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FRACTIONATION OF MITOCHONDRIAL MEMBRANES WITH SODIUM DEOXYCHOLATE

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SUMMARY

Treatment of mitochondrial cristae membranes with increasing concentrations of sodium deoxycholate in the presence of salt results in a differential distribution of cytochromes and proteins between a light supernatant fraction and a heavy membranous pellet. In thin sections, the mitochondrial membrane is seen splitting into two thinner membranes. At an optimal deoxycholate concentration a separation of the red (b and c_1) and green (aa_3) cytochromes between the pellet and supernatant fractions is achieved. Upon removal of deoxycholate by dialysis, each fraction assumes a characteristic vesicular arrangement. The membranes of each fraction are distinctive in both appearance and dimension. The triple-layered green membrane measures about 75 Å while the thinner red membrane, 50 Å, is uniformly electron dense.

The results also suggest the mode of action of deoxycholate on mitochondrial membranes.

INTRODUCTION

The detergent sodium deoxycholate has been used extensively in the fractionation and solubilization of membranes from various sources. Treatment of mammalian mitochondrial membranes[1] or of bacterial respiratory membranes[2] with deoxycholate results in the separation of the red and green cytochromes associated with these membranes. The morphology and biochemical composition of the resultant membrane fractions have been used to deduce the structural organization of the mitochondrial cristae membrane[3].

In the present paper we have treated mitochondrial cristae membranes with increasing concentrations of deoxycholate over the range normally utilized in fractionation studies. The ultrastructure of the supernatant material and the membranous pellet have been examined by means of negative staining and thin sectioning.

Abbreviation: ETP, electron transport particles.

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MATERIALS AND METHODS

General procedures

Beef heart mitochondria were prepared by a modification of the method of LÖW AND VALLIN[4]. Electron transport particles (ETP) obtained by sonication and centrifugation[5] were suspended in 0.25 M sucrose–0.1 M Tris·HCl buffer (pH 7.4) to a final protein concentration of 23 mg/ml. Enzyme grade deoxycholic acid purchased from Mann Research Laboratories was recrystallized twice from 95 % ethanol. 10 % (w/v) deoxycholate at pH 8.0 was added to the ETP suspension to give the desired final concentration expressed in mg deoxycholate per mg protein. Solid KCl was added to a final concentration of 1 M. The mixture was stirred in ice until the salt was completely dissolved then allowed to stand 10 min before centrifugation at 105000 × *g* for 20 min. The supernatant was carefully decanted and saved. The pellet was resuspended in a small volume of sucrose–Tris·HCl. When indicated, fractions were dialyzed for 15–20 h against 0.1 M Tris·HCl buffer (pH 8.0).

Chemical determinations

Protein concentration was determined by the modified biuret reaction of YONETANI[6].

Mitochondrial cytochromes were estimated from difference spectra (reduced *versus* oxidized) obtained on a Cary Model-14 recording spectrophotometer according to the method of WILLIAMS[7]. The extinction coefficients of VANNESTE[8] were used to calculate cytochrome concentrations.

Total phosphorus was determined by the method of BARTLETT[9] following ashing of the biological sample[10].

Electrophoresis

Disc-gel electrophoresis was performed on polyacrylamide gels according to the method of TAKAYAMA *et al.*[11]. The gels were stained with a 1.0 % amido black solution in 7 % acetic acid.

Electron microscopy

Samples were negatively stained with 2 % phosphotungstate (pH 6.9) as described by CUNNINGHAM AND CRANE[12]. For thin sectioning, samples were fixed in 4 % glutaraldehyde in 0.1 M phosphate buffer (pH 7.4), postfixed in 1 % OsO_4 in 0.028 M veronal acetate buffer[13] (pH 7.4), dehydrated in an acetone series, and embedded in Epon 812 according to LUFT[14]. Sections were cut with a diamond knife on a Porter-Blum MT-2 ultramicrotome and stained with uranyl acetate and lead citrate[15]. Samples were examined in a Philips EM 300 electron microscope.

RESULTS

Treatment of beef heart mitochondria or of ETP with increasing concentrations of deoxycholate in the presence of 1 M KCl results in the sequential extraction of the components of the electron transport chain from the cristae membranes. The extraction sequences of the various cytochromes, total protein, and phosphorus (as an indication of phospholipid) into the 105000 × *g* supernatant fraction are illustrated

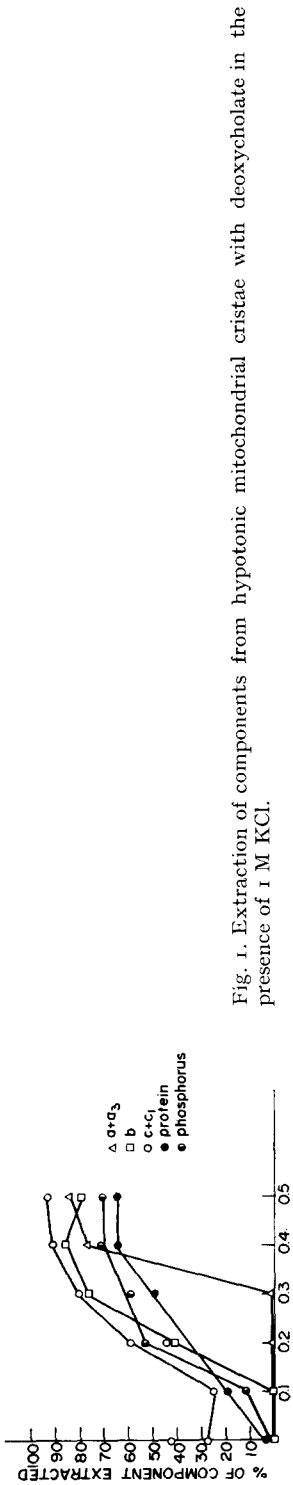

Fig. 1. Extraction of components from hypotonic mitochondrial cristae with deoxycholate in the presence of 1 M KCl.

