Clin. exp. Immunol. (1971) **9**, 407–418.

TECHNIQUES

PEROXIDASE CONJUGATES FOR DEMONSTRATION OF TISSUE ANTIBODIES: EVALUATION OF THE TECHNIQUE

VALERIE PETTS AND I. M. ROITT

Department of Immunology, Middlesex Hospital Medical School, London

(*Received* 8 *April* 1971)

SUMMARY

Conjugates of antibody with the enzyme marker, peroxidase, are relatively easy to prepare and can be shown to have a sensitivity in the indirect test for antinuclear antibodies which is entirely comparable to that obtained with fluorescein labelled material. Clear cut results were obtained in tests for autoantibodies on tissue sections and for bound IgG in biopsies using an antihuman immunoglobulin conjugate. Antibodies to *Treponema pallidum* were also readily demonstrable. Only a simple light microscope is required, slides can be read without fatigue and the morphology of the tissue section can be easily assessed. It is concluded that the peroxidase technique offers a useful alternative to immunofluorescence.

INTRODUCTION

Since its introduction by Coons & Kaplan (1950) the immunofluorescent method has had widespread use for the demonstration of tissue antigens, despite certain disadvantages associated with it. The technique involves conjugation of antibody to fluorescein isothiocyanate and visualization of the results by ultraviolet microscopy. The morphologic features of the resulting preparation are difficult to recognize, the immunofluorescence is prone to fading and permanent preparations cannot be made.

Recent introduction of enzyme-labelled antibodies by Nakane & Pierce (1967) and Avrameas (1969) presented a technique in which only a light microscope was needed and where permanent preparations, stainable by conventional methods were obtained. Studies successfully employing peroxidase labelling for the demonstration of tissue autoantibodies have been reported (cf. Davey & Busch, 1970; Benson & Cohen, 1970; Fukuyama *et al.*, 1970). The present study is an assessment of the relative convenience and sensitivity of peroxidase (Px−) as compared with fluorescent (Fl−) conjugates. Results of various applications of peroxidase labelled antibodies both by the direct and indirect techniques are presented.

Correspondence: Professor I. M. Roitt, Department of Immunology, Arthur Stanley House, Middlesex Hospital Medical School, London, W.1.

MATERIALS AND METHODS

Human sera

Sera sent for routine autoantibody testing in this Hospital, were stored at 4°C or −20°C until required.

Anti-immunoglobulin sera

A globulin fraction was prepared from pooled normal human serum by precipitation in 33% saturated ammonium sulphate and fractionation on a Sephadex G-25 column. Rabbits were immunized with 5 mg of the protein emulsified in Freund's complete adjuvant injected intramuscularly at four different sites and boosted with intravenous injections of 1 mg of alum precipitated protein given four times during weeks 3 and 4. Serum was taken 1 week later. Immunoelectrophoresis showed the antiserum to be polyvalent, with strong lines for IgG and IgA. No IgM arc was visible. This antiserum was stored at −70°C until required for conjugation.

Sheep antiserum raised against purified human IgG was obtained from Wellcome Reagents Ltd.

Labelling of antisera

The antisera were precipitated in 33% saturated ammonium sulphate, washed and layered onto a Sephadex G-25 column equilibrated against phosphate buffer 0·1 M, pH 6·8. The protein content of the eluate was calculated from the optical density at 280 mμ.

(a) *Peroxidase.* This was carried out following the method of Avrameas (1969). 12 mg horseradish peroxidase (Grade 1, Miles Seravac Ltd, Holyport, Maidenhead) were added to a solution containing 5 mg of the globulin fraction in 1 ml of 0·1 M phosphate buffer, pH 6·8. 0·05 ml 1% glutaraldehyde was then added while stirring and the mixture left at room temperature for 2 hr. After dialysis overnight against 5 litres of phosphate buffered saline at 4°C the conjugate was spun at 20,000 rev/min for ½ hr in the Rotor 50 head of a Spinco preparative ultracentrifuge. The conjugates were stored undiluted at 4°C for up to 3 months.

(b) *Fluorescein.* For the comparison of peroxidase and fluorescein conjugates, half of the globulins obtained from the sheep antiserum were dialysed against 0·25 M carbonate buffer and labelled with fluorescein isothiocyanate according to the method of Riggs *et al.* (1958).

