Br. J. exp. Path. (1969) **50**, 200.

MITOCHONDRIAL ANTIBODIES IN PRIMARY BILIARY CIRRHOSIS

II. THE COMPLEMENT FIXING ANTIGEN AS A COMPONENT OF MITOCHONDRIAL INNER MEMBRANES

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Received for publication November 6, 1968

SUMMARY.—The autoantibodies found in the sera of patients with primary biliary cirrhosis have been shown to react with a component of the mitochondrial inner membranes. Outer membranes were inactive. The purity of the inner and outer membrane fractions obtained by 2 different methods was assessed by electron microscopy and marker enzyme tests. Using negative-staining it was possible to visualize antibody binding to mitochondrial membranes. At high resolution it could be seen that the 90Å particles on the cristal membranes were not involved in the reaction with antibody, but it was not possible to establish in the present studies the precise antigenic site upon the mitochondrial inner membranes.

In previous reports it was shown that an antibody reacting with mitochondria from various tissues and animal species, is present in the serum of patients with primary biliary cirrhosis (PBC) (Walker, Doniach, Roitt and Sherlock, 1965; Doniach, Roitt, Walker and Sherlock, 1966; Goudie, Macsween and Goldberg, 1966). Evidence was obtained for the association of the antigen with mitochondrial membranes (Berg, Doniach and Roitt, 1967*a*; Berg, Muscatello, Roitt and Doniach, 1967*b*). The higher antigen content and brighter immunofluorescence in tissues known to have mitochondria with well developed cristae and the good correlation of these reactions with succinic dehydrogenase activity further suggested that the antigen was associated with the inner membranes. To provide more direct evidence for these findings, outer and inner membranes were separated from rat liver mitochondria by 2 different procedures, identified by electron microscopy and enzyme markers and assayed for antigen by complement fixation. Attempts were made to visualize the binding of the antibody to mitochondrial structures by electron microscopy of negatively stained preparations.

MATERIALS AND METHODS

Quantitative complement fixation (CF).—The CF test was performed as previously described (Berg *et al.*, 1967a) using the standard serum from a primary biliary cirrhosis patient (L.H.) having a high titre in the complement fixation test.

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Separations of outer and inner membranes.—This was accomplished by density gradient centrifugation following osmotic swelling of mitochondria (Parsons, Williams and Chance, 1966) or by the method of Sottocasa, Kuylenstierna, Ernster and Bergstrand, (1967) in which swelling and contraction of the mitochondria is followed by ultrasonication and separation of the membranes on sucrose gradients.

Enzyme assays.—Rotenone insensitive succinate-cytochrome *c* reductase and NADH-cytochrome *c* reductase were assayed spectrophotometrically (Sottocasa *et al.*, 1967). Succinate neotetrazolium reductase was estimated according to Slater and Planterose (1960). The succinate- and NADH-dependent reduction of dichlorophenolindophenol (DCPIP-reductase) was followed at 600 mμ (Cunningham, Crane and Sottocasa, 1965). The method of Schnaitman, Erwin and Greenawalt (1967) was employed for the measurement of monoamine oxidase.

Phospholipid estimations.—The phospholipid analysis was kindly undertaken by Dr. R. M. C. Dawson of the Agricultural Research Council Institute of Animal Physiology, Babraham (Dawson, 1960). The lipid phosphorus content was measured by the method of Bartlett (1959).

Electron microscopy.—Specimens prepared for examination in the electron microscope were negatively stained according to the methods previously described (Horne and Whittaker, 1962; Whittaker, 1963). Preparations were mounted either in a 2 per cent solution of potassium phosphotungstate, pH 7·0 or 2 per cent ammonium molybdate solution, pH 7·2. Sucrose was removed from the samples to be negatively stained by dialysis overnight at 0° against isotonic phosphate buffer, pH 7·2. When the effect of addition of antibody on a mitochondrial structure was to be studied, mitochondrial fractions were incubated in the presence of different dilutions of antibody for 1 hr at 30° in isotonic buffer or in CFT buffer. The fractions were centrifuged in the cold, washed twice with isotonic phosphate buffer and twice with distilled water and then processed for negative staining in the manner of the untreated controls. The specimens were examined in a Siemens Elmiskop I electron microscope and an A.E.I. EM6B electron microscope operating at instrumental magnifications within the range of X10,000–100,000. For maximum resolution of detail at high magnification it was found necessary to mount negatively stained specimens on to holey films of the type described by Huxley and Zubay (1960) and Harris (1962).

RESULTS

Separation of outer and inner membranes

Morphology.—The presence of the elementary particles described by Fernandez-Moran (1962) observed in negatively stained preparations, was used as a criterion for the identification of inner membrane fragments. The electron micrographs illustrated in Figs. 1 and 2 show the appearance of the outer and inner membranes of mitochondria isolated by the methods of Sottocasa *et al.* (1967) and that of Parsons *et al.* (1966) respectively. The outer membrane fractions were composed mainly of vesicles having smooth surface profiles but varying in shape and size (Figs. 1a and 2a). The inner membrane fractions consisted mainly of spheres and fragmented cristae on the surface of which the characteristic 90Å particles were clearly seen (Figs. 1b and 2b). Both procedures permitted a satisfactory separation of the 2 membranes.

The outer membrane fractions showed variable contamination with microsomes and although this appeared to be minor in low power fields (*e.g.* Fig 3) attention was paid to establishing the degree of contamination by differential counts of outer membrane and microsomal vesicles in random fields. The microsomes could be distinguished by their thick edges and generally smaller size (Figs. 4 and 5). At high magnifications the difference between the smooth profiles of outer membranes and the distinctive projecting small particles of the microsomal vesicles is apparent (Figs, 5 and 6). Eleven randomly selected fields from the outer membrane fraction prepared by Parsons' method (Exp. II Table II) were

examined. Of 1233 membrane fragments counted, 86 ± 6 per cent (S.D.) had the morphology of outer membranes, the others being mostly microsomes with some small vesicles which could not be identified owing to lack of penetration of the negative stain. The area of the outer membranes as estimated by planimetry was approximately 17 times greater than that of the contaminating vesicles. Fragments of inner membrane cristae were occasionally seen and were more numerous in fractions obtained by Sottocasa's method.

