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MITOCHONDRIAL ANTIBODIES IN PRIMARY BILIARY CIRRHOSIS

V. ULTRASTRUCTURAL LOCALIZATION OF THE ANTIGEN TO THE INNER MITOCHONDRIAL MEMBRANE USING A DIRECT PEROXIDASE CONJUGATE

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Summary.—The IgG fraction of a primary biliary cirrhosis serum with high titre of mitochondrial antibodies was conjugated with peroxidase and applied to 20 μm cryostat sections of various rat tissues (stomach, kidney and liver). Results at the ultrastructural level confirm the localization of the antigen to the inner mitochondrial membrane. It was found that fixation with 1% formaldehyde in 0·1 mol/l phosphate, pH 7·4, containing 0·25 mol/l sucrose for 40 minutes at 4° achieved reasonably good preservation of morphology without undue loss of antigen. Staining variations among mitochondria of different cell types are discussed with regard to antigenic distribution.

INTRACELLULAR constituents have been localized at the ultrastructural level using horseradish peroxidase as a label for both antibodies and antigens. Antibodies to peroxidase were localized in spleen plasma cells (Leduc, Avrameas and Bouteille, 1968) and lymph node immunocytes (Avrameas and Leduc, 1969) using peroxidase as antigen. Similarly, antibodies raised against human IgG were demonstrated in the spleen plasma cell line, using peroxidase conjugated human IgG (Avrameas and Bouteille, 1968). In 1967 Nakane and Pierce succeeded in demonstrating an intracellular antigen by enzyme labelled antibodies; in the same paper they suggested that peroxidase is superior to ferritin as a label for intracellular antigens because of its lower molecular weight and greater ease of penetration. The hormones of the anterior pituitary were localized by an indirect technique on ultrathin sections using peroxidase linked antibodies (Nakane, 1971) and albumin synthesis has been confirmed in liver cells using a direct peroxidase-antibody conjugate (Feldmann, Penaud and Grassous, 1972). In a study of membrane antigens in cryostat sections of tissue, Hoedemaeker and Ito (1970) were able to localize the site of binding of the parietal cell antibody from patients with pernicious anaemia by the direct technique.

We have for some time been concerned with the ultrastructural localization of the mitochondrial antigen which reacts with autoantibodies usually present in the serum of patients with primary biliary cirrhosis (PBC). This antibody was first clearly shown to be directed against mitochondria in 1967 by Berg, Doniach and Roitt using complement fixation (CFT) to assay the antigen in different tissue

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fractions. Subsequently strong evidence for an association with inner membranes was provided by complement fixation and negative staining E.M. techniques on mitochondrial subfractions (Berg *et al.*, 1967, 1969). In the present study we have attempted more precise localization of the antigen at the ultrastructural level using direct peroxidase conjugates of PBC autoantibody, particularly in view of a recent report suggesting that the antibody is directed against other cytoplasmic constituents, specifically ribonucleoproteins (Knieser and Jenis, 1972).

MATERIALS AND METHODS

Preparation of IgG–peroxidase conjugates.—Serum from a PBC patient with a CFT titre greater than 1 : 2048 was passed down a DEAE column equilibrated with 0·02 mol/l phosphate buffer, pH 6·5 and the IgG fraction was collected. After dialysis against 0·1 mol/l phosphate buffer, pH 6·8, 5 mg of IgG were mixed with 15 mg of peroxidase (as recommended for electron microscope studies) (Avrameas and Terninck, 1971) and conjugation was effected by stirring with 0·05 ml of 1% aqueous glutaraldehyde for 2 hours at room temperature (Avrameas, 1969). The mixture was then dialysed overnight against PBS at 4° and spun at 25,000 *g* to remove any precipitates. The conjugate of antimitochondrial IgG (Px-M) may be stored for 3–4 months at 4° without significant loss of activity. Normal serum IgG (Px-N) was prepared and conjugated in an identical manner.