Antigen substrates

Blocks of human and rat tissues were quenched in either precooled isopentane or in liquid nitrogen and stored at −70°C. Cryostat sections (4 μ) were cut and stored for up to 3 months at −70°C. The following tissues were used: human thyroid, stomach and kidney obtained at operation, and rat liver, stomach and muscle.

A suspension of *Treponema pallidum* supplied for the fluorescein treponemal antibody (FTA) test was obtained from B.B.L. Division of Bioquest (Cockeysville, Md, U.S.A.). Smears were prepared, fixed in 10% methanol for 20 sec and stored at −20°C.

Staining tissues with conjugated antisera

(a) *Indirect.* The method of Coons (1956) was followed in essence (Roitt & Doniach, 1969). Sections were treated for 20 min with test serum at room temperature, washed for

15 min with Coons' buffer (0·1 M sodium barbitone buffer, pH 7·2, containing 0·85% sodium chloride) and then covered with a drop of appropriately diluted conjugate for a further 20 min. The sections were finally washed in buffer for 15 min.

(b) *Direct.* Cryostat sections of kidney biopsies from patients with glomerulonephritis and of thyroid from a patient with thyrotoxicosis were stained with or without prior fixation in 1·25% paraformaldehyde in 0·1 M phosphate buffer, pH 7·2, for 5 min. After incubation in peroxidase conjugated anti-Ig at room temperature for 20 min the sections were washed for 20 min in Coons' buffer and then stained for peroxidase.

Peroxidase reaction

If desired, sections could be fixed in either 3% glutaraldehyde or in 1–5% paraformaldehyde for 5 min before the peroxidase reaction. The method of Graham & Karnovsky (1966) was followed using either 5 mg 3,3′-diaminobenzidine tetrahydrochloride or a saturated solution of 3,3′-diaminobenzidine free base (Sigma Chemical Co.) in 10 ml. 0·05 M tris buffer, pH 7·6. 0·01% H_2O_2 was added immediately before use. It was found that addition of 0·25 M sucrose to the enzyme substrate improved the morphology of the tissue, particularly in ultrastructural studies, without interfering with the enzymic reaction. A staining time of 4 min gave optimum discrimination of specific staining relative to the background. Sections were then rinsed in Coons' buffer. Staining of the reaction product could be enhanced by post-fixing in 2% osmium tetroxide for 5 min. This was only found necessary on sections with pale staining and is not recommended as a regular procedure.

Some sections were post-stained in 0·1% toluidine blue, 1% methylene blue or 1% methyl green.

All tissues were dehydrated and mounted in D.P.X.

RESULTS

Immunochemical specificity of peroxidase linked anti-Ig (Px-A) antisera

Two Ouchterlony plates were set up in duplicate using 1% agarose. Equal volumes of Px-A and rabbit antiovalbumin were placed in the centre well, with ovalbumin 5 mg/ml in one outer well and human IgG 5 mg/ml in the other. After precipitin lines had formed over-

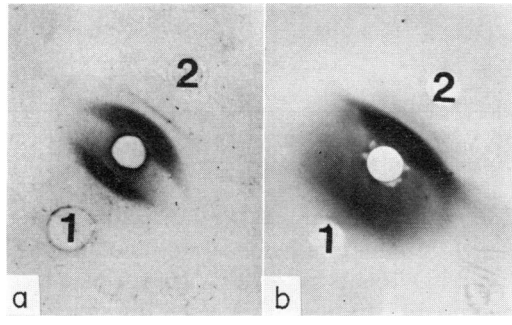

FIG. 1. Ouchterlony plates showing specificity of peroxidase-linked anti-Ig. Centre well: equal vol antiovalbumin and peroxidase conjugated anti-Ig; well 1: ovalbumin; well 2: human IgG. (a) Stained for protein; (b) stained for peroxidase.

night the plates were washed and dried; one was stained for protein with naphthylene black (Fig. 1a) and the other for peroxidase by the benzidine technique (Fig. 1b). It can be seen that positive staining for peroxidase was confined to the Px-A precipitin line. The specificity of this reaction was evident from the lack of adsorption of conjugate to the unrelated ovalbumin/antiovalbumin complex.

After conjugation with peroxidase the antibody titre, assessed by a slide agglutination test using IgG coated latex particles, fell from 1/80 to 1/20.

