Comparative Study of the Techniques for Ultrastructural Localization of Antienzyme Antibodies

W. D. KUHLMANN[1] AND H. R. P. MILLER[2]

Institut de Recherches Scientifiques sur le Cancer, Villejuif, France

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Different methods of fixation and tissue processing were employed to demonstrate intracellular antibody to horseradish peroxidase, *Escherichia coli* alkaline phosphatase and glucose oxidase in the popliteal lymph node of the rabbit, rat, guinea pig, and mouse. Fixation with 2.5 % glutaraldehyde for 1.5 hours, 1.25% glutaraldehyde and 1% formaldehyde for 1.5 hours and 4% formaldehyde for 24 hours all provided satisfactory ultrastructural conservation of the tissues. None of these fixatives appeared to inhibit the subsequent antigen-antibody reaction. After fixation the tissues were prepared for incubation with antigen in several different ways. Extremely small fragments of lymph node were cut either by hand with a razor blade or by using a tissue chopper and complete cross sections of the node were cut at 40 μ in a cryostat.

The 40 μ frozen sections gave the most consistently reproducible results in that antibodies to all 3 enzymes were demonstrable, the intracellular penetration of the enzymes was superior with this method, and specific areas in the lymph node could be selected by light microscopy prior to cutting thin sections. Finally, a technique is described whereby antibody antihorseradish peroxidase can be detected in ultrathin frozen sections.

INTRODUCTION

Because of their antigenic and catalytic properties, enzymes can be used both as antigens and as markers at the same time. Such systems have been employed to examine immunocompetent cells both by light microscopy *(3, 22)* and by electron microscopy (*1, 13, 18, 21)* it being necessary in the latter instance that the reaction product be electrondense. In studies which combine immunology, cytochemistry, and electron microscopy, the problems of satisf actory conservation of the tissue on the one hand and the demonstration of antibody with enzyme on the other must be reconciled. The description which follows, of the procedures for fixing and preparing tissues to demonstrate antibody-producing cells in animals, is based on the original work of Leduc et al. *(13),* Avrameas et al. *(4, 5),* and Scott et al. *(18, 19).*

We have reexamined the methodology of the immunoenzyme techniques for the following reasons: first, to obtain a method of fixation where the enzyme-antibody interactions are consistently reproducible. Second, to discover whether the commonly employed aldehyde

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[2] On study leave from the University of Glasgow, Scotland. In receipt of a grant from the Carnegie Trust for the University of Scotland.

fixatives do inhibit such interactions; and, finally, to improve the penetration of the enzymes and their substrates into the tissues. To these ends we have used several different enzymes of different molecular weights, derived from plant or bacterial sources.

MATERIAL AND METHODS

The following enzymes were employed: horseradish peroxidase (RZ 3) (Sigma Chemical Co. St Louis, Missouri), glucose oxidase from *Aspergillus niger* (140 units/mg) (C. F. Boehringer, Mannheim) and *E. coli* alkaline phosphatase (20 units/mg) (Worthington, New Jersey). Bovine serum albumin (Povite, Amsterdam) was also used.

Animals. 30 rabbits (Giant Flemish), 93 rats (WAG), 50 mice (C57 black), and 5 guinea pigs were used.

Immunization schedules. Injections into the hind foot pad were carried out with the enzymes dispersed in Freund's complete adjuvant (Difco Laboratories, Michigan). Rabbits were immunized with 5 mg of the respective enzyme, rats and guinea pigs with 1 mg and mice with 0.25 mg of enzyme. Animals used as controls were immunized either with equivalent doses of bovine serum albumin or with a heterologous enzyme.

The popliteal lymph nodes were carefully dissected out from animals under ether anesthesia between 6 and 30 days after primary immunization or 2-3 days after a secondary stimulation. Lymph nodes from rabbits, rats, and guinea pigs were placed in Hank's solution at 0°C, and cell suspensions were made or imprints were taken for examination by light microscopy (*3, 4).*

Fixation. Formaldehyde freshly prepared from paraformaldehyde (Merck) and purified and stabilized glutaraldehyde (TAAB) were used at varying concentrations. Fixation procedures were as follows: (a) 1 % formaldehyde in 0.2 *M* cacodylate with 0.25 *M* sucrose added, pH 7.2 for 20 minutes at 20°C and then 30 minutes at 3°C *(4, 14);* (b) 4% formaldehyde in 0.2 *M* cacodylate for 15 minutes, 1, 4, 8, and 24 hours at 3°C; (c) 10% formaldehyde in 0.2 *M* cacodylate for 1 and 4 hours at 3°C; (d) 1 % formaldehyde and 1.25% glutaraldehyde in 0.2 *M* cacodylate for 15 minutes, 1 and 1.5 hours at 3°C *(11);* (e) 2.5 % glutaraldehyde in 0.2 *M* cacodylate for 1.5 hours at 3°C.

The lymph nodes were chopped with razor blades into blocks 1-1.5 mm^3 in which case attempts were made to select blocks from the medullary region, or they were cut into slices approximately 2 mm in thickness. The nodes of mice from which 40 μ frozen sections were to be cut were halved and fixed without further slicing. Fixation was carried out under continuous agitation, and the tissues were then washed at 3°C for at least 24 hours during which the cacodylate buffer was changed several times.

Preparation of the tissues. Prior to treatment with antigen the small tissue blocks were reduced to extremely small fragments, either with a Sorvall tissue sectioner regulated to cut at 40 μ or, by hand, with a razor blade. Some of the larger slices were also cut with the tissue sectioner. Frozen sections from the larger slices of lymph nodes were cut at 40 μ in a cryostat (Dittes Duspiva). For the latter technique, the slices were placed in 10% dimethyl sulfoxide (*2, 7*) buffered with cacodylate for 1 hour, rapidly frozen with liquid nitrogen, and cut at-18°C to -20°C. The sections were gathered in several different ways. Some were dropped straight into

cacodylate buffer or 4% formaldehyde at room temperature, and in the latter instance they were allowed to fix for 0.5 hour prior to being washed. Other sections were collected in cacodylate-buffered 10% DMSO (at room temperature) to which the appropriate antigen (enzyme) had been added. Finally, some sections were placed in cacodylate buffered 40% DMSO at -18°C to -20°C and slowly warmed to room temperature. All sections were washed several times with buffer before the final incubation with antigen.