Enzyme tests.—In preliminary experiments, the outer and inner membrane fractions obtained by the two methods were analysed for succinate-cytochrome *c* reductase and rotenone insensitive NADH-cytochrome *c* reductase, generally considered as markers for inner and outer membranes respectively. Table I

TABLE I.—*Comparison of Enzyme Activities of Outer and Inner Mitochondrial Membrane Fractions*

	Parsons *et al.*		Sottocasa *et al.*	
Fractions	Succinate-cyt. *c* reductase μmoles cyt. *c* red./min/mg.	Rotenone insensitive NADH-cyt. *c* reductase	Succinate-cyt. *c* reductase μmoles cyt. *c* red./min./mg.	Rotenone insensitive NADH-cyt. *c* reductase
Input	0·165	0·410	0·115	0·189
Outer membrane	<0·001	13·4	0·065	9·82
Inner membrane	0·236	0·392	0·226	0·630

shows that the inner membrane subfraction contained almost all the succinate-cytochrome *c* reductase. Most of the rotenone insensitive NADH-cytochrome *c* reductase was present in the outer membrane preparation. Similar enzyme distributions were obtained in subsequent studies on antigen localization, using in addition succinate-DCPIP and -neotetrazolium reductases as inner membrane markers (Table II). In one experiment outer membranes were further identified by assay of monoamine oxidase activity.

TABLE II.—*Localization of Complement Fixing Antigen to Mitochondrial Inner Membranes: Correlation with Enzyme Activities*

Membrane separation according to:	Exp. No.	Enzyme assays	Gradient fractions: Input	Outer membrane	Inner membrane
Parsons *et al.*	I	Succinate neotetr. reduct.	0·19	0·005	0·24
		Antigen	65·0	<1·0	65·0
	II	Succinate neotetr. reduct.	0·06	0·017	0·204
		Antigen	5·4	<1·0	12·5
Sottocasa *et al.*	III	Succinate-cyt. *c* reduct.	—	0·20	0·35
		Monoamine oxidase	—	1·85	0·60
		Antigen	—	35·0	73·0
	IV	Succinate-DCPIP reduct.	—	0·12	0·23
		NADH-cyt. *c* reduct. (rot. insens.)	—	6·57	0·78
		Antigen	—	<1·0	212·0

All values are expressed as units/mg. protein except for succinate- and NADH-cytochrome *c* reductase which are given as μmoles cyt. *c* reduced/min./mg. protein.

Distribution of CF antigen in outer and inner membranes.—With both separation methods the CF antigen was largely confined to the inner membrane fractions although the antigenic specific activity varied considerably from one experiment to the other (Table II). With Parsons' method the activity of the inner membranes was equal to or greater than that of the gradient input whereas the antigen was barely detectable in the outer membrane fraction. The degree of contamination with inner membrane as evidenced by succinate dehydrogenase values and by morphological criteria was low. In contrast the fractions obtained with Sottocasa's procedure were more highly contaminated with inner membranes but nontheless the antigenic specific activity was low compared with that of the inner membrane fraction. Dissociation of antigen from succinate dehydrogenase activity as seen in the outer membrane fraction of Experiment IV has also been observed in subsequent studies following ultrasonic fragmentation of mitochondria (Berg, Roitt, Doniach and Horne, 1969).

Effect of antibody on fragmented mitochondria

Mitochondria were disrupted by swelling and sonication (Berg *et al.*, 1969) and the supernatant obtained after spinning at 50,000 r.p.m. for 40 min. (SN 50/0·7) was centrifuged for 14 hr at 25,000 r.p.m. on a 29–45 per cent sucrose gradient. A purified antigen-rich fraction was obtained in band 4 corresponding to a density of 1·141. On addition of immunoglobulin-G (IgG) from the standard PBC serum, a pellet containing 12 per cent protein was formed, with a corresponding loss of protein in the antigen band (Table III). In contrast, addition of

TABLE III.—*Effect of Mitochondrial Antibodies on Sedimentation of Antigen Rich Subfraction (SN 50/0·7)*

Sucrose gradient fractions	Per cent distribution Antigen SN 50/0·7 alone	Per cent distribution Protein SN 50/0·7 alone	Per cent distribution Protein SN 50/0·7 +Normal IgG	Per cent distribution Protein SN 50/0·7 +Antibody IgG	Per cent distribution Rot. insens. NADH cyt. *c* reductase SN 50/0·7 alone	Per cent distribution Rot. insens. NADH cyt. *c* reductase SN 50/0·7 +Normal IgG	Per cent distribution Rot. insens. NADH cyt. *c* reductase SN 50/0·7 +Antibody IgG
1	0	10	2	9	17	5	19
2	0	19	17	15	28	27	22
3	48	51	64	56	44	55	47
4	52	*12*	*11*	2	7	6	8
5	0	0	1·7	0	0	1	0
6	0	5	1	4	2	2	2
7	0	3	2	3	2	3	1
Pellet	No pellet	0	2	*12*	0	1	1

Input: 14 mg. of mitochondrial protein either alone or with 10 mg. of appropriate IgG fraction. Centrifuged in SW 25 head at 25,000 r.p.m. for 14 hr. (Spinco L2).

normal IgG caused no significant loss of protein from this band. The distribution of rotenone-insensitive NADH cytochrome *c* reductase in these fractions was not altered, showing that outer membranes are not involved in the reaction with antibody. Total phospholipid was assayed to estimate the amount of membrane material in the pellet sedimented by the addition of the two IgG preparations. The antibody pellet contained 5 times more phospholipid than the corresponding control. In a parallel experiment, using fluorescein-labelled IgG from a PBC and a normal serum, only the PBC fluorochrome sedimented in the pellet, further

suggesting that immune complexes were formed involving the mitochondrial antigen.

Phospholipid composition of purified antigen fraction

The antigen-rich subfraction 4 obtained after gradient centrifugation of SN 50/0·7 (cf. Table III), was analysed for phospholipid composition. Comparison of the results with data published by Parsons, Williams, Thompson, Wilson and Chance (1967) for purified outer and inner membranes further indicated the association of the antigen with inner membranes (Table IV).

EXPLANATION OF PLATES

FIGS. 1–9.—Electron micrographs of negatively stained unfixed preparations of rat liver mitochondria and subfractions. Preparations for Figs. 1, 2 and 9 were stained with 2 per cent potassium phosphotungstate and the remainder with 2 per cent ammonium molybdate.