Comparison of peroxidase and fluorescein conjugates

Preliminary experiments indicated that Px-A would detect antibodies to tissues, e.g. antinuclear antibody (ANA), with results comparable to those obtained with fluorescein (Fl) conjugates. It was decided to investigate their relative sensitivities and the optimum

TABLE 1. Checkerboard titration of Px- and Fl- anti-IgG conjugates: indirect test for antinuclear antibodies

Dilution of ANA	Dilution of conjugate 10	20	40	80	160	320
	PEROXIDASE					
5	+++	++	++	+	+	+
20	+++	++	++	+	+	+
80	+++	++	++	+	+	+
160	++	++	++	+	+	+
320	++	+	+	+	+	+
640	++	+	+	+	+	±
1280	+	±	±	+	+	−
C*	−	−	−	−	−	−
	FLUORESCEIN					
5	+++	+++	+++	++	+	+
20	+++	+++	++	++	+	+
80	+++	+++	++	++	+	+
160	+++	++	++	++	+	+
320	++	++	++	++	+	+
640	+	+	n.d.	+	+	±
1280	+	±	+	+	±	±
C*	−	−	−	−	−	−

n.d. = not done. Both conjugates were prepared from the same anti-IgG.
* Control with undiluted ANA negative serum.

dilutions which would give positive staining in the indirect test using fluorescein and peroxidase conjugates prepared from the same batch of antihuman IgG. Several positive ANA sera were pooled and stored in aliquots at −70°C. They were tested on sections of human thyroid tissue by a checkerboard titration using serial dilutions from 1/5 to 1/1280 of the ANA positive serum and from 1/10 to 1/320 of the conjugates. An undiluted ANA negative serum was included as a control. The slides were coded before examination.

As may be seen from Table 1, Px- and Fl-conjugates were of comparable sensitivity.

When the test was repeated on another occasion, identical results were obtained. A dilution of 1 in 20 of the Px-A gave the most clearly defined staining: this dilution was used in all subsequent applications of the indirect test. Readings made on coded photomicrographs of the Px-A stained slides correlated exactly with the readings shown in Table 1.

Absorption of a 1/5 dilution of Px-A with 100 mg/ml pig liver acetone powder did not affect the titre of the conjugate but marginally reduced background staining which in any case tended to be insignificant.

Stability of conjugates

After storage of the Px-A for 2 months at 4°C, no loss of titre was found using the same batch of ANA serum and thyroid tissue. Subsequent conjugates of the same antiserum made over a period of 6 months also gave identical results (i.e. staining of ± intensity at a dilution of 1/640 for the ANA serum and 1/320 for the Px-A). Conjugates stored at −20°C also retained their activity.