Incubation. Incubation of all tissues was carried out at room temperature for times varying between 1 and 24 hours. The concentration of horseradish peroxidase was 1 mg/ml cacodylate buffer and of glucose oxidase 3 mg/ml. Alkaline phosphatase was used as a solution 1 mg/ml phosphate buffered saline. After incubation the tissues were washed for 20-30 minutes in 3 changes of buffer and were immediately treated with the appropriate substrate. Some tissues were rinsed only once extremely rapidly in buffer before treatment with substrate and others were washed for 30 minutes, fixed for 15 minutes in 4 % formaldehyde and washed for a further 30 minutes.

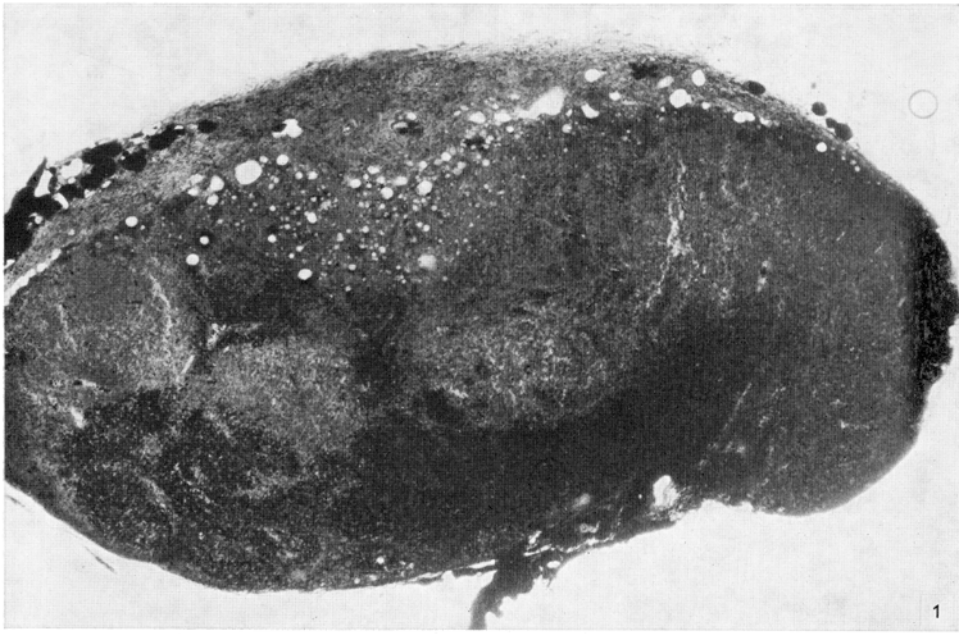
Enzyme activity and controls. The activities of the enzymes attached to their homologous antibodies were demonstrated as follows: horseradish peroxidase by the method of Graham and Karnovsky (*8*), incubation time varying between 20 and 60 minutes with several changes of substrate for the longer period of incubation. Glucose oxidase activity was revealed by a method described by Kuhlmann and Avrameas *(12).* Alkaline phosphatase was demonstrated using the substrate of Hugon and Borgers at pH 8.0 *(9, 10).*

The specificities of the reactions were examined by treating the tissues with the substrate without prior incubation in enzyme, by incubating tissues stimulated by an unrelated antigen (i.e., a heterologous enzyme or bovine serum albumin) with an enzyme and the appropriate substrate, and by omitting DAB or H_2O_2 from Graham and Karnovsky's medium or ß-glycerophosphate from Hugon and Borger's medium.

Dehydration and embedding: After postfixation in 2% cacodylate buffered OsO_4 the tissues were dehydrated through an ascending series of alcohols and embedded in Epon 812 (*15*). The small tissue fragments were polymerized in gelatin capsules and the 40 μ sections were flat embedded in the lids of BEEM capsules *(16).*

Ultrathin frozen sections. Ultrathin frozen sections were cut from lymph nodes fixed either in 4% formaldehyde for 24 hours or in 2.5% glutaraldehyde for 1.5 hours, using a Reichert OmU2 ultramicrotome with the freezing equipment FC-150 (Opt. Werke Reichert Wien). The tissues were embedded and cut according to the techniques described elsewhere *(6).* The following procedures were carried out to demonstrate antibody anti-horseradish peroxidase: the sections were floated on the surface of the incubating solution (0.5 mg peroxidase/1 ml cacodylate buffer) in Marinozzi rings. They were washed in 5 changes of buffer and immediately transferred to the substrate DAB H_2O_2 for 3 minutes and the substrate was changed 5 times during this period. After a brief rinse in buffer the sections were postfixed in 1 % buffered OsO_4 for 2 minutes.

Electron microscopy. Epon-embedded tissues were cut with Sorvall (MT-1) and LKB Ultrotomes and were mounted on carbon-coated 200-mesh copper grids or uncoated 300-mesh grids. Sufficient sections were cut from each block that some grids could be examined without being stained, some were stained 30 seconds with lead citrate *(17),* and others were double stained with both uranyl acetate and lead citrate. The sections were examined with a Siemens Elmiskop 1 operating at 80 kV and with a *50 μ* objective aperture.



