

# Use of Cryoprotectant to Maintain Long-term Peptide Immunoreactivity and Tissue Morphology

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WATSON, R. E., JR., S. J. WIEGAND, R. W. CLOUGH AND G. E. HOFFMAN. *Use of cryoprotectant to maintain long-term peptide immunoreactivity and tissue morphology.* PEPTIDES 7(1) 155-159, 1986.—Use of an ethylene glycol based cryoprotectant solution has been found to be effective for the long-term storage of brain tissue either in block form or as freely floating sections prior to immunocytochemical processing. Storage of tissue in the solution at  $-20^{\circ}\text{C}$  or  $4^{\circ}\text{C}$  for up to 3 months produced no adverse effects upon tissue morphology, nor was LHRH immunoreactivity diminished or accompanied by elevated non-specific staining. Furthermore, ultrastructural analysis of cryoprotected tissue revealed excellent preservation of cellular morphology. It is anticipated that this method can find use when it is necessary or desirable for the investigator to retain tissue for later immunocytochemical or electron microscopic processing.

Immunocytochemistry    Peptides    Morphology    Electron microscopy    Cryoprotectant

PROTOCOLS for immunocytochemical peptide localization have typically required tissue to be processed rapidly following perfusion of the experimental animal in order to maintain immunoreactivity and tissue morphology. This necessity has long hampered the design of studies involving immunocytochemical procedures, especially in situations when it is desired to compare staining across a number of groups. For example, in situations where many animals must be sacrificed simultaneously, simply sectioning much tissue can be demanding, while subsequent immunocytochemical processing of very large numbers of sections, with repetitive tissue rinses and incubations, can become virtually impossible. In addition, longitudinally designed studies can pose a problem to the investigator. Tissue from experimental groups which may be derived from animals sacrificed at different ages following a particular manipulation cannot be directly compared with confidence unless the tissue is processed simultaneously. However, it has been our experience that allowing tissue to remain for extended periods in either fixative or phosphate buffered hypertonic sucrose solutions results in substantial loss of peptide immunoreactivity. Thus, it is beneficial to have a means by which tissue can be stored until processing, for a long time if necessary, with neither a diminution of immunoreactivity nor of morphological integrity. We report here that use of an ethylene glycol based cryoprotectant solution, which is used to maintain horseradish peroxidase activity prior to histochemical reaction in tract tracing experiments [1,5], is suitable for longterm preservation of tissue morphology and peptide immunoreactivity. Portions of this work have been presented in abstract form [3].

## METHOD

### *Tissue Preparation—Immunocytochemistry*

Tissue was derived from 6 young adult male Sprague-Dawley rats (Charles River). Animals were deeply anesthetized with sodium pentobarbital, heparinized intracardially, and perfused first with 0.9% saline, followed by Zamboni's fixative. The brain was removed, postfixed for up to 18 hr in the same fixative at  $4^{\circ}\text{C}$  and then sectioned at  $30\text{ }\mu\text{m}$  on a vibrating microtome in a bath of potassium phosphate buffered saline (PBS, 0.05 M, pH 7.4). Freely floating sections were rinsed twice in PBS to remove excess fixative, and then immersed in cryoprotectant solution, or PBS, and stored for periods ranging from 2 to 12 weeks. Sections stored in cryoprotectant were maintained at either  $-20^{\circ}$  or  $4^{\circ}\text{C}$ . In addition, blocks of tissue, approximately 8 mm in rostro-caudal extent were cryoprotected for periods of up to 3 months. These blocks were mounted on vibratome chunks, sectioned at  $30\text{ }\mu\text{m}$ , and processed as above.

### *Cryoprotectant Solution*

As originally formulated by deOlmos and colleagues [1], one liter of cryoprotectant solution consists of 500 ml 0.1 M phosphate buffer, pH 7.2 (50% v/v) (1.59 g  $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ , 5.47 g  $\text{Na}_2\text{HPO}_4$  and 9.0 g NaCl in 500 ml distilled water), 300 g sucrose (30% w/v), 10 g polyvinylpyrrolidone (1% w/v; PVP-40, Sigma), 300 ml ethylene glycol (30% v/v; Fisher), with the final volume adjusted to 1000 ml with distilled water.