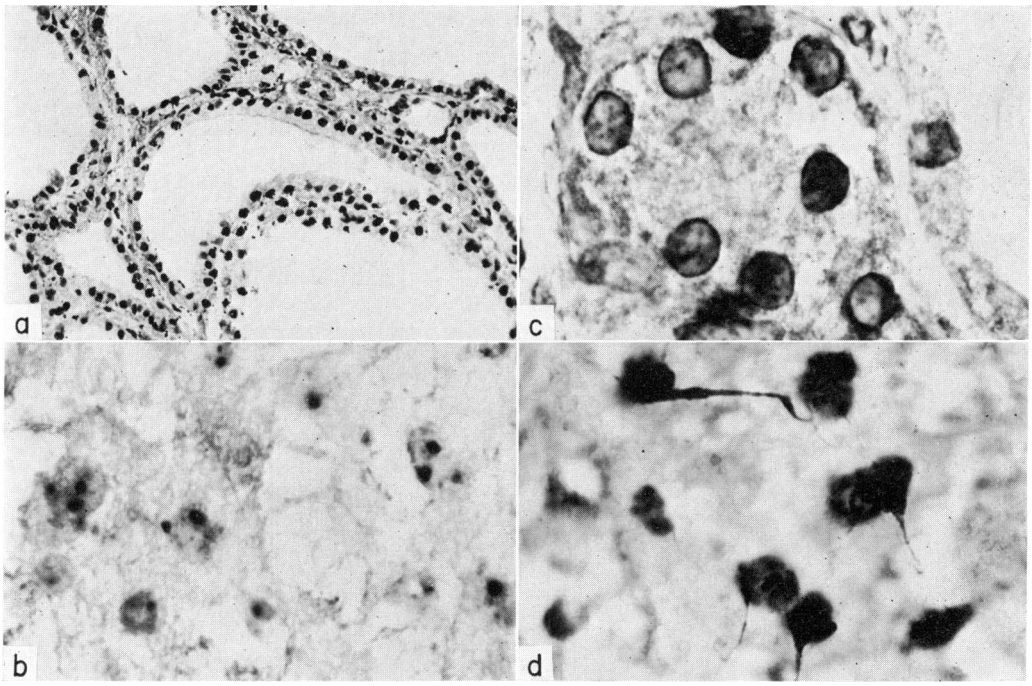

FIG. 2. Antinuclear antibodies. (a) Diffuse (human thyroid) × 250. (b) Nucleolar (rat liver) × 1600. (c) Membranous (human thyroid) × 1600. (d) Trailing (rat liver) × 1600.

Applications of the technique

The indirect test was used for the demonstration of human autoantibodies. The test serum was applied undiluted to the section and the Px-A at 1/20 dilution. Better defined results could sometimes be obtained by diluting the test sera and so reducing the non-specific background staining. Controls using serum negative for all autoantibody tests or peroxidase-linked normal rabbit IgG were included.

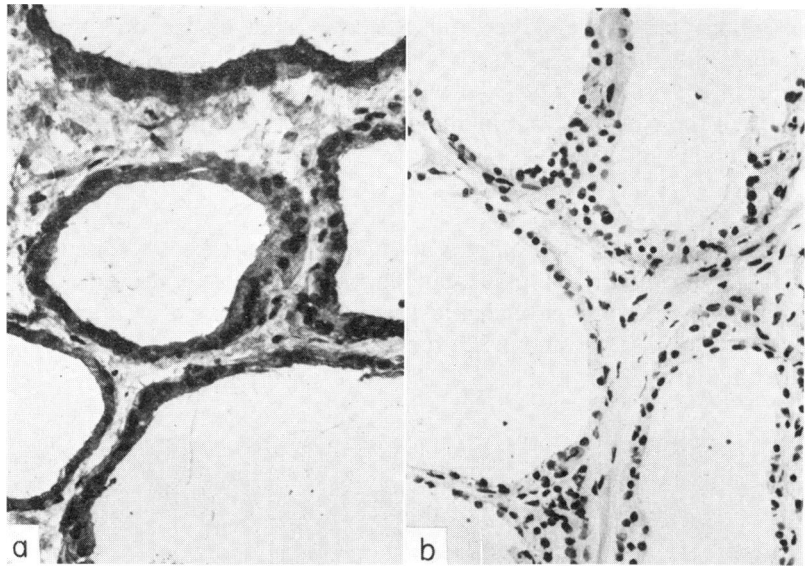

FIG. 3. (a) Thyroid cytoplasmic antibodies (human thyroid). (b) Control. Both with nuclear counterstain. ×200.

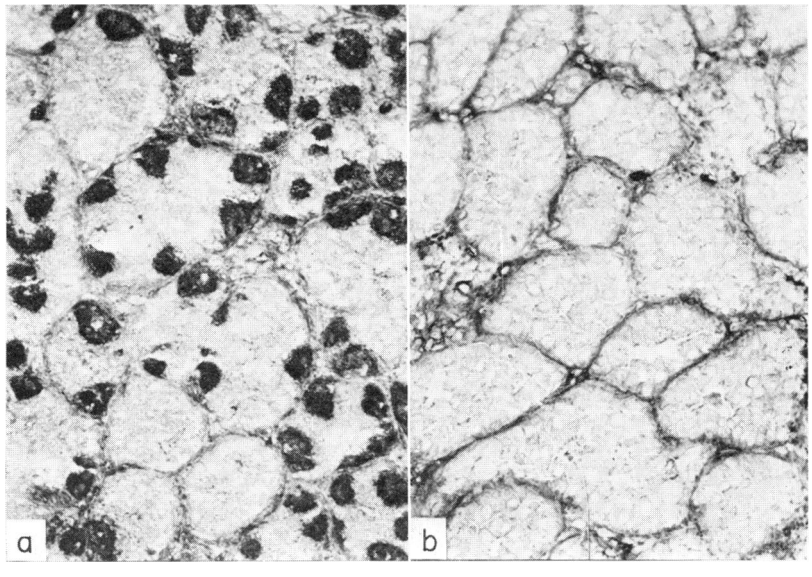

FIG. 4. (a) Parietal cell antibodies (human stomach). (b) Control. ×200.