FIG. 1a.—Typical appearance of outer membrane fraction from mitochondria prepared by the method of Sottocasa *et al.* (1967). The fraction appears to consist mainly of partially collapsed vesicles of different shapes and sizes, with a typically smooth surface (OM). No projecting 90 Å particles are seen. There is appreciable contamination with microsomes (ER). × 60,000.

FIG. 1b.—Inner membrane fraction prepared by the method of Sottocasa *et al.*, showing cristae on the surface of which the characteristic 90 Å projections can be recognized. × 100,000.

FIG. 2a.—Outer membrane fraction prepared from mitochondria by the method of Parsons *et al.* (1966). The vesicles resemble those obtained by Sottocasa's method (cf. Fig. 1a). Some contamination by microsomes (ER) and inner membrane fragments (IM) is present. × 40,000.

FIG. 2b.—Inner membrane fraction isolated by the method of Parsons *et al.* The morphology of the membranes closely resembles that seen in Fig. 1b. × 100,000.

FIG. 3.—Representative low power view of outer membrane fraction obtained by Parson's method consisting mainly of typical outer membrane vesicles. There is only a minor degree of contamination with microsomes (ER). × 20,000.

FIG. 4.—High power view of liver microsomal fraction showing small membrane structures with thickened edges. × 80,000.

FIG. 5.—High power view of outer membranes with smooth profiles compared with microsomal vesicles showing an array of regular projections on their surface. × 60,000.

FIG. 6.—Comparison of outer membrane and microsomal vesicles at very high magnification. The regularly spaced array of particles lining the surface of the microsomes contrasts with the smooth profile of the outer membrane. × 200,000.

FIG. 7a.—Mitochondrion treated with serum from normal individual. The mitochondrion appears as a large sphere in which the infoldings of the cristae are seen. The 90 Å particles can be clearly recognized on the surfaces of the cristae. × 46,000.

FIG. 7b.—Same mitochondrial preparation treated with serum from patient (L.H.) with primary biliary cirrhosis. The general organization of the mitochondrion appears unchanged, but penetration of negative stain is greatly reduced so that the structural details of the surface are obscured. The 90 Å particles are poorly resolved. × 46,000.

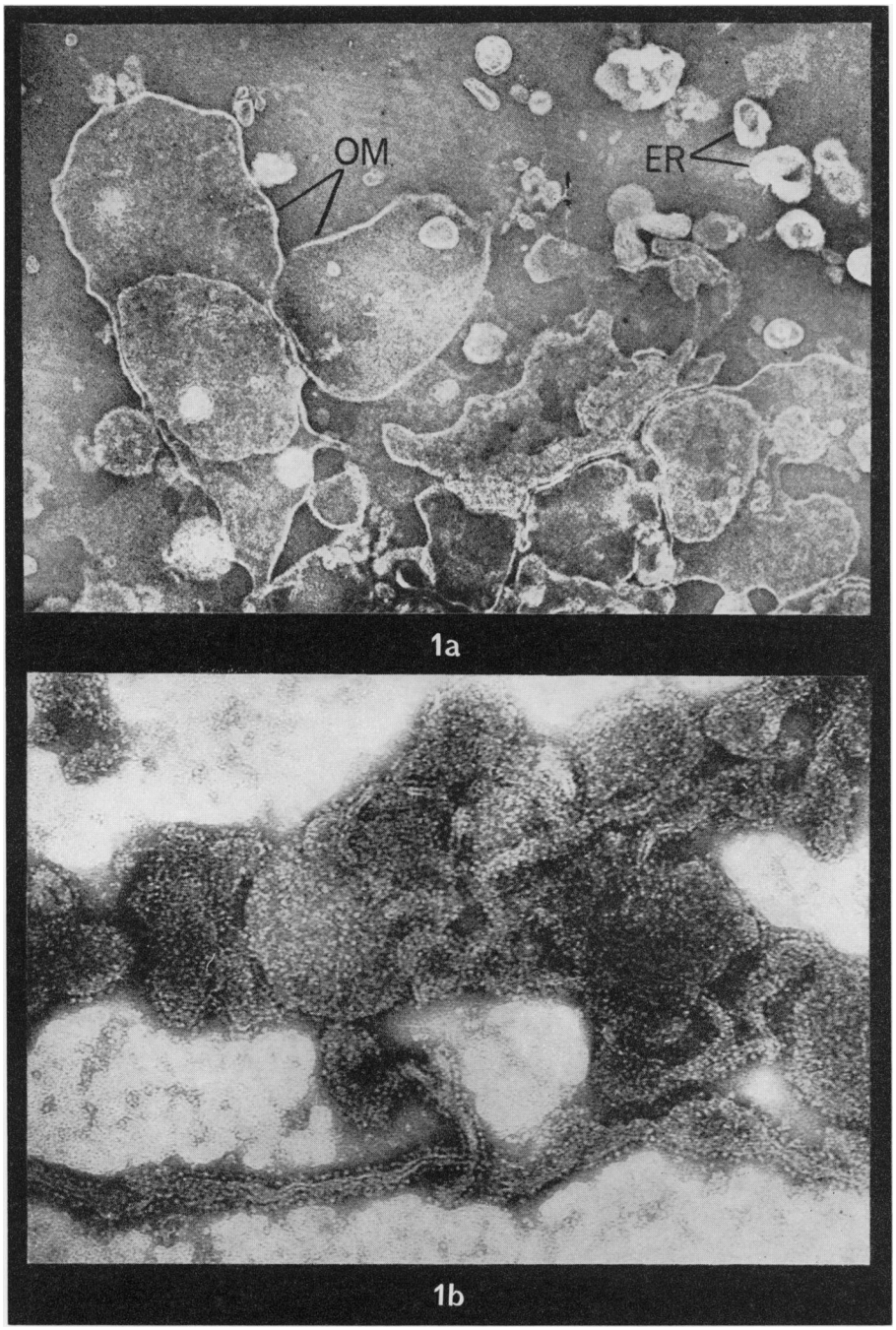
FIG. 8a.—Mitochondrion treated with control serum from patient with jaundice due to extra-hepatic obstruction. The negative stain has penetrated to the same degree as in the normal serum control in Fig. 7a. × 46,000.