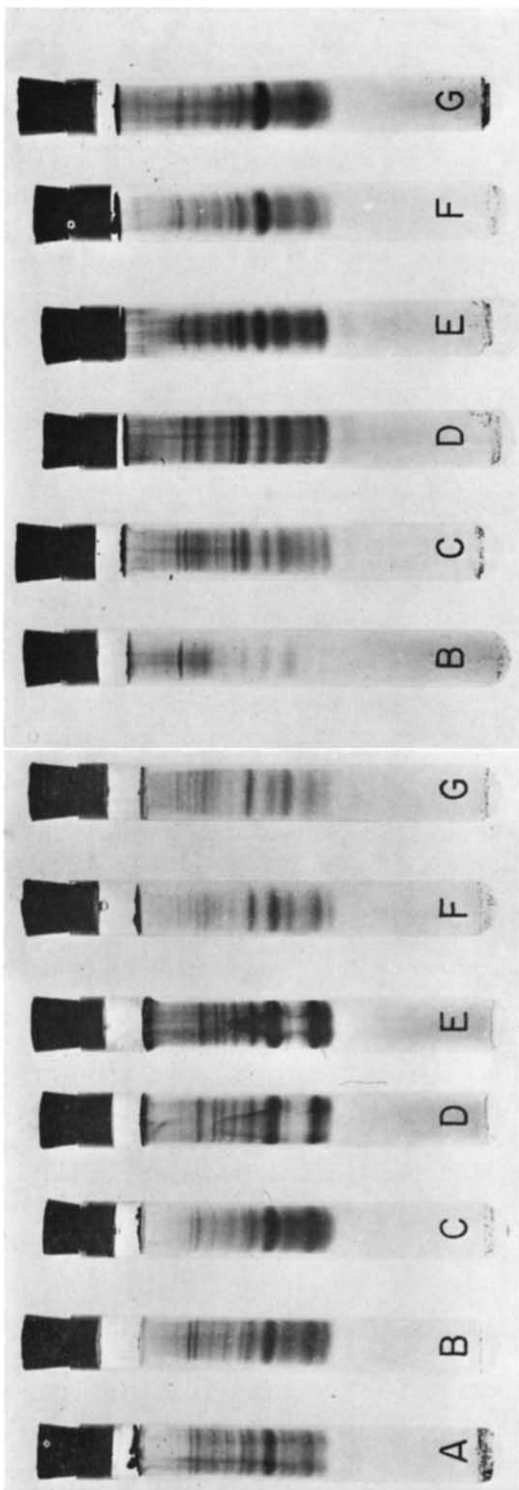

Fig. 2. Electrophoretic patterns of fractions from deoxycholate-treated mitochondrial cristae. Left gels, pellet fractions. Right gels, supernatant fractions. A. Hypotonic beef-heart mitochondria. B. 0 mg deoxycholate per mg protein. C. 0.1 mg deoxycholate per mg protein. D. 0.2 mg deoxycholate per mg protein. E. 0.3 mg deoxycholate per mg protein. F. 0.4 mg deoxycholate per mg protein. G. 0.5 mg deoxycholate per mg protein. All fractions except A were obtained in the presence of 1 M KCl.

graphically in Fig. 1. The deoxycholate concentration is increased from 0 to 0.5 mg deoxycholate per mg protein in the presence of 1 M KCl. Note the differences in the extraction patterns of the three types of mitochondrial cytochromes. There is regular increase in the total protein and phosphorus extracted with increasing deoxycholate concentration.

As the amount of deoxycholate is increased from 0.1 to 0.3 mg/mg protein, a red supernatant fraction and a greenish-brown pellet are obtained. The major portion of cytochromes b and $c + c_1$ are recovered in the supernatant fraction while all of the cytochromes $a + a_3$ remain in the pellet fraction. This separation of the red (b and c_1) and green ($a + a_3$) cytochromes is referred to as a red-green split[1]. At higher concentrations of deoxycholate, cytochromes $a + a_3$ are readily extracted into the reddish-green supernatant fraction leaving a gray residue pellet.

The distribution of specific proteins between the pellet and supernatant fractions at the various deoxycholate concentrations can be visualized by means of polyacrylamide-gel electrophoresis (Fig. 2). Those protein bands which are extracted into the supernatant fraction at low deoxycholate concentrations differ from those bands which remain associated with the pellet fraction at the higher deoxycholate concentrations. A mitochondrial protein represented by a major electrophoretic band is extremely resistant to deoxycholate extraction as evidenced by its persistence in the pellet fraction even at the highest deoxycholate concentration studied.

Negative staining reveals morphological changes in the pellet and supernatant material with increasing deoxycholate concentration. When ETP treated with

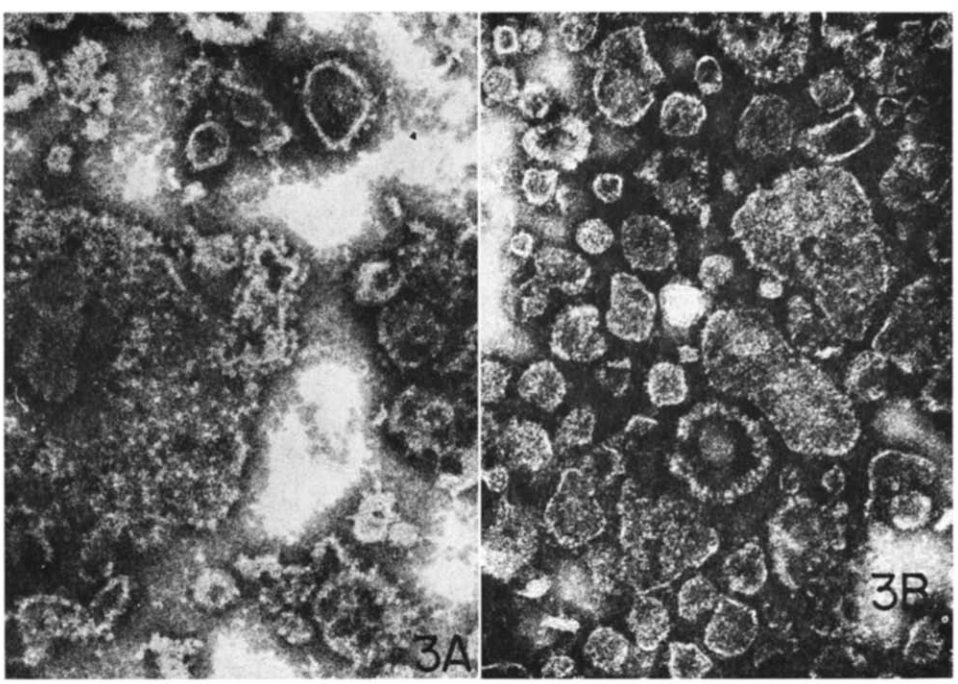

Fig. 3. 0 mg deoxycholate per mg protein. A. Pellet. B. Supernatant. × 100 000.
Figs. 3–8. Negatively stained pellet and supernatant fractions obtained from deoxycholate-treated ETP in the presence of 1 M KCl. Phosphotungstate stain.

1 M KCl but no deoxycholate are subjected to high speed centrifugation, the 105000 × *g* pellet (Fig. 3A) contains vesicles which are morphologically indistinguishable from the starting ETP. The F_1 (ATPase) particles[16] are seen projecting from the edges of the flattened vesicles. The clear yellow supernatant becomes progressively turbid down the length of the tube. The supernatant fraction (Fig. 3B) also contains small vesicles resembling ETP. Those vesicles found in the supernatant are, on the average, smaller in diameter than those in the accompanying pellet fraction.

When 0.1 mg deoxycholate per mg protein is added, the resulting pellet fraction (Fig. 4A) consists of membranous sheets or vesicles considerably larger in diameter than the original ETP. Small, indistinct particles are seen in the membrane. F_1 particles are no longer associated with the membrane edges but can be seen in the background either free or attached to membranifibrils[17]. The supernatant fraction (Fig. 4B) contains small vesicles or fragments of membranous sheets. The membranous areas have a finely granular surface rather than a particulate surface as seen in the membranes of the associated pellet fraction.

