

IMPROVED PROCEDURES FOR IMMUNOFERRITIN LABELING OF ULTRATHIN FROZEN SECTIONS

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ABSTRACT

In employing fixed frozen ultrathin sections as substrates for immunoferritin labeling of intracellular antigens, we have found that conventional glutaraldehyde fixation sometimes permits very little specific staining of the sections, either because it inactivates certain protein antigens, or because it renders them inaccessible to the antibody stains. We have developed several fixation procedures that are chemically milder and allow a uniform but less extensive cross-linking of the specimen. With these procedures and precautions in the handling of the more fragile frozen sections, excellent structural preservation and specific immunoferritin labeling has been achieved with several systems.

Immunoferritin and related specific labeling methods have great potential in the electron microscope study of the distribution of macromolecules in cells and cell organelles, but general application of the methods has required the development of new procedures of specimen preparation. In our technique of ultracryotomy for these purposes (14), cells and tissues were first fixed in 1–2% glutaraldehyde by standard methods and then infused with sucrose or other hydrophilic, chemically inert materials before freezing, which greatly improved the freezing and the frozen-sectioning characteristics of the specimens. Ultrathin frozen sections were then recovered from the cryobowl by adhering them onto a molten sucrose droplet, and were subsequently reacted with ferritin-antibody conjugates (10, 12). For general purposes, this technique is still highly satisfactory (12, 15, 16), but for immunoferritin labeling our experience of the last few years indicated that further refinements were necessary to increase the generality of the method.

The problems that we encountered, involving mainly the fixation of the specimen, were of two kinds. (a) 1–2% glutaraldehyde, although a relatively mild fixative chemically, did inactivate a significant number of protein antigens, such as

bovine pancreatic trypsinogen (5) and canine kidney Na, K-ATPase (6). This is not entirely unexpected, since reaction of soluble proteins with glutaraldehyde generally does lead to conformational changes in the protein molecules (7). (b) Even when such glutaraldehyde treatment did not produce a significant loss of antigenicity, however, we often observed much too low a level of immunoferritin labeling, which we came to realize was an *accessibility* problem. Apparently, such fixation often produced a network of cross-bridges that made a section impermeable to the relatively large antibody molecules and allowed only superficial labeling of the sections. For these reasons, we have investigated several alternative fixation procedures, together with modifications in other aspects of the technique required to accommodate to them, and these are described and illustrated in this paper. The basic approach was to decrease the extent of cross-linking of the specimen, and then to develop means to preserve intact the more fragile sections that resulted.

MATERIALS AND METHODS

Fixation

Aldehyde fixatives were always prepared in 0.1 M phosphate buffer, pH 7.4. Formaldehyde solutions were

prepared by heating suspensions of paraformaldehyde (Matheson, Coleman, and Bell, Los Angeles, Calif.) to about 60°C and adding a small amount of 1 N sodium hydroxide. Glutaraldehyde solutions were prepared by diluting 50% glutaraldehyde (Polysciences, Inc., Warrington, Pa.). Dimethylsuberimidate (DMS; refer to reference 2), dimethyl-3,3'-dithiobispropionimidate (DTP; refer to references 11 and 17), and ethylacetimidate (EAI; refer to reference 18) were synthesized in our laboratory. Imidates are readily destroyed by hydrolysis at alkaline pH, and their solutions must therefore be carefully neutralized. The pH of dibasic phosphate buffer was first raised to 10–11 by adding a small amount of 1–5 N sodium hydroxide so that the pH was near neutral, 7.2–7.6, upon the addition of the imidates. Solutions were made up immediately before use. Concentrations of imidates in experiments at 4°C were 6 mg/ml (or about 0.02 M) in 0.1 M buffer for DMS and DTP and 10–12 mg/ml (or about 0.1 M) in 0.1–0.12 M buffer for EAI. In experiments at room temperature, the concentration of DMS or DTP was raised to 12 mg/ml (or about 0.04 M) in 0.2 M buffer and that of EAI up to 44 mg/ml (or about 0.4 M) in 0.5 M buffer.

All fixation procedures, which are described in detail below, were done at 4°C unless stated otherwise. For fixing in a low concentration of glutaraldehyde such as 0.2%, it is important to use a large volume of the fixative to maintain the concentration nearly constant. For instance, a volume of specimen which would require 5–10 ml of 2% glutaraldehyde may be fixed in 50–100 ml of 0.2% glutaraldehyde. It is also advisable to agitate the fixative during the fixation. These precautions should also be taken for treating specimen blocks in an imidate solution or a mixture of an imidate and formaldehyde. In the present study, we used human erythrocytes and various tissues of mouse which were dissected in fixatives to blocks of 1 mm or smaller widths. After fixation, these were usually kept in 0.1 M phosphate buffer at 4°C overnight, or longer, before freeze-sectioning.

Handling of Frozen Sections

The general procedure of ultracryotomy was described in our previous paper (14). The Sorvall MT-2B ultramicrotome (DuPont Instruments, Sorvall Operations, Newton, Conn.) was used with the LTC-2 cryo-attachment. In the present experiments, the range of temperature for sectioning was –70 to –90°C, and the concentration range of sucrose for the prefreezing infusion into specimens was 0.8–1.2 M in 0.1 M phosphate buffer, pH 7.4.

The major problem with lightly fixed frozen sections is to maintain them morphologically intact through the various manipulations involved between retrieving them from the knife edge to the final drying on the grids. This problem can be solved by small changes in our previous procedures (14), which minimize the disruptive influence of surface tensions and other physical forces. These changes are as follows: (a) In retrieving frozen sections

from the knife edge in the cryobowl to the outside, we had previously used a small droplet of 2.3 M sucrose in an eyelash probe. We now use a droplet containing 2 M sucrose and 0.5–2% gelatin in 0.05 M phosphate buffer and employ a 1–1.5-mm loop of thin tin-plated copper wire to suspend the droplet, since the eyelash probe is inadequate to pick up a droplet out of the mixture of high viscosity. The gelatin serves to reduce the surface tension acting when the section melts on the liquid droplet, and lightly fixed sections thereby retain their integrity. On the other hand, for the same reasons, frozen sections cut in a wrinkled state tend to remain wrinkled when they melt on the sucrose-gelatin droplet, and careful cutting is therefore required.

(b) In order to wash out the sucrose, we have previously placed the grid with the sucrose droplet face down on a buffer surface. We now transfer the grid onto a series of drops containing successively lower concentrations of sucrose, each with 1–2% gelatin included. Alternately, the grid is placed directly onto a block of 2% gelatin–0.25% agarose, kept in a moist chamber, and left for 20–30 min until the sucrose is dissolved in the block. The grid can then be separated from the block surface by slowly adding buffer to the surface. Such measures are necessary to avoid a sudden detachment of the sucrose-containing droplet which often causes removal of parts of fragile sections from the grid.

