

# ULTRASTRUCTURAL LOCALIZATION OF INTRACELLULAR ANTIGEN USING ENZYME- LABELED ANTIBODY FRAGMENTS

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## ABSTRACT

The efficiency of small enzyme-labeled tracers for the demonstration of intracellular antigen was investigated in tissues fixed with picric acid-formaldehyde. The influence of fixation on the immunological activity was tested *in vitro* by radial immunodiffusion. The experimental model consisted of newborn pig jejunum after absorption of ferritin from the intestinal lumen. Ferritin was located after 1 hr in vacuoles scattered in the cytoplasm of the absorptive cells and represented an easily recognizable intracellular antigen. After immunohistochemical treatments with antiferritin preparations, the distribution of labeling enzyme reaction product was examined by morphometry. The ratio of the labeled volume to the total volume of vacuoles containing ferritin indicated the degree of specific labeling of the antigen. In both direct and indirect methods, the degree of labeling was low when enzyme-labeled immunoglobulin G was the tracer. With antigen binding fragments (Fab), the labeling was significantly increased. In the indirect method, the degree of labeling was influenced by the first-step reagents. Only when the serum titer was optimum was a high degree of labeling obtained. With antigen binding fragments or papain-digested serum the effect of the titer was negligible and maximum labeling was achieved. In both methods, with peroxidase as the labeling enzyme, a diffuse nonspecific deposition of reaction product was observed. This could be avoided by using cytochrome *c* instead.

## INTRODUCTION

The progressive development of immunohistochemical techniques for the localization of antigens at the ultrastructural level has been hampered by numerous problems, some of which are not yet satisfactorily solved. The earliest used tracer was ferritin-conjugated antibody (18, 27). To obviate some of the drawbacks of techniques involving conjugation, improved coupling methods have been developed (2) or substitute techniques avoiding conjugation have been proposed (8, 16). Fixation before immunological treatment in order to retain morphological detail has been found neces-

sary in all cases, excepting that of surface antigens of isolated cells (8). Whatever the tracer, nonspecific adsorption and insufficient penetration to the antigenic site may cause false positive as well as false negative results. These difficulties can be overcome by the use of two approaches, by reducing the thickness of the specimen or the size of the tracer. The first approach has been extensively investigated in recent years. Thin sections have been prepared from frozen tissues (14), from specimens embedded in methacrylate (12, 14), or from material embedded in polymerized serum albumin

(17). The second approach was investigated by Nakane and Pierce (21, 22) and Avrameas and Uriel (3) who introduced the use of enzyme-labeled antibodies. Other procedures, using heavy metals as labels (30, 31, 33), have had limited applications.

The purpose of this work was to investigate the influence of small tracers, obtained by reducing the immunological and enzymatic components, on intracellular antigen demonstration. This was done by comparing papainic 3.5S Fab<sup>1</sup> fragments with 7S antibodies and cytochrome *c* with peroxidase. The histochemical model consisted of the epithelial jejunal cell of the newborn pig (13) into which a recognizable antigen, i.e. ferritin, could be introduced by intestinal absorption. Localization of antigen was carried out on nonfrozen sections of fixed tissues. The interactions between fixatives and immunological activity were investigated *in vitro*.

## MATERIALS AND METHODS

### IMMUNOLOGICAL PROCEDURES

#### *Immunoplates*

The single radial immunodiffusion test (15) developed in a previous paper (13) was employed with various fixatives. Plates containing antiferritin antibodies incorporated in the agarose were immersed overnight at 4°C in 2.7% formaldehyde with or without 0.2% picric acid, in 3% glutaraldehyde, or in 95% ethanol. Formaldehyde and glutaraldehyde were buffered with 0.1 M, pH 7.4 cacodylate-HCl. Plates were rinsed for 48–72 hr in physiological saline. Some formaldehyde- and glutaraldehyde-treated plates were then immersed for 12 hr in 3% lysine or in 3% sodium bisulfite and rinsed overnight in saline. Wells were cut in the agarose and filled with ferritin at concentrations of 40, 20, 10, 5, and 2.5 mg/ml. After diffusing for 48 hr, the plates were rinsed overnight, dried, and stained with prussian blue. Precipitation rings were measured with a precision of ±0.05 mm. Plates containing control serum were immersed in the same fixatives and treated in

<sup>1</sup> Abbreviations used in the text: Rb, rabbit; Rt, rat; Sh, sheep; ab, antibody; a-, anti-; *c*, cytochrome *c*; F, ferritin; Fab, antigen binding fragment; Fc, crystallizable fragment; IgG, immunoglobulin G; P, peroxidase; S, serum; x<sup>c</sup>, substance x coupled to cytochrome *c*; x<sup>p</sup>, substance x coupled to peroxidase; CM-C, carboxymethyl-cellulose; DAB, 3,3'-diaminobenzidine; DEAE-C, diethylaminoethylcellulose; PAF, picric acid-formaldehyde; SDS, sodium dodecyl sulfate; THB, Tris-HCl buffer.

the same manner. Plates without fixative treatment were prepared similarly

#### *Antigens*

Horse spleen ferritin (Pentex Biochemical, Kankakee, Ill.), rabbit IgG, rabbit Fab and Fc fragments, sheep IgG, and sheep Fab fragments were used.

#### *Immunization Procedures and Antisera*

RABBIT ANTIFERRITIN SERUM (RbS A-F) was produced by weekly injections of 5 mg of ferritin incorporated in complete Freund's adjuvant (Difco Laboratories, Inc., Detroit, Mich.) into the hind footpads and subcutaneous tissue of the neck. Bleeding was started 1 wk after the third injection.

SHEEP ANTIRABBIT IGG SERUM (ShS A-RbIGG) AND SHEEP ANTIRABBIT FAB FRAGMENTS SERUM (ShS A-RbFAB) were obtained by immunization with 3 mg of purified antigen in complete Freund's adjuvant. Injections in the neck and thigh were repeated each week and bleeding was started 1 wk after the fourth injection.

RAT ANTIRABBIT SERUM (RtS A-RbS), RAT ANTISHEEP SERUM (RtS A-ShS), RAT ANTISHEEP IGG SERUM (RtS A-ShIGG), AND RAT ANTISHEEP FAB FRAGMENTS SERUM (RtS A-ShFAB) were prepared by injecting 150 µg of antigen in complete Freund's adjuvant into the four footpads. Two further injections without adjuvant were repeated at weekly intervals. 1 wk after the last injection, the rats were bled by cardiac puncture and sacrificed.

#### *Purification of IgG and Antibodies*

SHEEP AND RABBIT IGG: These were obtained by fractionation of the antisera on DEAE-Cellulose DE 22 (Whatman, Balston, England). Details of the method have been published (13).

SHEEP AND RABBIT ANTIBODIES: Sheep and rabbit antibodies were obtained by polyacrylamide immunoadsorption as described by Carrel et al. (4). For rabbit antiferritin antibodies, the concentration of the gel was 9 g acrylamide (BDH Chemicals Ltd., Poole, England) and 3 g bis-acrylamide per 100 ml H<sub>2</sub>O; for sheep antirabbit IgG or Fab antibodies, the ratio was 7.5 g acrylamide and 2.5 g bisacrylamide per 100 ml H<sub>2</sub>O. Purity and specificity of antibodies were tested by immunodiffusion and immunoelectrophoresis.