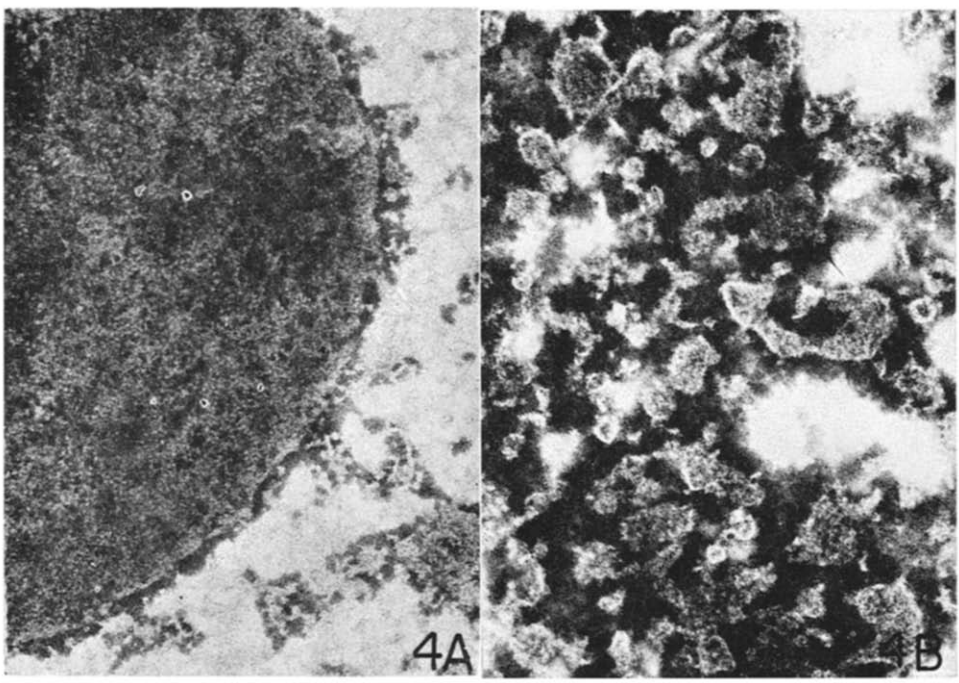

Fig. 4. 0.1 mg deoxycholate per mg protein. A. Pellet. B. Supernatant. × 100000.

At 0.2 mg deoxycholate per mg protein, the pellet fraction (Fig. 5A) consists of large membranous sheets or vesicles having distinct 50-Å particles in the surface. The particles are closely packed and appear randomly arranged. F_1 particles are not detected at the membrane edges or in the background. However, particles of this size, 90 Å, are recognized in the supernatant fraction (Fig. 5B) either free or associated with membranous areas.

At 0.3 mg deoxycholate per mg protein, the membranous areas in the pellet

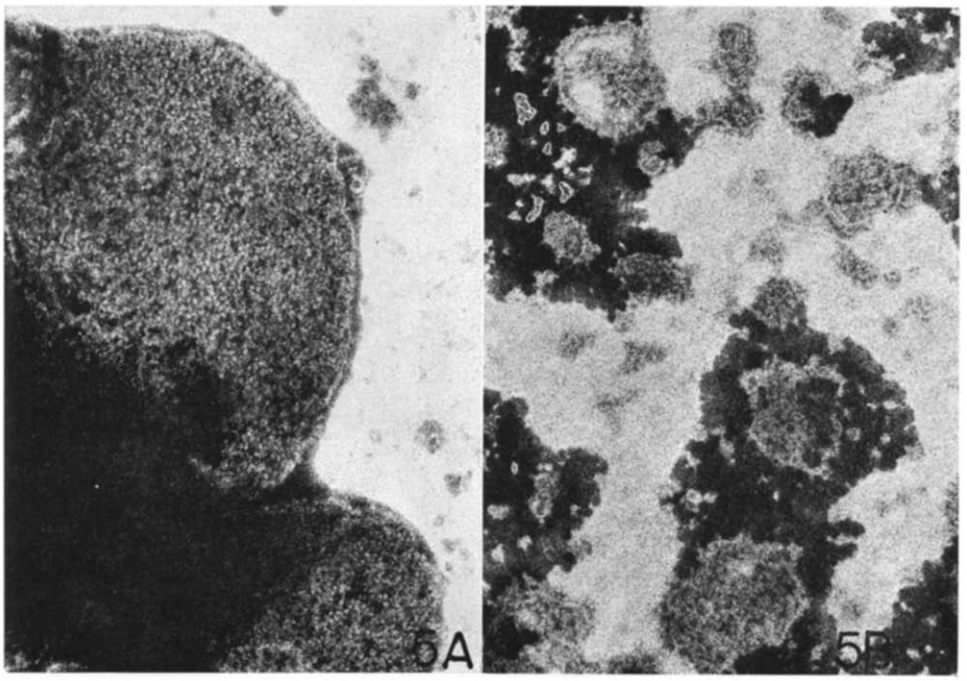

Fig. 5. 0.2 mg deoxycholate per mg protein. A. Pellet. B. Supernatant. × 100 000.

fraction (Fig. 6A) begin to show non-particulate regions or holes. The amount of membranous material in the supernatant fraction (Fig. 6B) is decreased while fibrous structures appear more predominant.

When 0.4 mg deoxycholate per mg protein is used, frayed membranous sheets or incomplete vesicles are seen in the membranous pellet (Fig. 7A). The asymmetrical membranous areas have both smooth, well-defined edges and irregular, disrupted edges suggesting deterioration of the membrane. Closely packed 50-Å particles are visible throughout most of the membrane. These particles are more distinct near the frayed edges of the membrane and are less distinct where smooth edges occur. The supernatant fraction (Fig. 7B) consists of fibrous and membranous or aggregated material. The membranous areas have a finely granular surface and irregular or frayed edges.

At the highest concentration of deoxycholate studied (0.5 mg deoxycholate per mg protein) apparent disorganization of the membranous material in the pellet occurs (Fig. 8A). The membranes have a disrupted appearance revealing fibrous structures. Membranous areas are not detected in the supernatant fraction (Fig. 8B). This fraction consists of loose aggregates of amorphous and particulate material. At higher magnification, the material appears to form a loose interconnected network (Fig. 8C).

Thin sectioning of the pellet fractions at the various deoxycholate concentrations confirms the alterations in the membrane morphology already described following negative staining. In addition, thin sectioning allows for a comparison of thickness and staining patterns as seen in membrane cross sections.

