

Use of Uranyl Acetate En Bloc To Improve Tissue Preservation and Labeling for Post-Embedding Immunoelectron Microscopy

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ABSTRACT We have developed a protocol for post-embedding immunoelectron microscopy that utilizes uranyl acetate, en bloc, as a secondary tissue fixative. Squirrel and cat retinas were fixed in 1% paraformaldehyde-1% glutaraldehyde for one hour. Secondary fixation was by 2% uranyl acetate, en bloc, (1 hour) during tissue dehydration. The tissue was embedded in LR White or Lowicryl K4M resin. Post-embedding immunoelectron microscopy (indirect immunogold) was performed on thin sections with antibodies to four different classes of proteins (filamentous, cytoplasmic, membrane, and extracellular matrix). The sections were then stained sequentially on drops of uranyl acetate and lead citrate, and by vapors of osmium tetroxide. Uranyl acetate fixation and/or staining of the sections by osmium tetroxide was omitted from the control experiments. Differences after secondary fixation with uranyl acetate and staining of the thin sections with osmium tetroxide were better overall preservation and enhanced contrast of the extracellular matrix, membranes, cytoplasm, and DNA. Antigenicity, as evidenced by the immunolabeling of the four proteins, was retained. Quantitation of the immunolabeling for the cytoplasmic and membrane proteins revealed significantly increased labeling densities in tissue postfixed with uranyl acetate. The improved tissue preservation and immunolabeling of proteins indicate that secondary fixation with uranyl acetate can be a valuable addition to post-embedding immunocytochemistry.

INTRODUCTION

For post-embedding immunoelectron microscopy, tissue preservation must often be sacrificed in order to retain adequate antigenicity. In conventional tissue processing for electron microscopy, osmium tetroxide is routinely used as a secondary fixative to provide better tissue preservation and contrast. Although osmium post fixation has been used for some immunolabeling experiments (Pelletier et al., 1981; Schwendemann et al., 1982; Smith and Keefer, 1982), its use as a fixative for immunocytochemistry is limited because it bonds covalently to proteins and alters their conformation (Lenard and Singer, 1968), usually to the "... extinction of antigenicity in the great majority of proteins" (Pearse, 1980).

Immersion of fixed tissue in a solution of uranyl acetate either before or during dehydration is often referred to as en bloc stain-

ing. Uranyl acetate used in this manner, however, also has properties of a fixative (Silva et al., 1968, 1971; Terzakis, 1968), and its action appears to be electrostatic (Hyatt, 1981), rather than covalent.

Following an ultrastructural study of normal and experimentally detached retinas (Anderson et al., 1983; Erickson et al., 1983) we were interested in repeating our study and preserving large pieces of retinal tissue for light and electron microscopic immunocytochemistry. Because we wanted better tissue preservation than simple aldehyde fixation yielded, with the possibility of immunolabeling as many proteins as possible, we tried uranyl acetate as a post fixative. In this paper we describe a simple and reliable pro-

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tozol that uses uranyl acetate as a secondary fixative for post-embedding immunoelectron microscopy.

MATERIALS AND METHODS

The procedures outlined below were performed at room temperature unless otherwise stated.

LR White: Tissue processing and polymerization

The retinas of eastern gray squirrels (*Sciurus carolenensis*) and domestic cats (*Felis domesticus*) were fixed by either intracardiac perfusion and then immersion (Anderson and Fisher, 1976), or by simple immersion in 1% paraformaldehyde–1% glutaraldehyde in 0.086M NaPO₄ buffer, pH=7.2. The tissue remained in this fixative for 1.0 hour. Following fixation, each retina was sectioned into quadrants, and these large tissue pieces were then washed three times in 0.137M NaPO₄ buffer (pH=7.2) for 10 minutes each wash. The tissue was dehydrated in 15%, 30%, and 50% methanol/water (MeOH/H₂O) for 10 minutes in each concentration. En bloc uranyl acetate fixation was for 1.0 hour in 2.0% uranyl acetate dissolved in 70% MeOH/H₂O. Dehydration continued in 85% and 95% MeOH/H₂O and 100% MeOH for 10 minutes each. The tissue was infiltrated by placing it in a 1:1 ratio of 100% MeOH:LR White resin (Polysciences, Inc., Warrington, PA) and rotating it overnight at 4°C. The vials were capped during this last step (note that this was the only stage where the tissue vials were capped). The next morning the tissue was placed in fresh 100% LR White resin in uncapped vials and rotated at 4°C for 2.0 hours, followed by 4.0 hours at room temperature. While the tissue was rotating at room temperature, fresh LR White resin (stored at 4°C) was poured into smooth aluminum pans (Fisher Scientific, Springfield, NJ) and allowed to equilibrate to room temperature. Because LR White decreases in volume during aerobic polymerization, care must be taken to place sufficient resin in the pans. After rotating for 4.0 hours, the tissue was transferred to the fresh LR White in the aluminum pans. Some tissue was embedded in 4- or 7-dram polyethylene vials (VWR, San Francisco, CA; tissue submerged in approximately 4 or 8 ml of LR White; vials capped for polymerization). The resin was polymerized at 52°C (+/-2°C) for 1–3 days. For the control experiments, uranyl acetate was omitted from the 70% MeOH/H₂O solution.