#### *Papain Digestion*

RABBIT IGG: Rabbit IgG was digested according to the method of Porter (24), and the fragments were purified on CM-cellulose 22 (Whatman, Balston, England), followed by exclusion chromatography. The purity of the Fab or Fc fragments was

checked by polyacrylamide electrophoresis. The antisera obtained after injection of fragments into sheep were shown by immunoelectrophoresis to be directed against only one type of fragment.

**TOTAL ANTIFERRITIN SERUM:** This serum was digested in the same way but without further purification. Digestion was checked by testing for the disappearance of precipitation properties by immunodiffusion.

**SHEEP IgG:** Sheep IgG was digested according to Heimer's (9) modification of the method of Porter. Some Fab fragments were purified on CM-cellulose, followed by exclusion chromatography. Purity was checked in the same manner as for rabbit Fab fragments but with rat antisera. Other batches of Fab were prepared by prolonged papain digestion without further purification (9).

### Conjugation

IgG, antibodies, or Fab fragments were coupled to peroxidase according to the technique of Avrameas (2). The ratio was 12 mg of peroxidase (90 U/mg, Fluka AG, Buchs, Switzerland) to 5 mg of IgG, ab, or Fab in 1 ml of 0.1 M, pH 6.8 phosphate buffer. 50  $\mu$ l of 1% glutaraldehyde purified by vacuum distillation were added. The solutions were then dialyzed overnight against 0.01 M, pH 7.4 phosphate buffer at 4°C, centrifuged at 15,000 rpm, and chromatographed on Sephadex G 200 (Pharmacia Fine Chemicals, Inc., Uppsala, Sweden). For cytochrome *c* (type VI, Sigma Chemical Co., St. Louis, Mo.), the same method was used but with the following ratio: 8 mg of cytochrome *c* to 5 mg of Fab fragment. After conjugation, the solutions were chromatographed on Sephadex G 100.

### Other Methods

Protein concentrations were determined by Folin reagents or by the micromethod of Kjeldahl.

**IMMUNODIFFUSION:** The *double immunodiffusion* technique of Ouchterlony (23) was done on microscope slides using 2% agar in pH 8.2 Veronal buffer at  $\Gamma = 0.05$ .

**IMMUNOELECTROPHORESIS:** Immunoelectrophoresis was performed on microscope slides as described by Scheidegger (25). The gel was the same as for immunodiffusion. A field strength of 3.5 v/cm for 90 min was applied across the agar.

**EXCLUSION CHROMATOGRAPHY:** Exclusion chromatography was performed on Sephadex G 200 or G 100. Samples of 1–2 ml were dialyzed against 0.01 M, pH 7.4 phosphate buffer and applied to columns measuring 2  $\times$  90 cm equilibrated with the same buffer. The rate of flow was 8 ml/hr. The void volume, calibrated with dextran blue, was 90 ml for Sephadex G 200 and 100 ml for Sephadex G

100. The optical density of fractions was read at 280 m $\mu$ .

**POLYACRYLAMIDE GEL ELECTROPHORESIS:** This was performed in the presence of sodium dodecyl sulfate (SDS) according to the procedure of Shapiro et al. (26), and molecular weights were calculated by comparison with proteins of known molecular weight (bovine IgG, alkylated reduced bovine IgG, cytochrome *c*, pepsine, bovine serum albumin, ovalbumin).

## MORPHOLOGICAL PROCEDURES

### Animals

The study was performed on seven newborn piglets which had been separated from the sow immediately after birth and before any ingestion of colostrum.

### Experimental Procedure

After an intraperitoneal injection of 30 mg of sodium pentobarbital (Vetanarcol, Veterinaria, Zurich, Switzerland) per kg of body weight, a laparotomy was performed and a solution of 10% horse spleen ferritin, dialyzed against isotonic saline, was introduced into the jejunum at known distances from the pylorus. The intestine was ligated above the site of injection.

### Fixation

1 hr after the ferritin injection, the anesthetized piglets were sacrificed by arterial perfusion of fixative (13), preceded by oxygenated Ringer's solution containing 0.1% procain (6). Both Ringer's solution and fixatives were cooled in an ice bath. The following fixatives were used: 2.7% formaldehyde freshly prepared by conversion of paraformaldehyde (10) with or without 0.2% picric acid (29), buffered at pH 7.4 with 0.075 M cacodylate-HCl; 2.2% glutaraldehyde purified by vacuum distillation and buffered at pH 7.4 with 0.1 M cacodylate-HCl. The fixatives contained 5 mEq/liter CaCl<sub>2</sub> and their final osmolarity was adjusted with NaCl to 1050 mosmols/liter for the formaldehyde fixatives, to 380 mosmols/liter for the glutaraldehyde. After perfusion, jejunal tissue was removed, cut into small pieces, further fixed by immersion in the respective fixative at 4°C for 2 hr and washed in several changes of cold 0.2 M, pH 7.4 cacodylate-HCl buffer or 0.2 M, pH 7.4 Tris-HCl buffer (THB) before being stored at 4°C in sealed glass vials. Some glutaraldehyde-fixed specimens were immersed for 3 hr at 4°C in 3% sodium bisulfite or in 3% lysine before being rinsed and stored in THB.

## Immunoenzyme Histochemistry

Nonfrozen sections were cut on a Sorvall TC-2 tissue sectioner (Ivan Sorvall, Inc., Norwalk, Conn.) (28) at 30–40  $\mu$ . Care was taken to orient the tissues so that the plane of section was parallel to the villi. The sections were treated by the direct or the indirect technique. Immunoenzymatic reagents were dialyzed overnight against 0.2 M, pH 7.4 THB or cacodylate-HCl before use. Incubations with immunological reagents were performed at room temperature during 5 hr.

A volume of 0.1–0.2 ml of reagent was sufficient for the incubation of one batch of TC-2 sections. All rinses in THB or in cacodylate-HCl were done in 0.2 M, pH 7.4 solutions at 4°C with constant agitation. For the direct method, TC-2 sections were incubated with the tracer and washed overnight in buffer. For the indirect method, the sections were incubated with the first-step reagent, rinsed overnight in buffer, incubated with the second-step tracer, and washed overnight in buffer. Some TC-2 sections were then immersed for 3 hr at 4°C in 3% glutaraldehyde in 0.1 M, pH 7.4 cacodylate-HCl buffer. After rinsing 4 hr in THB, enzyme activity was revealed. All sections were first immersed for 1 hr in 50 mg% 3,3'-diaminobenzidine (DAB) in 0.2 M, pH 7.4 THB. Peroxidase activity was demonstrated by incubation in 50 mg% DAB and 0.02% H<sub>2</sub>O<sub>2</sub> in 0.2 M, pH 7.4 THB at room temperature for 10 min (7). Cytochrome *c* activity was revealed by incubation in 50 mg% DAB and 0.05% H<sub>2</sub>O<sub>2</sub> in 0.05 M, pH 3.9 citrate buffer corrected with NaCl to 350 mosmols/liter for 45 min at 37°C (11). After the enzymatic activity had been revealed, sections were rinsed repeatedly in THB and rinsed overnight in 0.2 M, pH 7.4 cacodylate-HCl buffer at 4°C with constant agitation.