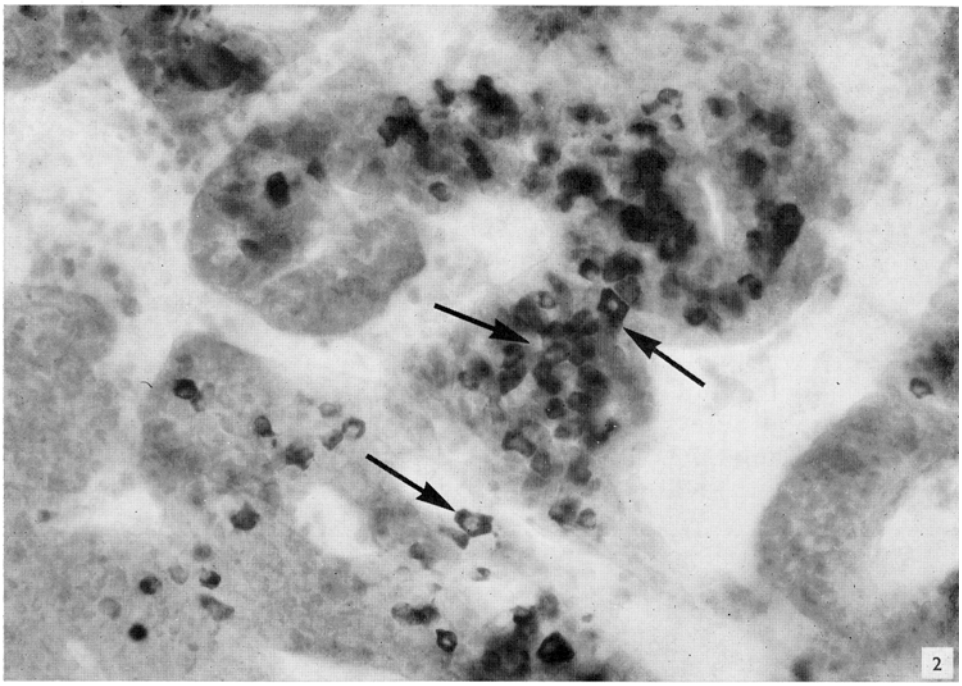


FIG. 1. Light micrograph of a 40 μ frozen section of a popliteal lymph node from a rat 13 days after a single injection of horseradish peroxidase. Fixation 4% formaldehyde. x 37.5.

FIG. 2. Light micrograph of a 40 μ frozen section showing the medullary region of the popliteal lymph node of a mouse 28 days after immunization with alkaline phosphatase. There are numerous plasma cells containing antibody to alkaline phosphatase (arrows) in the medullary cords. Fixation 4% formaldehyde. x 375.

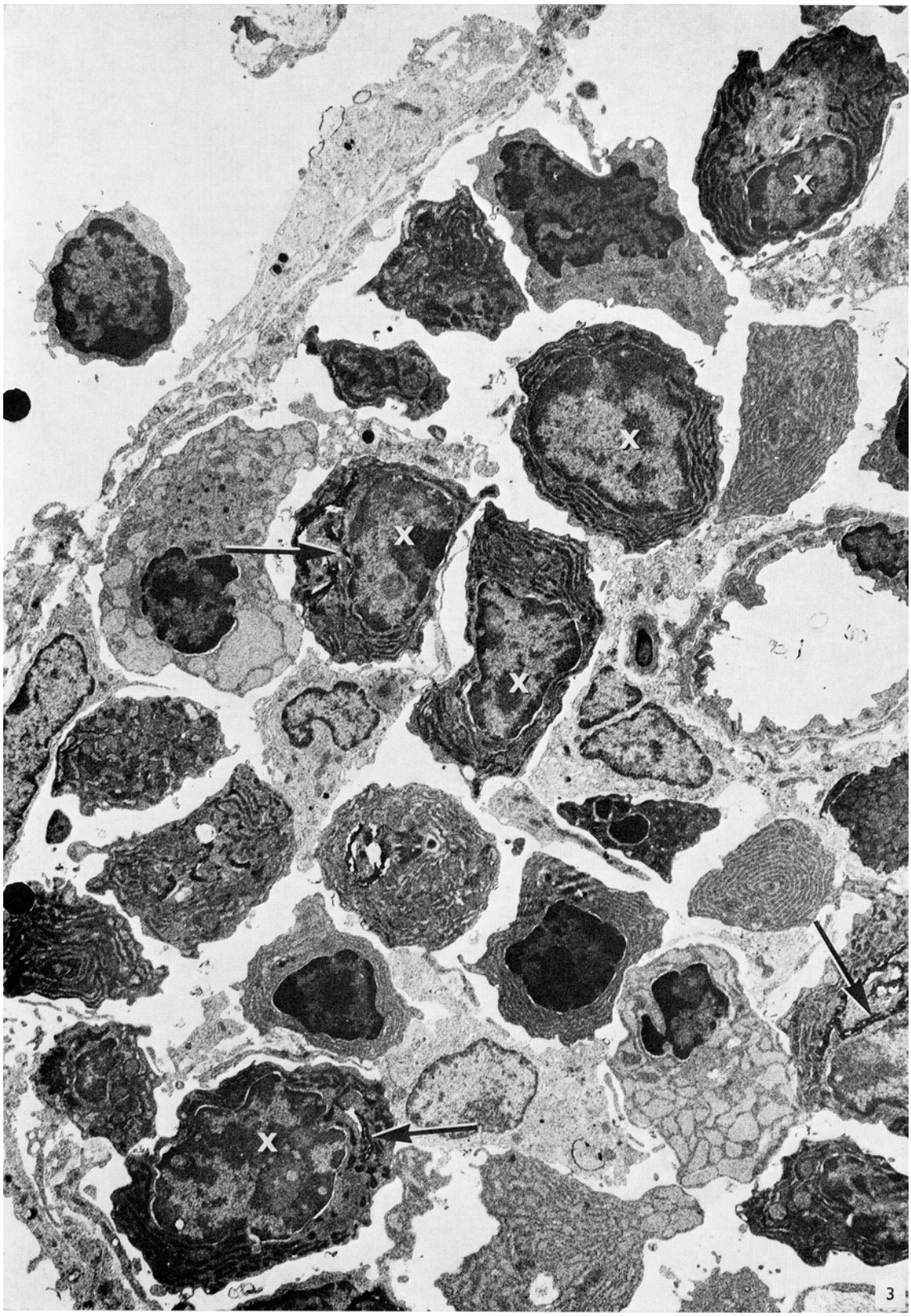


FIG. 3. Low power electron micrograph of a medullary cord in the popliteal node of the mouse 14 days after immunization with horseradish peroxidase. A number of antibody-producing cells (stars) are interspersed among macrophages and antibody-free plasma cells. Note that antibody is concentrated in the Golgi regions of several cells (arrows). Fixation 4% formaldehyde. 40 μ frozen section. Uranyl acetate and lead citrate. x 5 800.