Lowicryl K4M: Tissue processing and polymerization

Tissue, primary fixation, and buffer washes were the same as listed in the LR White protocol. Tissue was dehydrated in 15%, 30%, and 50% n,n-dimethyl formamide/water (DMF/H₂O) for 10 minutes in each concentration. Secondary fixation, for 1.0 hour, was in 2.0% uranyl acetate in 70% DMF/H₂O (mixed fresh just prior to use). The tissue dehydration continued in 85% DMF/H₂O (10 minutes) and 95% DMF/H₂O (10 minutes × 2 washes). The tissue was infiltrated in a 1:1 ratio of DMF:Lowicryl K4M (Polysciences, Inc., Warrington, PA) and rotated overnight in capped vials. The next morning the tissue was placed in fresh 100% K4M and rotated for 30 minutes in uncapped vials; this step was repeated once. The tissue was then placed in cold (4°C) K4M in cold (4°C) plastic petri dishes or aluminum pans, and allowed to equilibrate at 4°C for 30 minutes. Plastic wrap (Saran wrap) was floated on top of the resin to reduce exposure of the resin to room air. Some tissue was embedded in 0.5 or 1.5 ml polypropylene microcentrifuge tubes (Out Patient Services, Inc., Petaluma, CA; tubes completely filled with K4M; capped). The covered resin was then exposed to ultraviolet light (two G.E. 15 Watt, black lights at a distance of 10 cm) overnight at 4°C. For the control experiments, uranyl acetate was omitted from the 70% DMF/H₂O solution.

Immunocytochemistry

Thin sections (silver to gold) were placed on carbon-coated Formvar films on nickel or copper grids. The antibodies were diluted in phosphate buffered saline (PBS) that contained 0.125 g/liter sodium azide and was supplemented with 0.5% bovine serum albumin (BSA). The grids were incubated on drops of diluted normal goat serum (Vector Laboratories, Inc., Burlingame, CA) for 15 minutes. Without drying or rinsing, the normal goat serum was blotted from the sections and the grids were incubated on drops of primary antibody. Four polyclonal antibodies were tested: anti-glial fibrillary acidic protein (GFAP, rabbit anti-bovine IgG fraction, 1:400 dilution, from DAKO Laboratories, Santa Barbara, CA); anti-interphotoreceptor retinol binding protein (IRBP, rabbit anti-bovine IgG fraction, 1:300 dilution); anti-cellular retinaldehyde binding protein (CRALBP, rabbit anti-bovine IgG fraction, 1:400 dilution); anti-opsin (rabbit anti-bovine

whole serum, 1:500 dilution). The sections were incubated with the primary antibodies overnight. The grids were then rinsed with several drops of PBS/BSA, immersed in PBS/BSA for 5–15 minutes, and then rinsed dropwise again. Next, grids were incubated on drops of secondary antibody-gold complexes (goat anti-rabbit IgG-5 or -20 nm gold, 1:20 dilution; Janssen Pharmaceutica, Beerse, Belgium) for 1.0 hour. The grids were rinsed, as above, and then washed with several drops of double-distilled water. The water was blotted from the grids and the grids were allowed to air dry. The tissue was then stained in 1.0% uranyl acetate (10 min), lead citrate (10 min), and with osmium tetroxide vapors (2.0% OsO₄ in 0.086M NaPO₄ for 1.0 hour). Staining of the thin sections by the OSO₄ vapors was omitted as an experimental control. Immunocytochemistry controls included replacement of the primary antibodies with pre-immune IgG (1:400 dilution), non-immune rabbit IgG (1:400 dilution), or PBS/BSA.