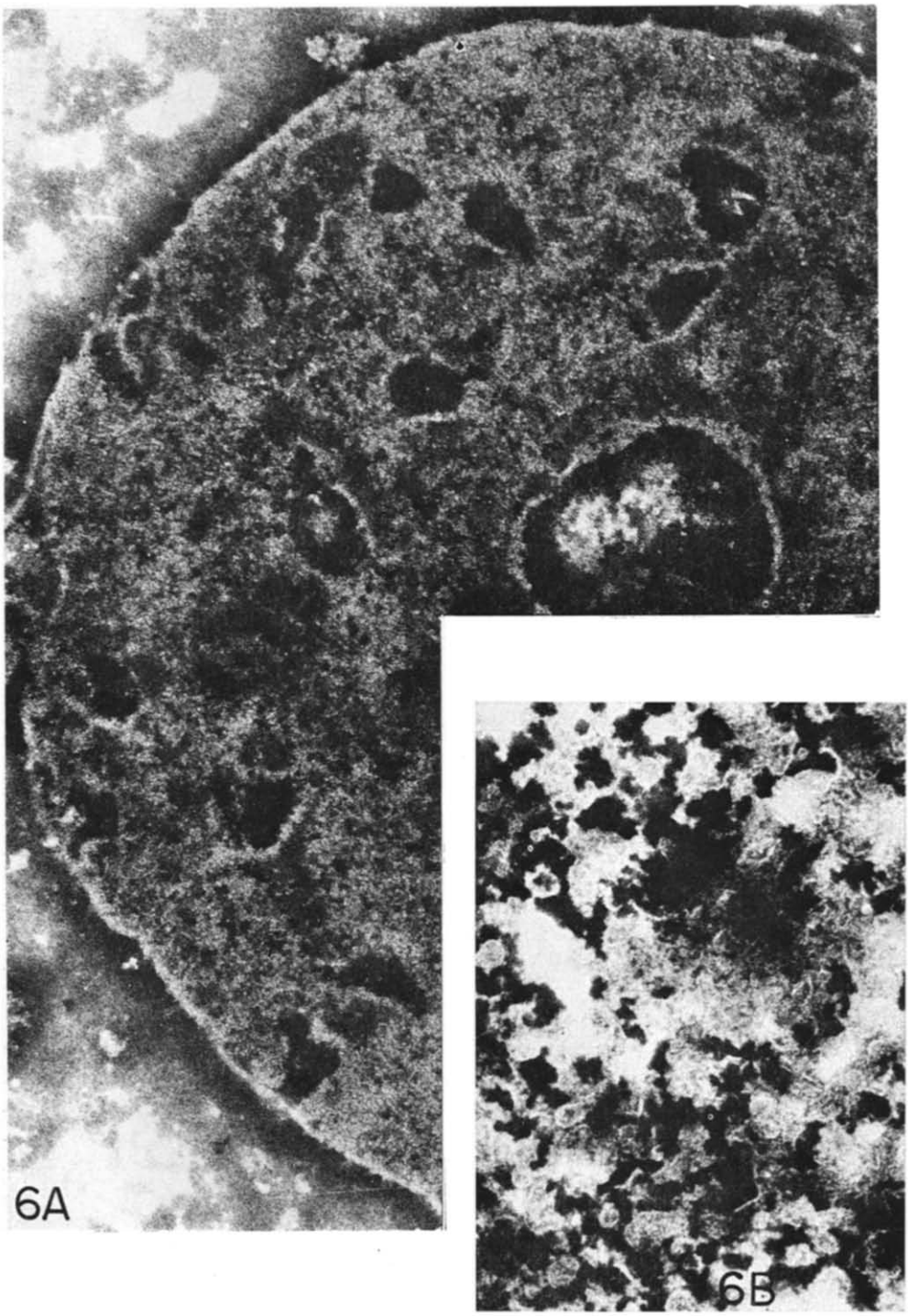

Fig. 6. 0.3 mg deoxycholate per mg protein. A. Pellet. B. Supernatant. × 100 000.

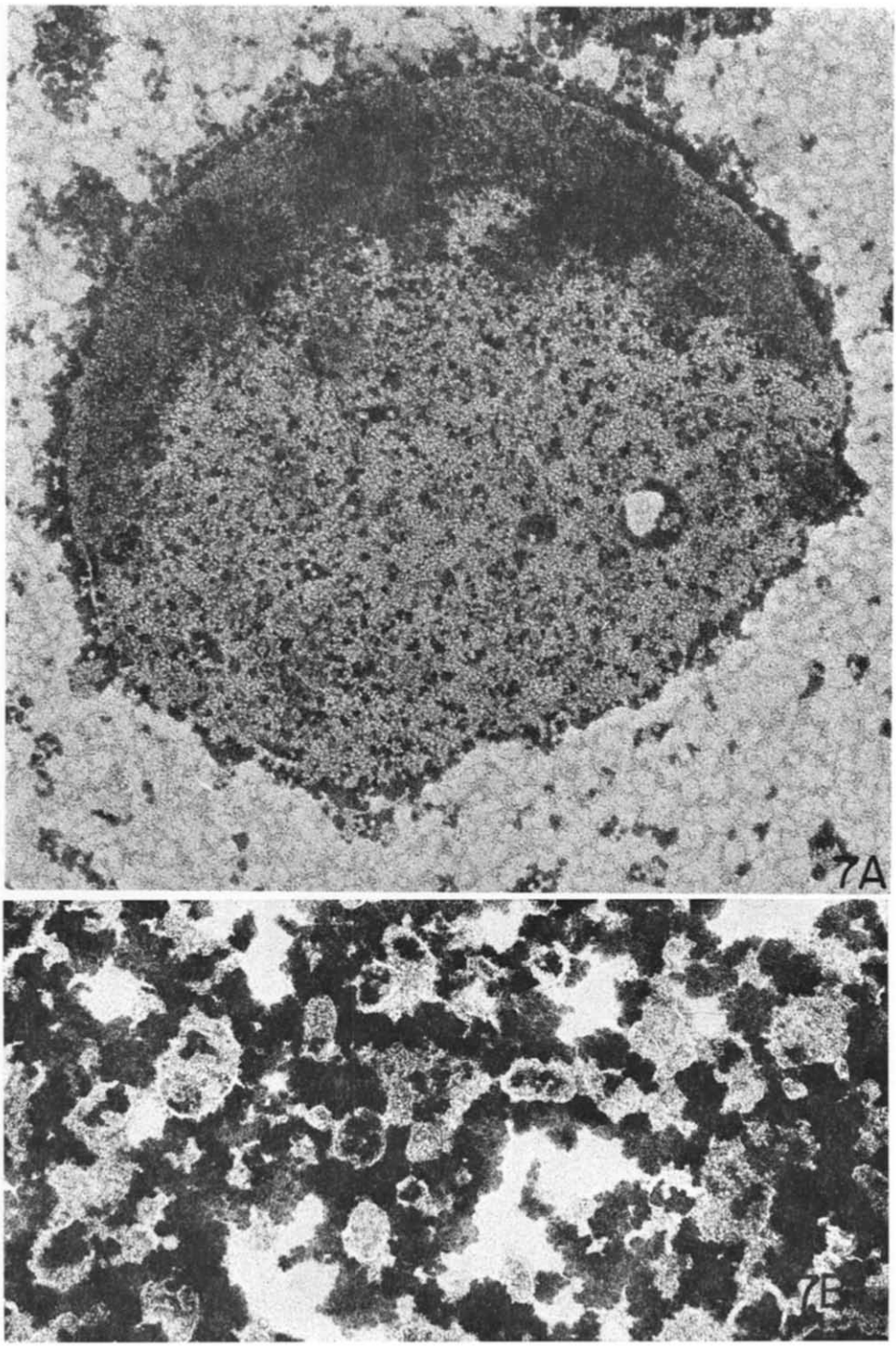

Fig. 7. 0.4 mg deoxycholate per mg protein. A. Pellet. B. Supernatant. × 100 000.

