Immunology, 1974, **26**, 311.

Further Purification of the Mitochondrial Inner Membrane Autoantigen Reacting with Primary Biliary Cirrhosis Sera

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(*Received* 25*th May* 1973; *accepted for publication* 10*th July* 1973)

Summary. The mitochondrial inner membrane autoantigen reacting with sera from patients with primary biliary cirrhosis depends on lipid and protein moieties for complement-fixing activity, but its chemical analysis requires some degree of solubilization. Attemps to achieve this by citraconylation led to anticomplementary effects and inactivation, but treatment with 8 M urea fragmented the membranes sufficiently to allow gel filtration and estimation of its mol. wt at 180,000–200,000. The antigen was further purified by affinity chromatography using Igs from patients with anti-mitochondrial antibodies (AMA) coupled to Sepharose 4B, as immunosorbent. The 8 M urea eluate was about 100 times more active than crude inner membranes and showed a single band on polyacrylamide electrophoresis. Liver and brown fat gave the same band, brown fat having four times the potency of liver. Electron microscopy of the purified antigen from the two organs showed that it reaggregated into membranous vesicles when urea was removed. The purified antigen may be of use if an automated radioimmunoassay were to prove sensitive and specific for the detection of AMA as this antibody is an important marker for 'autoimmune' chronic liver disorders.

INTRODUCTION

Previous purification of the mitochondrial inner membrane lipoprotein autoantigen reacting with primary biliary cirrhosis sera (cf. Doniach, 1972), was based mainly on differential centrifugation and gradient separation of fragmented mitochondria (Berg, Muscatello, Horne, Roitt and Doniach, 1969a; Berg, Roitt, Doniach and Horne, 1969b). By these physical methods a 50-fold concentration was achieved. The purified antigen was still membraneous, activity being lost when lipid was removed or when protein was digested with enzymes (Berg, Roitt and Doniach, 1969c). In the present experiments efforts were made to solubilize the active component of this antigen by chemical means. Its approximate molecular weight was assessed by gel filtration and affinity chromatography was employed to purify it still further and observe its ultrastructure.

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MATERIALS AND METHODS

Patients' sera

The sera of four cases of classical primary biliary cirrhosis (PBC) were pooled. All gave a clearcut mitochondrial (AMA) fluorescence pattern on various tissues in solutions of 1:500 and reacted by CFT in equally high titres. Immunoglobulin fractions prepared by precipitation with 40 per cent ammonium sulphate were used in all experiments, while similar fractions prepared from normal sera served as controls.

Reagents

Citraconic anhydride (British Drug Houses) Sepharose 2B and 4B, Sephadex G-25, G-150 and G-200 (Pharmacia) polyacrylamide gel (BDH) 1251, 80–140 mCi/ml(Radiochemicals, Amersham).

Mitochondria

These were prepared from rat liver and neonatal rabbit brown fat as described in Berg *et al.* (1969b). Inner membrane subfractions (Parsons, Williams and Chance, 1966) were prepared from liver but whole mitochondria were used in the case of brown fat.

Solubilization of mitochondrial antigen

This was attempted by several methods. (1) Citraconylation according to Dixon and Perham (1968). (2) Butanol extraction and urea treatment modified from Ward and Nairn (1967) as follows. Mitochondrial inner membranes or whole mitochondria at a concentration of 100 mg protein/ml were suspended in 8 M urea solution buffered to pH 7·2. The mixtures were dialysed for 6 hours against repeated changes of buffered urea. The contents of the dialysis bag were then centrifuged at 40,000 ***g*** for 30 minutes and the supernatant diluted twenty times with distilled water to make antigen–antibody interaction possible. The diluted mixture was centrifuged as before and the supernatant applied to the immunosorbent column. Butanol extraction was carried out in the same way by using 6 per cent *n*-butanol in CFT buffer and mitochondrial membranes at 100 mg protein/ml.

Immunosorbents

The citraconylated mitochondrial membranes were dialysed against 0·1 M glycine/HCl buffer pH 3·5 to remove the citraconic groups and then conjugated to activated Sepharose 2B (cyanogen bromide method, Porath, Axen and Ernboek, 1967). Control columns were prepared with bovine serum albumin. Immunoglobulins from patients with PBC and normal subjects were passed through test and control columns. After suitable washing until no further protein could be detected in eluates, the antigen–antibody complexes were dissociated with 0·1 M acetic acid.