Fixation procedures.—The effects of 2 fixative solutions on antigenic and morphological preservation were investigated: they were 1% glutaraldehyde and 1% formaldehyde (freshly prepared from paraformaldehyde) both in 0·1 mol/l phosphate buffer, pH 7·4, containing 0·25 mol/l sucrose. They were used for periods of 30 and 60 minutes at 4°. The effects on antigenic preservation were studied at the light level on 5 μm cryostat sections with 1 : 10 dilutions of Px-M, and Px-N as a negative control, and by indirect fluorescence. Sections from unfixed blocks were used as positive controls. With 1% formaldehyde and 30 minutes fixation no significant loss of reaction intensity or specificity was found. After 60 minutes with this fixative the staining was reduced. Using 1% glutaraldehyde for 30 minutes staining was severely reduced, and was completely abolished after 60 minutes. We decided to use 1% formaldehyde in 0·1 mol/l phosphate buffer, pH 7·4, containing 0·25 mol/l sucrose at 4° for 40 minutes.

Tissues were selected on the basis of quantity of antigen and availability.

(a) *Rat stomach.*—Strips of stomach wall were cut and fixed by dipping. To facilitate fixative penetration the muscular layer was removed. (b) *Rat kidney.*—Blocks of cortex and medulla were cut to approximately 2 × 4 × 0·5 mm dimensions and fixed by dipping. The segments of the kidney tubules were identified according to Maunsbach (1966). (c) *Rat liver.*—The dipping method of fixation was inadequate for this tissue. Reasonable morphology was obtained by perfusion with the fixative through the portal vein for 5 minutes. The best perfused lobe, as judged by surface discolouration, was excised, cut into 1 mm cubes and dipped in the fixative for a further 35 minutes.

Processing.—(1) After fixation tissues were washed in 0·1 mol/l phosphate buffer, pH 7·4, containing 0·25 mol/l sucrose for 1 hour at 4°, with 2 changes, on a mechanical stirrer. (2) Freezing with liquid nitrogen showed no significant advantage over the usual procedure involving isopentane cooled by an acetone-solid-CO_2 mixture; therefore the latter method was used. (3) 20 μm sections were cut on a cryostat and transferred to the buffer (as in (1)). (4) 5–10 sections were completely immersed in 0·5 ml of undiluted conjugate solutions (Px-M and Px-N) and incubated at room temperature for 1 hour. No substantial advantage regarding penetration of conjugate was achieved by longer incubation. Additional sections for use as controls of endogenous peroxidase activity were kept in the buffer for the same period. We noticed that control sections which had been kept in the buffer and not exposed to the conjugate had a superior morphology at the ultrastructural level. We decided to investigate therefore whether this was due to the difference in osmolality between the solutions. The conjugates were dialysed against PBS and 0·1 mol/l phosphate buffer and used both with and without 0·25 mol/l sucrose. Morphology was not improved by the presence of sucrose, neither was there any difference between PBS and 0·1 mol/l phosphate buffer. (5) After incubation, the sections were washed in the buffer (as in (1)) for either 1 hour (3 × 20 minutes) or 15 minutes (3 × 5 minutes). No improvement was found with prolonged washing. (6)

Postfixation with 2·5 % glutaraldehyde in 0·1 mol/l phosphate buffer, pH 7·4, which stabilizes the binding of the conjugate antibody without impairing the peroxidase reactivity (Nakane and Pierce, 1967), was carried out for 30 minutes at 4°. This was followed by overnight washing in the buffer (as in (1)). (7) The method of Graham and Karnovsky (1966), as modified by Petts and Roitt (1971), was used in the peroxidase reaction. The reaction times were 10 and 40 minutes. After diaminobenzidine (DAB) treatment, sections were washed in the buffer (as in (1)), treated with 1% OsO_4-Palade for 30 minutes, dehydrated and embedded in Araldite. Sections were pressed flat in tin embedding dishes by prepolymerized square resin blocks.

0·5 μm sections were studied at the light level with and without toluidine blue counterstain. Thin sections were examined unstained on unsupported grids on an AEI EM 6B electron microscope at 50 kV.

RESULTS

With 1% formaldehyde in 0·1 mol/l phosphate, pH 7·4, containing 0·25 mol/l sucrose for 40 minutes at 4° as fixative, a compromise between antigenic and morphological preservation in the electron microscope was achieved. For stomach and kidney, fixation by dipping was adequate but for liver, penetration with the same fixative gave superior morphology, presumably due to the poor penetration of this fixative in compact tissue with the dipping technique.