(c) In the final drying step before electron microscopic examination, the mechanical support for the sections is provided by drying in 0.2–1% aqueous solution of a protein such as gelatin or a polysaccharide such as dextran, with or without an additional low concentration of phosphotungstic acid (PTA) or uranyl acetate (UA). This is to overcome the difficulty that the embedding of sections in such heavy metal compounds alone often results in too strong a contrast for the detection of ferritin particles. Proteins tend to form aggregates when mixed with heavy metal compounds: it is therefore advisable to embed sections first in a protein alone and after drying, to negatively stain the embedded sections lightly with a heavy metal compound. Care should be taken not to expose sections too long to a UA solution or its low pH will damage structures. An extensive OsO₄ positive staining, such as a few hours in a 2% solution, will make the sections less susceptible to damage by a subsequent positive staining with UA or lead citrate, but the increased overall density of the sections is not necessarily advantageous for the present purposes.

Immunoferitin Labeling

The procedure to isolate human erythrocyte spectrin, and to prepare and purify the rabbit anti-spectrin antibody are described elsewhere (9). Rabbit antibodies against human uterine smooth muscle myosin and chicken skeletal muscle myosin, prepared by similar procedures, were gifts of Dr. J. F. Ash and Dr. Kuan Wang of our laboratory, respectively. Immunochemical studies showed that the anti-human smooth muscle myosin anti-

body is highly cross-reactive with mouse smooth muscle myosin but not at all with mouse skeletal muscle myosin and that the anti-chicken skeletal muscle myosin antibody cross-reacts with mouse skeletal muscle myosin to some extent but not at all with mouse smooth muscle myosin.

In the present study, we employed the indirect immunolabeling method in which the labeling of an antigen by its antibody is followed by the labeling of the antibody by the ferritin-goat anti-rabbit IgG conjugate. The rabbit anti-spectrin antibody and the goat anti-rabbit IgG were affinity-chromatography-purified proteins, while other rabbit antibodies were contained in a γ -globulin fraction of the antiserum. The conjugation of ferritin to the goat antibodies was done as previously described (6) or by the method of Kishida et al. (4). The anti-spectrin antibody and the γ -globulin fraction containing anti-human smooth muscle myosin antibodies were used at a concentration of 0.2 mg protein/ml, while the fraction containing antibody to chicken skeletal muscle myosin was used at a concentration of 1 mg protein/ml to label mouse skeletal muscle myosin. The concentration of the ferritin-goat anti-rabbit IgG conjugate was 1–2 mg protein/ml.

All steps of immunoferritin labeling, except the stages of incubating the grids on the labeling solutions, were taken on 1.5–2.5 cm wide drops arranged on sheets of Parafilm (American Can Co., New York, N. Y.). Grids were first floated on 2% gelatin in 0.1 M phosphate buffer or 5–10% bovine serum albumin (BSA) or sometimes on both in sequence, to condition the grid surface to minimize the level of nonspecific labeling. Directly from the protein solution, a grid was floated on a small droplet suspended on a 4-mm diam loop. This was simply achieved by lifting the loop from below the grid floating on the solution. The loop was either a commercially available one (Ladd Research Industries, Inc., Burlington, Vt.) or a loop made of 0.3-mm thick stainless steel wire and held in an inoculating needle holder (VWR Sci., Los Angeles, Calif.). The loop was then turned over and after removing most but not all of the droplet by filter paper absorption, a droplet of the antibody solution (10–20 μ l) was added from above. The loop was again inverted so that the grid floated on the

top surface and was held in a moist chamber for 5–10 min at room temperature. The grid was then transferred through a series of several drops of phosphate-buffered saline containing 0.01 M glycine, to wash unbound antibodies away. This was achieved by simply immersing the loop into a drop and leaving the grid on its surface. The grids were subsequently pooled on the surface of a large drop of the buffer and sometimes washed further by gently swirling them on the surface. The process was repeated to label the antibodies with the ferritin-goat anti-rabbit IgG conjugates.

Sections on the grids, sometimes after a 1% glutaraldehyde–10 min post-fixation, were quickly washed on a distilled water surface to remove salts and dried in the manner described above. Observations were made in a Philips EM-300 electron microscope.