RESULTS

Only those lymph nodes in which antibody-containing cells were found by light microscopy were used for subsequent ultrastructural examination of the small fragments. For the latter technique semithin sections (1-2 μ) were cut, were examined by light microscopy, and blocks containing positive cells were thin-sectioned for electron microscopy. The 40 μ frozen sections were examined directly by light microscopy (Fig. 1), and thin sections were cut from an appropriate area (Fig. 2).

Fixation

Formaldehyde. There was a general tendency for the cells to shrink in all the tissues examined with a subsequent increase in the intercellular spaces (Fig. 3). Fixation with 4% formaldehyde for 24 hours at 3° gave consistently good results, although the plasmalemmata of the more fragile cells tended to be poorly preserved and membrane whorls were frequently observed in the endoplasmic reticulum of the plasma cells. However, the penetration of antigen and the localization of specific antibody was satisfactory with this method (Figs. 3-5). Eight hours fixation was also adequate whereas 4 hours appeared to be too short because many of the plasma cells had swollen and indistinct cisternae and with fixation times of 1 hour or 15 minutes very few cells remained intact. The quality of the fixation with 1 % formaldehyde 0.25 *M* sucrose approximated to that of 4 % formaldehyde for 1 hour. With 10 % formaldehyde and fixation time of 1-4 hours both cell shrinkage and swelling were seen but specific antibody-containing cells were readily demonstrable.

Formaldehyde-glutaraldehyde. Fixation for 1.5 hours with 1 % formaldehyde / 1.25% glutaraldehyde, gave good conservation of the tissues. The localization of antibody and enzyme activity was indistinguishable from that obtained with formaldehyde alone (Fig. 7). Fixation for 15 minutes provided poor conservation of the cells.

Glutaraldehyde. Fixation for 1-1.5 hours with 2.5% glutaraldehyde gave the best tissue preservation, and the localization of the antibody was similar to that observed with formaldehyde fixation.

Preparation of the tissues for incubation with antigen

It was possible to identify specific antibody activity to horseradish peroxidase and to glucose oxidase with all the procedures employed, but only frozen sections were satisfactory for the demonstration of antibody-antialkaline phosphatase.

Lymph nodes fragments. With this method the blocks could be cut to a sufficiently small size that a complete cross section of the fragment could be examined ultrastructurally. Thus all the areas of maximal penetration by the enzyme (i.e., the peripheries of the fragments) were included in the section. However, the fragments were not of a uniform size, the extent of enzyme penetration was not known with any certainty, and the relationship of the fragments to the lymph node structure in general were difficult to judge.

Sorvall TC2. The tissue chopper was used to facilitate the penetration of the enzyme. However, we were unable to obtain uniform sections of the node and the mechanical damage of the more fragile cellular elements appeared to be greater with this method than with the hand-cut blocks.

Frozen section. We achieved the most consistent results with this method. Complete serial cross sections of lymph nodes were obtained (Fig. 1), and, by altering the advance of the specimen, thinner sections could be cut for light microscopic cytochemistry.

The thickness of the sections (40 μ) were such that the penetration of the enzymes was ensured and, as will be discussed below, there also appeared to be better intracellular

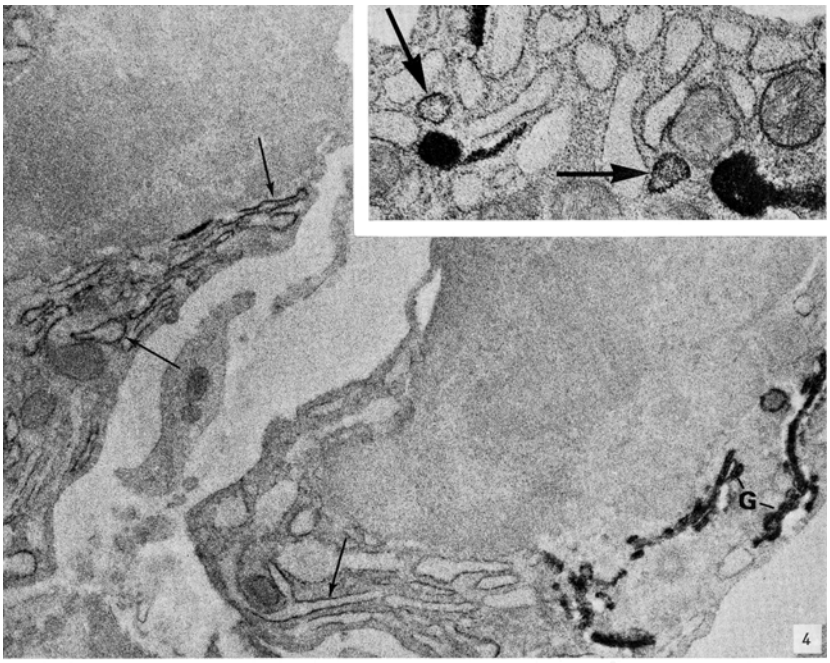
penetration of the enzyme than with the other techniques. The specific antibodies remained well localized in the intracellular compartments (Figs. 4, 4 inset, and 5) although a greater degree of membrane damage was observed in the more fragile cells such as macrophages (Fig. 3). Good results were obtained with 4% formaldehyde for 24 hours and the mixture of 1 % formaldehyde/1.25% glutaraldehyde for 1-1.5 hours. The preservation of the cell membranes was best with 2.5% glutaraldehyde for 1.5 hours but using light microscopy there was a more intense background staining of the 40 μ sections, and it was difficult to locate structures in the lymph node when this fixative was used.