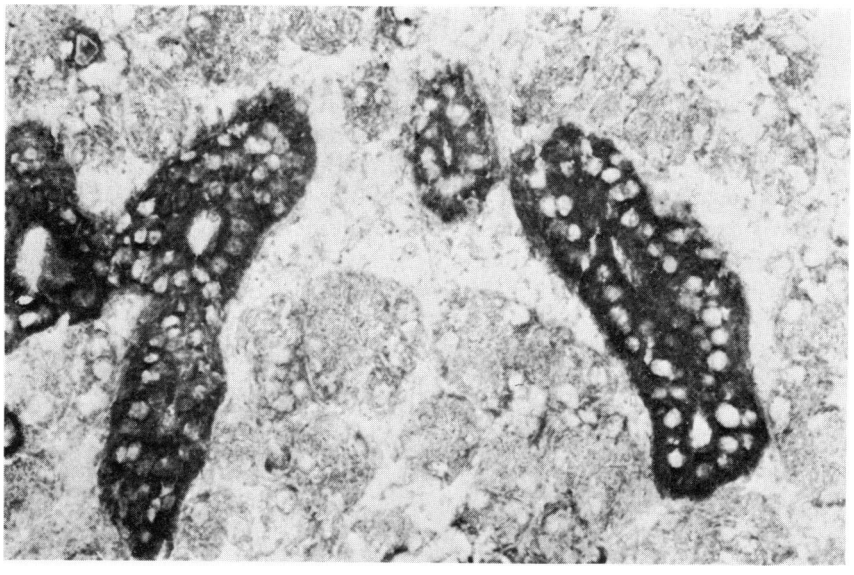

FIG. 5. Mitochondrial antibodies staining duct cells in rat submaxillary gland. ×290.

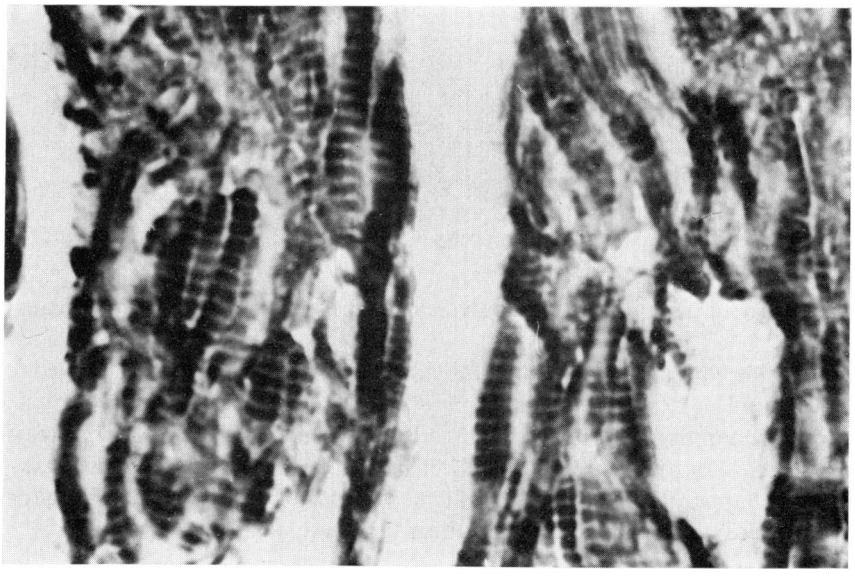

FIG. 6. Striated muscle antibodies (rat skeletal muscle). ×1800.

The results showed that tissue autoantibodies gave clear staining patterns which were easily evaluated. A number of examples are given in Figs 2–6. All photographs were taken and printed under identical conditions. Only in the case of fixed thyroid sections stained with anti-thyroglobulin* were the results inferior to those obtained with immunofluorescence.