The immunological reagents used are summarized in Table I. Controls for the direct method consisted of sections incubated with enzyme-labeled rabbit serum, IgG, or Fab without antiferritin activity. Control sections for the first step of the indirect method were incubated with rabbit serum without antiferritin activity and with RbS a-F absorbed with ferritin. For the second step, control sections were first exposed to specific antiferritin serum, then incubated in unlabeled ShFab a-RbIgG or ShFab a-RbFab, and finally incubated in the same reagents coupled to enzyme. All controls were negative.

Endogenous peroxidase- and cytochrome *c*-like activities were checked by examining tissues without previous immunological treatment. In both cases, activity was present in erythrocytes and in granules of polynuclear leucocytes. Cytochrome *c*-like activity was weak on mitochondrial cristae. No reaction product was found in relation to ferritin.

TABLE I  
Immunological Reagents

| Direct Method           |                              |
|-------------------------|------------------------------|
| Rb S <sup>P</sup> a-F   |                              |
| Rb IgG <sup>P</sup> a-F |                              |
| Rb ab <sup>P</sup> a-F  |                              |
| Rb Fab <sup>P</sup> a-F |                              |
| Indirect Method         |                              |
| Ist step                | 2nd step                     |
| Undiluted Rb S a-F      | Sh IgG <sup>P</sup> a-Rb IgG |
|                         | Sh ab <sup>P</sup> a-Rb IgG  |
|                         | Sh Fab <sup>P</sup> a-Rb IgG |
| Diluted Rb S a-F        | Sh Fab <sup>P</sup> a-Rb IgG |
|                         | Sh Fab <sup>c</sup> a-Rb IgG |
| Rb Fab a-F              | Sh Fab <sup>P</sup> a-Rb Fab |
| Digested Rb S a-F       | Sh Fab <sup>P</sup> a-Rb Fab |
|                         | Sh Fab <sup>c</sup> a-Rb Fab |

## Preparative Procedures for Light and Electron Microscopy

Specimens were postfixed during 60 min in 2% OsO<sub>4</sub> buffered with 0.2 M, pH 7.4 sodium cacodylate. Some tissues were treated for 45 min with 0.5% uranyl acetate. The sections were then dehydrated in ethanol and flat-embedded in Epon 812. Under a dissecting microscope, blocks were selected in which complete villi were oriented parallel to the plane of section of the ultramicrotome. 1- $\mu$  thick sections, parallel to the axis of the villi, were stained in 1% azur II and 1% methylene blue in 1% borax and examined by light microscopy. Some thick sections were also examined unstained. Thin sections cut on a Sorvall MT-2 Porter-Blum microtome with a diamond knife were picked up onto grids covered with a Parlodion film (Mallinckrodt Chemical Works, St. Louis, Mo.) reinforced with carbon. Some sections were examined unstained while others were double-stained with uranyl acetate followed by lead citrate. Micrographs were taken on a Philips EM 300 electron microscope at 80 kv with a 40  $\mu$  thin metal aperture in the objective.

## Volumetric Analysis of Ferritin-Containing Vacuoles

The degree of labeling of ferritin contained in vacuoles by the immunoenzyme tracers was determined by a stereological method (32). Random micrographs of epithelial cells in villi sectioned parallel to their axis were taken at the same original magnification (2500) on 35 mm film. 35 micrographs were taken for each preparation, and samples

consisted of six preparations. A minimum of 500 vacuoles per sample were analyzed. Positive films prepared by contact were examined on a projector unit fitted with a multipurpose 168 point test screen (Institute of Anatomy, Bern). Points on vacuoles were counted. The ratio of the number of points on labeled vacuoles to the number of points on all vacuoles was calculated and gave the relative labeled vacuolar volume which was expressed as per cent of total vacuolar volume.

## RESULTS

### *Effects of Fixatives*

**EFFECTS ON IMMUNOPLATES:** On all immunoplates, the diameter of the precipitation rings increased with increasing ferritin concentra-

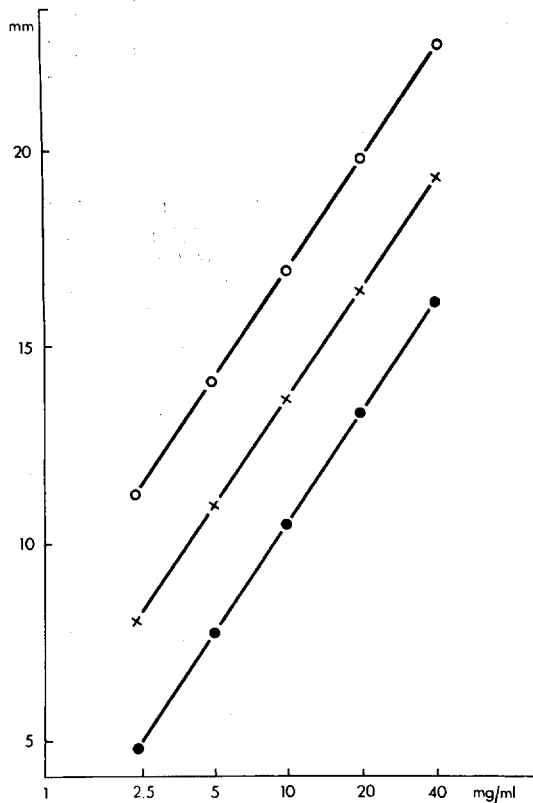


FIGURE 1 Influence of fixation on antibody activity. Precipitation rings were obtained by radial immunodiffusion of ferritin in agarose containing antiferritin antibodies. On the graph, the diameter of the ring in millimeters is plotted against the logarithm of ferritin concentration. Standard immunoplates (●—●); plates treated with ethanol (X—X); plates treated with formaldehyde or PAF (○—○).

tion. Straight lines were obtained by plotting the diameter against the logarithm of the concentration (Fig. 1). Each point represents the mean of measurements on eight separate plates. The standard error of the mean was lower than 0.1 mm for all points on the graph. On immunoplates treated with fixatives, the diameter of the rings, for any concentration of ferritin, was larger than on standard plates. This shift of the line was smaller after ethanol treatment than after formaldehyde. The addition of picric acid to formaldehyde did not modify the shift. The three lines were parallel. When control sera were incorporated into the agarose, no specific adsorption of ferritin was observed on plates fixed with ethanol, formaldehyde, picric acid-formaldehyde (PAF), or on standard plates. In contrast, after glutaraldehyde fixation, nonspecific adsorption was marked and did not change after lysine or bisulfite treatment. For this reason, glutaraldehyde-treated plates were not included in the results shown on Fig. 1.

**EFFECTS ON TISSUES:** In specimens fixed by perfusion of glutaraldehyde, tissue preservation was good. After addition of picric acid, formaldehyde provided an adequate preservation of ultrastructure. However, after incubations with immunological reagents and repeated rinsings, tissues were less well preserved. Histochemical demonstration of peroxidase or cytochrome *c* caused further swelling of organelles and extraction of cytoplasmic matrix. These alterations due to diaminobenzidine and  $H_2O_2$  treatment were less prominent when tissues had been immersed in glutaraldehyde immediately before.