FIG. 8b.—Same mitochondrial preparation as used for extra-hepatic jaundice control, treated with serum from patient (C.W.) with primary biliary cirrhosis. The degree of stain was markedly reduced. × 46,000.

FIG. 9a.—High magnification of a crista from an untreated mitochondrion. The unit membrane structure and the 90 Å particles projecting from the surface, are clearly seen. × 200,000.

FIG. 9b.—Fragment of mitochondrial crista treated with normal serum. The appearance of the 90 Å particles as well as that of the unit membrane is unchanged relative to the untreated control. × 200,000.

FIG. 9c.—Crista from mitochondria treated with PBC serum. The unit membrane of the crista is unresolved, although the 90 Å particles are still visible. × 200,000.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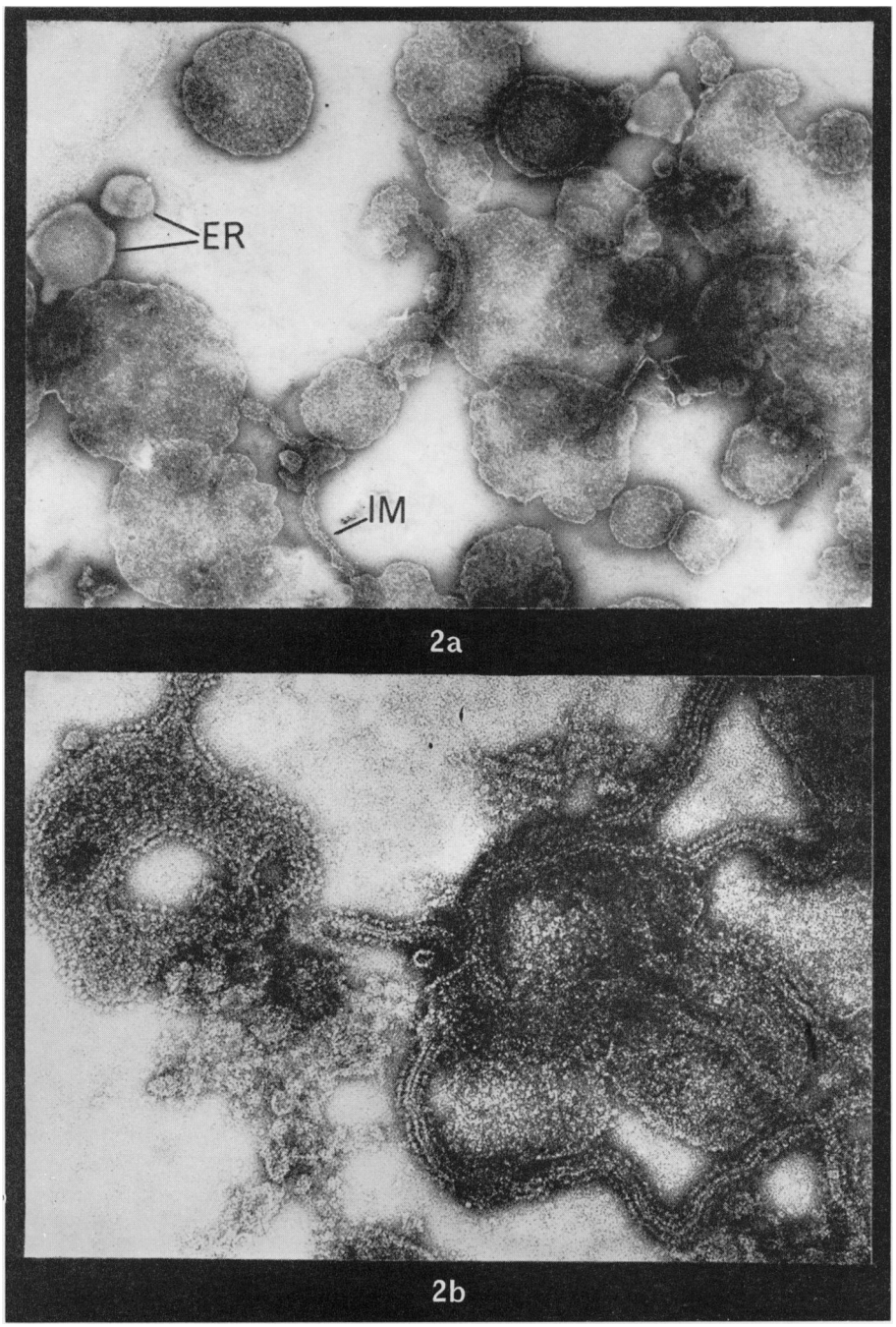
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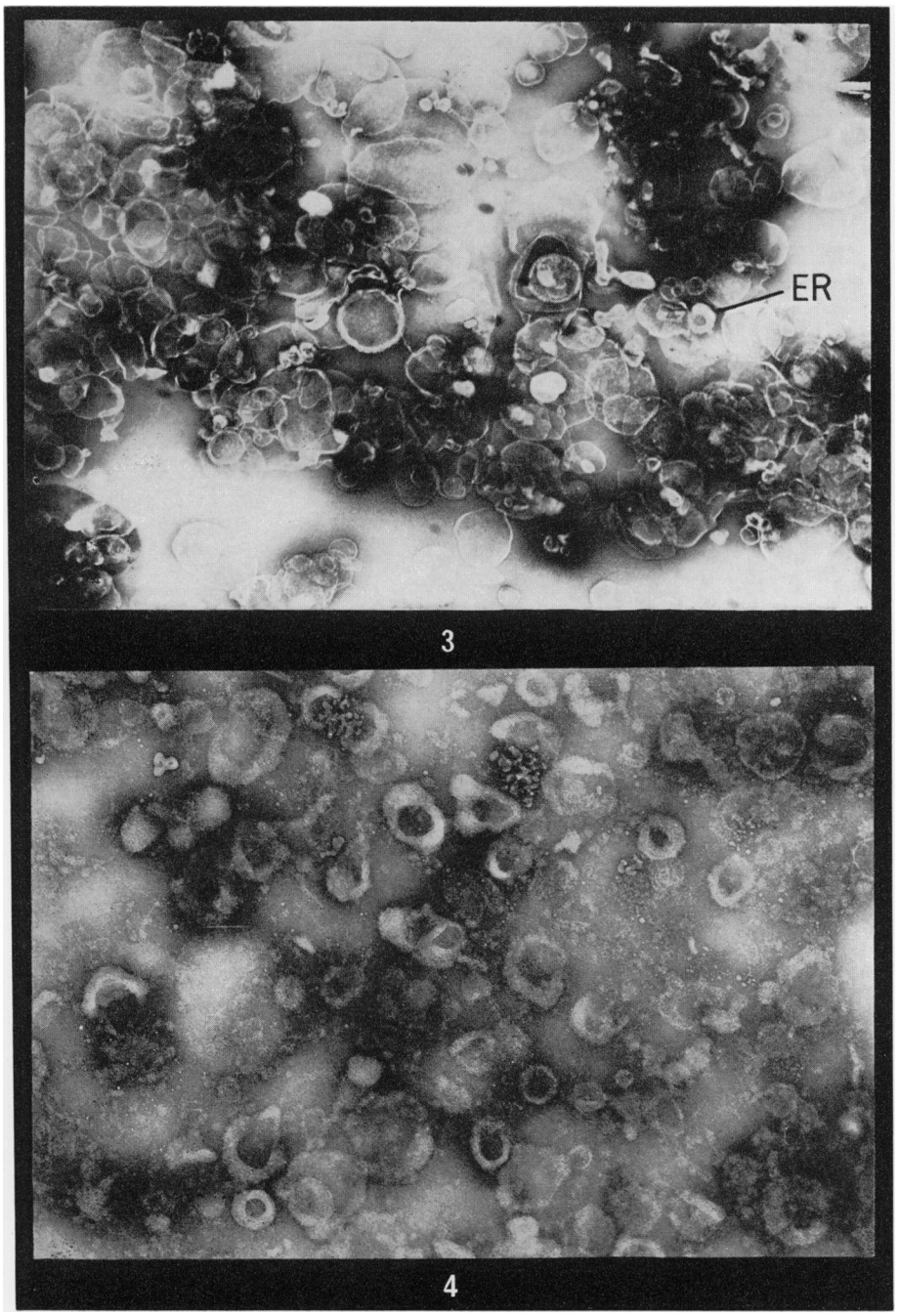