Ultrathin frozen sections. It was possible to demonstrate cells containing antibody anti-horseradish peroxidase on ultrathin frozen sections (Fig. 9), however, the conservation of the tissues was less satisfactory than with the other techniques. There was some extraction of the nuclei, enlargement of the perinuclear spaces, detachment of cytoplasmic fragments and, in some cases, severe damage to entire cells. Furthermore, the section thickness was not always regular and only 10-20 % of the sections were usable. After treatment with the substrate a nonspecific granular precipitate was often present.

Cellular localization of the antibody

With all the techniques where the fixation was satisfactory, there was good localization of enzyme activity. The site of antibody localization does, however, depend on the stage of synthesis in each cell and this will be described elsewhere, but with all the methods used, cells were detected which varied from being replete with antibody (Figs. 3 and 5-8) to those which had only one or two cisternae showing antibody reactions (Figs. 4 and 5). In some cells the enzyme reaction also occurred in the perinuclear space (Figs. 5 and 8). At all stages after immunization, plasma cells were detected which had a mixture of antibody-positive and antibody-negative cisternae (Figs. 4 and 5). Moreover, with antibody anti-horseradish peroxidase, specific staining of the membranes and ribosomes of the rough endoplasmic reticulum were observed either when the sections were not counterstained or when they were lightly stained with lead citrate (Figs. 4 and 5). With alkaline phosphatase, a light, nonspecific staining of the membranes did not permit the same detailed localization of antibody as was observed with peroxidase. Occasionally, a nonspecific staining of the plasmalemmata of some cells was observed with the glucose oxidase procedure. One striking feature was that a far greater proportion of cells in the 40 μ frozen sections showed intense specific antibody reaction in the Golgi cisternae (Figs. 3, 4, 6, and 8) than was the case with the other techniques used for tissue preparation. This was true for all the enzymes employed although no comparisons could be made for alkaline phosphatase since tissue fragments were unsatisfactory for the demonstration of its specific antibody.

The other variables examined in this study, for example, the duration of incubation with enzyme or the number of times and the length of time that the tissues were washed after incubation, did not affect the distribution or the intensity of the subsequent reaction. Doubling the incubation time with the diaminobenzidine substrate for horseradish peroxidase caused a very slight increase in the intensity of the reaction, but did not affect its distribution within the cells.



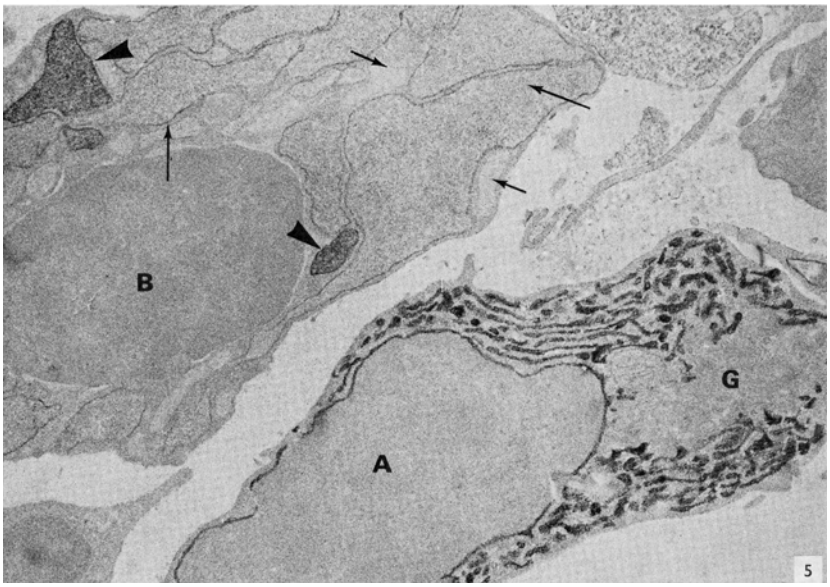


FIG. 4. Two plasma cells in the early stages of antibody production to horseradish peroxidase. In one there is antibody in the Golgi complex (G) and the membranes of some cisternae of RER (arrows) are stained in both cells. Mouse popliteal node. Ten days after immunization. Fixation 4% formaldehyde. 40 µ frozen section. Not counterstained. x 17 500. INSET: Part of the cytoplasm of a plasma cell containing antibody to horseradish peroxidase. Some cisternae are negative, others contain small quantities of antibody and their membranes and ribosomes are strongly contrasted (arrows) and some cisternae are intensely stained. Mouse popliteal node 12 days after immunization. Fixation 4% formaldehyde. 40 µ frozen section. Lead citrate 30 seconds. x 27 000.

FIG. 5. Electron micrograph of two plasma cells synthesizing anti-horseradish peroxidase antibody in the rat. In one cell (A) there is a heavy concentration of reaction product in the RER and the perinuclear cisternum, but the Golgi region (G) is antibody-free. A nearby cell (B) has distended cisternae of which some are antibody-free (short arrows). Some have faint reaction in the lumina of the cisternae and marked staining of the cisternal membranes (long arrows), and others are strongly stained for antibody (arrow heads). 25 days after immunization. Fixation 4% formaldehyde. Small tissue fragment. Section not counterstained. x 12000.