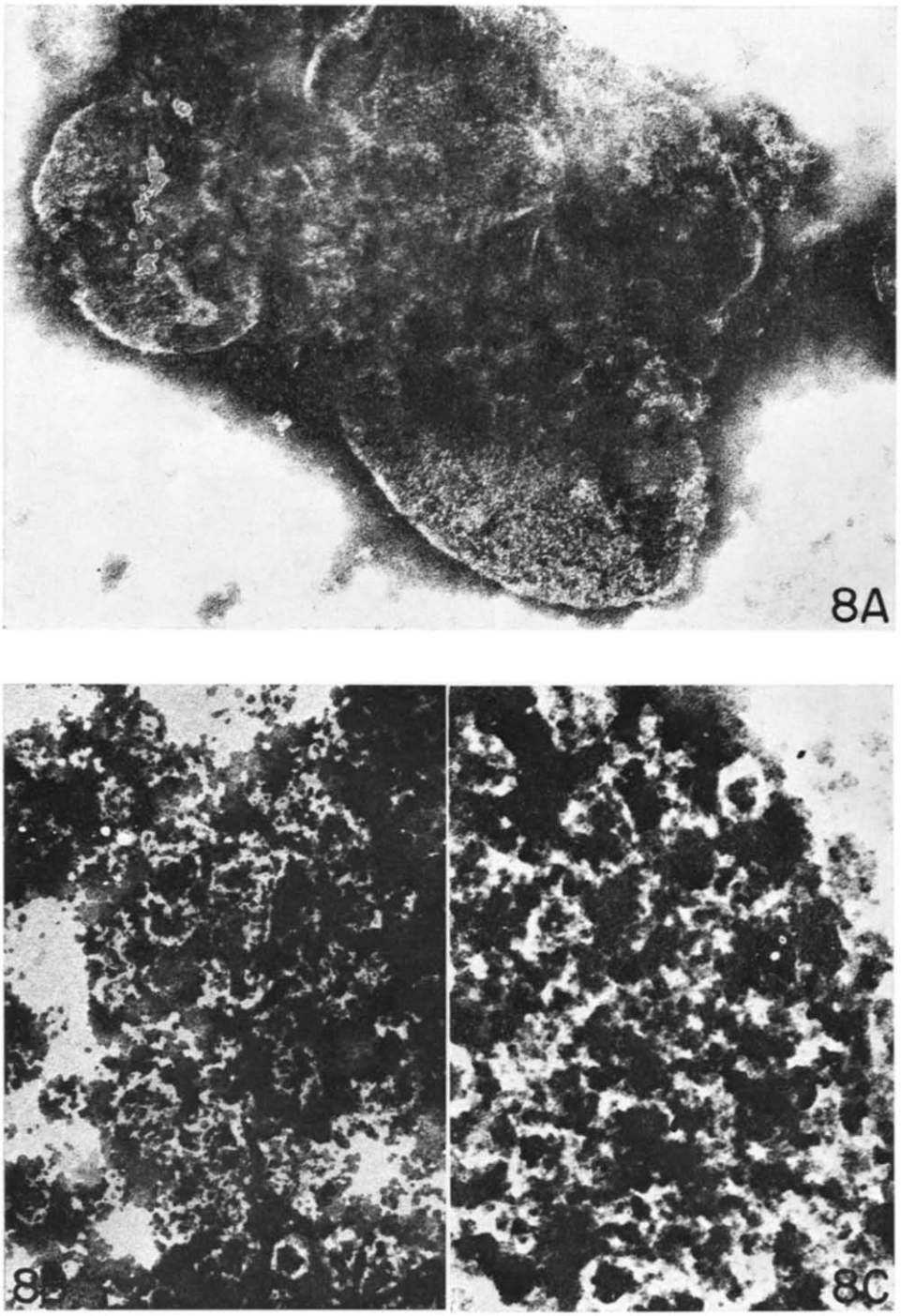

Fig. 8. 0.5 mg deoxycholate per mg protein. A. Pellet. × 100 000. B. Supernatant. × 100 000. C. Supernatant. × 200 000.

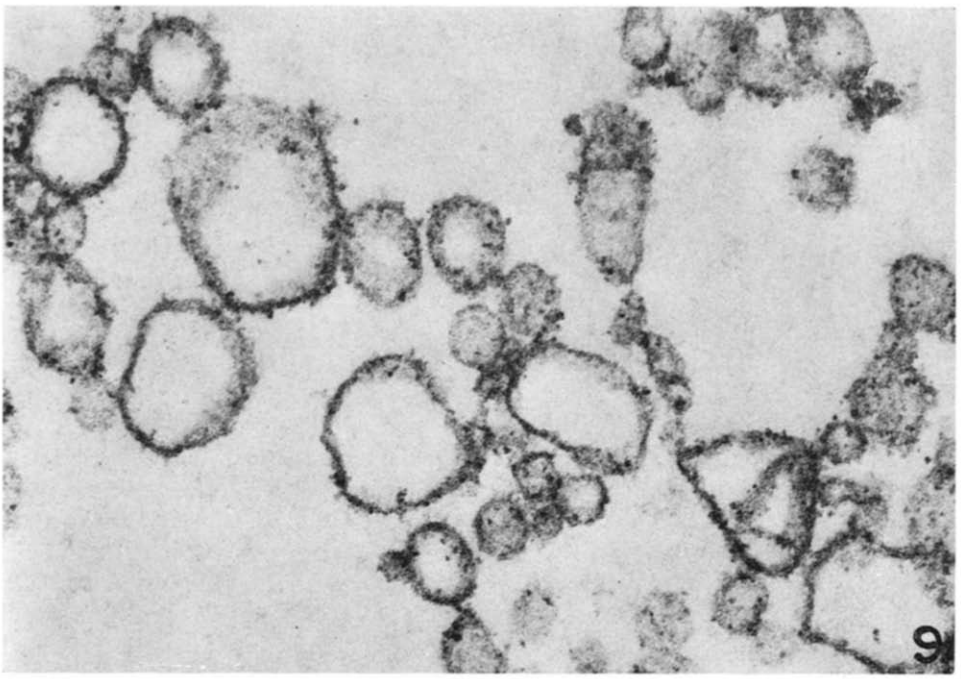

Fig. 9. 0 mg deoxycholate per mg protein. × 100 000.
Figs. 9–13. Thin sections of pellet fractions obtained from deoxycholate-treated ETP in the presence of 1 M KCl. Glutaraldehyde and OsO_4 fixation. Uranyl acetate and lead citrate poststain.

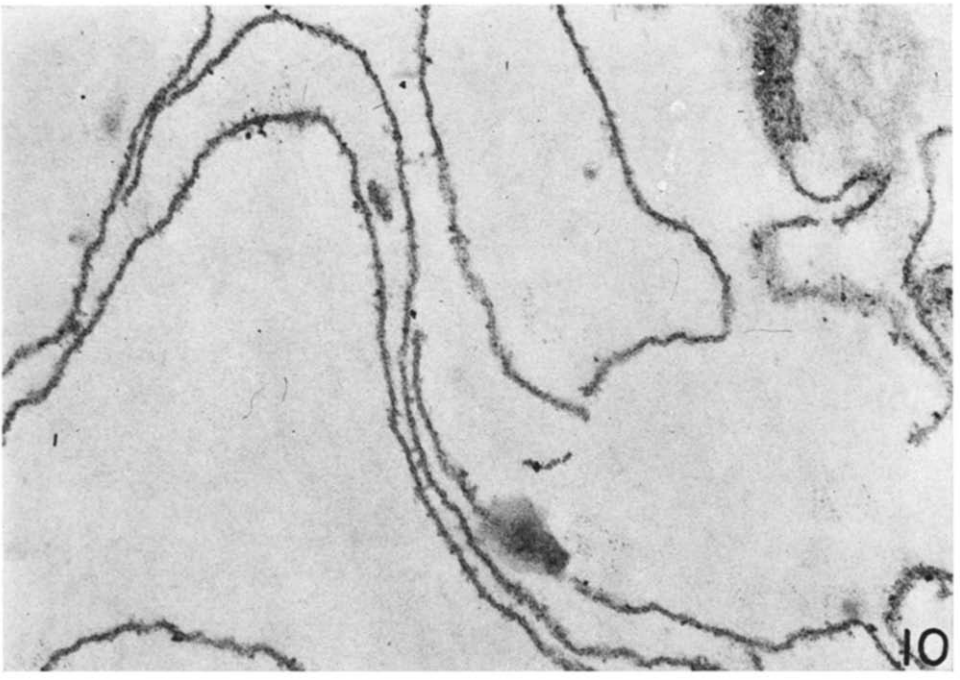

Fig. 10. 0.1 mg deoxycholate per mg protein. × 100 000.

Sectioning of the 105000 × *g* pellet fraction obtained following treatment of ETP with salt but no deoxycholate (Fig. 9) reveals vesicles similar in size and morphology to the original ETP. The single membrane bound vesicles appear empty inside when cut in cross section and appear electron dense throughout all or part of their area when sectioned tangentially. The electron dense granules associated with the membranous areas are not thought to represent the F_1 particles as F_1 particles are not readily demonstrated in sectioned mitochondrial material.