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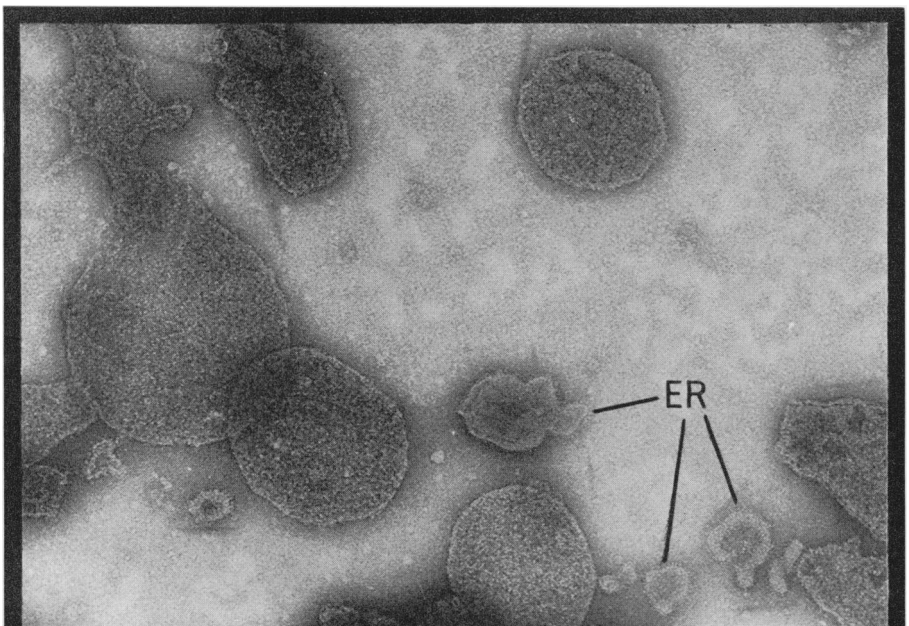

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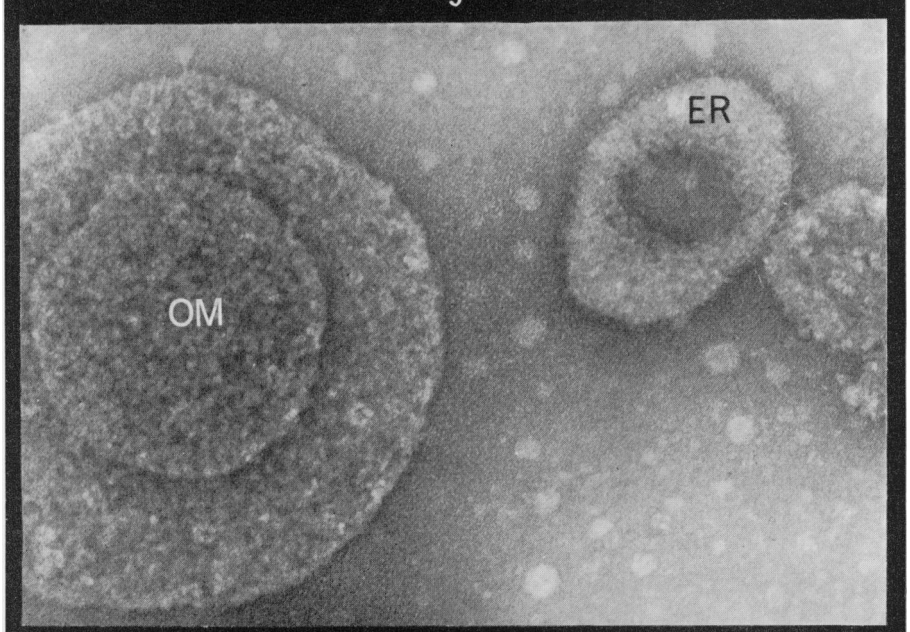