At the light microscope level penetration by the Px-M was confined to approximately 3–4 μm on each side of the 20 μm section. All the tissues treated with Px-M which we examined showed a brown granular stain within the cytoplasm. Staining was never seen within the nucleus. In the stomach, with respect to the topography of the glands, cells in the position normally occupied by parietal cells showed more numerous granules than those with the distribution of zymogenic cells. In the kidney, a comparison between control sections stained with toluidine blue and those treated with Px-M showed positive elongated organelles perpendicular to the basement membrane after the peroxidase reaction, corresponding to the mitochondria in the toluidine blue stained sections. Control sections treated with Px-N were always negative.

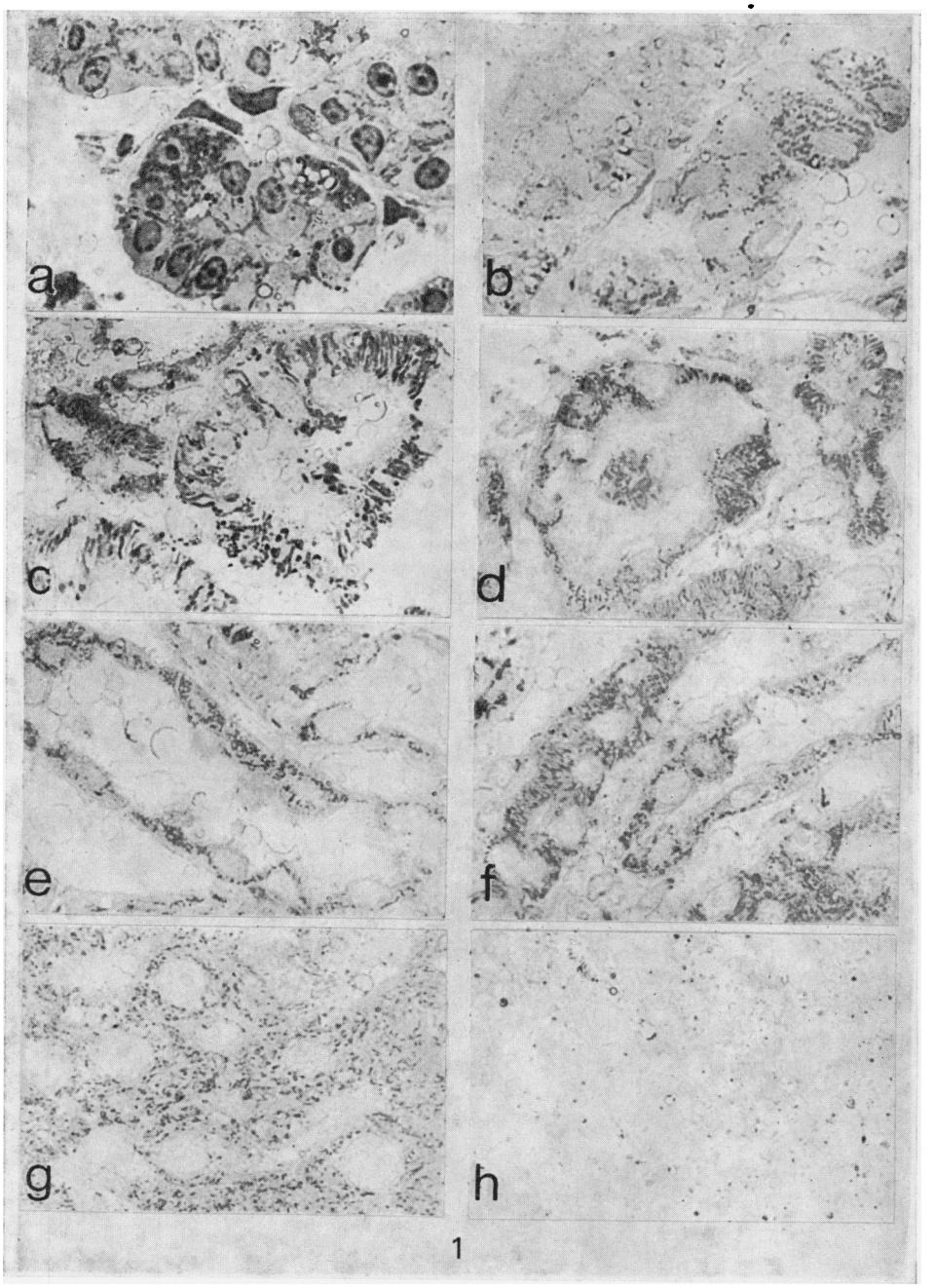
At the ultrastructural level binding of conjugate was manifested as a marked increase in contrast of membranes in the Px-M treated tissue, relative to that seen in the controls with Px-N. Antimitochondrial conjugate was specifically localized to the inner mitochondrial membrane and cristae. In addition to the