Direct staining

As with the immunofluorescent technique, antibodies conjugated with peroxidase can be applied directly to sections for the demonstration of tissue antigens. For example, antibody raised in rabbits against human kidney proximal tubules (provided by Dr B. Phillips), was linked to peroxidase and used to stain sections of human kidney, either unfixed or fixed in 1·25% paraformaldehyde. The conjugate was used at a 1 in 5 dilution to give the best definition. Staining was localized to the brush border of the proximal tubules (Fig. 7). The

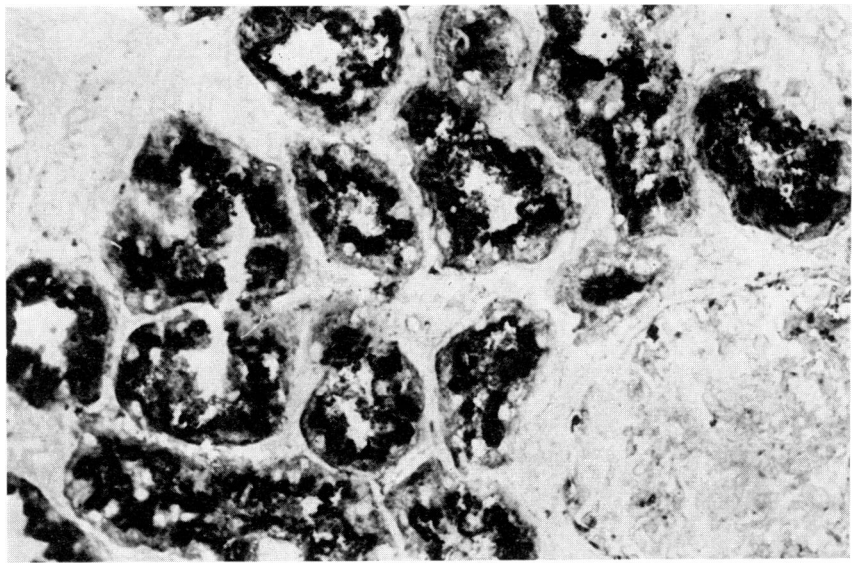

FIG. 7. Human kidney section showing peroxidase-linked antibody directly staining the brush border. ×290.

staining pattern obtained with an equivalent fluorescein conjugate was somewhat more diffuse.

Direct staining with an anti-immunoglobulin conjugate is frequently employed to visualize antibodies bound or deposited in tissues *in vivo*. Fig. 8 shows an unusual feature found on reacting a section of thyrotoxic thyroid directly with Px-A. The staining indicates deposition of IgG at the base of the acinar epithelial cells possibly in the form of complexes since β_{1C} can also be demonstrated. (Material kindly supplied by Dr D. Doniach.) Control sections reacted with peroxidase-linked normal rabbit IgG were negative.

Treponemal antibody test

Sera for testing were diluted 1 in 5 in FTA-ABS Test Sorbent (B.B.L. Laboratories) and

* Tanned red cell agglutination is the test normally employed for detection of these antibodies.

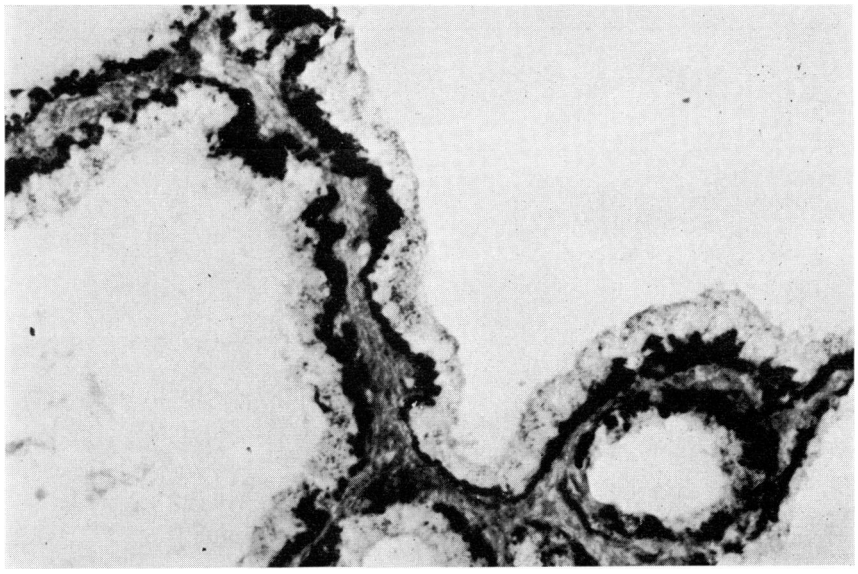

FIG. 8. Section of human thyroid stained by peroxidase-linked anti-Ig demonstrating unusual deposits of immunoglobulin. ×400.