In later experiments the opposite sequence was followed. Immunoglobulins from patients with PBC and normals were covalently linked to activated Sepharose 4B as before, and the urea-treated mitochondria stirred with the immunosorbent for 6 hours at 4°. After washing, elution was carried out with Tris-buffered 8 M urea, pH 7·2 at 4°, since acetic acid proved less effective. Ten-millilitre aliquots of buffered 8 M urea, preheated to 50° were added to the immunosorbent–antigen complex and thoroughly mixed for 15 minutes, then eluted under negative pressure. This was repeated three to four times until most of the protein was recovered.

Complement fixation (CFT) and immunofluorescence

The microtitration method described in the WHO manual (Roitt and Doniach, 1969) was used, with a standard PBC serum for monitoring antigenic activity in various treated mitochondrial fractions and in those eluted from Sepharose 4B columns. In the citraconylation experiments the same CFT test was made with eluted antibody using lyophilized rat liver mitochondrial antigen. Indirect immunofluorescence tests were applied to antibody eluted from the antigen-conjugated Sepharose 2B columns.

Radioiodination

The purified antigen dissociated from its antibody immunosorbent was dialysed free of urea, lyophilized, resuspended in PBS and labelled with ^{125}I (0·5 mCi/2 mg protein/ml) by the chloramine T method of Hunter and Greenwood (1962).

Chromatography and gel filtration

The citraconylated mitochondria were chromatographed on a DEAE–cellulose column and each protein peak was examined by polyacrylamide gel electrophoresis.

Urea-treated mitochondrial inner membranes were separated by gel filtration on Sephadex G-200 (1·2 × 56 cm in 8 M urea) and eluted protein peaks were tested for antigenic activity by CFT. Two purified antigen preparations from liver and brown fat, eluted from the insoluble antibody columns (each radio-labelled and unlabelled), were also examined on the same Sephadex G-200 column in order to determine their approximate molecular weight. Protein markers of known size included catalase mol. wt 225,000, human IgG mol. wt 150,000, chymotrypsin mol. wt 28,000 and cytochrome C mol. wt 13,000.

Polyacrylamide electrophoresis

This was carried out in three ways: (1) in plain gels according to Davis (1964); (2) in gels containing 1 per cent lauryl sulphate; (3) in gels in 8 M urea according to Reisfeld and Small (1966). The gels were stained for protein with Naphthol Blue and prestained for lipid with Sudan Black.

Protein and lipid estimations

Protein content was measured by Lowry's method (1951) and lipid content was determined by weighing the dried methanol–ether extract of the purified antigen.

Electron microscopy

Antigens and column eluates were examined in an AEI 6B electron microscope. The samples were suitably diluted with distilled water dried on to formovar-coated copper grids and negatively stained with 2 per cent ammonium molybdate at pH 7·3. The grids were stabilized with a liquid nitrogen 'cold finger'. Photographs were taken at 80 kV (100,000 magnification).

RESULTS

CITRACONYLATION

After this procedure it was not possible to estimate antigen activity by CFT owing to anticomplementary effects which persisted after removal of the citraconic acid residues.

The solubilized mitochondrial preparation was covalently linked to Sepharose 2B immunosorbent, in the hope that Ig containing AMA could be examined by CFT and immunofluorescence after dissociation at acid pH from antigen on the column. The eluted antibody was again anticomplementary. Immunofluorescence was negative in the column washings before treatment with acetic acid and become positive after it, but the same results were obtained in the control column conjugated with BSA, suggesting that some antibody activity was bound non-specifically to these columns.