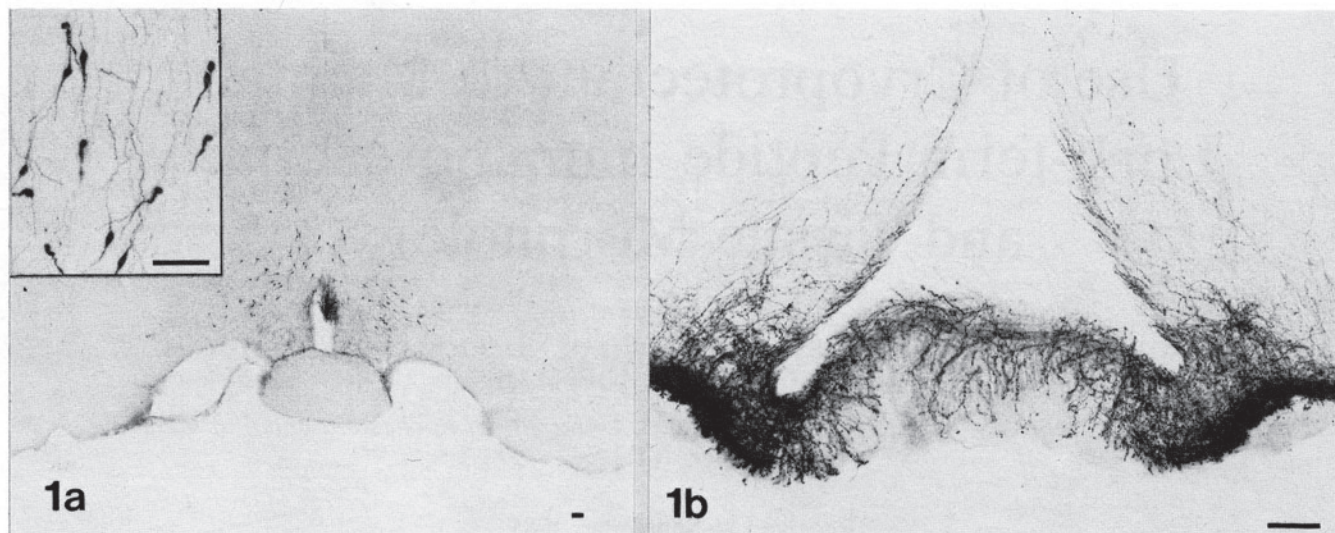


FIG. 1. LHRH immunoreactivity: processed immediately. a. The low magnification photomicrograph at the level of the organum vasculosum of the lamina terminalis (OVLT) shows the typical pattern of LHRH immunoreactivity seen in sections processed immediately after sectioning. The insert shows some of the cells at a higher magnification. b. At the level of the median eminence, the dense plexus of LHRH axons is seen. Note the discreteness of the immunoreactive labeling in both cells and fibers and the low background staining associated with rapid processing. Bars indicate 100  $\mu$ m.

#### Immunocytochemistry

Prior to immunocytochemical processing, tissue stored in both PBS and cryoprotectant underwent three 10 min rinses in large volumes (50 ml) of PBS. This step was necessary for tissue stored in cryoprotectant since immediately upon being replaced in PBS, the tissue shrank upon itself rather markedly. However, following 30 min in PBS, the sections reassumed their normal appearance, presumably as cryoprotectant diffused from the tissue.

Immunocytochemical localization of LHRH was performed using either the unlabeled antibody (PAP) technique, as previously described [2], or the avidin-biotin-peroxidase complex (ABC) method [4] (Vectastain kit, Vector Laboratories, Burlingame, CA). Anti-LHRH was used at a concentration of 1:30,000 and was a generous gift of Drs. Benoit and Guillemin (#LR1). This antiserum was produced in rabbits and showed no significant cross reactivity with other substances.