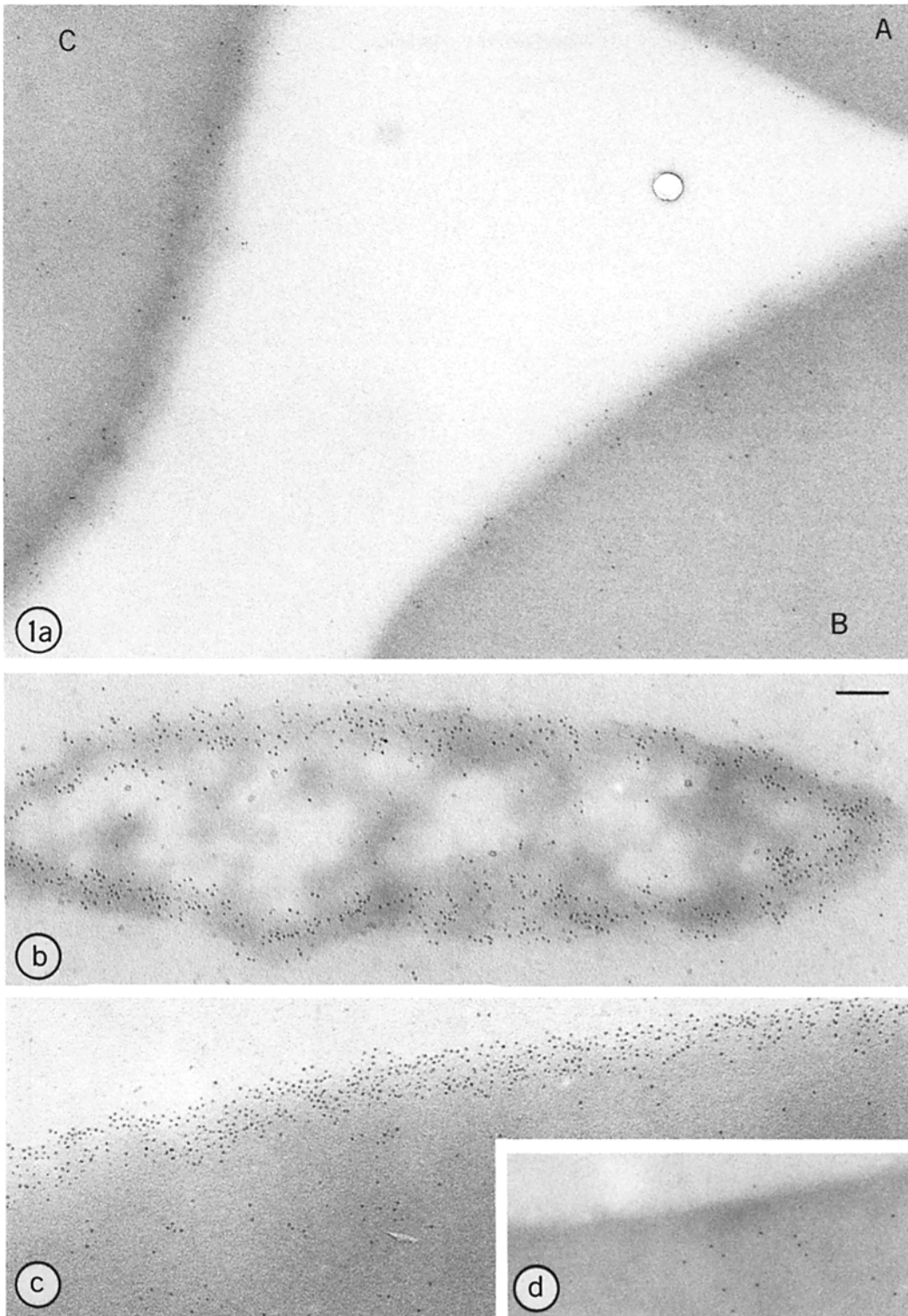
RESULTS

When intact human erythrocytes were fixed in 1–2% glutaraldehyde for 1 h, very little labeling of spectrin was observed (Fig. 1 *a*), although *in vitro* complement fixation studies (R. G. Painter and S. J. Singer, unpublished observation) as well as immunoferritin labeling studies on erythrocyte ghosts (Fig. 1 *b*) indicated that spectrin substantially retained its ability to bind antibody after such treatment. With milder fixation such as 15 min in 0.2% glutaraldehyde and the use of the improved specimen handling procedures described above, the erythrocyte structure remained intact in frozen sections, and the specific immunoferritin labeling of spectrin was increased more than 10-fold (Fig. 1 *c* and *d*). Since this improvement is not due to any substantial difference in the antigen's intrinsic binding capacity, it must be due to increased accessibility of the antigen to the antibody.

While 0.2% glutaraldehyde fixation may be adequate for single cells under these conditions, it does not adequately fix most tissue blocks. When the blocks were treated with 0.2% glutaraldehyde

All figures (1–10) are electron micrographs of ultrathin frozen sections. Scales with and without end marks represent 1 and 0.1 μ m, respectively.

FIGURE 1 (*a–d*) Human erythrocytes. Spectrin is only very little immunoferritin-labeled with its specific antibody in frozen sections of intact cells fixed in 1% glutaraldehyde for 1 h (*a*) but significantly in those of similarly fixed ghosts (*b*). In part (*a*), portions of three cells (*A*, *B*, and *C*) are included to show that the level of labeling is low regardless of the obliqueness of the section relative to the cell surface, which increases in the order from *A* to *C*. In frozen sections of intact cells fixed in 0.2% glutaraldehyde for 15 min (*c*), spectrin is extensively labeled along the cell periphery. A control for (*c*), using anti-human smooth muscle myosin antibody followed by the ferritin-goat anti-rabbit IgG conjugate, is shown in (*d*). All are embedded in thin layers of gelatin, except (*b*) which is negatively stained with a mixture of 0.4% dextran and 0.05% PTA. All; \times 80,000.



for 1 h, only 0.1-mm thick surface layers were suitably fixed, and sections of such layers showed mechanical damage that was inevitably caused during the initial dissection of fresh tissues. To overcome this difficulty, we studied the characteristics of formaldehyde fixation and the possibility of combining formaldehyde and glutaraldehyde.

Characteristics of Formaldehyde

Fixation

Formaldehyde, which is apparently a chemically milder fixative than glutaraldehyde, allowing greater retention of antigenic activity of susceptible proteins (5, 6), is generally considered to be inadequate for an accurate ultrastructural preservation. Yet, a hypertonic mixture of formaldehyde and glutaraldehyde was reported to be a highly effective fixative, and it was surmised that fast penetrating formaldehyde may serve as a temporary stabilizer for a more permanent fixation by glutaraldehyde (3).

A characteristic feature of formaldehyde fixation that we found distinguishes it from glutaraldehyde fixation is its apparent reversibility. For instance, a 20% hemoglobin solution in a dialysis bag becomes quite rigid when fixed in 2% formaldehyde for 1 h but subsequently returns to a fluid state in formaldehyde-free buffers within a few hours. Similarly, erythrocytes are totally resistant to lysis by H₂O treatment immediately after a formaldehyde fixation but when transferred into formaldehyde-free buffers begin to hemolyze within a short period of time. The duration of the lysis-resistant state after the termination of fixation becomes longer as the fixation is extended, e.g., about 1 h after a 1-h fixation but a few hours after a 15-h fixation. Many tissues and cells fixed in 2% formaldehyde for overnight or up to a few days can in fact be preserved intact in frozen sections, when 4–8% formaldehyde is included in the sucrose solution for the prefreezing infusion and if retrieved sections are immediately covered with gelatin. Nevertheless, formaldehyde fixation alone does not generally serve for the present purposes. Intact frozen sections that can be obtained only after such an extensive fixation often show very poor antigen accessibility. A partial reversal of cross-linking, which occurs when such sections are exposed to buffers, causes a gain in accessibility but inevitably also causes a loss in structural delineation.

Sequential Formaldehyde-Gutaraldehyde Fixation

This partial reversibility of formaldehyde fixation suggests that if such fixation is followed by a treatment with dilute glutaraldehyde, extensive washing should then reverse many of the remaining formaldehyde-produced cross-linkages, without affecting the stable glutaraldehyde-produced ones. In this manner, the retention of both antigenicity and antigen accessibility might be achieved. This scheme was the basis for the following fixation procedure. Tissues were dissected into 1-mm blocks in 1–2% formaldehyde and incubated for 1–2 h. The hardened blocks were then further dissected to 0.5 mm or smaller pieces and fixed in 0.2% glutaraldehyde, with or without 1–2% formaldehyde, for 1 h. After the specimens were then well washed in aldehyde-free media, frozen sections were prepared and were immunoferritin-labeled.