EXPLANATION OF PLATES

FIG. 1.—Light micrographs of 0·5 μm sections, *a*, *b*, *c*, *d*, *e*, *f*: Px-M treated tissue; *g*: Px-N treated tissue. Rat stomach *a*: toluidine blue counterstain. Cross section of glands: parietal cells are distinguishable from chief cells by the more numerous mitochondria. *b*: no counterstain. Rat kidney. *c*: upper proximal tubule; *d*: lower proximal tubule; *e*: thin loop; *f*: thick loop and thin loop (see text). Rat liver *g*: mitochondrial pattern of hepatocytes; *h*: Px-N control. Original magnification × 400.

FIG. 2.—Rat stomach. *a*, *b*, *c*: Px-M treated tissue; *d*: Px-N treated tissue (control). *a*: parietal cell; all the mitochondria show uneven distribution of stain; the nucleus (N) is unstained. Original magnification × 7500. *b*: parietal cell mitochondria. Original magnification × 30,000. *c*: chief cell mitochondria, showing even stain of the inner membrane (circle). Original magnification × 30,000. *d*: showing absence of stain. *P* = parietal cell; C = chief cell. Original magnification × 30,000.

FIG. 3.—Rat kidney. *a*, *b*, *c*: Px-M treated tissue; *d*: Px-N treated tissue (control). *a*: upper proximal tubule mitochondria (see text). Original magnification × 30,000. *b*: thin loop mitochondria. Original magnification × 30,000. *c*: distal tubule mitochondria (see text). Original magnification × 30,000. *d*: distal tubule mitochondria; note absence of stain. Mitochondrial granules are clearly visible. Original magnification × 20,000.



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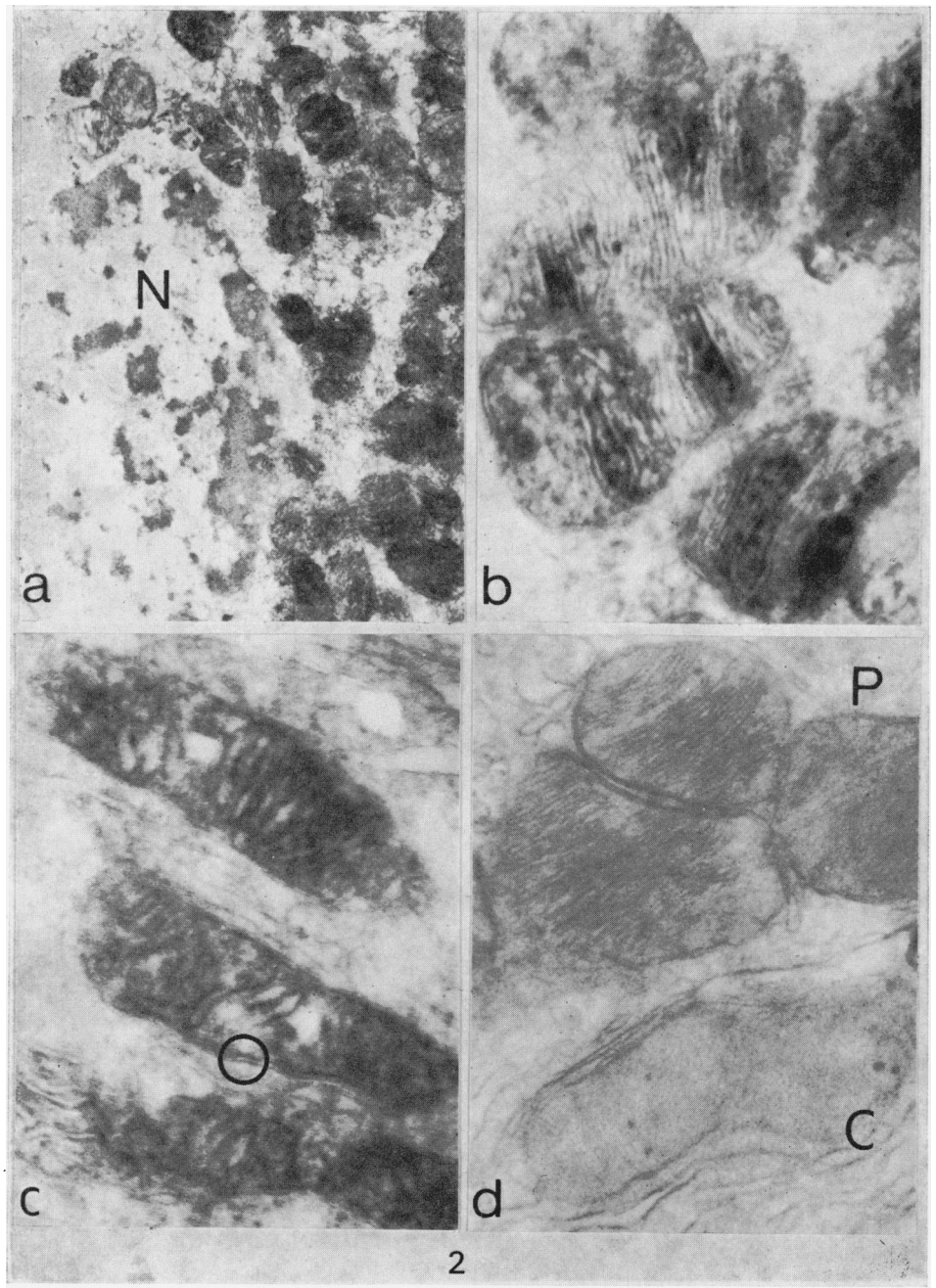