Localization of immunoreactivity using the ABC technique was performed in the following manner. Freely floating sections were rinsed in PBS, incubated in normal goat serum diluted in PBS (Vectastain kit, Vector Laboratories) for 30 min at room temperature, rinsed twice in PBS, and then incubated in antisera in PBS containing 0.4% Triton X-100 (TX) for 15–18 hr at 4°C. Sections were then rinsed 10 times (10 min each) in PBS + 0.02% TX and incubated for 1 hr at room temperature in biotinylated affinity purified goat anti-rabbit IgG at the dilution (in PBS + 0.02% TX) specified by the Vectastain kit. Four rinses in PBS + 0.02% TX (15 min each) followed, after which the tissue was incubated for 1 hr at room temperature in avidin-biotinylated horseradish peroxidase complex, again at the dilution (in PBS + 0.02% TX) specified in the instructions supplied with the Vectastain kit. The tissue was rinsed twice in PBS + 0.02% TX (15 min each) and then rinsed twice in Tris buffered saline (0.05 M, pH 7.2, Sigma). The sections were then transferred into fresh

Tris buffered saline to which was added an equivalent volume of Tris buffered saline containing 0.043% 3-3' diaminobenzidine and 0.04%  $H_2O_2$ . Incubation occurred at room temperature for 6–7 min, after which sections were transferred into large volumes of normal saline, mounted on subbed glass slides, mounted with Permount, and coverslipped.

#### Electron Microscopy

In order to assess the ultrastructural preservation of cryoprotected brain tissue, 2 male rats were anesthetized and perfused with heparinized saline followed by freshly made fixative consisting of 2% paraformaldehyde and 1.25% glutaraldehyde in 0.15 M PBS (pH 7.2; perfused at 50 mm Hg pressure; 5 ml/min for 30 min). The brain was removed and blocked coronally into 1 mm slices. Slices containing the medial preoptic area (MPOA) were sagittally bisected. Each MPOA was dissected out, trimmed to approximately 1 mm<sup>3</sup> and placed in the primary fixative (total time 90 min). Following primary fixation, the MPOA blocks were either post-fixed in osmium tetroxide (1%, at 4°C, 1.5 hr), or placed into cryoprotectant solution. The osmicated blocks were immediately dehydrated in ascending series of alcohols and embedded in araldite 502. Those blocks immersed in cryoprotectant were stored at –20°C for 2 weeks. Following this, the blocks were osmicated as described above for the control blocks, dehydrated and embedded in araldite. Thin sections (silver interference color) were cut using a diamond knife, mounted on 200 mesh copper grids and stained with sequential uranyl acetate and lead citrate. Sections were then examined using a Zeiss model EM 10 electron microscope.

#### RESULTS

A comparison of tissue processed for immunocytochemistry of LHRH immediately after cutting, after storage for 6 weeks in PBS, and after storage for 3 months in cryoprotectant is shown in Figs. 1–3, respectively.