### *Enzyme-Labeled Tracers*

**CHARACTERISTICS OF TRACERS:** In order to obtain well-defined tracers of known molecular weight, rabbit or sheep IgG, ab, or Fab, coupled with enzymes, were purified by exclusion chromatography.

**IgG<sup>P</sup> and ab<sup>P</sup>:** The elution patterns on Sephadex G 200 of solutions of IgG or ab conjugated to peroxidase were similar and characterized by two peaks, one at 95 and the other at 220 ml, the former being eluted with the void volume of the column.

The pooled fractions of the first peak were examined by immunoelectrophoresis. Against anti-total serum, a single line was obtained; against anti-IgG serum, a single line was observed, which was shifted towards the anode when compared to

unlabeled IgG or ab; both lines were stained after incubation for peroxidase. When electrophoresis on 6% SDS-polyacrylamide was performed, the material of the first peak did not penetrate the gel. These results showed that the first peak contained peroxidase-labeled IgG or ab of a molecular weight higher than 200,000.

*Fab<sup>P</sup>*: When a solution of Fab conjugated to peroxidase was chromatographed on Sephadex G 200, a two-peak elution pattern was also obtained, but the first peak was at 130 ml. Immunoelectrophoretic analysis of the pooled fractions of the first peak showed a single line against anti-total serum; against anti-Fab serum, a single line was seen, which was shifted towards the anode when compared to unlabeled Fab; both lines were stained after incubation for cytochrome *c*. When the material of the first peak was electrophoresed in 6% SDS-polyacrylamide gel, a band was obtained which corresponded to a molecular weight of approximately 120,000. This band was stained after DAB and H<sub>2</sub>O<sub>2</sub> treatment. These data confirmed that peroxidase-labeled Fab of a molecular weight of approximately 120,000 was present in the first peak.

The material contained in the second peak of eluates, of conjugated IgG, ab, or Fab did not penetrate the polyacrylamide gel. It should be noted that noncoupled peroxidase did not penetrate either. The material of the second peak contained noncoupled peroxidase with enzymatic activity, as revealed by immunoelectrophoretic analysis.

*Fab<sup>c</sup>*: Solutions of Fab conjugated with cytochrome *c* were chromatographed on Sephadex G 100. The pattern of elution (Fig. 2) showed five peaks; the fractions were pooled accordingly, and analyzed by immunoelectrophoresis and SDS-polyacrylamide electrophoresis. Details of techniques and documents are given in Fig. 2.

Pool I contained mainly Fab<sup>c</sup> of a molecular weight of approximately 70,000 and some labeled components of higher molecular weight but no lighter proteins. Pool II contained labeled fragments of IgG and cytochrome polymers. Pool III contained mainly cytochrome tetramers (mol wt ~ 50,000) and weakly labeled fragments of IgG. Pool IV contained mainly cytochrome dimer. Pool V contained noncoupled cytochrome of molecular weight 12,000 and some cytochrome dimer (mol wt ~ 24,000).

IN VITRO BEHAVIOR: The capacity of en-

zyme-coupled Fab fragments to label the antigen was checked on immunoelectrophoresis slides. This model allowed the in vitro testing of both direct and indirect immunohistochemical methods. Fig. 3 illustrates the case of peroxidase-labeled Fab used as the second step with antiferritin IgG as the first step. The lines between RbIgG a-F and F fixed ShFab<sup>P</sup> a-RbIgG, as demonstrated by the superposition of ferritin staining and peroxidase activity. When the first step was RbFab a-F, the previously described technique was modified as follows. A line was obtained between ShFab<sup>P</sup> a-RbFab and RtS a-ShFab. This line fixed the subsequently added RbFab a-F and ferritin as demonstrated by the superposition of ferritin staining and peroxidase activity. Similar results were obtained using cytochrome *c* instead of peroxidase. Enzyme-labeled IgG was also tested and similar results were obtained.

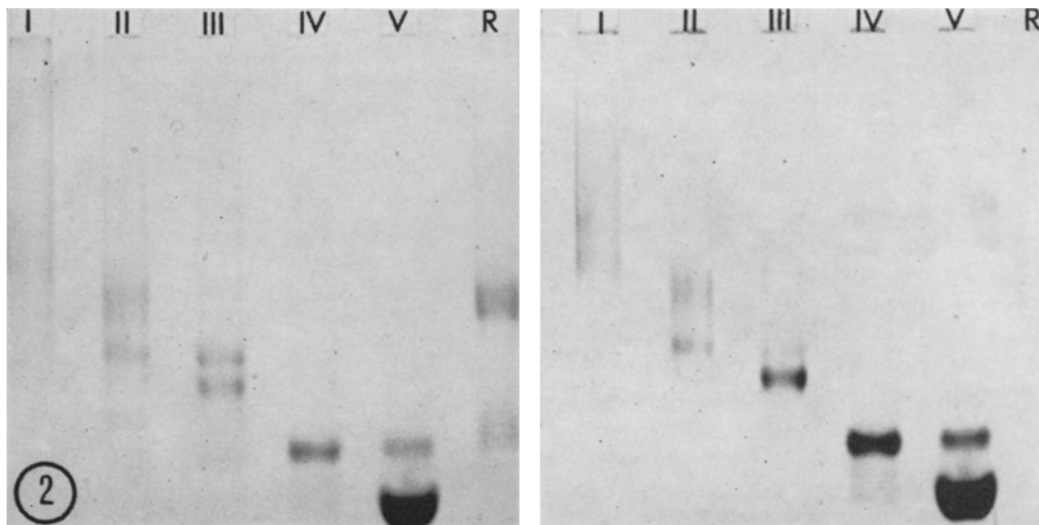
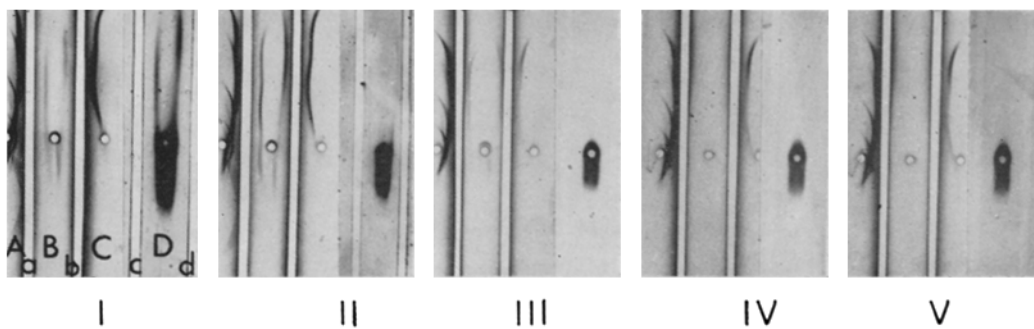
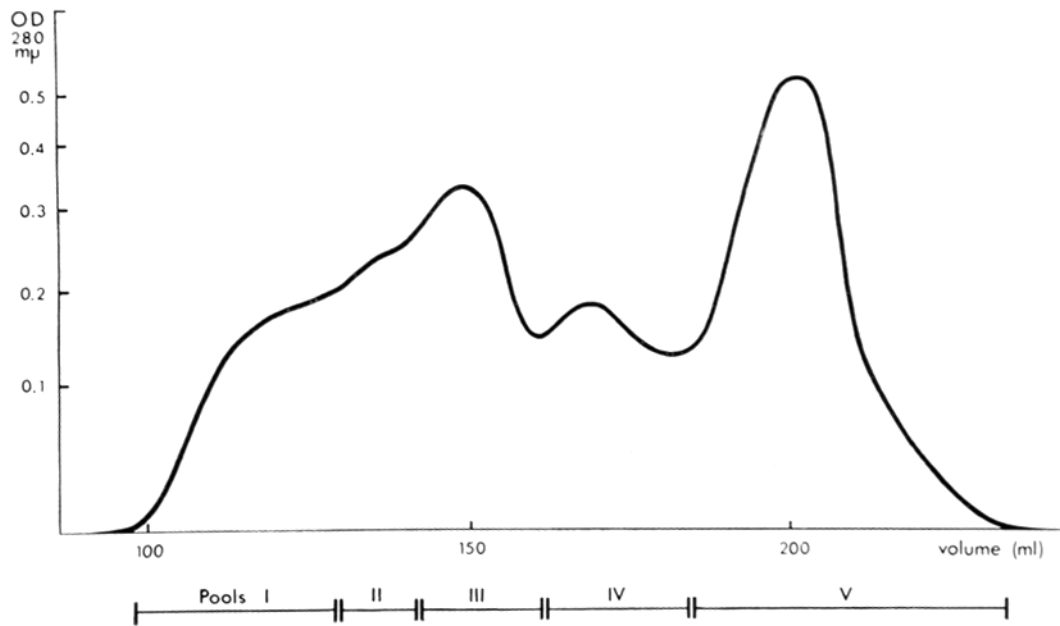
### Immunohistochemical Results