The pellet obtained with 0.1 mg deoxycholate per mg protein and 1 M KCl (Fig. 10) consists of long stretches of single membranes, presumably representing membranous sheets. Often, the free ends of the membranous sheets are diffuse and electron dense throughout their thickness. This could reflect an altered membrane composition or could merely result from a tangential section. Occasional clusters of electron dense, nonmembranous material are also seen. Although the membrane thickness does not vary significantly from that of ETP membranes, membrane structure is more clearly seen. Frequent diffuse regions occur along the length of the membranes. While at first these regions were thought to represent deviations in the angle of sectioning, subsequent micrographs suggest an alternative interpretation.

If ETP is incubated with 0.1 mg deoxycholate per mg protein but not centrifuged, thin sectioning reveals very large, single membrane-bound vesicles. Membranous sheets are seen only after the treated ETP are centrifuged at high speed.

After treatment of ETP with 0.2 mg deoxycholate per mg protein and 1 M KCl, the 105000 × *g* pellet contains large membranous sheets (Fig. 11). These appear as long, single membranes in section. Broad, diffuse areas occur frequently at random intervals along the membranes. Electron-dense clusters of nonmembranous material

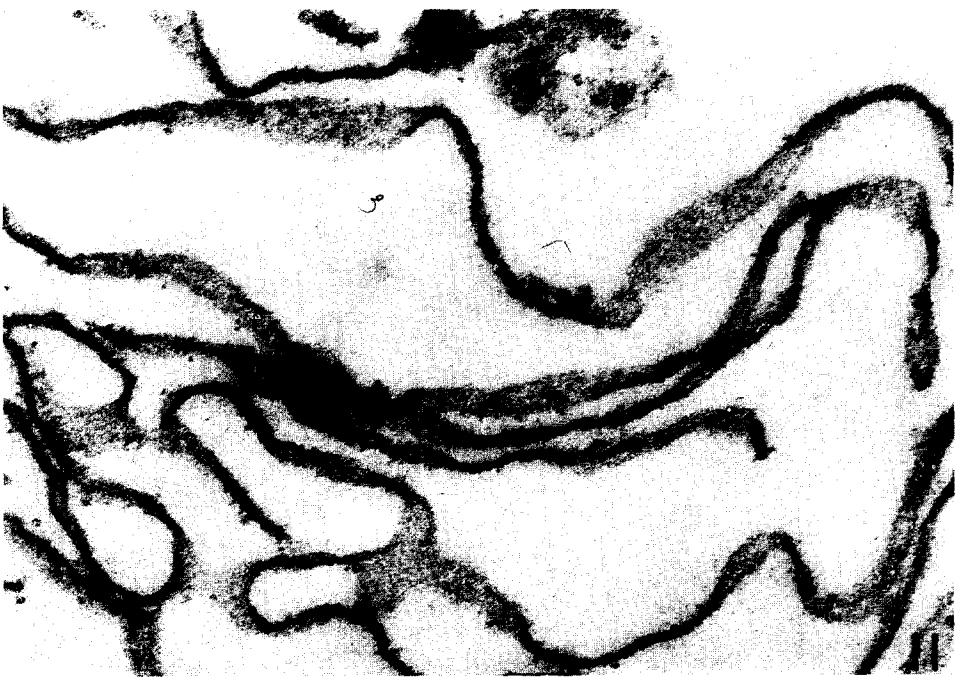

Fig. 11. 0.2 mg deoxycholate per mg protein. × 100000.

are also seen. At several places the membrane appears to be splitting into two thinner membranes (Fig. 12). Each of these thinner membranes shows unit membrane[18] structure, *i.e.* electron-dense layers at both surfaces of the membrane separated by an electron transparent region within the membrane. In addition, these thinner membranes show some indication of globular substructure.

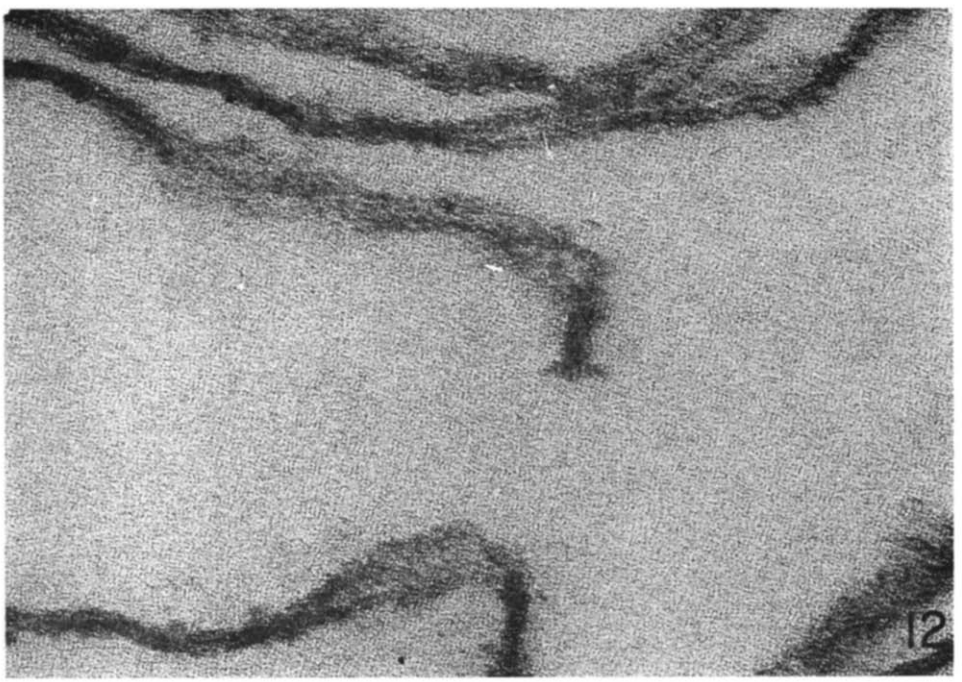

Fig. 12. 0.2 mg deoxycholate per mg protein. × 240 000.

Treatment of ETP with 0.3 mg deoxycholate and 1 M KCl results in a pellet fraction which in section resembles the preceding fraction. However, the lengths of the membrane stretches are shorter and the diffuse areas along the membrane are more frequent. More clusters of electron-dense material are seen. The phenomenon of membrane splitting is occasionally detected.

As the higher concentrations of detergent are used (0.4 mg deoxycholate and 0.5 mg deoxycholate (Fig. 13) per mg protein) the amount and length of membranous material in the pellet continue to decrease. The amount of nonmembranous material and the frequency of diffuse regions along the membrane increase. Even at these highest concentrations of deoxycholate where the majority of membrane components are recovered in the supernatant fraction, the membranous material in the pellet fractions when seen in cross section appears similar in thickness and structure to the pellet membranes obtained at the lowest deoxycholate concentration studied.

When ETP vesicles are treated with high concentrations of deoxycholate so that all of the mitochondrial cytochromes are extracted into the supernatant, a grayish-white residue pellet is obtained upon centrifugation. Examination of this pellet by negative staining reveals amorphous material and fibrous elements forming a

net-like pattern. In thin section no membranous structures are seen. The non-membranous material appears to be arranged in a loosely woven network.