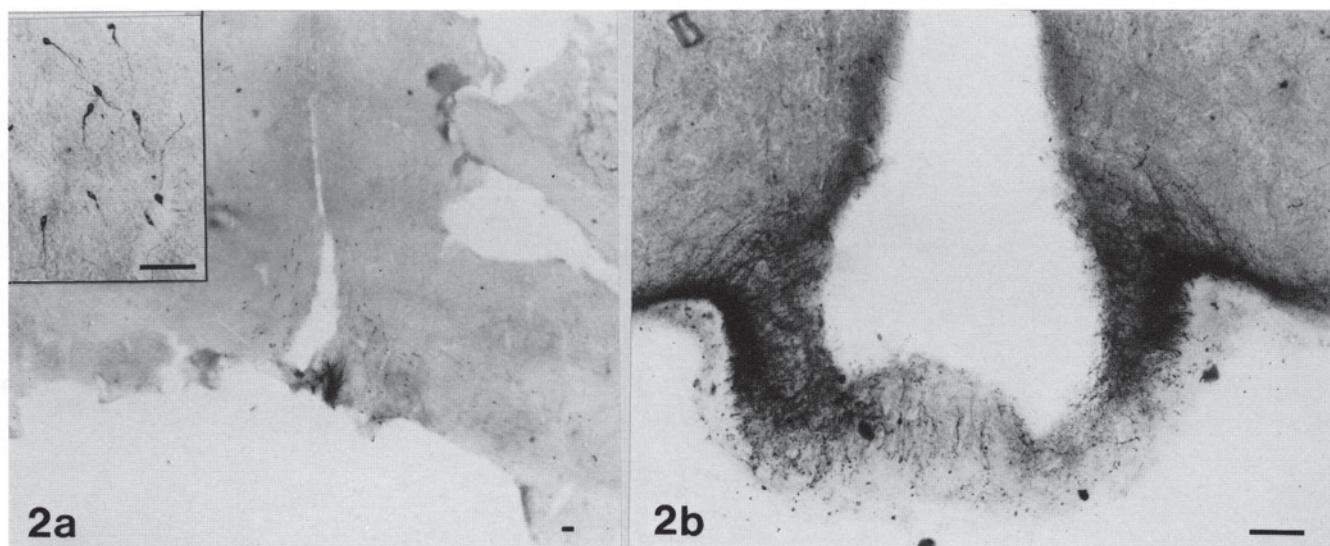


FIG. 2. LHRH immunoreactivity: processed after 6 weeks storage in PBS. a. OVLT (the insert shows some of the cells at a higher magnification). b. Median eminence. These micrographs depict LHRH immunoreactivity at levels similar to those seen in Fig. 1, but from tissue sections that were stored in PBS. Note that while some immunoreactive structures still remain following long term storage in PBS, the tissue is badly torn and non-specific background staining is greatly increased. Bars indicate 100  $\mu$ m.