DISTRIBUTION OF ANTIGEN IN TISSUE: After 1 hr of absorption ferritin was located mainly in vacuoles dispersed in the cytoplasm and in the tubulovesicular system under the brush border of jejunal absorptive cells of the villi (Fig. 4). Maximum absorption of ferritin took place at approximately 120 cm of the pylorus.

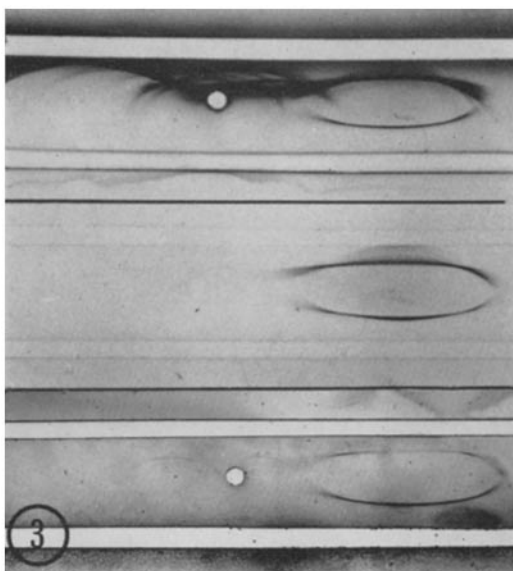
DISTRIBUTION OF ENZYMIC REACTION PRODUCT: This was examined on thick sections, stained or unstained, as well as on thin sections. After glutaraldehyde fixation, reaction product was abundantly deposited on the edge of nonfrozen sections and practically no labeling of ferritin was obtained, even after lysine or sodium bisulfite treatment. All the results reported below were obtained on PAF-fixed tissues.

When incubations with immunological reagents were performed in cacodylate-HCl buffer, the preservation of tissues was relatively good, but the penetration of tracers was poor and reaction product was deposited at the edge of the TC-2 sections. When THB was used during the incubations, the degradation of tissues was more pronounced but the penetration of tracers was better and non-specific deposition at the edge of nonfrozen sections was seldom observed. All results reported below were obtained on tissues incubated with immunological reagents in THB.

In ferritin-containing vacuoles, the distribution of enzyme reaction product varied with the tracer used. Some vacuoles were completely labeled,



**FIGURE 2** Characteristics of sheep Fab labeled with cytochrome *c*. See text for description. *Top*: Elution pattern of a solution of sheep Fab coupled with cytochrome *c*. Five peaks, numbered I through V, are seen. *Middle*: Immunoelectrophoretic analysis of pools I to V. Wells (from left to right), A and C, sheep serum; B and D, pool. Troughs (from left to right), RtS a-ShS (a and c); RtS a-Sh Fab (b and d). Cytochrome *c* activity was revealed with DAB and H<sub>2</sub>O<sub>2</sub> on slides cDd. *Bottom*: SDS-polyacrylamide electrophoresis of pools I to V. Left, after amido-Schwartz staining; right, after incubation for cytochrome *c* with DAB and H<sub>2</sub>O<sub>2</sub> in pH 3.9 citrate buffer. Reference (R), heavy and light chains from alkylated reduced bovine IgG.



**FIGURE 3** In vitro demonstration of labeling of Rb IgG a-F by Sh Fab<sup>P</sup> a-Rb IgG. Central well, Rb S a-F; upper trough, Rt S a-Rb S; lower trough, ferritin. After 1 hr of diffusion, Sh Fab<sup>P</sup> a-Rb IgG was added in the lower trough. Upper slide, amido-Schwartz; middle slide, prussian blue; lower slide, DAB + H<sub>2</sub>O<sub>2</sub> in pH 7.4 THB.

some were not labeled at all, and a few vacuoles were partly labeled. The distribution of labeled vs. unlabeled vacuoles in any given tissue appeared to be random. To obtain a quantitative estimate, the ratio of labeled vacuole volume to total vacuole volume was measured by morphometry. The principal results are reported in Table II and Table III.

*Peroxidase-labeled tracers* were used in most experiments. With the direct as well as with the indirect method, low percentages of labeling were observed when the molecular weight of the peroxidase-labeled tracer was larger than 200,000; the use of purified antibodies instead of IgG did not modify these results. High degrees of labeling were obtained when peroxidase-coupled Fab (mol wt approximately 120,000) was used in direct methods (Fig. 5). With indirect methods, using peroxidase-coupled Fab for the second step, the degree of labeling depended on the composition of the first step: 22% of vacuolar volume was labeled when undiluted antiferritin serum containing 6 mg/ml specific antibodies was used, 60% when the serum was diluted 1:10, and 98% if papain-digested antiferritin serum or antiferritin Fab was used

(Fig. 6). The homogeneity of the labeling, as observed on nonfrozen sections of 30–40  $\mu$  thickness, varied also: labeling was more marked at the periphery of the sections if undiluted serum was used for the first step, whereas it was homogeneous if digested serum or Fab fragments were used. Nonspecific deposition of enzyme reaction product was marked when large tracers were used by the direct method: mitochondria and nuclei were heavily stained, and membranes and cytoplasm were diffusely labeled (Fig. 8). Background was lower when the tracer was smaller and when the indirect method was used. In all experiments, at least a diffuse nonspecific labeling of cytoplasm and membranes was observed.