In sections of smooth muscle of *mouse* uterus or small intestine fixed in 2% glutaraldehyde for 1 h, smooth muscle myosin can be immunolabeled with rabbit anti-*human* uterine myosin but the level of labeling is low (Fig. 2*a* and *b*). When the tissue was first fixed with 1% formaldehyde for 1 h and then with 0.2% glutaraldehyde for 1 h, however, the level of labeling was markedly increased (Fig. 3*a*). The specificity of the labeling is demonstrated by the observation of very low nonspecific labeling when normal rabbit IgG was used in place of the rabbit antibody (Fig. 3*b*). Furthermore, in sections of *mouse* skeletal muscle, no evidence of labeling with the anti-*human* smooth muscle myosin antibody was recognized (Fig. 4), as expected from the absence of cross-reaction between smooth and striated muscle myosin.

In sections of highly contracted *mouse* skeletal muscle, fixed with formaldehyde and glutaraldehyde in the same manner as above, a significant labeling of myosin with the anti-*chicken* skeletal muscle myosin antibody was recognized in all areas except on Z-lines (Fig. 5*a* and *b*), which is in agreement with the observations that the mouse skeletal muscle myosin and the anti-*chicken* skeletal muscle myosin antibody cross-react to a certain degree (K. Wang and S. J. Singer, unpublished observation). This finding also confirms that the absence of labeling with the anti-*human* smooth muscle myosin antibody in sections of mouse skeletal muscle is not due to antigen inaccessibility or

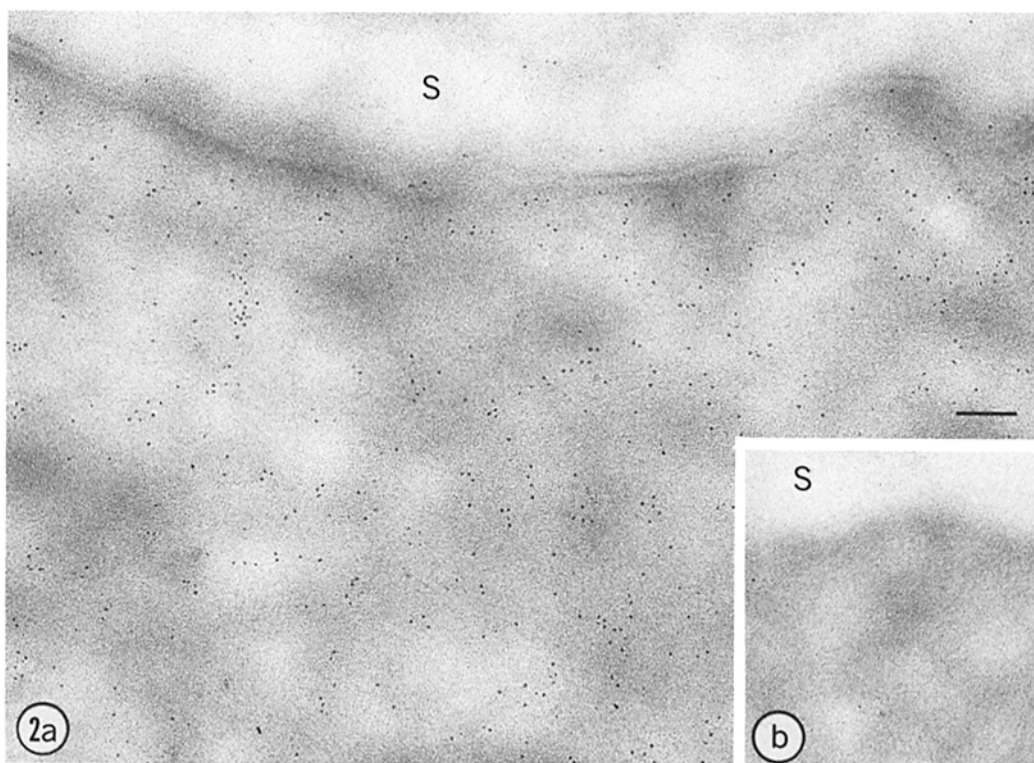


FIGURE 2 (a and b) Mouse uterine smooth muscle, fixed in 2% glutaraldehyde for 1 h. Immunolabeling of myosin using rabbit anti-human smooth muscle myosin antibody in the first stage of immunolabeling in (a) is found to be significant, when compared with the control in (b) treated with rabbit anti-human spectrin antibody in the first stage. S, extracellular space. Negatively stained with a mixture of 0.4% dextran and 0.05% PTA. Both, $\times 80,000$.

the loss of antigenicity, but due to the different antigenic specificity of the smooth and skeletal muscle myosins.