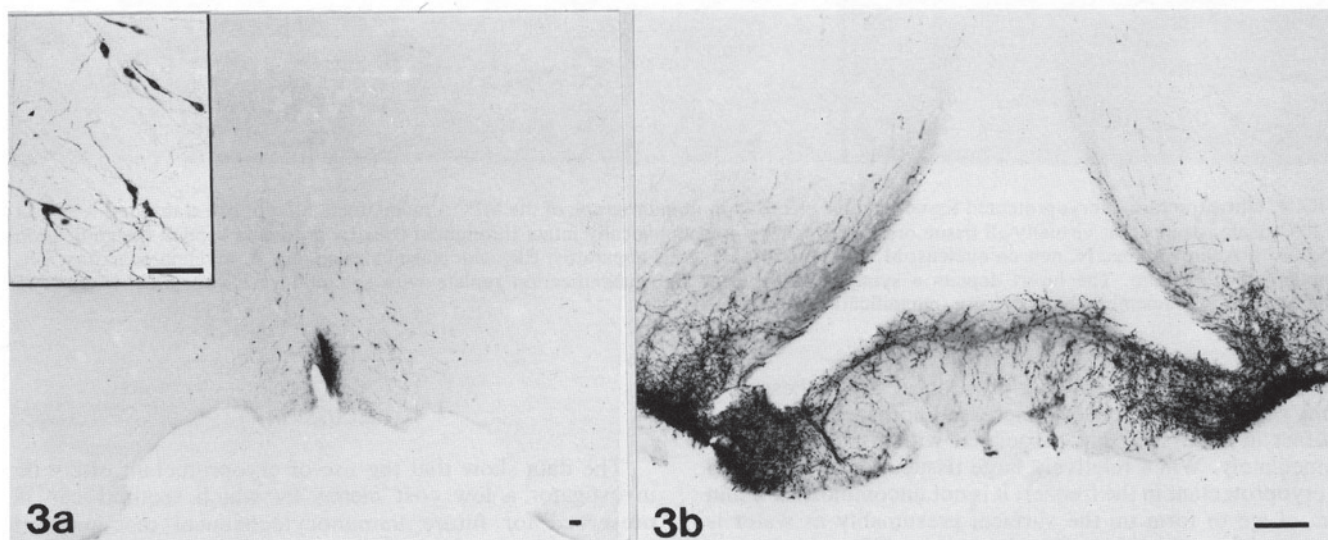


FIG. 3. LHRH immunoreactivity: processed following 3 months in cryoprotectant. a. OVLT (the insert shows some of the cells at a higher magnification). b. Median eminence. These micrographs depict LHRH immunoreactivity at levels similar to those seen in Fig. 1, but from tissue sections that were stored in cryoprotectant. Note that reaction of this tissue after long term storage is associated with undiminished immunoreactivity, well-preserved tissue morphology, and no elevation in non-specific staining. Similar results were obtained with tissue stored in cryoprotectant *en bloc*. Bars indicate 100  $\mu$ m.

The figures demonstrate LHRH immunoreactivity at the level of the organum vasculosum of the lamina terminalis (OVLT) and the median eminence associated with these different treatments of the tissue. Immediate reaction of the tissue (Fig. 1) produced the typical dense immunoreactivity within cells and fibers, and background staining was minimal. In contrast, while immunocytochemical labeling of neuronal elements was still detectable in tissue stored for up to 6 weeks in PBS, the tissue was typically unworkable in that it tore and was shredded easily while undergoing im-

munocytochemical processing. Also, non-specific staining was markedly increased following long term storage in PBS (Fig. 2). However, storage of tissue blocks or freely floating sections in cryoprotectant solution at  $-20^{\circ}\text{C}$  for periods of up to 3 months produced no observable diminution of LHRH immunoreactivity (Fig. 3). In addition, immunostained cryoprotected sections showed no evidence of loss of morphological integrity which characterized sections stored for long periods in PBS. Sections stored in cryoprotectant were much more resistant to damage and typically remained intact