*Cytochrome-labeled tracers:* Cytochrome *c* was substituted for peroxidase in conditions where the specific labeling was high and the background low, i.e. in the indirect method with labeled Fab. The degree of labeling was as high as with peroxidase, and the same influence of the first step was noted (Fig. 9). The principal difference consisted in the almost total absence of background (Fig. 7), if Fab<sup>c</sup> from the first pool after Sephadex chromatography (Fig. 2) was used. When Fab<sup>c</sup> was contaminated by noncoupled cytochrome polymers from the other pools, nonspecific deposition of reaction product was observed.

## DISCUSSION

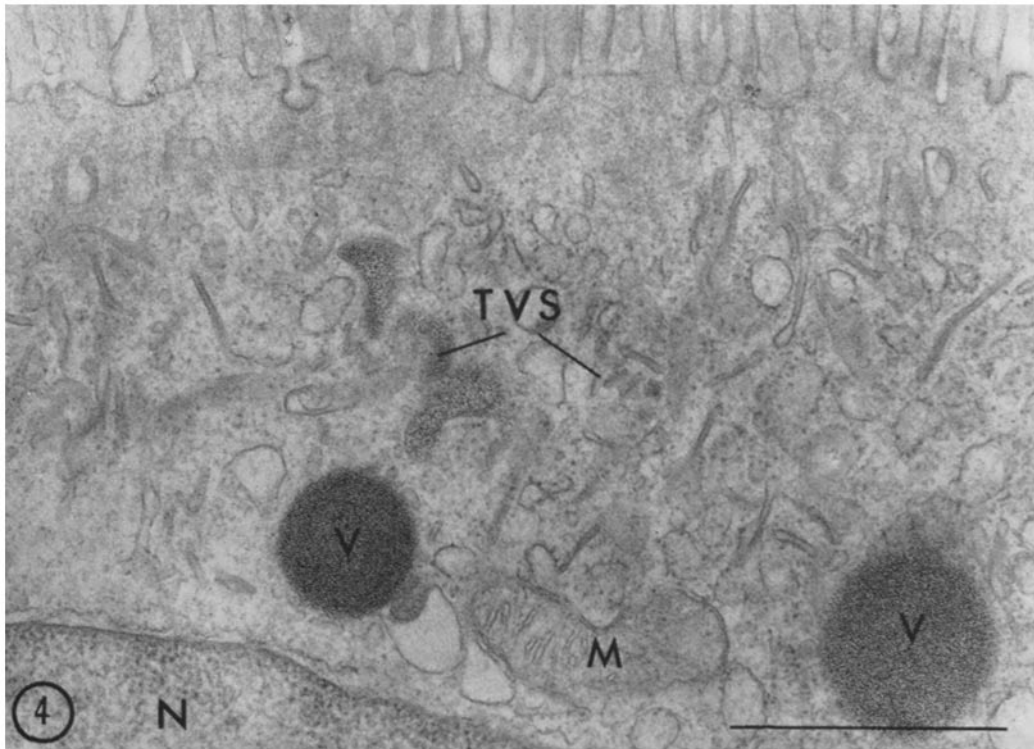
### *Experimental Model*

The capacity of newborn pig jejunal cells to absorb proteins into vacuoles provides a means of introducing well-characterized and readily recognizable antigens, such as ferritin, into defined areas of the cell. Under the conditions used in the present study, the fixed cells were relatively undamaged, as freezing procedures were avoided. In all cases, a complete villus was included in the 30–40- $\mu$  chopper sections so that the cells remained intact. Consequently, the tracers had to cross both the plasma membrane and the membrane limiting the vacuole in order to reach the antigen. This contributed in making the model a valid one for testing the localization of intracellular antigens, even though the jejunal epithelium is in essence a monolayer of cells.

### *Influence of Fixatives*

The radial immunodiffusion technique of Mancini et al. (15) provides an in vitro system al-





*Key to Symbols Used in the Micrographs*

M, mitochondria

N, nucleus

TVS, tubulovesicular system

V, vacuole containing ferritin

V<sub>L</sub>, vacuole containing ferritin labeled by enzymatic reaction product

FIGURE 4 Newborn pig; jejunal absorptive cell, 60 min after injection of ferritin into the intestinal lumen. Ferritin is located in the tubulovesicular system and in vacuoles. No immunohistochemical treatment; uranyl acetate and lead citrate.  $\times 37,000$ .

lowing a quantitative estimation of the interaction between various fixatives and the antiferritin antibodies. These rather than the ferritin were incorporated into the gel and exposed to the fixatives for the following reasons. Diffusion of the ferritin from the wells allowed the staining of the precipitation rings by the prussian blue reaction. Furthermore, the greater susceptibility of antiferritin antibodies to fixatives allowed more informative comparisons. As shown by Mancini et al. (15), the diameter of the precipitation ring is a measure of the relative concentrations of antigen and antibody if conditions are appropriate. Fig. 1 shows the semilogarithmic relation found between the antigen concentration and the diameter of the precipita-

tion ring. Preliminary treatment by fixatives caused a shift of the line but all lines remained straight and parallel. This similarity of slope allows the calculation of the inactivation caused by the fixatives in the system under study. For a given diameter, the inactivation is given by the ratio of ferritin concentration in the fixed plate to ferritin concentration in the standard plate. After treatment with ethanol the inactivation is 50%; after formaldehyde it reaches 80%. Addition of picric acid to formaldehyde does not increase the inhibition of immunological activity. The inactivation due to glutaraldehyde is higher but cannot be calculated precisely; some activity, however, is still present. Anderson (1) studied the inactivation of

various enzymes by formaldehyde and glutaraldehyde; his results indicate that in most cases inhibition of enzymatic activity also is lower after formaldehyde than after glutaraldehyde. It must be stressed that the immunoplate test was used to obtain comparative data on various fixatives, and that the calculated values of antibody activity inhibition are only applicable to the system studied here.

The best morphological results are obtained with glutaraldehyde, but the labeling in this case is known to be lower and less homogeneous than after formaldehyde fixation (14), presumably because of a poor penetration of tracers. In order to improve these results, some glutaraldehyde-treated immunoplates and tissues were immersed in 3% lysine or 3% sodium bisulfite, with the hope of blocking the remaining free aldehyde groups. These attempts were unsuccessful, possibly because

the lysine or sodium bisulfite concentrations adequate for morphological purposes were too low to inactivate the aldehyde groups.

The lowest inhibition of antibody activity was observed after ethanol fixation but this fixative does not allow a sufficient preservation of ultrastructure. The comparison of immunoplates suggested that the next best choice was formaldehyde, with or without picric acid. The addition of picric acid improved slightly the preservation of tissue structure. After PAF fixation, however, the immunological procedures and the demonstration of enzyme activity had deleterious effects on the ultrastructure. The morphological preservation was slightly better when the incubations with immunological reagents were performed in cacodylate-HCl, but this buffer has the great disadvantage when compared to THB of hindering the penetration of tracers. The good penetration observed when THB was used could be explained by an interaction between Tris and remaining free aldehyde groups. The damage due to revelation of enzyme activity was partly avoided if tissues were treated with glutaraldehyde immediately before (22).