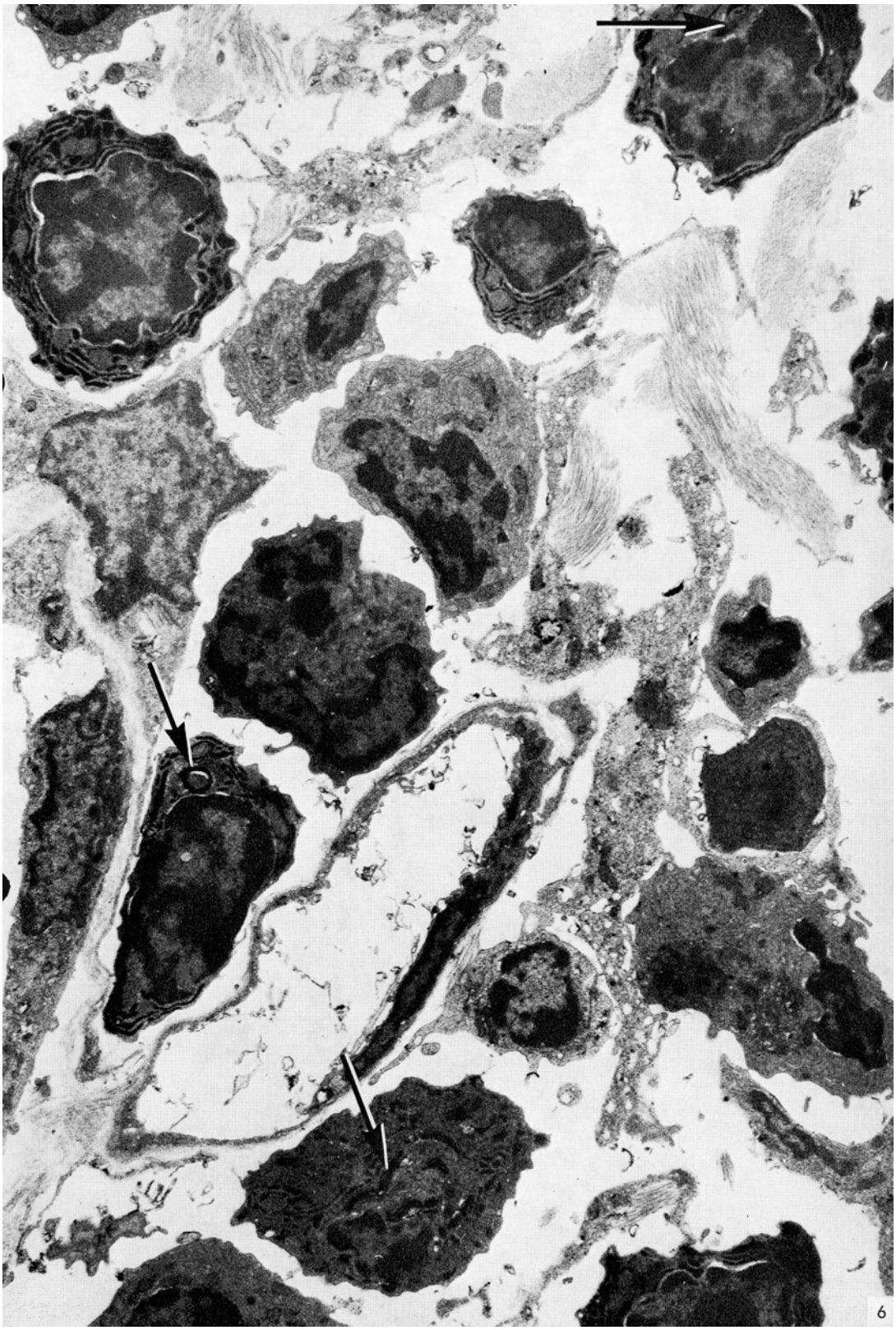


FIG. 6. Plasma cells synthesizing anti-alkaline phosphatase antibody are present in the medullary regions of a mouse popliteal node 12 days after immunization. Antibody is present in the Golgi regions of several cells (arrows). Fixation 4% formaldehyde. 40 μ frozen section. Uranyl acetate and lead citrate. x 8 250.