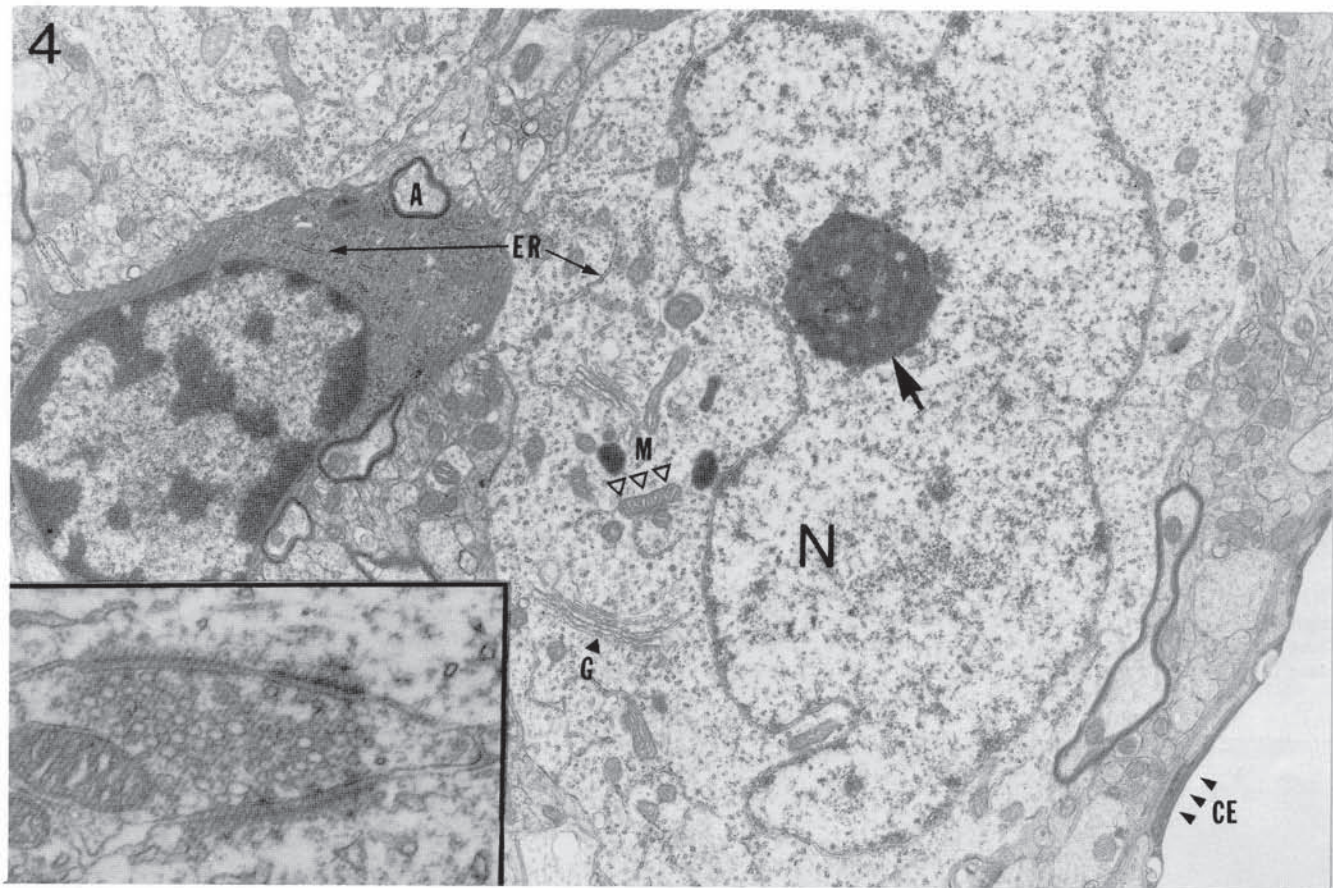


FIG. 4. Ultrastructure of cryoprotected tissue. Sample electron photomicrograph of the MPOA maintained in cryoprotectant for 2 weeks at  $-20^{\circ}\text{C}$  demonstrates that virtually all tissue organelles remain morphologically intact throughout the cryoprotected section (magnification  $15,000\times$ ). Abbreviations: N, neuron nucleus; M, mitochondria; G, golgi apparatus; ER, endoplasmic reticulum; A, myelinated axons; CE, capillary endothelium. The insert depicts a synaptic terminal at high magnification replete with synaptic vesicles, mitochondria and characteristic paramembraneous density (magnification  $60,000\times$ ).

throughout the entire staining procedure. Also, these sections showed no evidence of increased non-specific staining artifact in excess of that associated with tissue processed immediately. When relatively large tissue blocks are stored in cryoprotectant in the freezer, it is not uncommon for a thin film of ice to form on the surface, presumably as water is extracted from the tissue. This has not been found to pose a problem to either retention of peptide immunoreactivity or tissue morphology.

Figure 4 is representative of the general ultrastructural profile of MPOA tissue maintained in cryoprotectant at  $-20^{\circ}\text{C}$  for 2 weeks. It is apparent in this photomicrograph as well as from our analysis of the remaining MPOA tissue that the ultrastructure of the region is preserved extremely well in the cryoprotectant solution and does not differ from tissue processed immediately after perfusion. There was no loss of membrane integrity nor any apparent swelling within the neuropil of the MPOA. It was expected that there may be morphological disturbances near the surface of the cryoprotected blocks; however our analysis revealed that even ependymal cells of the third ventricle, including their characteristic microvilli, cilia, junctional complexes, and other organelles, remained remarkably well preserved in the cryoprotectant (Fig. 5).