DEAE–cellulose chromatography was done in the hope of removing anticomplementary effects. Four protein peaks were obtained (Fig. 1). Peaks 1 and 2 were anticomple-

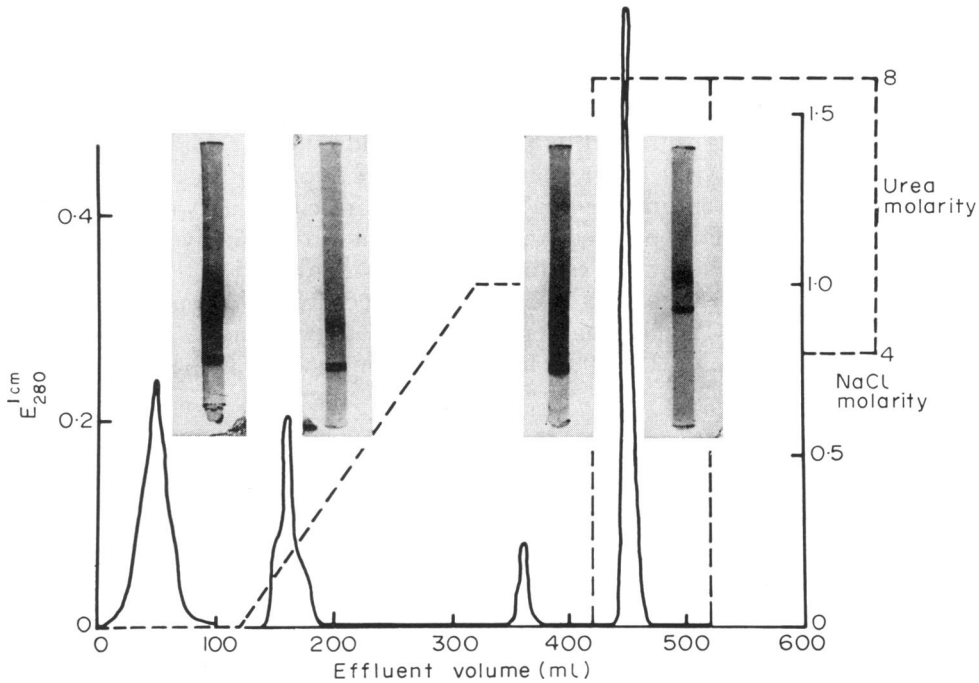

FIG. 1. Chromatography of rat liver mitochondrial inner membranes on DEAE–cellulose (30 × 1 cm) in 0·05 M phosphate buffer at pH 6·0 with NaCl gradient followed by 8 M urea.

mentary while peaks 2 and 4 were inactive. Polyacrylamide electrophoresis showed multiple components, none of which corresponded in mobility to the purified antigen obtained in later experiments.

From these results it was concluded that active mitochondrial antigen cannot be extracted from mitochondria by citraconylation.

BUTANOL EXTRACTION

Liver mitochondrial inner membranes extracted with 6 per cent *n*-butanol were antigenically active and not anticomplementary. The method was not pursued since it was thought that urea treatment would yield a more soluble preparation.

UREA EXTRACTION

Concentrations of 4 M, 6 M and 8 M urea were investigated with mitochondrial protein concentrations up to 100 mg/ml. The best antigen yield was obtained after extraction with 8 molar urea. Anticomplementary effects disappeared on dilution and antigen activity could be detected in the extract up to concentrations of 0·2 mg protein/ml. The 8 M urea extract was passed on Sephadex G-150 and G-200 columns in 8 M urea and gave rise to three protein peaks on each column. The entire complement-fixing activity was recovered in the first part of the main protein peak of both columns as shown on Fig. 2. Although a

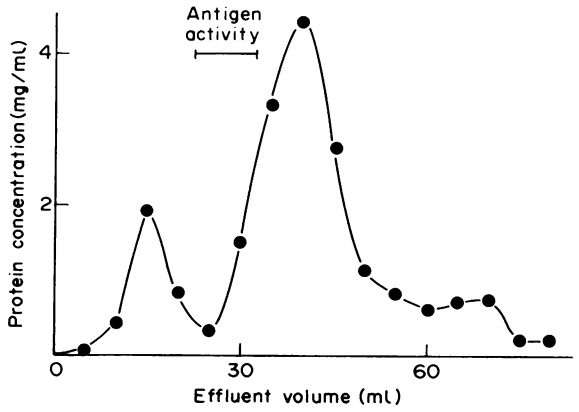

FIG. 2. Gel filtration of rat liver mitochondrial inner membrane urea extract on Sephadex G-200 (50 × 1·2 cm) in 8 M urea at pH 7·2.

poor separation was achieved by this technique, the antigen molecular weight could be estimated at about 200,000. When urea was removed from the extract by dialysis, most of the antigen became insoluble again, but when the urea was diluted up to twenty times most of the antigen remained soluble.