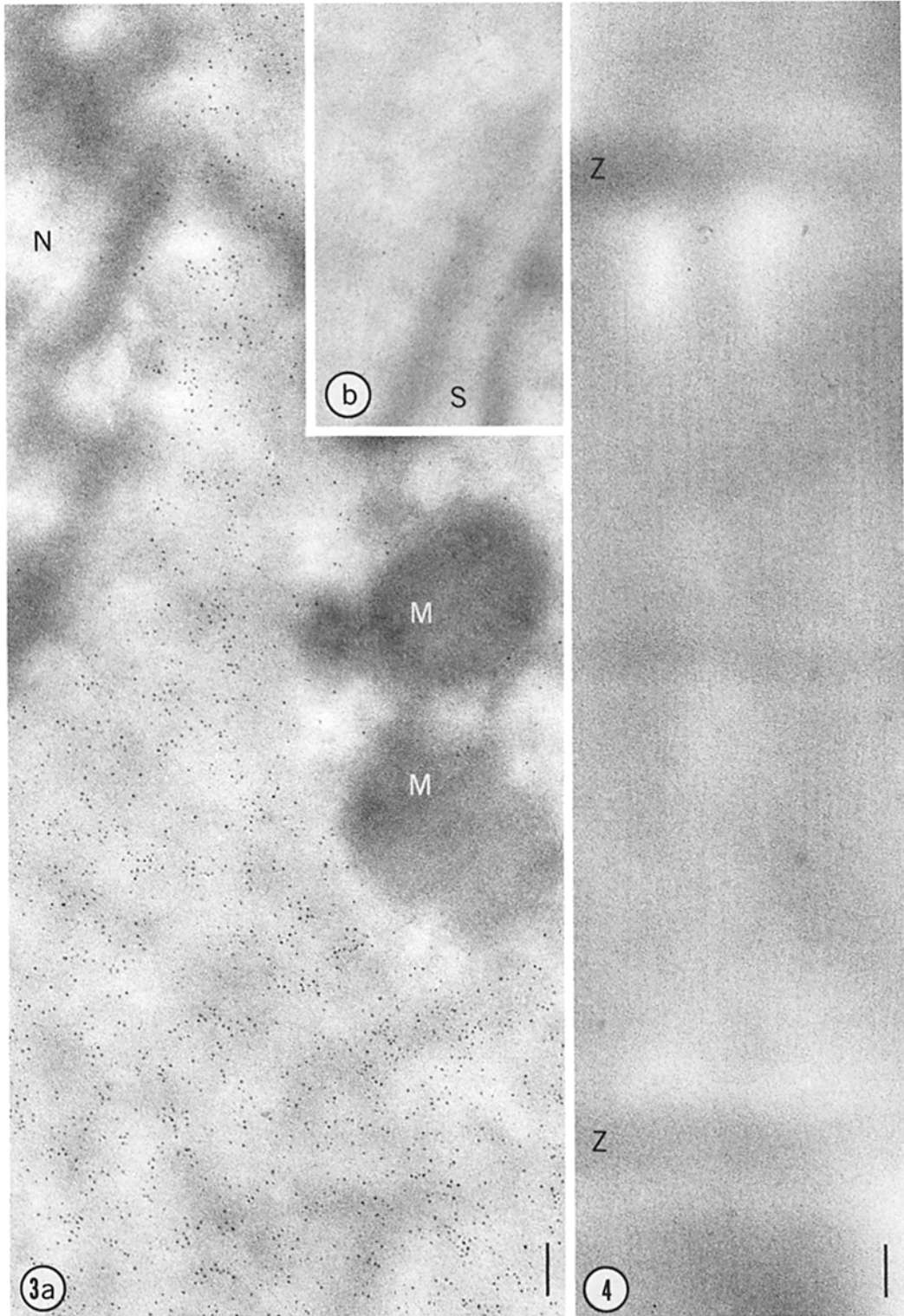
Utilization of Imidates

Some years ago, our laboratory introduced the use of bifunctional imidates as fixatives for electron microscopy (8), in a study of immunoferritin labeling of the hemoglobin in intact erythrocytes. In that case, we used diethylmalonimidate (DEM, refer to reference 1). Imidates are chemically even milder than aldehydes because, when they react with free amino functions on proteins, they do not seriously alter the ionic charge distribution on the protein molecule as aldehydes do (1). Bifunctional imidates might, therefore, be expected to allow better retention of antigenic activity than glutaraldehyde. On the other hand, as Hassel and Hand also observed (2), bifunctional imidates like DMS are not highly reactive near pH 7, and autolysis of

specimens can occur before fixation. Although DTP is more reactive than DMS, we have found that the degree of cross-linking attained at pH 7-8 is too low to preserve cellular structures in frozen sections, even after a prolonged fixation such as 8 h.

One might overcome this difficulty of slow reaction near pH 7 by designing other kinds of bifunctional imidates. Although loss of antigenic activity might thereby be avoided and ultrastructures retained, the problem of antigen accessibility would still have to be dealt with, and we have therefore tried other approaches.

In particular, we have investigated the possibility that pretreatment of a tissue block with a *mono-functional imidate*, followed by fixation with glutaraldehyde, might serve the purpose. The mono-functional imidate, by converting a large fraction of free amino groups to unreactive amidines without cross-linking them, would restrict the subse-



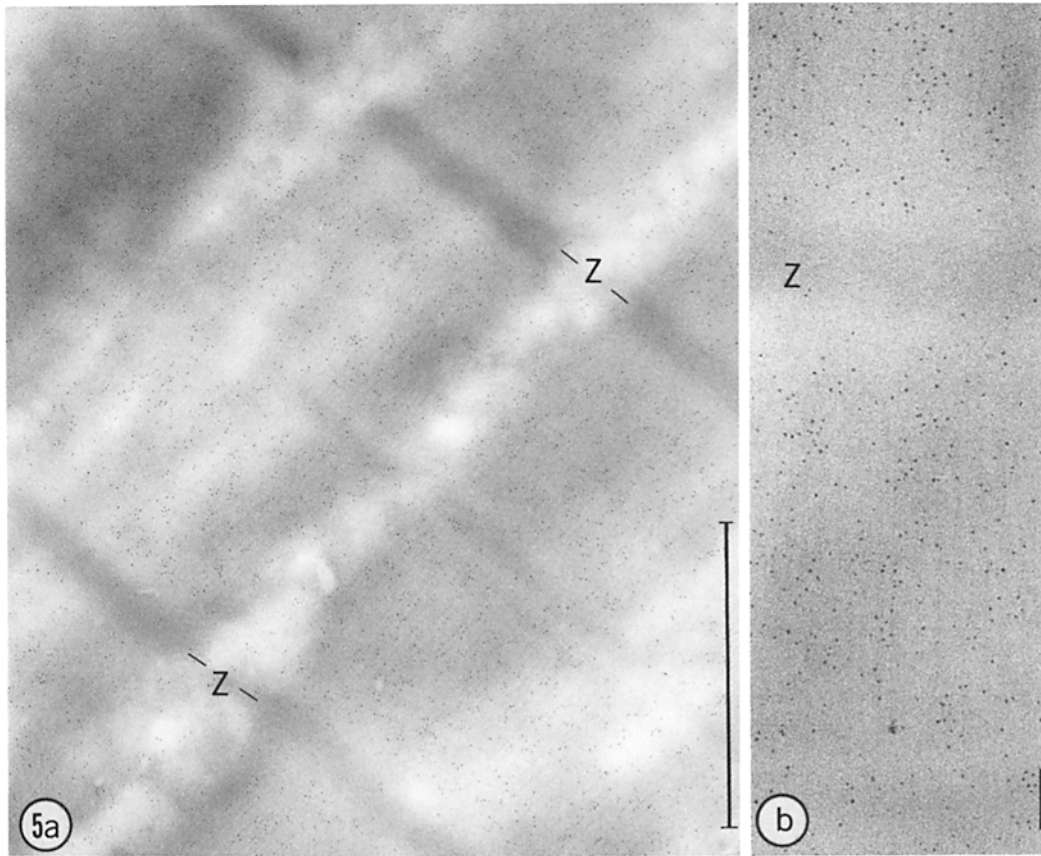


FIGURE 5 (*a* and *b*) Contracted mouse skeletal muscle, fixed in 1% formaldehyde for 1 h and then in 0.2% glutaraldehyde for 1 h. A significant labeling of myosin with the anti-chicken skeletal muscle myosin antibody is found in the entire area, except on Z-lines (*Z*). (*b*) an enlarged portion of (*a*). Negatively stained with a mixture of 0.4% dextran and 0.05% PTA. (*a*) $\times 40,000$. (*b*) $\times 80,000$.

quent degree of reaction and of cross-linking with glutaraldehyde. We chose EAI as the monofunctional imidate in this study.