#### DISCUSSION

The data show that the use of cryoprotectant offers the investigator a low cost means by which sections can be preserved for future immunocytochemical reaction with neither a decrement in staining quality, nor of tissue morphology. The tissue preservation lasts for at least 3 months, and probably longer, if not indefinitely. It is important to note that in recent work in our laboratory, cryoprotectant has been found to preserve immunoreactivity for a number of additional peptides, including corticotropin releasing factor, substance P, met-enkephalin, vasoactive intestinal polypeptide, neurotensin, cholecystokinin, as well as oxytocin, vasopressin and their associated neurophysins (unpublished observations). This technique therefore should prove useful to investigators who: (1) must react large amounts of tissue which cannot practically be combined into a single procedure, or conversely; (2) wish to compare tissue derived from different experimental groups by processing in a single reaction; and (3) wish to retain tissue for electron microscopy or potential immunocytochemical processing in the future, when, for example, new antisera become available.

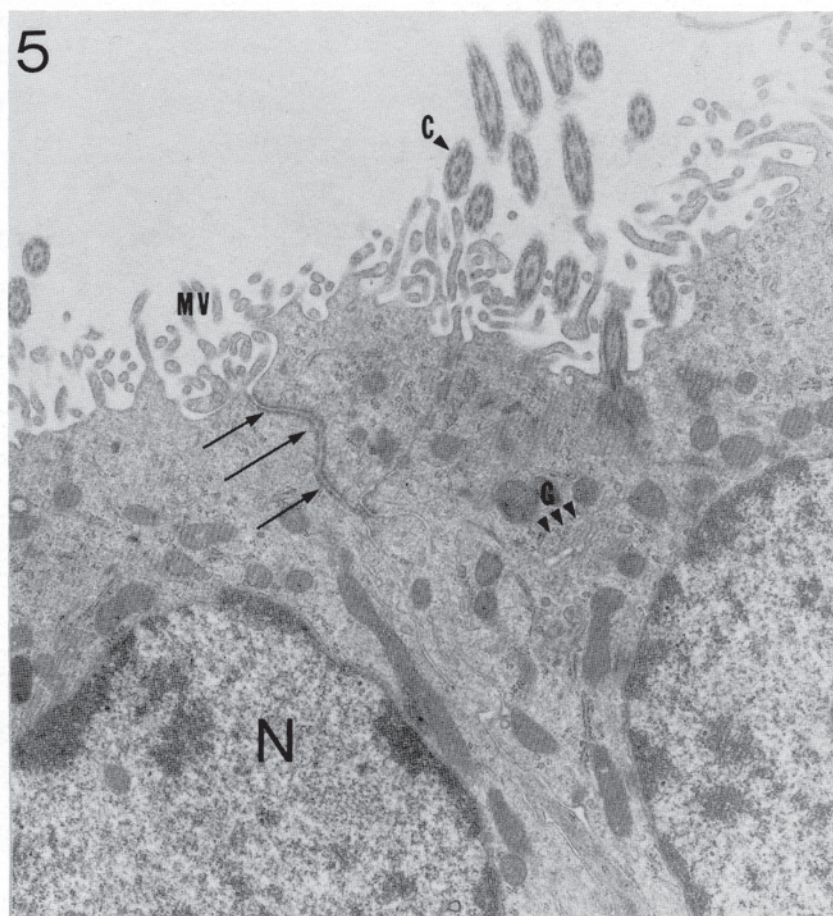


FIG. 5. Ultrastructure of MPOA ependymal surface. Sample electron micrograph showing the ventricular ependyma in the MPOA from tissue stored in cryoprotectant. It illustrates the excellent degree of preservation of the delicate organelles found on the ependymal surface (magnification 15,000 $\times$ ). MV, microvilli; C, cilia; N, nucleus; G, golgi; arrows demarcate junctional complexes.

#### ACKNOWLEDGEMENTS

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