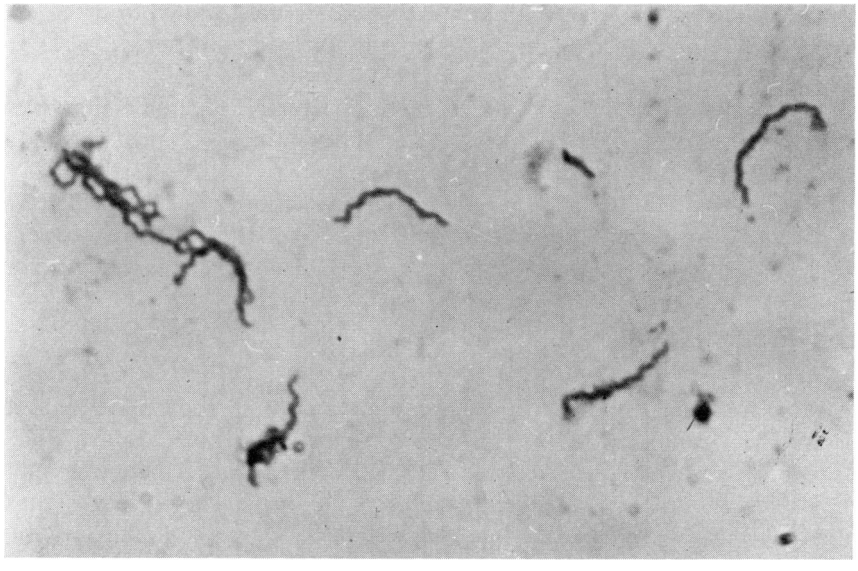

FIG. 9. *Treponema pallidum* stained for antibody by the indirect technique. ×2000.

TABLE 2. Comparison of Px-A and Fl-A in test for treponemal antibodies

Serum	Px-A (EC)*	Px-A (VP)*	Px-A (VP)†	Fl-A (EC)
CONTROLS				
Non-spec. Pos. 1/10 P.B.S.		++		++
Non-spec. Pos. 1/20 P.B.S.		+		+
Non-spec. Pos. 1/5 Sorbent		−		−
TEST SERA				
No. 1	+	+	+	+
2	+++	+++	+++	+++
3	n.d.	n.d.	+++	++
4	n.d.	n.d.	++	++
5	+++	+++	++	++
6	+++	+	+	++
7	+	++	+	+
8	+++	+++	+++	+++
9	±	±	+	−
10	+	+	+	±
11	n.d.	n.d.	±	−

n.d. = not done.
* Different observers reading the same test.
† Tested and read on a separate occasion.

incubated for ½ hr at 37°C on fixed smears of *T. pallidum*. Subsequent steps followed those already outlined for the ANA specificity test. Following the normal routine testing procedure, non-specific positive control serum was also tested, diluted with either P.B.S. (1 in 10 and 1 in 20) to give + or ++ readings respectively, or with sorbent (1 in 5) to give a negative reading.

The slides were coded and read by two individuals independently and compared with the readings previously obtained with fluorescent antibody. Smears stained for endogenous peroxidase were negative.

It can be seen from Table 2 that a very good correlation was obtained between the Px-A test and the Fl-A test and between readings of two observers in all except one of the sera tested (No. 6). In the routine FTA test a ± or + reading is considered inconclusive and is repeated.

DISCUSSION

The peroxidase linked antibody method appears to provide a convenient and adaptable technique for the localization of tissue antigens. Several substrates are available for the peroxidase staining reaction in tissues but the benzidine-brown technique was chosen as being simple and providing a permanent preparation, although Straus (1964) claims that it is not as intense or sensitive as the benzidine-blue technique. Other methods using 3-amino-9-ethyl carbazole (Graham & Karnovsky, 1965) α-naphtholpyronin and 4-Cl-1-naphthol (Nakane, 1968) have been successfully employed for the localization of multiple tissue

antigens (Nakane, 1968). The benzidine-brown stained reaction product is very stable so permitting modification of the method to demonstrate other cell constituents or functions. For example Straus (1967) has shown that acid phosphatase and peroxidase activities can be demonstrated in the same section. This technique could no doubt be applied to other enzymes. Work is in hand in this laboratory to combine the Px-A method with autoradiography for double-labelling experiments. Although fixation in 2% osmium tetroxide enhances the staining reaction this step was only found necessary in sections where the reaction product was weak. Use of a blue counterstain was found to intensify the brown stain and to increase the contrast relative to the background.