If erythrocytes were first treated with 0.4 M EAI in 0.5 M sodium phosphate buffer for 1 h at room temperature and then with 1% glutaralde-

hyde for 1 h at 4°C, spectrin could be specifically labeled at a high level in frozen sections (Fig. 6*a* and *b*). Since a direct 1% glutaraldehyde-1 h treatment inhibited the immunoferritin labeling (Fig. 1*a*), this result suggests that the glutaraldehyde fixation was indeed significantly restricted by

FIGURE 3 (*a* and *b*) Mouse uterine smooth muscle, fixed in 1% formaldehyde for 1 h and then in 0.2% glutaraldehyde for 1 h. Immunolabeling of myosin in (*a*) is much higher than that in Fig. 2*a*. No significant labeling is found in the nucleus (*N*) or mitochondria (*M*). Control, treated with normal rabbit IgG in the first stage of immunolabeling is shown in (*b*). *S*, extracellular space. Negatively stained with a mixture of 0.4% dextran and 0.05% PTA. Both, $\times 80,000$.

FIGURE 4 Contracted mouse skeletal muscle, fixed in 1% formaldehyde for 1 h and then in 0.2% glutaraldehyde for 1 h. Little labeling is seen when the first stage staining is with the anti-human smooth muscle myosin antibody. Negatively stained with a mixture of 0.4% dextran and 0.05% PTA. *Z*, Z-lines, $\times 80,000$.

the pretreatment with the monofunctional imidate.

A basic difficulty in such a procedure, however, is that the lack of cross-linking during the treatment with the monofunctional imidate can cause serious deterioration of structures in many cells and tissues. This can be overcome by using a mixture of EAI and formaldehyde instead of an EAI solution for the pretreatment. Indeed, in sections of mouse uterine smooth muscle there was a greater increase (fivefold or more) in immunoferritin labeling of myosin when the tissue blocks were pretreated in the mixture of 0.1 M EAI and 1% formaldehyde for 2 h at 4°C before 0.2% glutaraldehyde-1 h fixation, than when the blocks were pretreated in 1% formaldehyde alone for the same period (cf. Figs. 7-9 with Fig. 3a). Such a fixation procedure, which is believed to provide an even milder and more uniform fixation than the formaldehyde-glutaraldehyde fixation, is quite effective in preserving cell ultrastructure in frozen sections (e.g., Fig. 10a-c).

DISCUSSION

In our experience, fixed frozen ultrathin sections are generally the most satisfactory preparations for the immunoferritin labeling of intracellular macromolecules (10, 12) and are better than those prepared by our previous procedure using specimens embedded in cross-linked BSA (8, 5). However, we have encountered a significant number of instances where conventional (1-2%) glutaraldehyde fixation inactivates a protein antigen, or, as described in this paper, where it so tightly cross-links the intracellular matrix as to render an antigen in the thawed ultrathin frozen section inaccessible to indirect immunoferritin staining. The main purpose of this paper is to show that these difficulties can be circumvented by any one of several procedures which we have described. They each involve a first stage treatment of a specimen under chemically mild conditions that generally should preserve antigenicity better than direct fixation with glutaraldehyde. It is well known that formaldehyde treatment (6) or reaction with imidates (18, 1), as compared with glutaraldehyde, generally have little or no effect on antigenicity of proteins. The first stage treatment, by modifying a significant fraction of available free amino residues, restricts the extent of modification that occurs upon the second stage treatment with glutaraldehyde. This not only minimizes the antigen-inactivating effect of glutaraldehyde, but also re-

duces the extent of stable cross-linking that is achieved. The lesser extent of cross-linking allows greater access of intracellular antigens to antibody. Despite the decreased cross-linking, ultrastructure is generally well preserved and well delineated (see Figs. 7a and 10). The apparently poor structural delineation at high magnifications is not a reflection on the technique. Either no poststaining was employed (Figs. 7-9), or only dilute PTA treatment, so as to allow maximum contrast between individual ferritin molecules and the cellular matrix. For other purposes, as desired, the contrast within the cellular matrix can readily be increased by other poststaining procedures (14).

Although the decreased cross-linking renders the frozen ultrathin sections more fragile, the simple precautions described above in the handling of such sections have allowed intense immunoferritin staining of several different specimens to be carried out routinely. The experience we have had so far is sufficiently diverse to indicate that these procedures are of general applicability, but they should not be viewed as immutable prescriptions. For some systems, the antigen in question may not be adequately fixed by these procedures, and its translocation may not be completely prevented; for other systems which are highly organized, even lesser cross-linking may be desirable. Modifications in the first and second stage fixation treatments may therefore be indicated in specific instances.