IMMUNOSORBENT EXPERIMENTS

In the first trial, immunoglobulins precipitated from 10 ml normal serum and 10 ml of serum from patients with PBC containing AMA were linked to Sepharose 4B. 100 mg of rat liver mitochondrial inner membrane protein treated with 8 M urea were mixed with the immunosorbent and stirred for 6 hours at 4°. The test and control mixtures were then placed on sintered glass funnels and washed with PBS under negative pressure until the wash was free of protein. CF activity was present in the first filtrate of the control column but the parallel filtrate from the antibody column was negative, suggesting that some antigen became bound to its insoluble antibody. Dissociation of the immune complexes with 8 M urea resulted in CF active antigen being recovered from the antibody column while the control column gave negative results for both antigen and protein. Antigen potency has not yet been assessed quantitatively but in the microtitre test, positive CFT was obtained at 2–4 μg protein/ml with the purified antigen while mitochondrial inner membranes had to be used at a concentration of 200 μg/ml for comparable results.

A larger batch was then set up with immunoglobulins from 50 ml of serum from PBC patients to obtain sufficient purified antigen for further analysis. 500 mg of rat liver

mitochondrial protein were applied and after dissociation, 15 mg of purified antigen could be recovered. This was dialysed free of urea and lyophilized. After reconstitution of the immunosorbent, 100 mg of brown fat mitochondrial protein was similarly applied and in this experiment 11 mg of purified antigen was recovered. The ratio of antigen in the two organs was 11:3, i.e. brown fat contains approximately four times more mitochondrial antigen than liver, confirming data obtained in previous experiments by quantitative CFT (Berg *et al.*, 1969b). A rough estimate showed a lipid content of about 20 per cent in each of these antigens.

GEL FILTRATION

Each purified antigen was passed through Sephadex G-200 in 6 M urea with the protein markers as shown in Fig. 3 and the estimated molecular weight was found to be approximately 180,000. The radioactivity of purified labelled brown fat and liver antigens was recovered at identical elution volumes, using the same Sephadex G-200 column. This corresponded to the protein peak obtained on the column with unlabelled liver antigen as seen in Fig. 4.

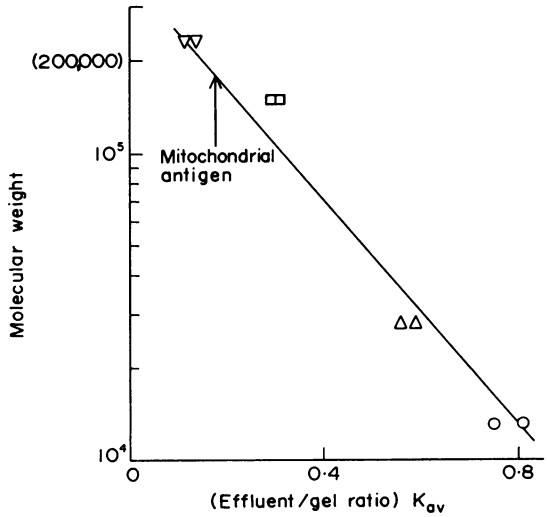

FIG. 3. Assessment of molecular weight of mitochondrial antigen by comparison with known protein markers applied to the same Sephadex G-200 column. ▽; Catalase. □, Human IgG △, Chymotrypsin. ○, Cytochrome c.

POLYACRYLAMIDE ELECTROPHORESIS

The purified antigen gave a single protein band in detergent and in plain polyacrylamide gels, the antigen from brown fat showing the same mobility as that extracted from liver (Fig. 5). In 8 M urea gels, a major protein band and two minor bands could be seen. Pre-staining of the liver antigen with Sudan Black showed faint lipid staining in the position of the main band on urea polyacrylamide.