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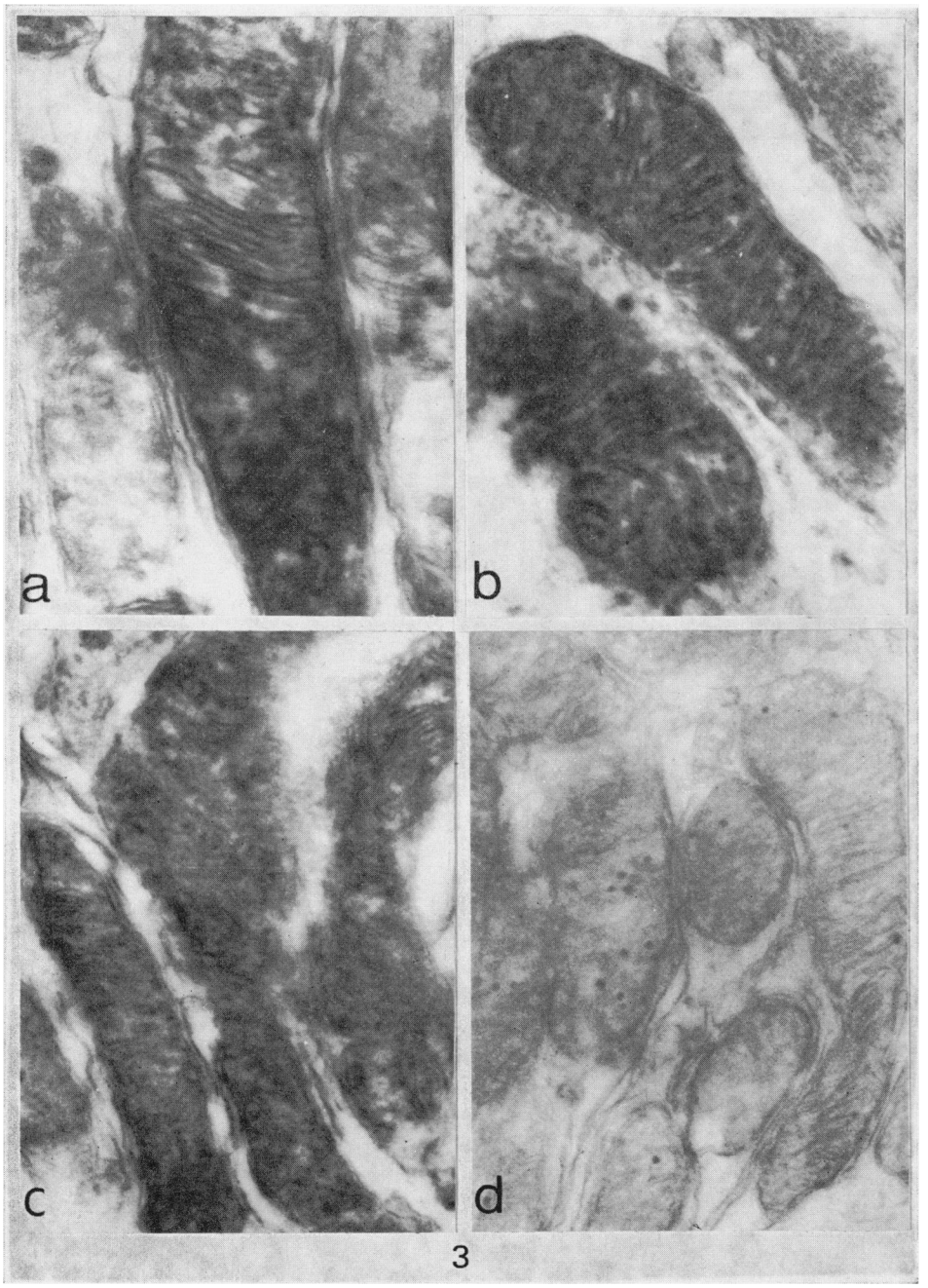