Quantitation

Adjacent pieces of perfused squirrel retina, taken from the superior central region, were used for quantitative immunocytochemistry. One of these pieces of tissue was fixed in aldehydes and the other was fixed in aldehydes and postfixed in uranyl acetate. Both were embedded in LR White resin. Grids, with tissue sections to be used for quantitation, were processed simultaneously throughout the immunocytochemistry protocol to assure identical processing times and solutions. Antibody labeling density was determined by counting gold particles/ μm^2 (mean \pm standard deviation; Kraehenbuhl et al., 1980). For each value five cells were selected by computer-generated random numbers, from each of three grids ($n=15$). Micrographs of anti-opsin-labeled rod photoreceptor outer segments were obtained at a primary magnification of $\times 40,000$ and those of anti-CRALBP-labeled retinal pigment epithelial cells at $\times 20,000$. Counts were made on photographic prints enlarged to $\times 100,000$ (anti-opsin; average of $2.0 \mu\text{m}^2$ counted per cell) and $\times 50,000$ (anti-CRALBP; average of $10.0 \mu\text{m}^2$ counted per cell). Counts and areas were determined using a Zeiss MOP-3 digitizing tablet.

RESULTS

Exposure of tissue sections to OsO₄ vapors alone resulted in moderately improved tissue

contrast compared to unexposed sections. The preservation of tissue fixed in aldehydes (Fig. 1) was inferior to tissue fixed in aldehydes and uranyl acetate (A + UA; Fig. 2). The better overall preservation after A + UA fixation was especially noticeable in the extracellular matrix, membranes, cytoplasm, and DNA (Figs. 3–7).

In addition to better tissue preservation with A + UA fixation, sufficient antigenicity is retained to allow antibody labeling. We localized anti-CRALBP to the nucleoplasm and cytoplasm of both retinal pigmented epithelial cells (Fig. 1–5) and Muller cells, anti-opsin to the rod outer segment membranes (Fig. 6), anti-IRBP to the interphotoreceptor matrix (Fig. 7), and anti-GFAP to the 10 nm intermediate filaments of astrocytes and Muller cell end feet (Fig. 8). The labeling patterns we observed were the same as those described by previous investigators (IRBP and CRALBP: Bunt-Milam and Saari, 1983; opsin: Bok, in press; GFAP; Erickson et al., 1986). The immunocytochemistry controls were essentially devoid of labeling.

Quantitation of immunogold labeling density revealed a significantly higher labeling, with anti-opsin and anti-CRALBP, on tissue that had been postfixed with uranyl acetate (Table 1). IRBP labeling was also significantly higher in the A + UA-treated tissue owing to increased preservation of extracellular matrix (Fig. 7). The extracellular matrix material is much less prominent in tissue fixed only with aldehydes (Fig. 7). IRBP labeling was not quantitated because 1) there was very little labeling observed in tissue not postfixed in uranyl acetate, and 2) there was a tremendous increase in immunolabeling owing to the preservation of additional extracellular matrix in the tissue that was postfixed in uranyl acetate.

Tissue that was fixed with both protocols contained anti-GFAP-labeled filaments in astrocytes and Muller cells (A + UA; Fig. 8) with no apparent difference in the location or quantity of GFAP-positive filaments (data not shown). Tissue labeling of the filaments was not quantitated because of the difficulty in identifying regions in different tissue samples with comparable filament densities.