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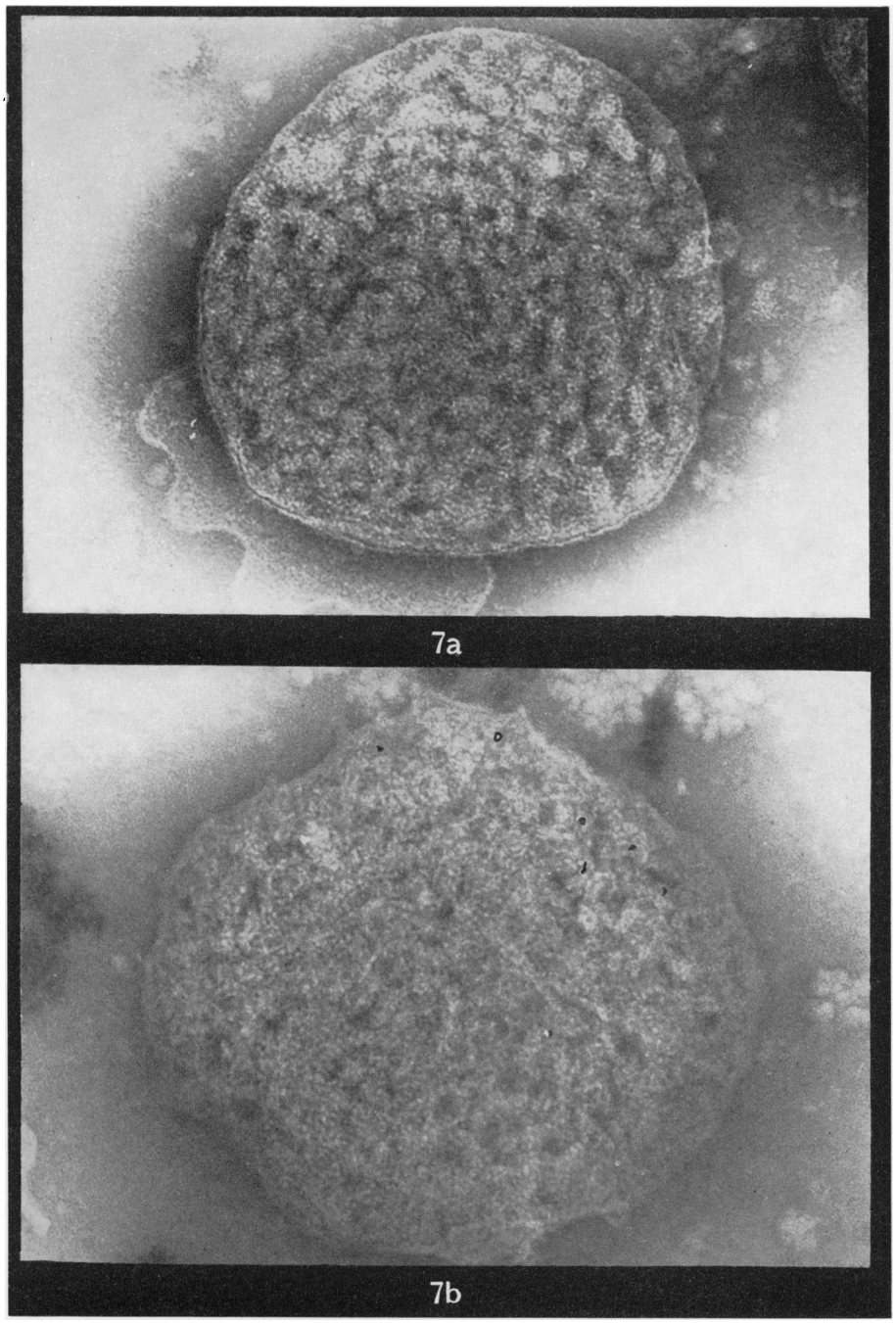

7a

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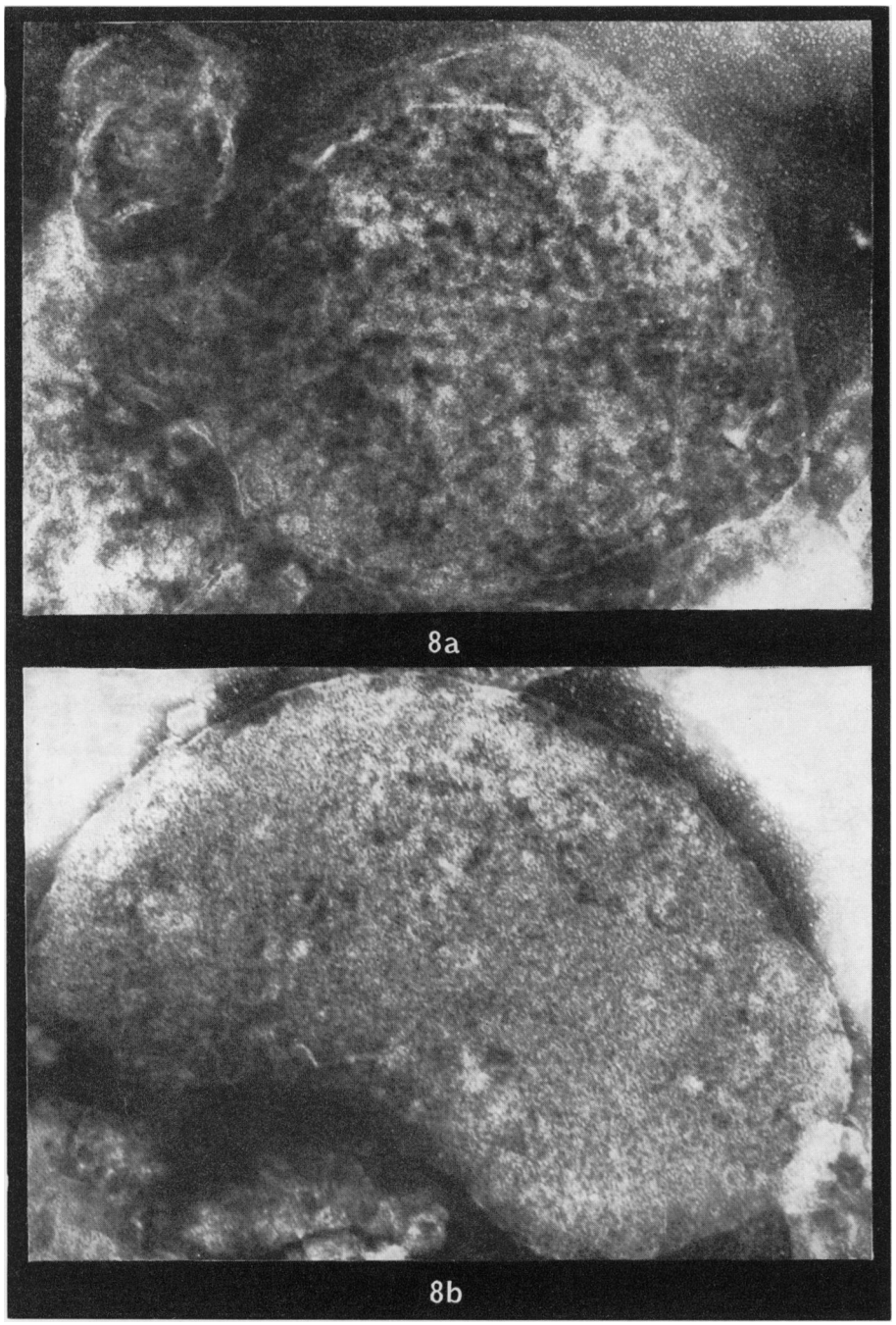