#### *Efficiency of Immunohistochemical Labeling*

The reduction of the molecular weight of the enzyme-labeled tracer, achieved by using Fab fragments instead of 7S antibodies, allowed a striking increase in the degree of labeling, whereas no such increase could be obtained with purified antibodies. The preparation of labeled fragments

TABLE II  
*Volumetric Analysis of the Degree of Labeling in the Direct Method*

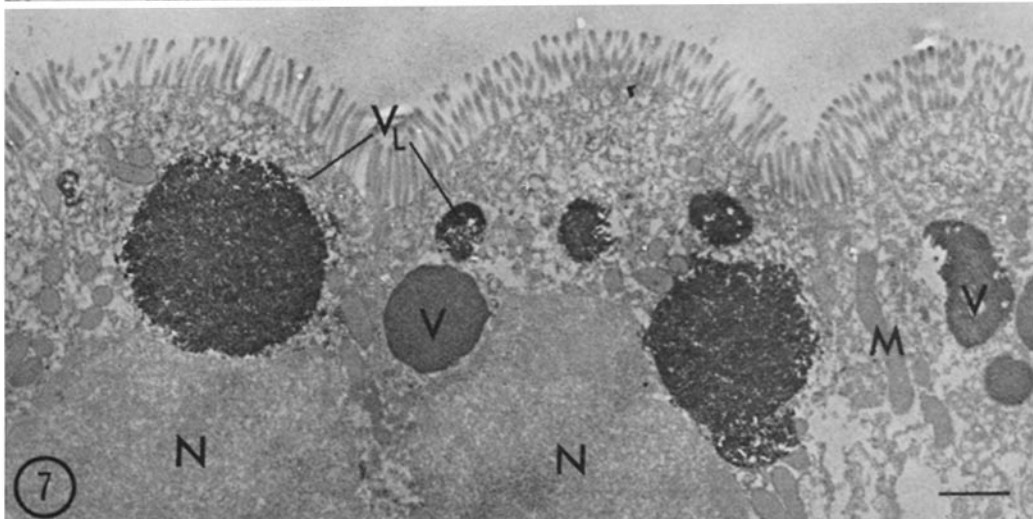
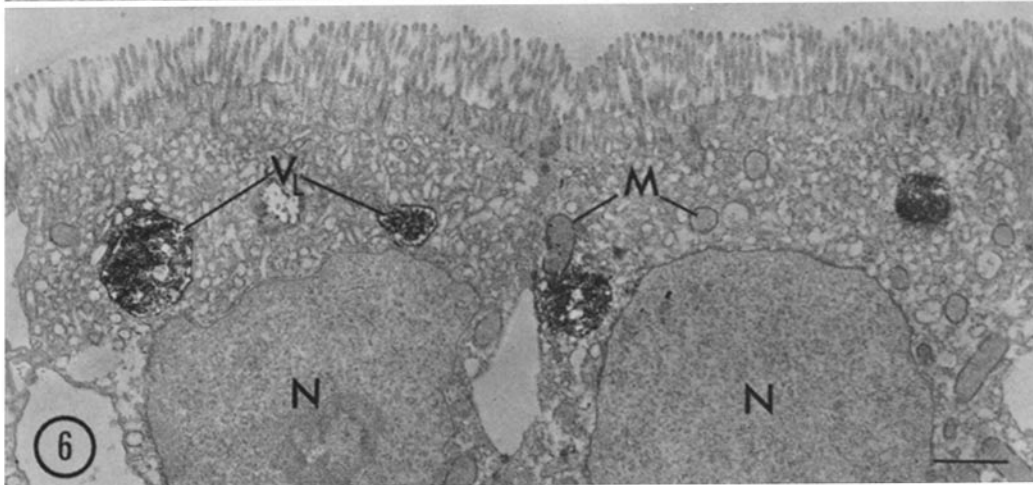
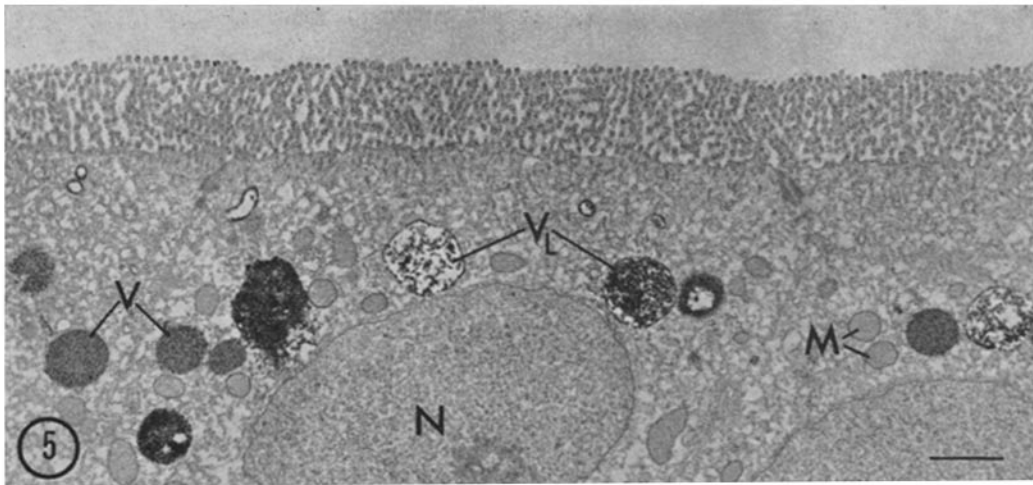
| Peroxidase-labeled tracers | Mol wt of tracers | Labeling (relative labeled vacuolar volume) |
|----------------------------|-------------------|---|
|                            |                   | %   |
| Rb S <sup>P</sup> a-F      | over 200,000      | 10  |
| Rb IgG <sup>P</sup> a-F    | over 200,000      | 36  |
| Rb Fab <sup>P</sup> a-F    | ~120,000          | 68  |

With the  $\chi^2$  test, the difference between the percentages is significant ( $P < 0.005$ ).

TABLE III  
*Volumetric Analysis of the Degree of Labeling in the Indirect Method*

| First-step reagents                     | Second-step tracers          | Mol wt of tracers | Labeling (relative labeled vacuolar volume) |
|---|------------------------------|-------------------|---|
|   |                              |                   | %   |
| Undiluted Rb S a-F<br>(6 mg/ml ab)      | Sh IgG <sup>P</sup> a-Rb IgG | over 200,000      | 11  |
| Undiluted Rb S a-F<br>(6 mg/ml ab)      | Sh Fab <sup>P</sup> a-Rb IgG | ~120,000          | 22  |
| Rb S a-F diluted 1:10<br>(0.6 mg/ml ab) | Sh Fab <sup>P</sup> a-Rb IgG | ~120,000          | 60  |
| Rb Fab a-F                              | Sh Fab <sup>P</sup> a-Rb Fab | ~120,000          | 98  |
| Digested Rb S a-F                       | Sh Fab <sup>P</sup> a-Rb Fab | ~120,000          | n.s.<br>98                                  |

With the  $\chi^2$  test, the difference between the percentages is significant ( $P < 0.005$ ) with the exception of the two last values.



**FIGURE 5** Direct method; incubation with Rb Fab<sup>P</sup> a-F. Peroxidase reaction product labels vacuoles containing ferritin. Some vacuoles with ferritin are unlabeled. Membranes and cytoplasm are diffusely labeled by nonspecific deposition of reaction product. Unstained section.  $\times 10,000$ .