At an optimal concentration of deoxycholate (0.3 mg deoxycholate per mg protein and 1 M KCl) there is a fairly clean separation of the red cytochromes, *b* and c_1, and the green cytochromes, *a* and a_3, into the supernatant and pellet fractions,

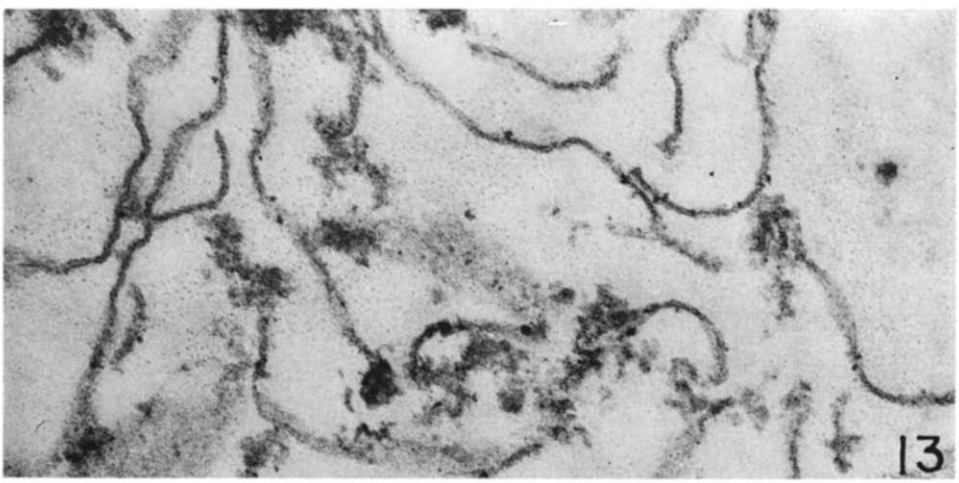

Fig. 13. 0.5 mg deoxycholate per mg protein. × 100 000.

respectively. These two fractions are distinct in their enzymatic properties as well as their cytochrome content. The effect on structure of the removal of deoxycholate from these fractions by dialysis can be followed by negative staining and thin sectioning.

The green pellet membranes (Fig. 14A) show no detectable change upon dialysis (Fig. 14B) when viewed after negative staining. Flattened, symmetrical vesicles or membranous sheets containing 50-Å particles are seen. However, thin sectioning reveals changes in membrane structure upon dialysis. Before dialysis (Fig. 14C) concentric vesicles or irregular lamellar arrangements of membranes are seen. The membranes vary in thickness and have frequent diffuse regions along their lengths. After dialysis (Fig. 14D) the membranes occur in orderly lamellar arrangements or concentric vesicular arrangements. Diffuse regions along the membranes are no longer seen except where it is clearly a tangential section of the membrane. The membranes are uniform in thickness, about 75 Å, and thinner than the original membranes. Unit membrane structure is clearly seen.

Dialysis of the red supernatant fraction (Fig. 15A) results in the formation of a flocculent red precipitate which is collected by centrifugation. Negative staining of this red pellet (Fig. 15B) reveals flattened vesicles having irregular outlines. The vesicular nature is confirmed by thin sectioning. Both smooth and particulate regions occur in the membranes. Occasional groups of 90-Å particles are attached to the edges of the membranes.

Thin sectioning of the red supernatant fraction before dialysis (Fig. 15C) reveals diffuse linear structures possibly representing membrane fragments. Particulate and nonmembranous material is also seen. After dialysis (Fig. 15D) the red

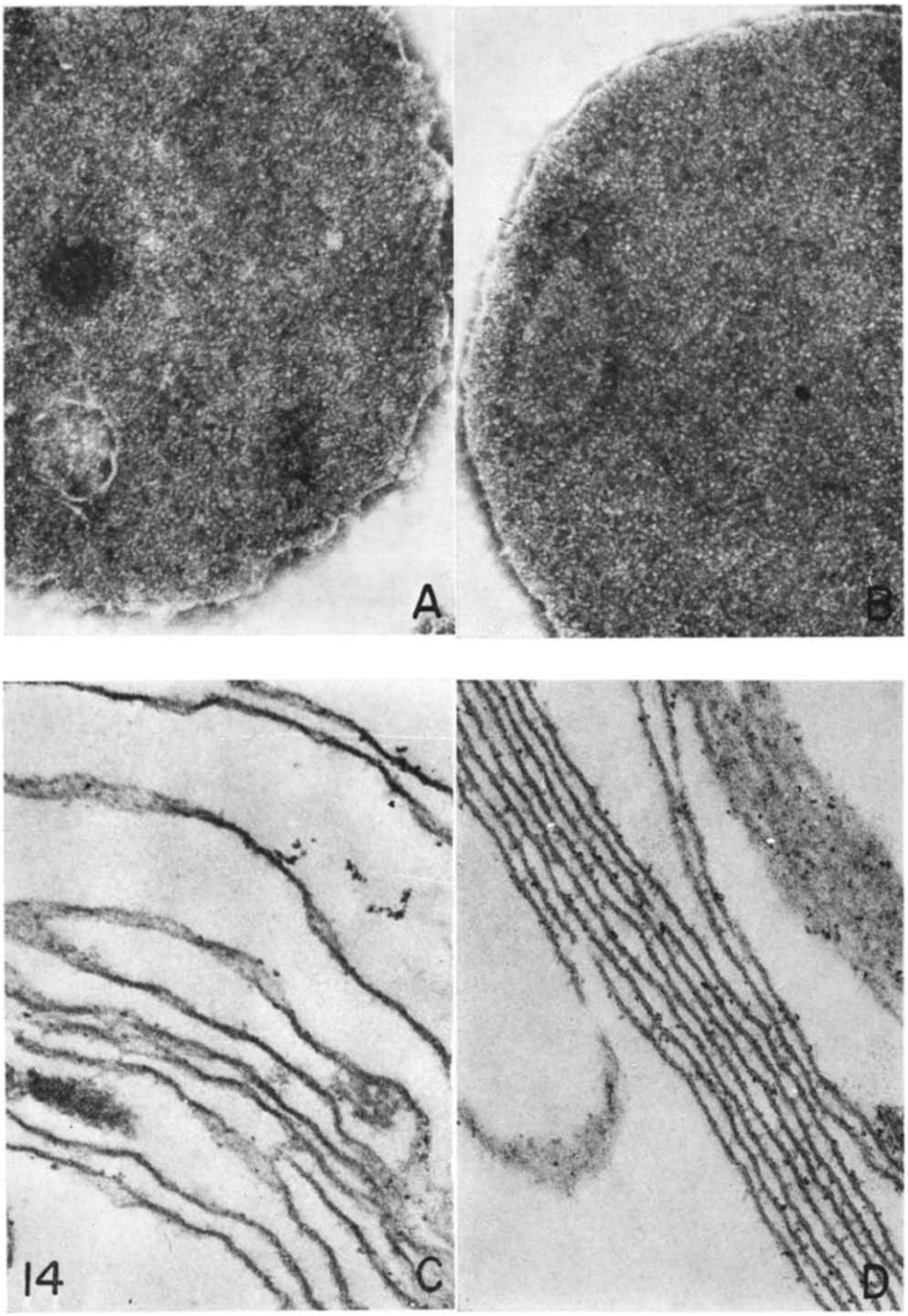

Fig. 14. Green pellet obtained at 0.3 mg deoxycholate per mg protein in the presence of 1 M KCl A. Not dialyzed. Phosphotungstate stain. B. Dialyzed. Phosphotungstate stain. C. Not dialyzed Thin section. D. Dialyzed. Thin section. × 100 000.