DISCUSSION

The development of LR White and Lowicryl K4M resins has allowed significant improvements in post-embedding immunoelectron microscopy (Altman et al., 1983, 1984; Carlemalm et al., 1982; Newman et al., 1982,

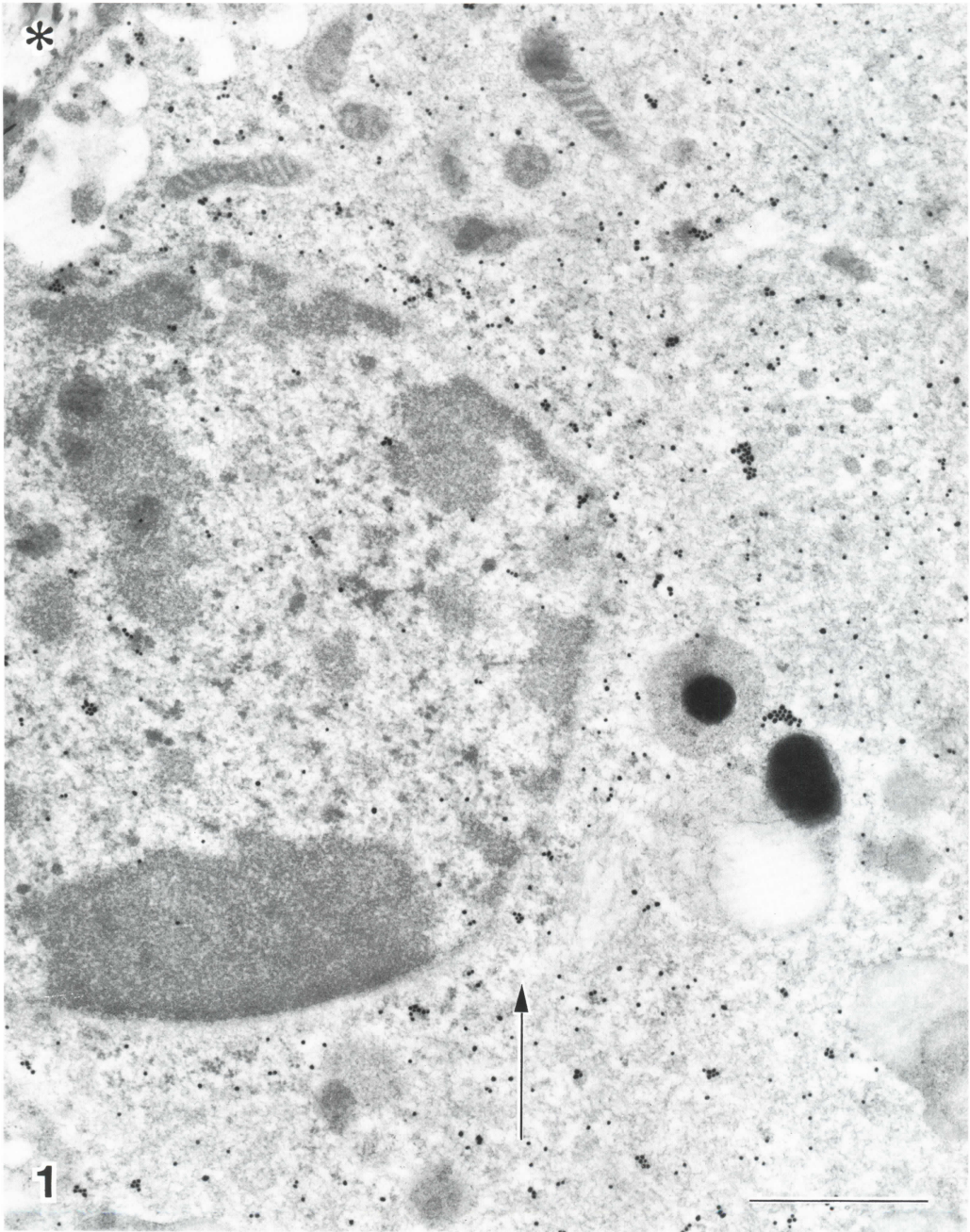


Fig. 1. Low-power immunoelectron micrograph showing a portion of a pigmented epithelial cell from a squirrel retina fixed with aldehydes and embedded in LR White resin. Tissue section exposed to OsO₄ vapors. Compare the overall anti-CRALBP immunolabeling and

fixation quality of this pigment epithelial cell with a similar cell in Figure 2. The area near the arrow is enlarged in Figure 3a. Asterisk indicates the choroidal vasculature. $\times 25,000$; bar = 1.0 μm .