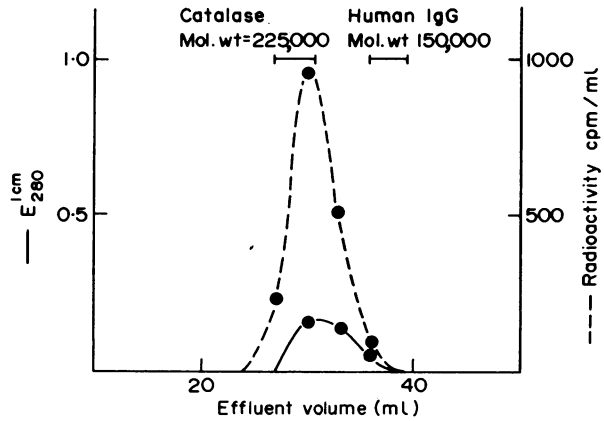

FIG. 4. Gel filtration of mitochondrial antigen. Immunoglobulin fraction from PBC serum containing AMA was covalently bonded to a Sepharose 4B column. Rat liver mitochondrial inner membranes were treated with 8 M urea. The partially solubilized fraction was diluted 1:20 and passed through the antibody-laden column. After washing, the antigen–antibody complexes were dissociated with 8 M urea. The eluted antigen was dialysed, freeze dried and passed through Sephadex G-200 column. Part of the recovered antigen was labelled with ^{125}I and treated similarly. All the protein and radioactivity were recovered in one peak of approximate mol. wt 200,000 by comparison with catalase and human IgG applied to the same column as markers.

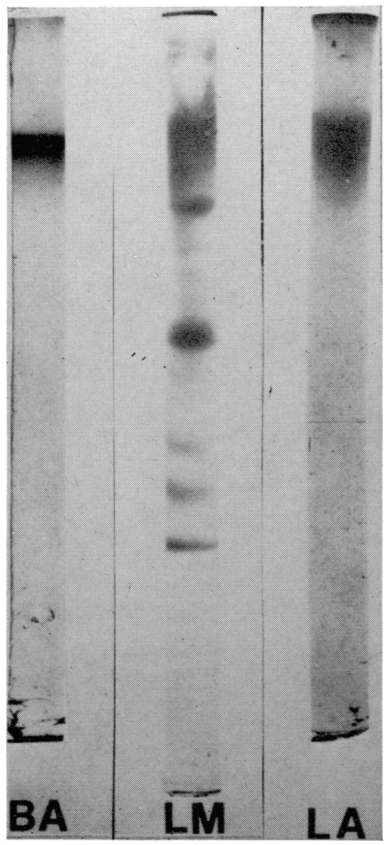

FIG. 5. Polyacrylamide gel electrophoresis of solubilized mitochondrial inner membrane (LM), purified antigen from brown fat (BA) and from liver (LA). (Tris–glycine buffer pH 8·5, 2 hours room temperature, constant current 5 mA/gel).

ELECTRON MICROSCOPY

A representative view of the rat liver mitochondrial inner membrane preparation applied to the immunosorbent is shown on Fig. 6a. It contained mainly crystal fragments studded on either side with regularly spaced 90 Å Fernandez–Moran particles and was contaminated with a number of smooth outer membrane and microsomal vesicles. After