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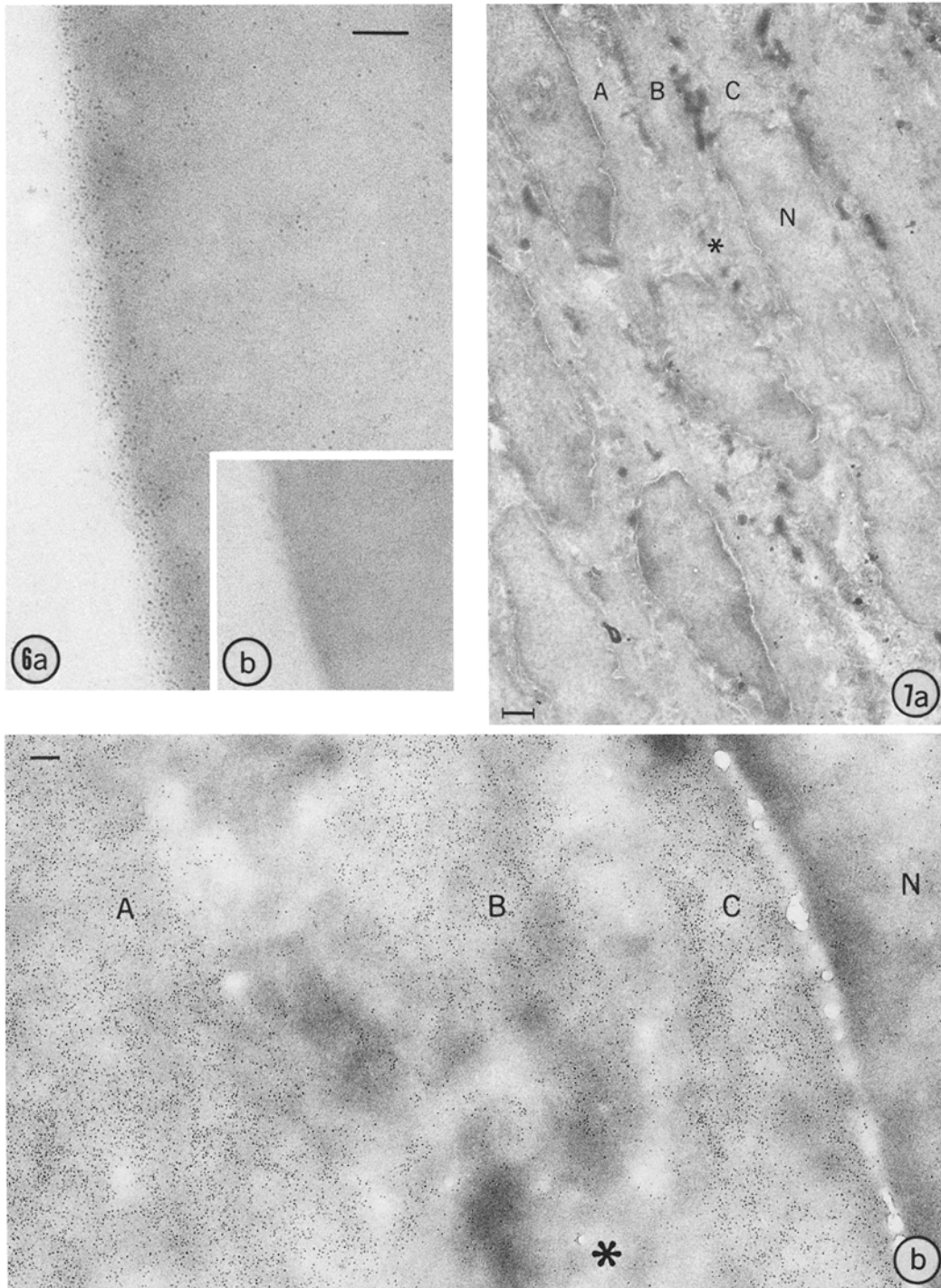


FIGURE 6 (*a* and *b*) Human erythrocytes, first treated with 0.4 M EAI for 1 h at room temperature and then fixed in 1% glutaraldehyde for 1 h at 4°C. An extensive labeling of spectrin is found along the cell surface in (*a*). Control, treated with the anti-human smooth muscle myosin antibody in the first stage, is shown in (*b*). Embedded in thin layers of gelatin. Both, $\times 80,000$.

FIGURE 7 (*a* and *b*) Mouse uterine smooth muscle, fixed in a mixture of 0.1 M EAI and 1% formaldehyde for 1 h and then in 0.2% glutaraldehyde for 1 h, both steps at 4°C. In (*a*), the general morphology of the tissue is seen to be well preserved. In (*b*), an enlarged part of (*a*), an extensive level of labeling of myosin makes it possible to differentiate A, B, and C cells (the same cells are indicated in (*a*)). Asterisk in (*a*) or (*b*) indicates the site where the three cells meet. N, nucleus of C cell. Embedded in a thin layer of gelatin. (*a*) $\times 4,500$. (*b*) $\times 40,000$.