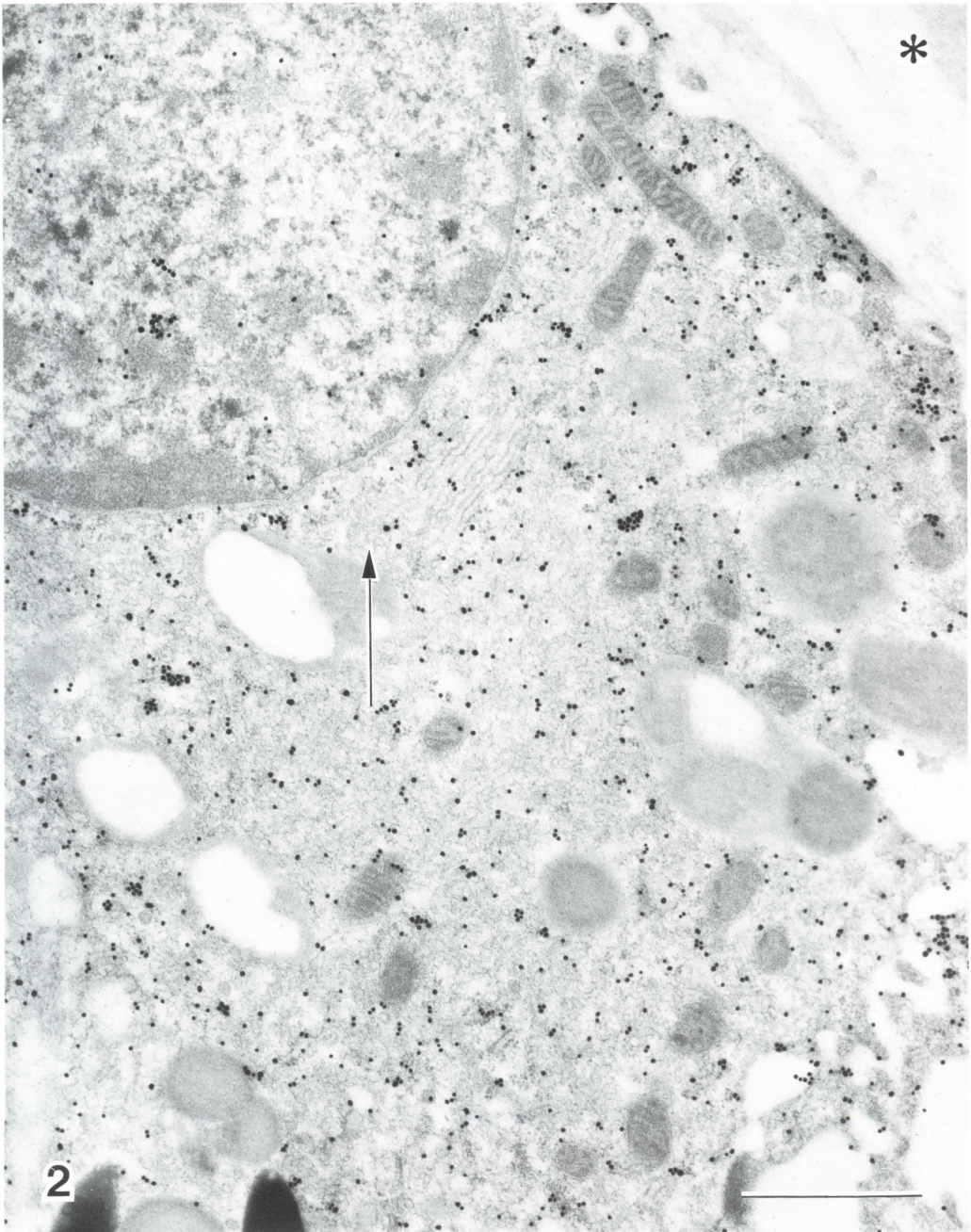


Fig. 2. Low-power immunoelectron micrograph of a pigmented epithelial cell from a squirrel retina fixed first with aldehydes and then en bloc with uranyl acetate; tissue embedded in LR White resin. Tissue section exposed to OsO_4 vapors. Compare the overall anti-

CRALBP immunolabeling and fixation quality of this cell with that in Figure 1. The area near the arrow is enlarged in Figure 3b. Asterisk indicates the choroidal vasculature. $\times 25,000$; bar = $1.0 \mu\text{m}$.