**FIGURE 6** Indirect method; 1st step, digested Rb S a-F; 2nd step, Sh Fab<sup>P</sup> a-Rb Fab. All vacuoles containing ferritin are labeled with peroxidase reaction product. Nonspecific staining of membranes and cytoplasm; unstained section.  $\times 10,000$ .

**FIGURE 7** Indirect method; 1st step, diluted Rb S a-F; 2nd step, Sh Fab<sup>c</sup> a-Rb IgG. Most vacuoles containing ferritin are labeled with reaction product of cytochrome *c*. Membranes and cytoplasm are almost free of nonspecific deposition. Unstained section.  $\times 10,000$ .

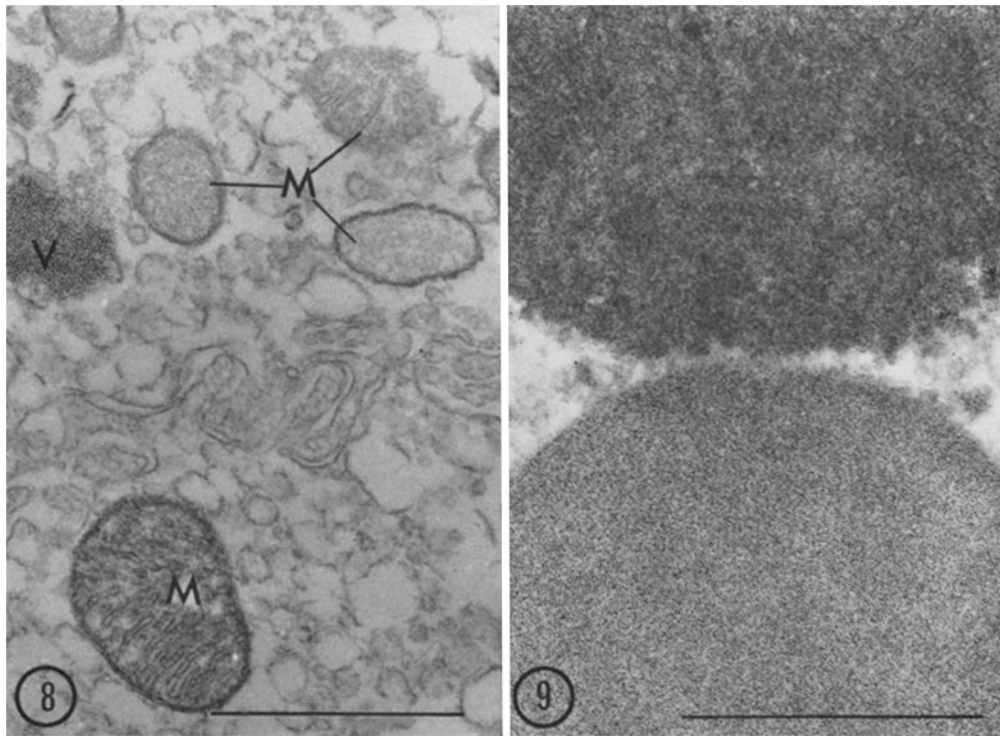


FIGURE 8 Direct method; incubation with Rb IgG<sup>P</sup> a-F; nonspecific deposition labels mitochondria. A vacuole containing ferritin remains unlabeled. Unstained section.  $\times 34,000$ .

FIGURE 9 Indirect method; 1st step, diluted Rb S a-F; 2nd step, Sh Fab<sup>c</sup> a-Rb IgG. The upper vacuole is labeled. Ferritin is visible in the deposits of cytochrome *c* reaction product. The lower vacuole is unlabeled. Unstained section.  $\times 41,000$ .

requires relatively large amounts of serum and is time consuming as it involves separation and digestion of IgG, conjugation of the enzyme, and purification of the conjugated complexes. For this reason, the indirect method is preferable as the labeled Fab of the second step can be prepared in large batches and used to demonstrate any number of different antigens. The sheep was chosen for the preparation of the second-step Fab because large quantities of serum could be harvested and, as shown recently (9), sheep Fab can be obtained easily by prolonged papain digestion. As most specific antisera are prepared by immunizing rabbits, this species was chosen for the first-step reagents. It is known, from light microscope studies (19), that the titer of the first-step serum is critical and that the optimum concentration should be determined by using a range of different dilutions. In the system used in this study, similar observations were made when 7S antibodies consti-

tuted the first step. With a high titer, undiluted antiferritin serum containing 6 mg/ml specific antibodies, the degree of labeling was low (22%); this was increased to 60% when the serum had been diluted 1:10. No such influence of the concentration of the first-step reagent was observed when Fab was used, perhaps because the steric conditions for antigen-antibody binding are less stringent for univalent molecules. Purity of the first-step Fab is not critical and papain-digested sera can be used as successfully as purified Fab.

Nonspecific deposition of the reaction product was evaluated by light microscopy on stained or unstained 1- $\mu$  sections as well as by electron microscopy. Focalized deposition of the reaction product on cellular structures such as nuclei and mitochondria was observed mainly with the direct method when peroxidase-labeled total serum or IgG was used. This background is probably due to nonspecific adsorption of tracers and is strongly

reduced in the indirect method, in agreement with immunofluorescence studies (19). In both direct and indirect methods, the reduction of peroxidase-labeled tracer size brings about a decrease of nonspecific deposition. To further improve results, cytochrome *c* (mol wt ~ 12,500) was substituted for peroxidase (mol wt ~ 40,000). This modification resulted in an almost complete disappearance of background. This improvement does not, however, seem to be primarily caused by the reduction of the molecular weight of the enzyme, but rather by the elimination of nonconjugated cytochrome *c* polymers, as shown by the analysis of eluted fractions of conjugates (Fig. 2). After conjugation of peroxidase to Fab or 7S antibodies, it is not possible, with the methods available, to separate nonconjugated peroxidase polymers from the enzyme-labeled IgG or Fab. These contaminating polymers are probably responsible for some of the nonspecific deposits.

### Conclusions

In the system used in this study, fixation with picric acid in formaldehyde (PAF) appears to allow sufficient activity to remain for the detection of immunological sites and does not hinder the penetration of tracers; preservation of ultrastructure, if not of high quality, is adequate and tends to be improved if tissues are postfixated in glutaraldehyde before incubation for enzyme. Indirect methods, in agreement with immunofluorescence studies, also reduce nonspecific adsorption when enzyme-labeled tracers are used. The titer and purity of Fab fragments used as first-step reagents are not critical; this makes it possible to use relatively small volumes of papain-digested sera. High degrees of labeling can also be obtained with undigested sera as first-step reagents, provided that their titer is appropriate. With Fab instead of IgG in the enzyme-labeled tracer, higher degrees of labeling are achieved in both the direct and the indirect techniques. Sheep Fab can be prepared for the second step simply by prolonged papain digestion of IgG; this should, however, be directed against highly purified Fab of the species used in the first step. Cytochrome *c* as an enzymatic label is superior to peroxidase because nonconjugated cytochrome *c* can be separated from the tracer; this allows an almost complete disappearance of nonspecific deposition.

The methods described and discussed should be

of help in increasing the specificity and reproducibility of the ultrastructural localization of intracellular antigens. An application of these techniques is reported elsewhere (5).

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