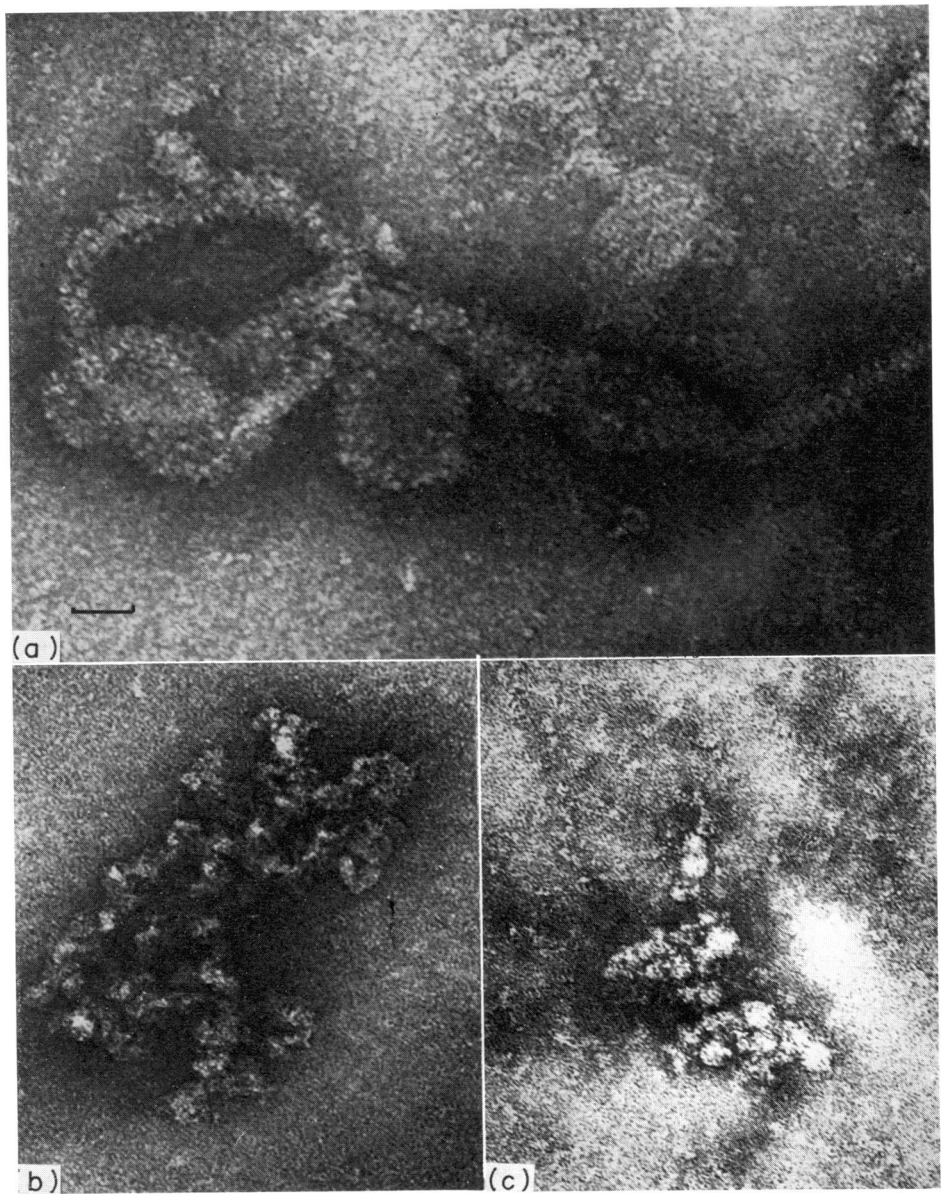

FIG. 6. Electron micrographs of mitochondrial antigen. (a) Rat liver inner membrane preparation before application to immunosorbent. (b) Same antigen after dissociation with 8 M urea from AMA-conjugated Sepharose 4B column. (c) Brown fat mitochondrial antigen after elution from same immunosorbent column as (b). This antigen had four times more activity/mg/protein than equivalent eluate of rat liver.

combination with AMA and subsequent urea elution the antigen reaggregated into amorphous membranous structures no longer showing 90 Å particles but which appeared to contain small globular subunits about 100 Å in diameter (Fig. 6b). Similar eluates from the control column conjugated with normal immunoglobulins showed none of these membranous structures. In the experiment with brown fat mitochondria, the eluate from the antibody column again consisted of reaggregated membranous fragments with possible globular subunits similar in appearance to those seen in the rat liver antigen but having a larger diameter, about 200 Å (Fig. 6c).

DISCUSSION

In previous studies a 40–50-fold purification of the mitochondrial antigen was achieved by differential and sucrose gradient centrifugation. The antigen was found to be a lipoprotein requiring both protein and phospholipids for activity and which retained a membranous appearance on electron microscopy in its purified state. It was also shown that the antigen was not involved in any known respiratory chain subunit of the inner mitochondrial membranes (Berg *et al.*, 1969a, b and c). In the present work it was hoped to obtain a more homogeneous and highly purified preparation suitable for chemical characterization. Citraconylation was attempted first in view of the results obtained by this method with cell surface antigens on lymphocyte membranes. Eshhar, Gafni, Givol and Sela (1970, 1971) reported that 40 per cent of the antibody-binding capacity against anti-lymphocytic serum was retained in these solubilized surface antigens. In the case of mitochondrial membranes no antigen activity could be demonstrated in citraconylated preparations, or else the entire activity was masked by anticomplementary effects, which could not be removed by DEAE–cellulose chromatography.