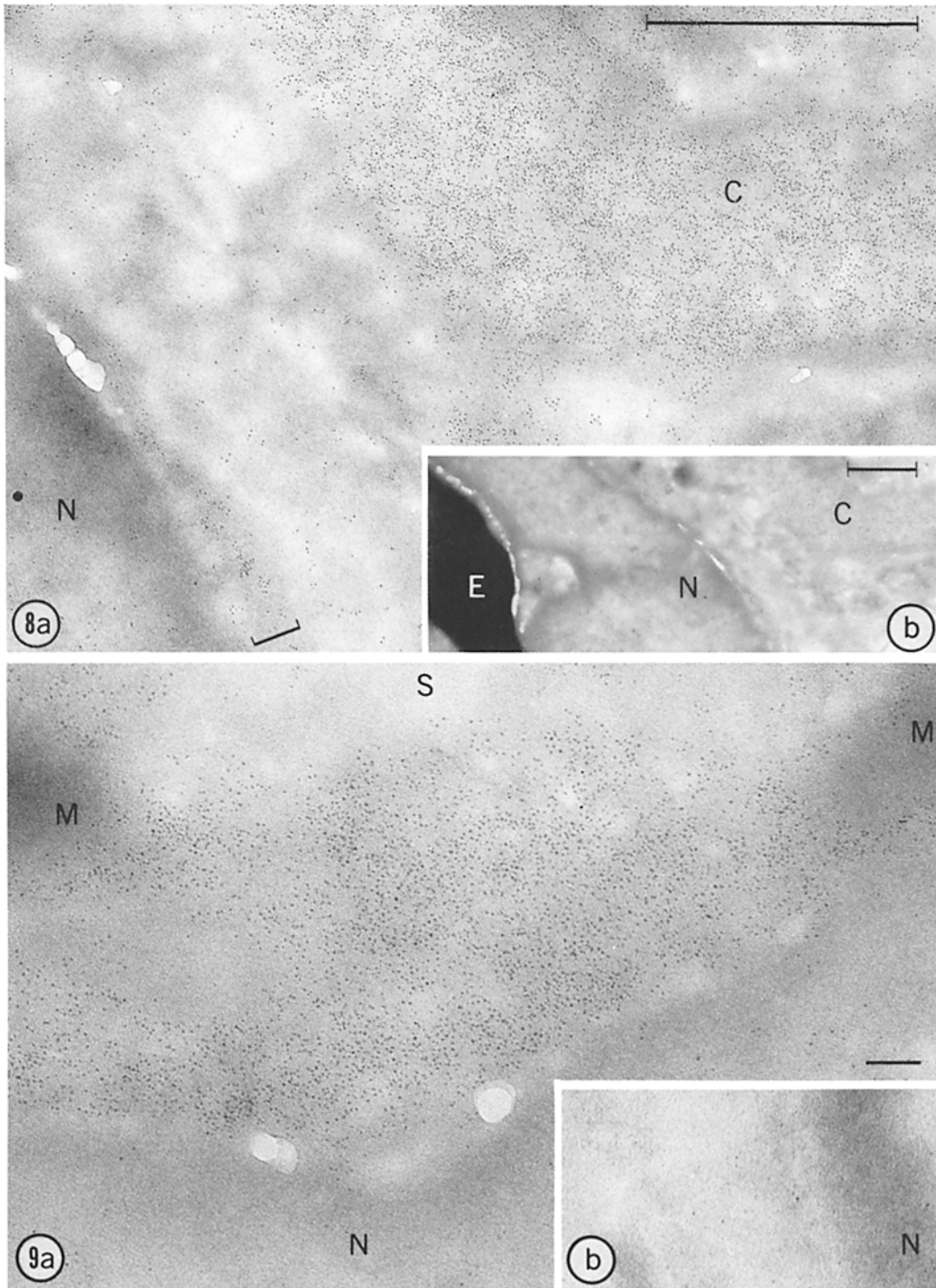


FIGURE 8 (*a* and *b*) Mouse uterine smooth muscle, fixed in the manner as indicated in the legend of Fig. 7. In (*a*), an enlarged part of (*b*), an extensive labeling of myosin in a smooth muscle cell (*C*; same cell indicated in (*b*)) is strongly contrasted with the little labeling found in the extracellular area. The level of labeling in the thin layer of cytoplasm (marked with a bracket) of an endothelial cell (its nucleus marked with *N* both in (*a*) and (*b*)) seems to be slightly higher than in the extracellular area, but its significance is not clear at present. *E*, erythrocyte in the lumen of the capillary. Embedded in a thin layer of gelatin. (*a*) $\times 40,000$. (*b*) $\times 10,000$.

FIGURE 9 (*a* and *b*) Mouse uterine smooth muscle, fixed in the manner as indicated in the legend of Fig. 7. In (*a*), magnified more highly than Figs. 7*b* and 8*a*, the labeling is found to be extensive in cytoplasm but virtually absent in mitochondria (*M*), the nuclear periphery (*N*) or the extracellular space (*S*). Embedded in thin layers of gelatin. Both, $\times 80,000$.

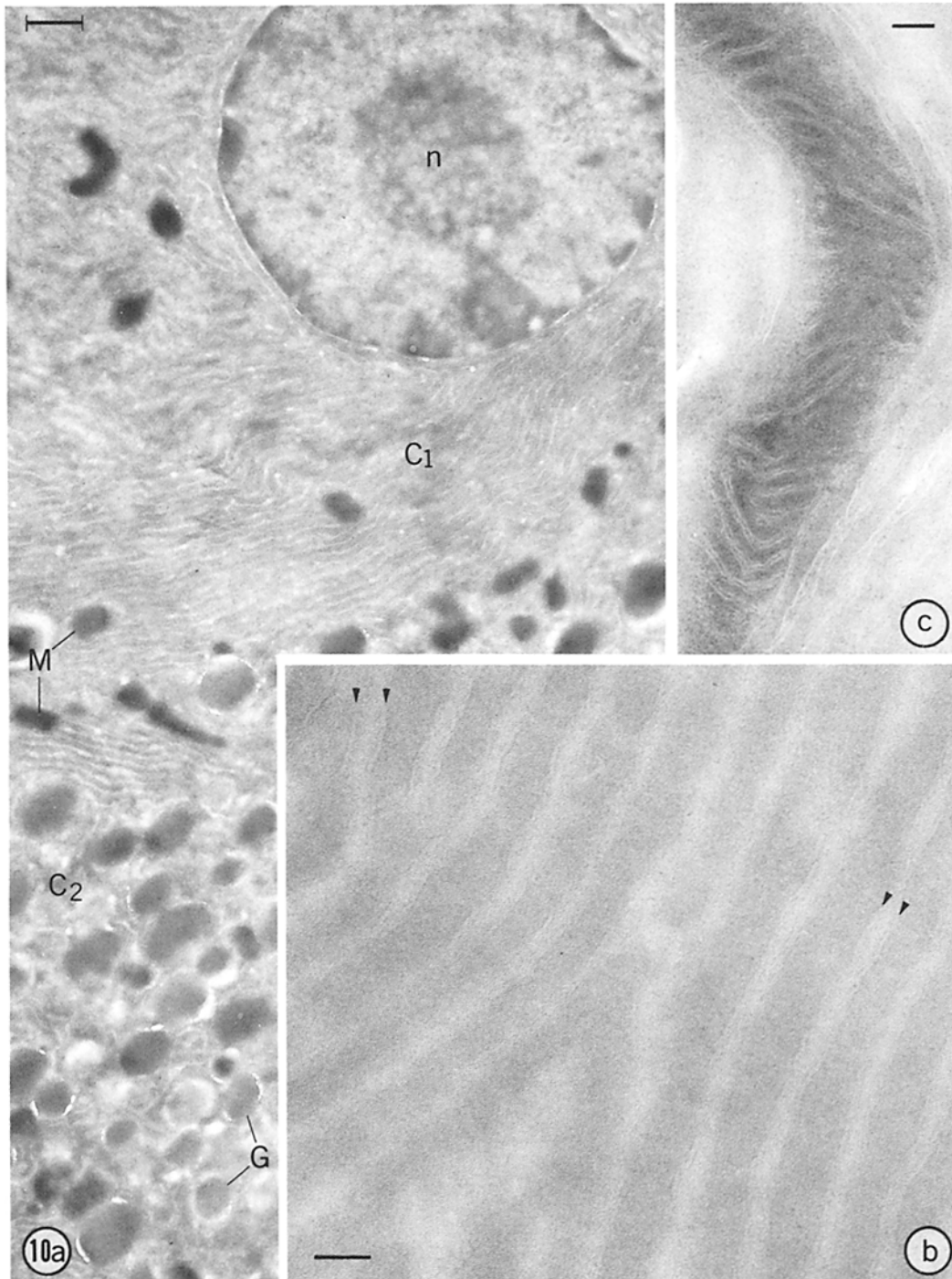


FIGURE 10 (a-c) Mouse pancreas, fixed in a mixture of 0.1 M EAI and 1% formaldehyde for 1 h and then in 0.2% glutaraldehyde for 1 h, both steps at 4°C. In (a) showing parts of two cells (C_1 and C_2), various structures such as endoplasmic reticulum, zymogen granules (G), mitochondria (M) or nucleolus (n) are seen to be well preserved. Membranes of endoplasmic reticulum (arrowheads) in (b) and mitochondrial cristae in (c) are well defined by negative staining. All are negatively stained with a mixture of 0.2% Ficoll and 0.2% PTA. (a) $\times 8,000$. (b) $\times 80,000$. (c) $\times 60,000$.

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