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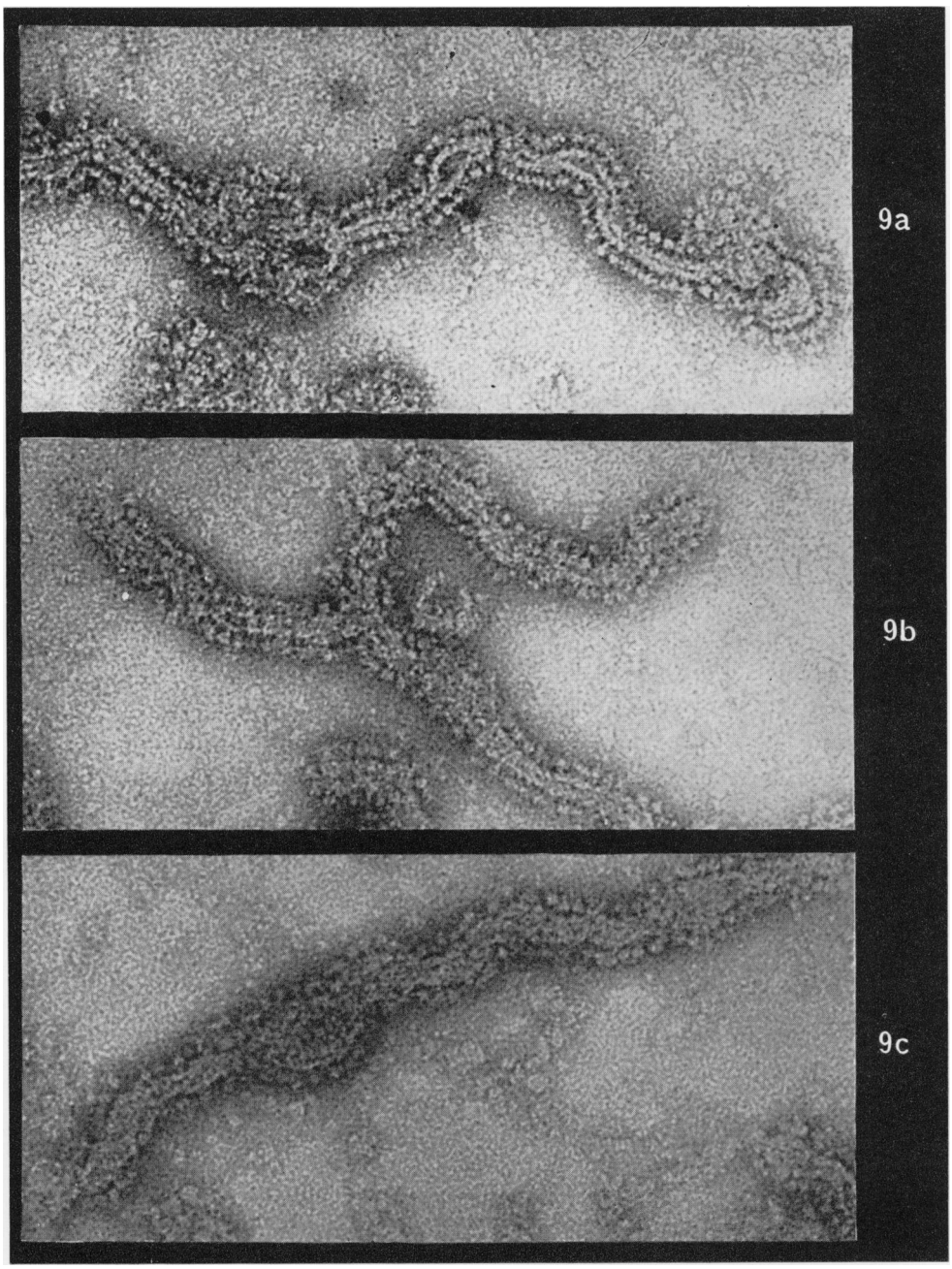

Berg, Muscatello, Horne, Roitt and Doniach.

TABLE IV.—*Comparison of Phospholipid Composition of Purified Mitochondrial Antigen with Data for Outer and Inner Membrane Fractions Published by Parsons* et al. (1967).

Phospholipids	Per cent total phospholipid P		
	Purified antigen*	Inner membrane	Outer membrane
Phosphatidylcholine	43·4	44·5	55·2
Phosphatidylethanolamine and plasmalogen .	29·1	27·7	25·3
Phosphatidyl inositol	7·1	4·2	13·5
Cardiolipin	20·4	21·5	3·2

* Gradient subfraction 4 (cf. Table III) with specific antigen activity of 457 units/mg. protein representing a 15-fold purification relative to intact mitochondria.