Urea treatment was successfully used by Ward and Nairn (1967) to separate the microsomal autoantigen of gastric parietal cells which is known to be a lipoprotein membrane component. Our results suggest that this also solubilizes or fragments the mitochondrial lipoprotein without destroying its antigenic activity. Immunosorbents are widely employed to fix antibodies which can subsequently react selectively with their antigen in a mixture of proteins. The selected antigen is then dissociated again at acid pH or at high urea concentrations and this sometimes leads to a degree of purification. The mitochondrial antigen was purified about 100-fold by this means but experiments have not yet been performed on a sufficiently large column to permit quantitative CFT studies which require relatively large amounts of reagents. The molecular weight of the purified antigen could be assessed at about 180,000 by gel filtration on Sephadex G-200 in the presence of four marker proteins of known size and it is of interest that the antigenic complement-fixing activity was found in a corresponding fraction of the crude antigen (Fig. 2). Polyacrylamide separation produced only one protein peak except in 8 M urea where two other minor components were observed, possibly due to dissociation of the antigen into subunits. Lipid was still attached in the polyacrylamide protein peak and electron microscopic observations suggested that the antigen reaggregated into membrane-like structures. Brown fat and liver behaved very similarly in the gels, indicating a common antogenic component, in both organs. The greater activity of brown fat was reflected in the 4-fold yield of purified antigen obtained from the immunosorbent. This was also reflected morphologically in the larger size of membrane subunits seen on EM (Fig. 6b and c).

It has not yet proved possible to find any enzyme activity related to the mitochondrial autoantigen, but this is partly due to the incomplete state of knowledge regarding the enzymes of mitochondrial inner membranes. In this context it is of interest to mention that AMA fluorescence was seen not only in all vertebrate tissues but also in the flight muscles of blue bottle fly, bee and locust (Doniach, unpublished). These insect muscles were tested as it is known that their mitochondria contain only some of the enzyme systems of higher animals and it was hoped that if the human antibody failed to react with these incomplete mitochondria, this would eliminate whose groups of substances as being involved in this antigen. It turned out that, although insects reacted more feebly than mammalian tissues, only plant chloroplasts proved completely non-reactive.

Purification of the mitochondrial autoantigen could be of use in developing a radioimmunoassay for the detection of AMA which might prove more sensitive than the immunofluorescence test and more suitable for automation. AMA immunofluorescence can be confused with four other antibodies found in pathological sera. One of these, the cardiolipin fluorescent antibody gives a similar staining pattern since its antigen is situated in the same part of the mitochondrial inner membranes though it is distinct from the antigen reactive in PBC (Wright and Doniach, 1971). The second is a microsomal antibody recently characterized in some patients with active chronic hepatitis (Doniach, Lindqvist and Berg, 1971; Rizzerro, Swana and Doniach, 1973). This reacts mainly with proximal renal tubules and hepatocytes but can be confused with AMA (which reacts more strongly with the ascending limb of Henle's loop and distal tubules) if only renal cortex is used as substrate or if the antibodies are of low titre. The third is a ribosomal antibody found in some cases of systemic lupus, which stains all renal tubules, thyroid cells, gastric chief cells and exocrine pancreas (Homberg, Rizzetto and Doniach, 1974). A fourth pattern giving rise to confusion is the brush border fluorescence caused by a heterophile antibody (Ireton, Muller and McGiven, 1971). It is unlikely that radioimmunoassay will eliminate false reactions due to cardiolipin antibodies since this diphospholipid is still intimately bound to fragmented inner membranes (Berg *et al.*, 1969c). Purified antigen will be more important for distinction of AMA from the microsomal antibody as crude mitochondrial fractions are contaminated with 10–30 per cent microsomal vesicles, even when washed repeatedly and it is likely that this will be overcome in the purified antigen.

ACKNOWLEDGMENTS

We wish to thank Mr Philip Penfold for preparing the electron micrographs and Professor I. M. Roitt for useful discussions. Miss J. Richardson helped with the manuscript.

Work in the department of Immunology is supported by the Medical Research Council and the World Health Organization.

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