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increase in contrast of the cristae and inner membrane, a further criterion for assessing the degree of staining was the ease of identification of the intramitochondrial granules which become obscured in positive mitochondria although they are clearly visible in control mitochondria.

In the parietal cells of the stomach all the mitochondria were positive but in every case zones of varying intensity of staining could be seen. The mitochondria of the chief cells were all strongly positive, with uniform staining of the inner membrane and cristae. Similarly, all the mitochondria of the liver cells were stained with an even intensity. However, in the upper part of the proximal tubules of the kidney not all the mitochondria were stained to the same extent. Variations of staining also occurred, even within individual mitochondria where some groups of cristae showed less reaction product than others. In the lower segment of the proximal tubule all the mitochondria exhibited a strong uniform staining of cristae and inner membrane. The mitochondria of the thin loop of Henle were similar in staining properties to those of the lower proximal tubules The thick loop and the distal tubules showed identical mitochondrial staining, which was uniform in distribution about the cristae and inner membrane but of only moderate intensity and more diffuse profile.

Endogenous peroxidase activity within the mitochondria (presumably due to cytochrome oxidase), was present in all tissues but was seen only after 40 minutes incubation with DAB and H_2O_2, whereas the bound conjugate was apparent after 10 minutes incubation. Nonspecific staining was insignificant in the Px-N and Px-M treated tissues.

DISCUSSION

The above results suggest the following points of discussion:

The fixation procedure must be adapted for the antigen in question, which involves analysis of type and concentration of fixative and time of exposure. At the same time the fixation must preserve adequate morphology. In our case 1% glutaraldehyde, applied even for a short time, caused marked loss of antigenic activity.

Penetration of conjugate was limited to 3–4 μm on each side of the 20 μm cryostat sections. Thus, high titre conjugates are essential not only to give more distinct staining but also to help visualize a larger area of the cell. In tissues where uneven distribution of antigen occurs, either at cellular or subcellular levels, problems in interpreting antigenic distribution arise. In rat parietal cells and the upper segment of the proximal tubule of rat kidney, mitochondria showed an uneven distribution of staining. This may be due to unevenness either in the distribution of antigen, the penetration of conjugate or the preservation of antigenic reactivity by the fixative. In the parietal cell mitochondria, where the staining is consistently uneven, a genuinely uneven distribution of antigen is suggested. Work is now in progress to resolve these possibilities.

The mitochondrial antibody is essentially defined by its fluorescence staining pattern, *i.e.* the strong positivity of cells with a well developed mitochondrial system, such as gastric parietal cells, distal tubules of the kidney and hypertrophic thyroid cells. The complement fixing mitochondrial antibodies can be titred in CFT, using mitochondria as antigen, but the microsomal fraction is always far weaker than the mitochondrial fraction in this test. Using an IgG preparation

from a serum with these fluorescence and CFT characteristics, the antigen has been localized to the inner membranes of the mitochondria. The pattern described by Knieser and Jenis (1972) at the ultrastructural level is not analogous to the accepted fluorescence staining pattern, which is an integral part of the definition of the mitochondrial antibody.