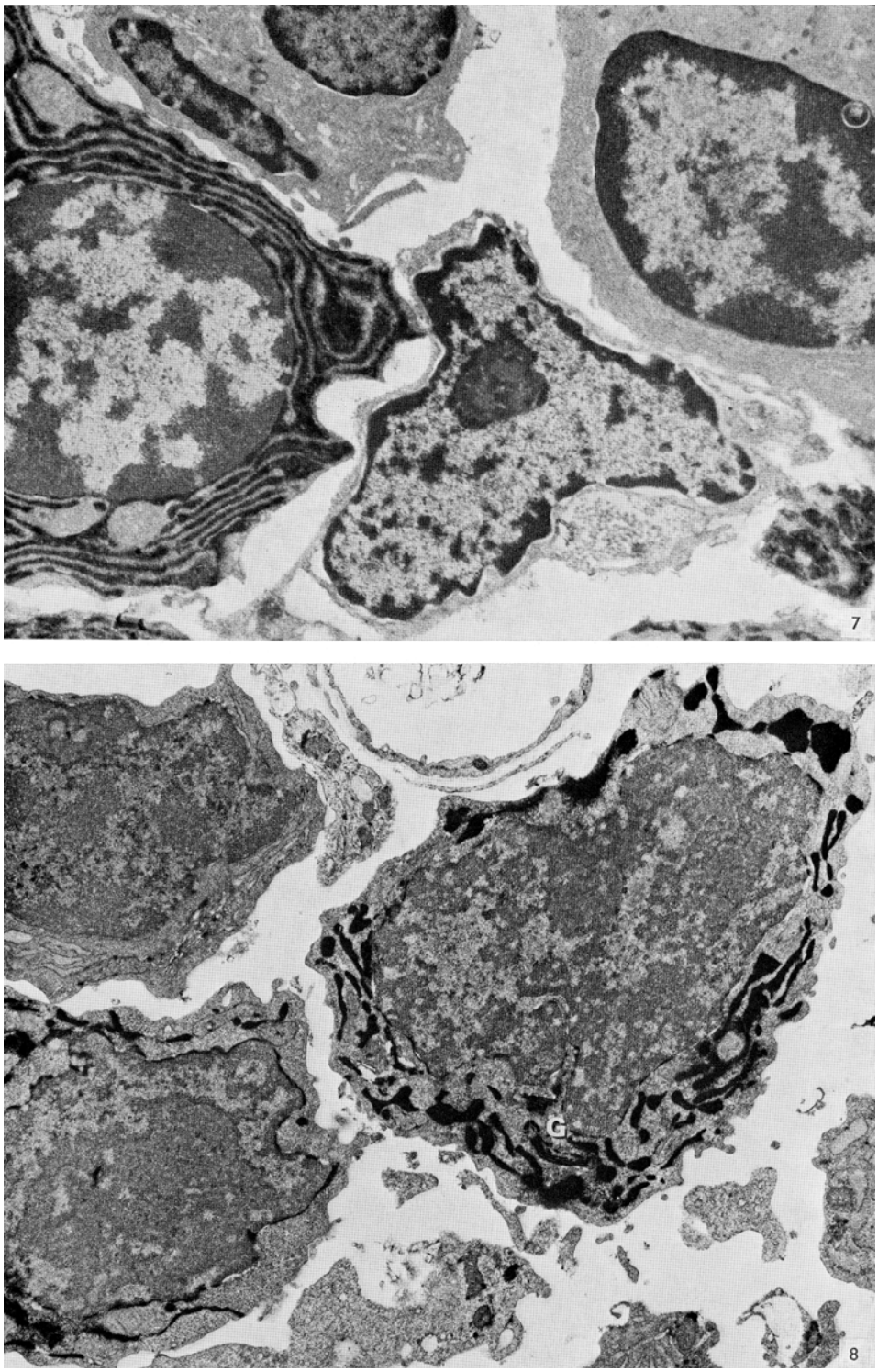


FIG. 7. Several plasma cells containing anti-horseradish peroxidase antibody in the popliteal lymph node of the rat. Fixation 1% formaldehyde and 1.25% glutaraldehyde. 40 µ frozen section. Uranyl acetate and lead citrate. x 12 000.

FIG. 8. Plasma cells synthesizing anti-alkaline phosphatase antibody. Reaction product is present in the cisternae of the RER, the Golgi complex (G) and in the perinuclear space. Mouse popliteal node 12 days after immunization. Fixation 4% formaldehyde. 40 µ frozen section. Uranyl acetate and lead citrate. x 10 400.

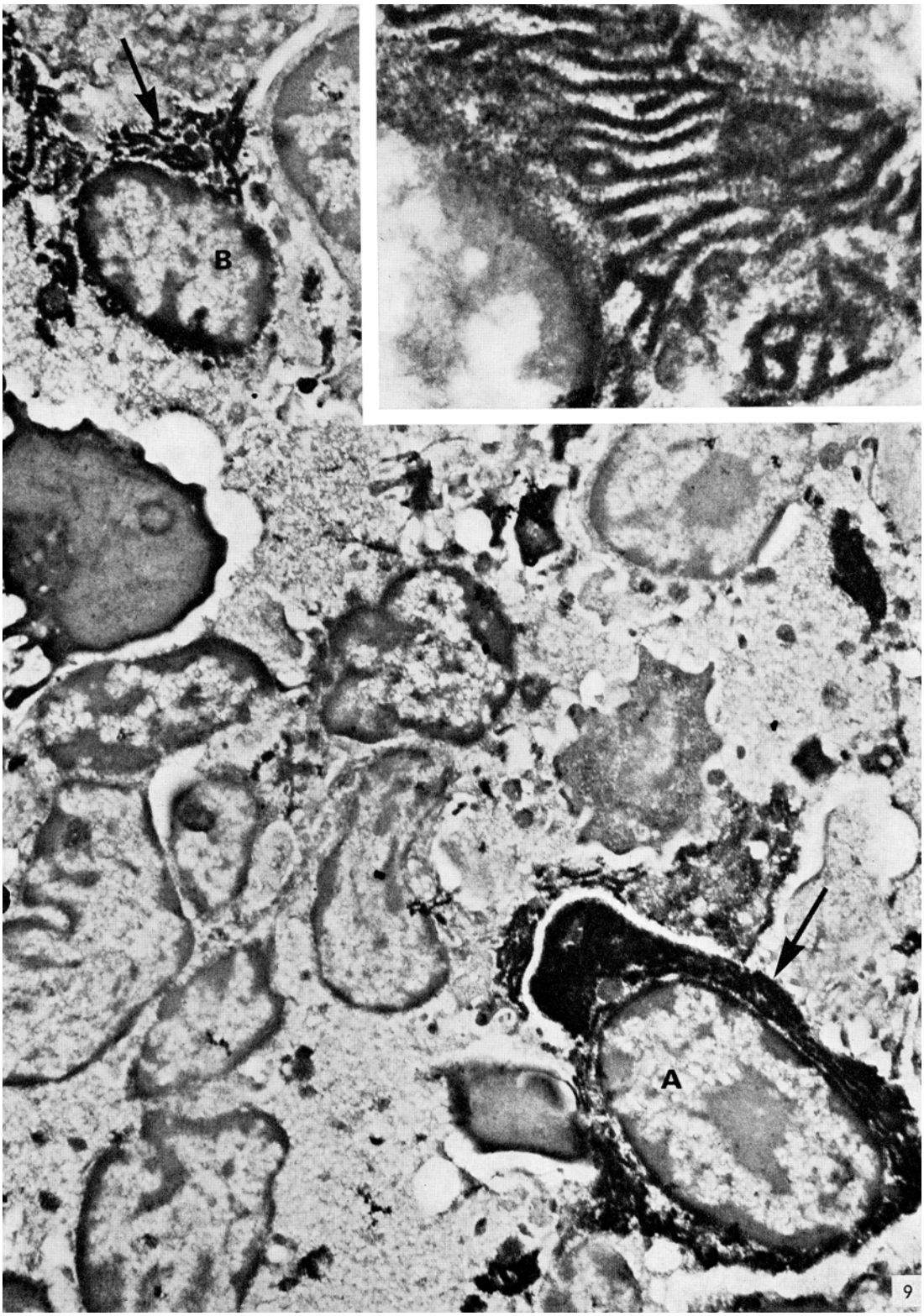


FIG. 9. Two cells (*A* and *B*) containing anti-horseradish peroxidase antibody (arrows) in an ultrathin frozen section of rat lymph node. 3 days after a second injection of peroxidase. Fixation 2.5% glutaraldehyde. x 7 000.
INSET: A high power view of part of a plasma cell. Note the localization of reaction product in the cisternae of RER. x 18 000.