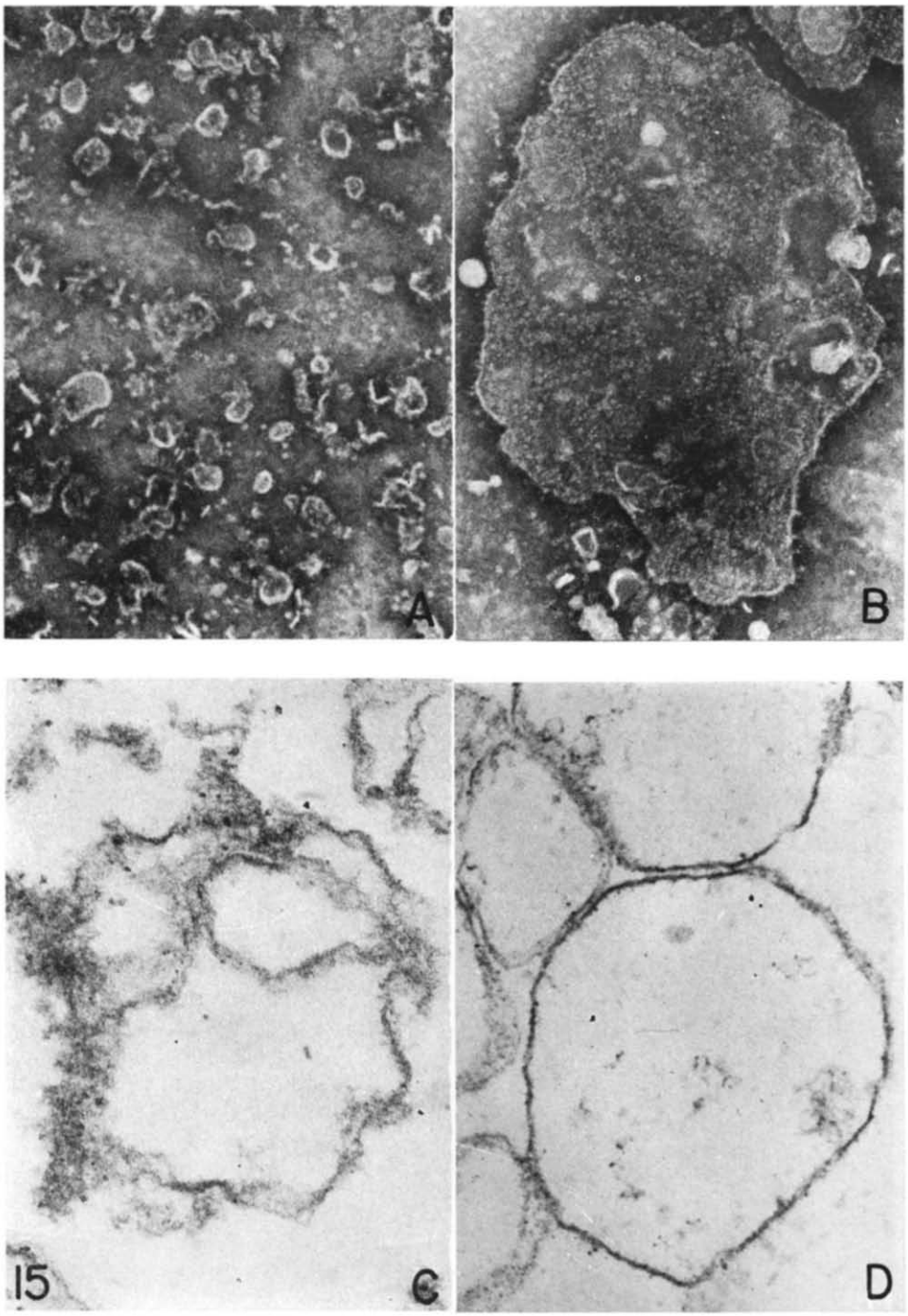

Fig. 15. Red supernatant obtained at 0.3 mg deoxycholate per mg protein in the presence of 1 M KCl. A. Not dialyzed. Phosphotungstate stain. B. Dialyzed. Phosphotungstate stain. C. Not dialyzed. Thin section. D. Dialyzed. Thin section. × 100 000.

pellet consists of single membrane bound vesicles of varying size. Rarely are concentric vesicles seen. The membranes are thin, about 50 Å, and stain uniformly throughout their thickness.

DISCUSSION

Exposure of mitochondrial cristae membranes to low concentrations of deoxycholate selectively extracts some membrane components into the supernatant fraction while leaving a membranous residue pellet. The membranes in the pellet do not appear to be reaggregation products. Although the possibility of membrane reorganization cannot be eliminated, drastic reorganization is precluded by the fact that the separate fractions retain characteristic electron transport functions.

Electrophoresis and cytochrome analysis support the sequential extraction of specific components from the membrane. This difference in extraction patterns has been interpreted by KLINGENBERG[19] as reflecting differences in the binding and/or accessibility of the various components of the electron transport chain.

The observed changes in ETP membrane structure following treatment with deoxycholate suggest the mode of action of deoxycholate on the membrane. Low concentrations of deoxycholate and salt, without centrifugation, results in the formation of vesicles larger than the starting ETP vesicles. Thus, the detergent allows for or causes the breakage and fusion of membranes. When these treated vesicles are subject to high-speed centrifugation membranous sheets are formed. This suggests that the treated membranes are more fragile than the untreated ETP membranes.

Deoxycholate appears to be incorporated into the membranes as suggested by the increase in diffuse regions along the membranes of the pellet with increasing deoxycholate concentration. Support for this comes from the observation that dialysis, which removes deoxycholate, leads to the disappearance of these diffuse regions. STOECKENIUS[20] has suggested that bile salts such as deoxycholate can be taken up preferentially into the lipid layers of membranes.

The fact that membrane length in the pellet fraction decreases as deoxycholate concentration is increased suggests that (1) the membranes are being progressively solubilized at their edges or (2) localized regions are being solubilized while the surrounding areas fuse together into a more tightly packed membrane. The biochemical data showing selective extraction of components favors the latter process.

The apparent splitting of the membrane into two thinner membranes as seen by thin sectioning in Fig. 12 suggests a separation of membrane components along natural division lines. The possibility that this represents an optical artifact of oblique sectioning cannot be eliminated entirely. However, one would expect to see an artifact with equal frequency at all levels of deoxycholate concentration as well as in untreated ETP. This is not the case. Since the deoxycholate concentration at which this phenomenon is observed corresponds to a level at which there is a partial separation of cytochromes b and c_1 from cytochromes $a + a_3$, it seems likely that these cytochromes are located on different sides of the membrane and may even occupy separate layers within the membrane. The asymmetrical location of mitochondrial cytochromes within the cristae membranes has been previously proposed on the basis of permeability studies[19], and antibody and diazobenzene sulfonate labeling[21].

GREEN[3] has postulated the existence of four biochemically active electron

transfer complexes which are the morphological units of the mitochondrial membrane. It has been shown that each of these isolated complexes has the capacity to form membranes, thus supporting the role of the complexes as membrane forming elements. However, the dispersed state of the complex was shown only by negative staining. Our studies indicate that the red supernatant fraction is not totally dispersed into morphological subunits. Both negative staining and thin sectioning reveal the presence of membrane fragments in this fraction.

Treatment of mitochondrial cristae membranes with an optimal concentration of deoxycholate and salt results in the separation of red and green cytochromes into two distinct functional fractions. The fractions differ in the following respects: the characteristic shape and arrangement of vesicles, the appearance and dimensions of the membranes in section, the distribution and size of particles observed in the membrane with negative staining, the major electrophoretic protein bands, the exposed freeze–etch surfaces[22], and the associated electron transport functions[22].

The splitting phenomenon observed in deoxycholate-treated ETP membranes, the apparent stripping of a membranous layer into the red supernatant fraction, and the formation of two distinct membranous fractions are consistent with the existence of two separate layers within the mitochondrial cristae membrane as previously proposed for a binary membrane structure[22].

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