Finally, the point may be made that the high resolution achieved using 0·5 μm sections of Araldite embedded tissue may often be of value in problems involving immunohistochemical localization of antigens at the light microscope level.

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REFERENCES

AVRAMEAS, S. (1969) Coupling of Enzymes to Proteins with Glutaraldehyde. Use of the Conjugates for the Detection of Antigens and Antibodies. *Immunochemistry*, **6**, 43.

AVRAMEAS, S. & BOUTEILLE, M. (1968) Ultrastructural Localization of Antibody by Antigen Label with Peroxidase. *Expl cell. Res.*, **53**, 166.

AVRAMEAS, S. & LEDUC, E. H. (1969) Detection of Simultaneous Antibody Synthesis in Plasma cells and Specialized Lymphocytes in Rabbit Lymph Nodes. *J. exp. Med.*, **131**, 1137.

AVRAMEAS, S. & TERNINCK, T. (1971) Peroxidase Labelled Antibody and Fab Conjugates with Enhanced Intracellular Penetration. *Immunochemistry*, **8**, 1175.

BERG, P. A., DONIACH, D. & ROITT, I. M. (1967) Mitochondrial Antibodies in Primary Biliary Cirrhosis. I. Localization of the Antigen to Mitochondrial Membranes. *J. exp. Med.*, **126**, 277.

BERG, P. A., MUSCATELLO, U., HORNE, R. W., ROITT, I. M. & DONIACH, D. (1969) Mitochondrial Antibodies in Primary Biliary Cirrhosis. II. The Complement Fixing Antigen as a Component of Mitochondrial Inner Membrane. *Br. J. exp. Path.*, **50**, 200.

BERG, P. A., MUSCATELLO, U. ROITT, I. M. & DONIACH, D. (1967) Antibodies to Mitochondrial Inner Membranes in the Serum of Patients with Primary Biliary Cirrhosis. In *Protides of the Biological Fluids*. Amsterdam: Elsevier Publishing Co. vol. 15, p. 341.

FELDMAN, G., PENAUD, J. & CRASSOUS, J. (1972) Albumin Synthesis by the Human Liver: its Morphologic Demonstration. Abst. Fifth Meeting I.A.S.L., Versailles p. 28.

GRAHAM, R. C. JR & KARNOVSKY, M. J. (1966) The Early Stages of Absorption of Injected Peroxidase in the Proximal Tubules of Mouse Kidney: Ultrastructural Cytochemistry by a New Technique. *J. Histochem. Cytochem.*, **14**, 291.

HOEDEMAEKER, P. H. & ITO, S. (1970) Ultrastructural Localization of Gastric Parietal Cell Antigen with Peroxidase-coupled Antibody. *Lab. Invest.*, **22**, 184.

KNIESER, M. R. & JENIS, E. H. (1972) Immunoultrastructural Localization of Antimitochondrial Antibody from the Sera of Patients with Chronic Liver Disease. *Lab. Invest.*, **27**, 412.

LEDUC, E. G., AVRAMEAS, S. & BOUTEILLE, M. (1968) Ultrastructural Localization of Antibody in Differentiating Plasma Cells. *J. exp. Med.*, **127**, 109.

MAUNSBACH, A. B. (1966) Observations on the Segmentation of the Proximal Tubules in the Rat Kidney. Comparison of Results from Phase Contrast, Fluorescence and Electron Microscopy. *J. ultrastruc. Res.*, **16**, 239.

NAKANE, P. K. (1971) Application of Peroxidase Labelled Antibodies to the Intracellular Localization of Hormones. *Acta endocr. Copenh.*, **153**, (Suppl.), 190.

NAKANE, P. K. & PIERCE, G. B. JR. (1967) Enzyme-labelled Antibodies for the Light and Electron Microscopic Localization of Tissue Antigens. *J. cell. Biol.*, **33**, 307.

PETTS, V. & ROITT, I. M. (1971) Peroxidase Conjugates for Demonstration of Tissue Antibodies: Evaluation of the Technique. *Clin. & exp. Immunol.*, **9**, 407.