Direct comparison of peroxidase and fluorescein conjugated antisera by checkerboard titration in an indirect system for antinuclear antibodies, established that the sensitivities of both the methods were identical. Although the preparation of slides by the immunofluorescent technique is slightly quicker and has already been widely applied, the peroxidase method offers certain advantages:

(a) conjugation with peroxidase using glutaraldehyde is relatively straightforward;

(b) the preparations are permanent and may be stored for reference;

(c) they are examined in a conventional light microscope so that specialized and relatively expensive u.v. microscopes are not required (a significant consideration for work at high magnification), and the tissue structure may be easily identified and photographed. Furthermore the examination of large numbers of slides is less fatiguing because the results are more easily read, particularly by inexperienced observers, and there is no need to work in a darkened room, making it easier to record the results;

(d) preparations can be further studied by cytochemical techniques or by staining with a second antibody labelled with radioisotope or a different enzyme marker;

(e) ultrastructural studies may also be carried out on stained preparations because the reaction product is electron dense. It has the advantage over ferritin conjugates of smaller size and therefore presumably more readily penetrates to intracellular antigens.

It is felt therefore that the advantages and scope of peroxidase linked antibody techniques are considerable. When the ease and speed of reading slides without fatigue are of particular benefit as in the treponemal antibody test, the peroxidase technique offers a useful alternative to immunofluorescence.

ACKNOWLEDGMENTS

We wish to thank Mrs E. Chipperfield for her assistance with the treponemal antibody test, and Mrs G. Stead for preparing the manuscript. The work was supported by grants from the Medical Research Council and the World Health Organization.

REFERENCES

AVRAMEAS, S. (1969) Coupling of enzymes to proteins with glutaraldehyde. *Immunochemistry*, **6**, 43.

BENSON, M.D. & COHEN, A.S. (1970) Antinuclear antibodies in systemic lupus erythematosus. Detection with horseradish peroxidase conjugated antibody. *Ann. Intern. Med.* **73**, 943.

COONS, A.H. (1956) Histochemistry with labelled antibody. *Int. Rev. Cytol.* **5**, 1.

COONS, A.H. & KAPLAN, M.H. (1950) Localization of antigen in tissue cells. *J. exp. Med.* **91**, 1.

DAVEY, F.R. & BUSCH, G.J. (1970) Immunohistochemistry of glomerulonephritis using horseradish peroxidase and fluorescein labelled antibody—a comparison. *Amer. J. clin. Path.* **53**, 531.

FUKUYAMA, K., DOUGLAS, S.D., TUFFANELLI, D.L. & EPSTEIN, W.L. (1970) Immunohistochemical method for localization of antibodies in cutaneous disease. *Amer. J. clin. Path.* **54,** 410.

GRAHAM, R.C. & KARNOVSKY, M.J. (1965) Cytochemical demonstration of peroxidase activity with 3-amino-9-ethyl carbazole. *J. Histochem. Cytochem.* **13,** 150.

GRAHAM, R.C. & KARNOVSKY, M.J. (1966) The early stages of absorption of injected horseradish peroxidase into the proximal tubules of mouse kidney. *J. Histochem. Cytochem.* **14,** 291.

NAKANE, P.K. (1968) Simultaneous localization of multiple tissue antigens utilizing the peroxidase labelled antibody method. 19th Ann. Meeting Histochem. Soc., New Orleans, 1968.

NAKANE, P.K. & PIERCE, G.B. (1967) Enzyme-labelled antibodies for the light and electron microscopic localization of tissue antigens. *J. Cell Biol.* **33,** 307.

RIGGS, J.L., SELWALD, R.J., BURCKHALTER, J.H., DOWNS, C.M. & METCALF, T.G. (1958) Isothiocyanate compounds as fluorescent labelling agents for immune serum. *Amer. J. Path.* **34,** 1081.

ROITT, I.M. & DONIACH, D. (1969) *Immunofluorescent Tests for the Detection of Autoantibodies.* W.H.O. Handbook.

STRAUS, W. (1964) Factors affecting the cytochemical reaction of peroxidase with benzidine and the stability of the blue reaction product. *J. Histochem. Cytochem.* **12,** 462.

STRAUS, W. (1967) Method for the study of small phagosomes and their relationship to lysosomes with horseradish peroxidase as a 'marker protein'. *J. Histochem. Cytochem.* **15,** 375.