Ultrastructural appearance of mitochondria after addition of antibody

The electron micrograph illustrated in Fig. 7a shows a single mitochondrion treated with normal serum. It was observed that under the conditions of negative staining described earlier, little swelling was produced and the mitochondria were seen as membranous spheres partially penetrated by the electron dense stain which allowed the infolding of the cristae to be recognized. The cristae were interpreted as being tubular structures on the surface of which could be seen the characteristic 90Å diameter particles. The effect of the addition of 2 different PBC sera on the structural components of mitochondria is shown in Fig. 7b and 8b. The general morphological features of the mitochondria were similar to those illustrated in Fig. 7a, but it was apparent that the amount of negative stain penetrating into the mitochondria and between the inner components was greatly reduced. The individual 90Å diameter particles associated with the inner membranes were difficult to resolve, although the membranes still possessed a granular structure. Mitochondria treated with the serum of a patient with jaundice due to extrahepatic biliary obstruction (Fig. 8a) had the same appearance on negative staining as the normal serum control. The electron micrographs shown in Figs. 7 and 8 are representative of the small but important differences observed in a large number of mitochondria obtained from several experiments.

Attempts were made to visualize directly with the aid of the electron microscope the precise site of the antibody reaction and the changes induced by the addition of PBC serum. Even under optimum instrumental operating conditions for high resolution electron microscopy, the problem is fraught with difficulties due to the fact that the relative sizes of the mitochondria in depth and the dimensions of the antibody molecules limit the detail capable of being resolved. For these reasons the separated cristae components were examined at high resolution and a careful comparison made between cristae obtained from an untreated control, from mitochondria treated with normal serum and from components treated with PBC serum. The structural features observed in the three different preparations are illustrated in Figs. 9a, b and c. In the case of the untreated control cristae shown in Fig. 9a, the 90Å diameter particles were clearly resolved as being regularly spaced and separated from each other. There was also evidence from the electron micrographs that the negative stain had penetrated the interior of the tubules revealing the unit membranes. Essentially no difference in the appearance of the cristae was found on addition of serum from normal individuals (Fig. 9b). After treatment with PBC serum, the unit membrane of the cristae appeared unresolved

due perhaps to the presence of material attached to its surface (Fig. 9c). Whether antibody is bound to the outer or the inner face of the cristae has not yet been established. No significant changes were observed in the shape or size of the head-pieces associated with the 90Å particles nor could antibody molecules be seen attached to these particular components. The spaces between the 90Å particles were still penetrated by electron dense stain.

DISCUSSION

Using 2 different methods for separating inner and outer mitochondrial membranes, it was shown that the complement fixing antigen reacting with primary biliary cirrhosis serum was associated with the inner membranes. The two methods differed in terms of yield and effectiveness of morphological separation of the structures; the procedure of Sottocasa *et al.* (1967) gave a higher yield of outer membranes than did that of Parsons *et al.* (1966), but the purity of the fractions was inferior. The fractions were characterized by electron microscopy and the degree of contamination of the outer membranes with microsomes and fragments of cristae were assessed by differential counts of the vesicular structures in random fields. On this basis contamination with microsomes accounted for approximately 15 per cent of the outer membrane fraction obtained by Parsons' method. The low content of inner membrane material in this fraction was confirmed by estimation of succinic dehydrogenase activities. Virtually no antigen was found in these outer membrane fractions in contrast with the high antigen concentration in the inner membranes. In the experiments using the separation procedure of Sottocasa *et al.* (1967), rotenone insensitive NADH-cytochrome *c* reductase and monoamine oxidase activities were used as markers for outer membranes (Sottocasa *et al.*, 1967; Parsons *et al.*, 1967; Tipton, 1967; Schnaitman and Greenawalt, 1968) although on the basis of their work on subfractionation of phospholipase-treated beef heart mitochondria, Green, Allmann, Harris and Tan (1968) have challenged the validity of these enzymes for this purpose. The specific activity of the enzymes was 3–10 times higher in the outer than in the inner membrane fractions, whereas the antigen content of the outer membrane fraction was markedly lower in one case and undetectable in another. Phospholipid analysis of a purified antigen fraction showed a high content of cardiolipin and a relatively low value for phosphatidyl-inositol, a pattern characteristic of inner membranes (Parsons *et al.*, 1967).

In studies to be published separately (Berg *et al.*, 1969), purified antigen could be obtained regularly on sucrose gradients at densities of 1·14–1·17 by the methods illustrated in Table III. Whereas under these conditions no pellet was formed, addition of PBC antibody to the gradient resulted in a conspicuous pellet having a high phospholipid content indicative of membrane components. There was a corresponding loss of protein in the antigen-rich band 4. Although this loss of protein could not be equated with disappearance of antigen from this fraction owing to anti-complementary effects, the sedimentation of fluorochrome labelled antibody in the pellet suggests that the sediment is composed of antibody complexed with membrane-bound antigen.

Attempts were made using negative staining to assess the site of antibody reaction on the inner membranes by electron microscopy. When gently disrupted mitochondria were treated with IgG antibody prepared from the standard PBC

serum the outer membrane structures appeared unchanged as compared with the control, whereas material was associated with the inner membranes. From the evidence produced by examination of the high resolution electron micrographs it appears that the elementary 90Å particles were not involved since their morphology and dimensions remained unchanged. The precise site of interaction between the antibody molecules and inner membrane components is still uncertain and further detailed studies are required to establish this point. This type of experiment presents special problems for very high resolution electron microscopy as the protein when attached to the cristae tends to obscure the structural features to be resolved. It should be possible to extend these observations by attempting a series of experiments to obtain critical dilutions of antigen and antibody and observe the attachment of individual antibody molecules to antigen as demonstrated with viral antigens (Lafferty and Oertilis, 1963; Almeida and Howatson, 1963).

Many of the functions of the mitochondrion such as respiration, oxidative phosphorylation and substrate transport appear to be linked to the inner membranes (cf. Chappell, 1968; Tubbs and Garland, 1968). The localization of the autoantigen to these structures raises the question of whether it represents one of the enzymes involved in these biochemical processes. Experiments have been performed to examine the relationship of the antigen to respiratory enzymes (Berg *et al.*, 1969). The enzyme systems of the electron transport chain appear not to be directly involved in the reaction with the autoantibodies found in patients with liver diseases.

We thank Dr. D. E. Green and Professor H. Baum for a useful discussion. We wish to acknowledge the excellent technical assistance of Miss Heather M. Cooper and we also thank Mrs. G. Stead for help in preparation of the manuscript. The work was supported by grants from the Medical Research Council of Great Britain, the Nuffield Foundation, the Arthritis and Rheumatism Council and the World Health Organization.

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