DISCUSSION

The purpose of this work was to find a technique which satisfactorily preserved the tissue without blocking the antibody-antigen reaction but which, at the same time, permitted maximal penetration of the enzymes concerned. As regards fixation, adequate tissue preservation was observed with 4 % f ormaldehyde (24 hours) with the mixture of 1% formaldehyde/1.25% glutaraldehyde (1-1.5 hours) and with 2.5% glutaraldehyde (1-1.5 hours). None of these fixatives appeared to influence the subsequent intensity of the antibody staining reactions in the cells, and it would seem theref ore that they probably do not inhibit the interaction of antibody with antigen to any significant degree.

However, the preparation of the tissues after fixation and prior to incubation with the enzyme is more critical. Thus we were able to demonstrate cells containing antibody-anti-alkaline phosphatase in frozen sections but not in tissue fragments. Furthermore, judging by the high frequency with which the Golgi apparatus was stained in the cells in frozen sections, the intracellular penetration of enzyme and substrate is probably superior with this method.

Only aldehydes were used as fixatives, formaldehyde being freshly prepared from paraformaldehyde and glutaraldehyde from a purified concentrated stock solution *(20).* Little difference was noted between the two except that there was a better conservation of the membranes, particularly of the more fragile cells, when glutaraldehyde was used. Although hypertonic fixatives were applied, the subsequent embedding techniques caused some tissue shrinkage, especially in formaldehyde-fixed preparations. However, intimate relationships between cells could still be recognized.

Longer periods of fixation were required with formaldehyde (4-24 hours) than with glutaraldehyde or with formaldehyde-glutaraldehyde mixture (1-1.5 hours). With formaldehyde neither the concentration of the fixative nor duration of fixation impeded the subsequent reaction of antibody with antigen, for example, tissues fixed as long as 1 week in 4% formaldehyde showed no diminution in their reaction with antigen. These results are contrary to previous findings where fixation of short duration at feeble concentrations was recommended *(4).* Although weak fixation may be of some help in obtaining adequate penetration of enzyme-antibody conjugates *(14),* it is disadvantageous in the immunoenzyme technique because the cells are so poorly preserved.

The localization of antibody-producing cells by the immunoenzyme method would therefore appear to be governed by two factors: penetration of the enzyme, and the method of tissue sampling. One way of facilitating the penetration of the enzymes was to cut the tissues into extremely small fragments either by hand or by using a tissue chopper, and there are two main drawbacks with these methods. In the first place the enzyme penetrates only to a depth of 4-5 cells from the surface *(13)* so that great care must be taken when thin-sectioning the blocks. Second, because antibody-producing cells tend to be localized in certain areas in lymphoid tissues, there is a possibility that few or no cells will be found unless large numbers of samples are examined. To minimize these difficulties, we attempted to dissect out the medullary region and to chop the tissues into extremely small fragments by hand so that thin sections could be cut which completely traversed the blocks. Furthermore, by increasing the incubation time to 24 hours it was hoped to increase the penetration of the enzymes. Although many antibody-producing cells were found using this method, there remained the uncertainty as to whether cells toward the centers of the blocks were genuinely negative or whether enzymes and substrates had failed to penetrate to that depth. In our hands the tissue chopper did not overcome either of the above problems; furthermore we observed considerable cell damage using this method. Finally, with

both of these sampling techniques it was difficult to be sure of the location of each fragment in relation to the rest of lymph node structure.

Further points which should be considered are that different components of the substrate may diffuse at different rates in the tissues and that enzymes of high molecular weight probably do not penetrate into the cell so readily as those of low molecular weight. Nor is it known what effect the size, shape, and electrical charge of molecules may have on their ability to penetrate into fixed tissues since cell membranes may still act as barriers. Under these circumstances techniques which break down such barriers, without allowing diffusion of antibody, are probably of some advantage. It was for these reasons that we adapted 40 μ frozen sections for the immunoenzyme methods.

Frozen sections have been employed by de Petris et al. (*7*), who demonstrated antibody antiferritin in immunocompetent cells and by Scott et al. *(18)* for the demonstration of antibody anti-alkaline phosphatase. The method facilitates the penetration of substrates into the tissues although it is often stated that the ultrastructural conservation of the cells suffers considerably from such a treatment. We did observe damage to cell membranes in 40 μ frozen sections but, because DMSO was used prior to freezing the tissues and suitable precautions were taken the sections were recovered, little or no diffusion of the antibody occurred and the conservation of the majority of the cells and their relationships to one another were similar to those obtained using other methods.

Theoretically, the penetration of both enzymes and Substrates into 40 μ sections should be highly satisfactory, and, in effect, varying the time of incubation with enzyme from 1 to 24 hours made no difference to the subsequent intensity of the staining reactions. As mentioned earlier, a much higher proportion of cells showed intense staining for antibody in the Golgi regions in frozen sections than was the case with tissue fragments. We conclude from these observations that penetration of enzyme and substrate is superior in the frozen sections.

Frozen sections 40 μ thick offer several advantages; they are of regular thickness, complete cross sections of virtually a whole lymph node may be processed for electron microscopy, and alternate sections may be used for experimental and control purposes. Flat embedding techniques enable the sections to be examined by light microscopy prior to selection of areas for ultrastructural examination, and, for systematic studies of lymph node changes during immune responses, the 40 μ frozen sections are extremely useful.

Scott and Avrameas *(19)* were the first to use ultrathin frozen sections to demonstrate antibody anti-alkaline phosphatase within plasma cells. As pointed out by these authors the penetration of both enzyme and substrate is no longer a problem with this technique. However, the method is not yet sufficiently developed to be of routine use. The tissues are embedded in gelatin, the sections are floated on dimethyl sulfoxide, and all the subsequent reactions are carried out in the rings of Marinozzi *(6)*. When the immunocytochemical techniques have been completed the sections are placed on Formvar-coated grids.

We were able to demonstrate antibody anti-horseradish peroxidase in immunocompetent cells but despite numerous precautions, there was often an accompanying nonspecific granular precipitate which could, fortunately, be distinguished from the specific intracellular reaction. Apart from technical difficulties, the ultrathin frozen sections suffer from the same drawbacks as the tissue fragments in that many different blocks may have to be sampled to find specific antibody producing cells. However, with the rapid progress that is being made with this technique it may eventually be of great value in immunocytochemistry.

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