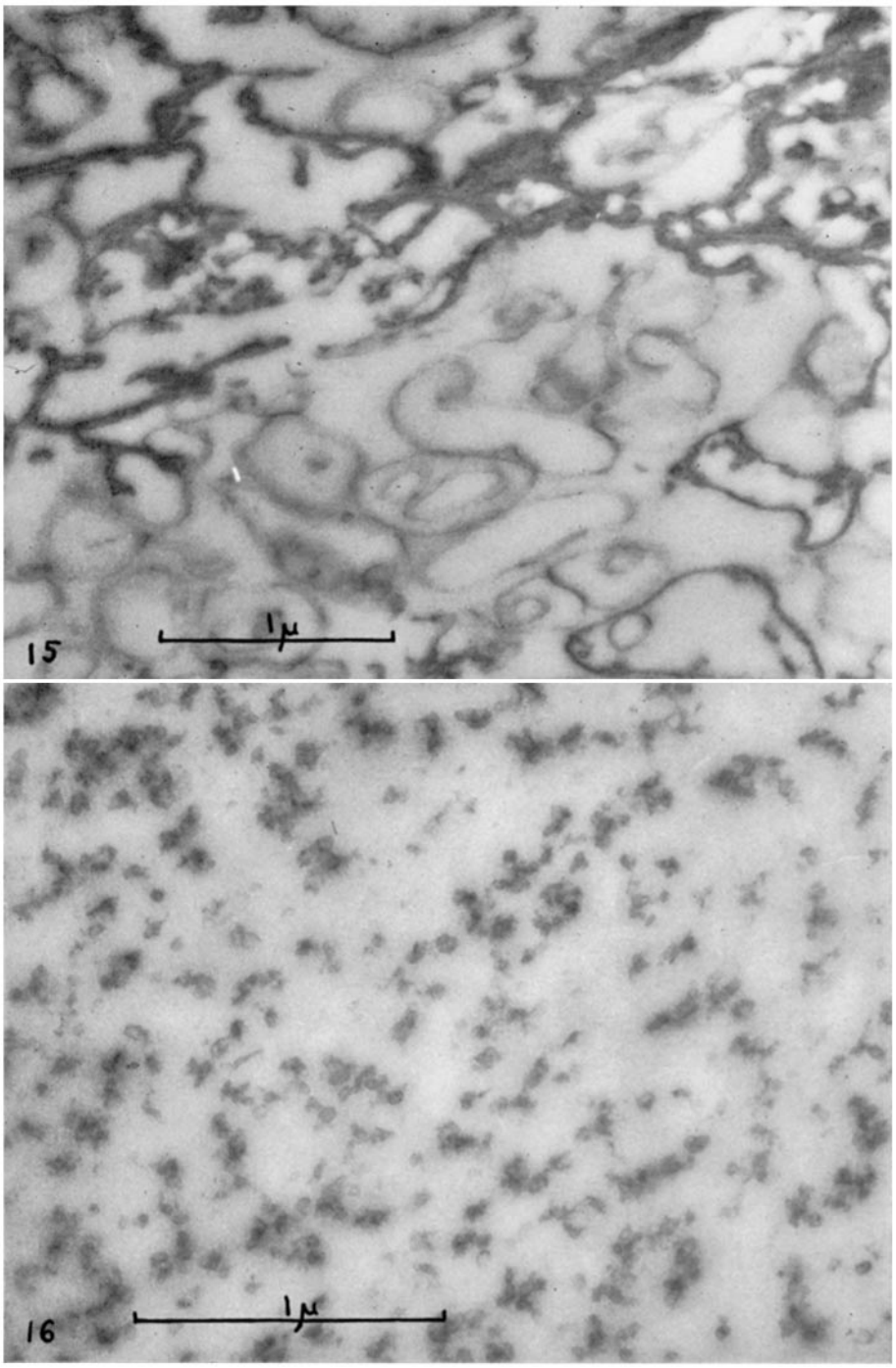


(Watson and Siekevitz: Cytochemical studies of mitochondria. I)

PLATE 169

FIG. 15. Isolated mitochondria after resuspension in water and blending at high speed. Many mitochondria were broken by blending and the resulting material formed the rather dense, thick films shown here. It is believed that this may consist of membranes with adhering matrix. Since the origin of such material is difficult to determine it was not investigated further. Magnification 30,000.

FIG. 16. Mitochondria isolated in 0.44 M sucrose and treated 20 minutes in the sonic oscillator (11). This fraction obtained at 105,000 *g* consisted of a rather uniform preparation of small vesicles ranging from 200 A to 500 A in diameter. Their rather high density suggested that matrix was present within the vesicles. This was supported by biochemical examination in which little concentration of enzyme was observed (11). Magnification 42,000.



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