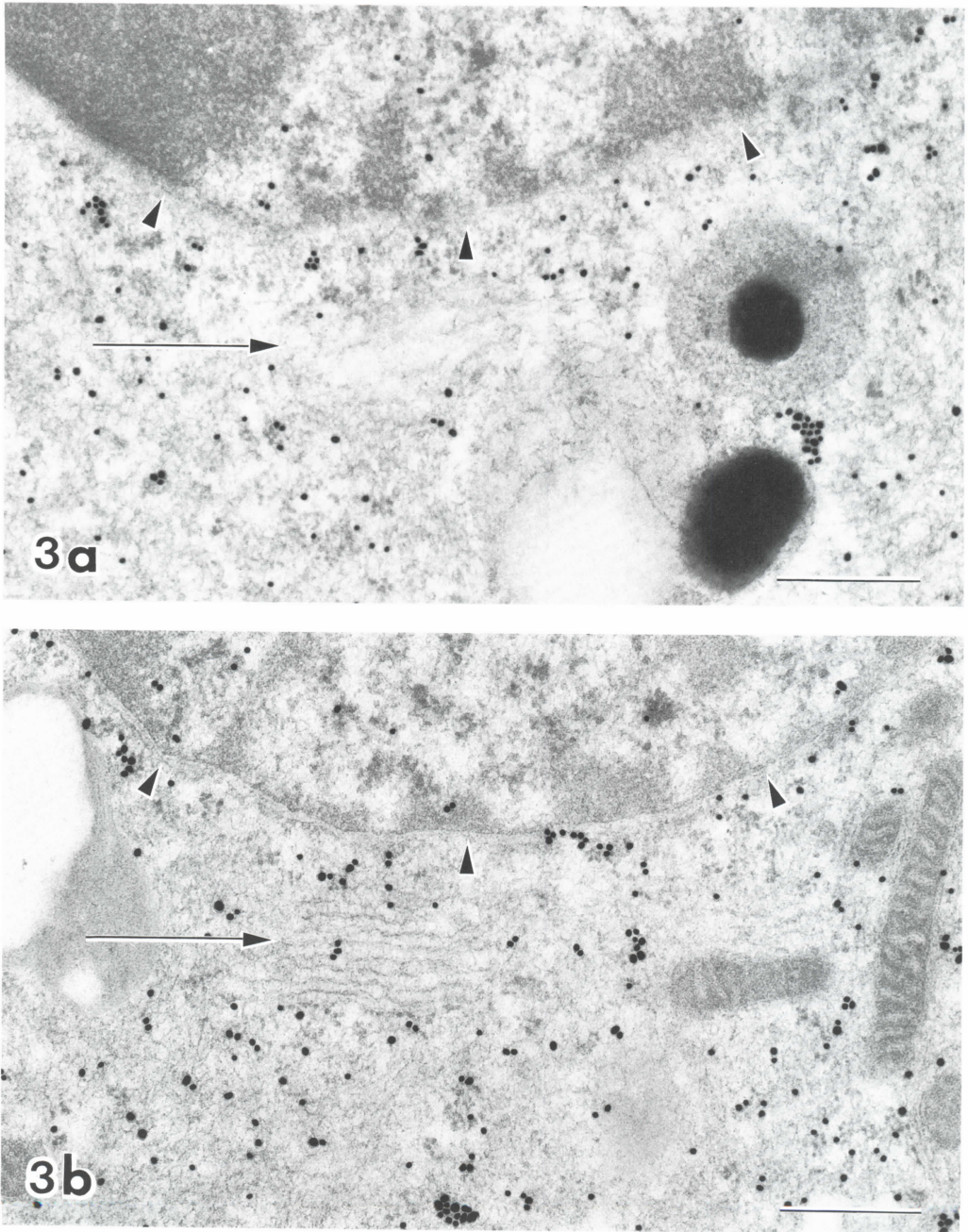


Fig. 3. Immunoelectron micrographs of CRALBP localized in squirrel retinal pigment epithelium (LR White embedment; tissue sections exposed to OsO_4 vapors). a: Tissue fixed only with aldehydes. This micrograph is an enlargement of the region near the arrow in Figure 1. b: Tissue fixed with aldehydes and postfixed with uranyl

acetate; enlargement of the region near the arrow in Figure 2. The nuclear membranes (arrowheads), smooth endoplasmic reticulum (arrows), and cytoplasm are much more distinct in the uranyl acetate treated tissue. Both = $\times 40,000$; bar = $0.5 \mu\text{m}$.

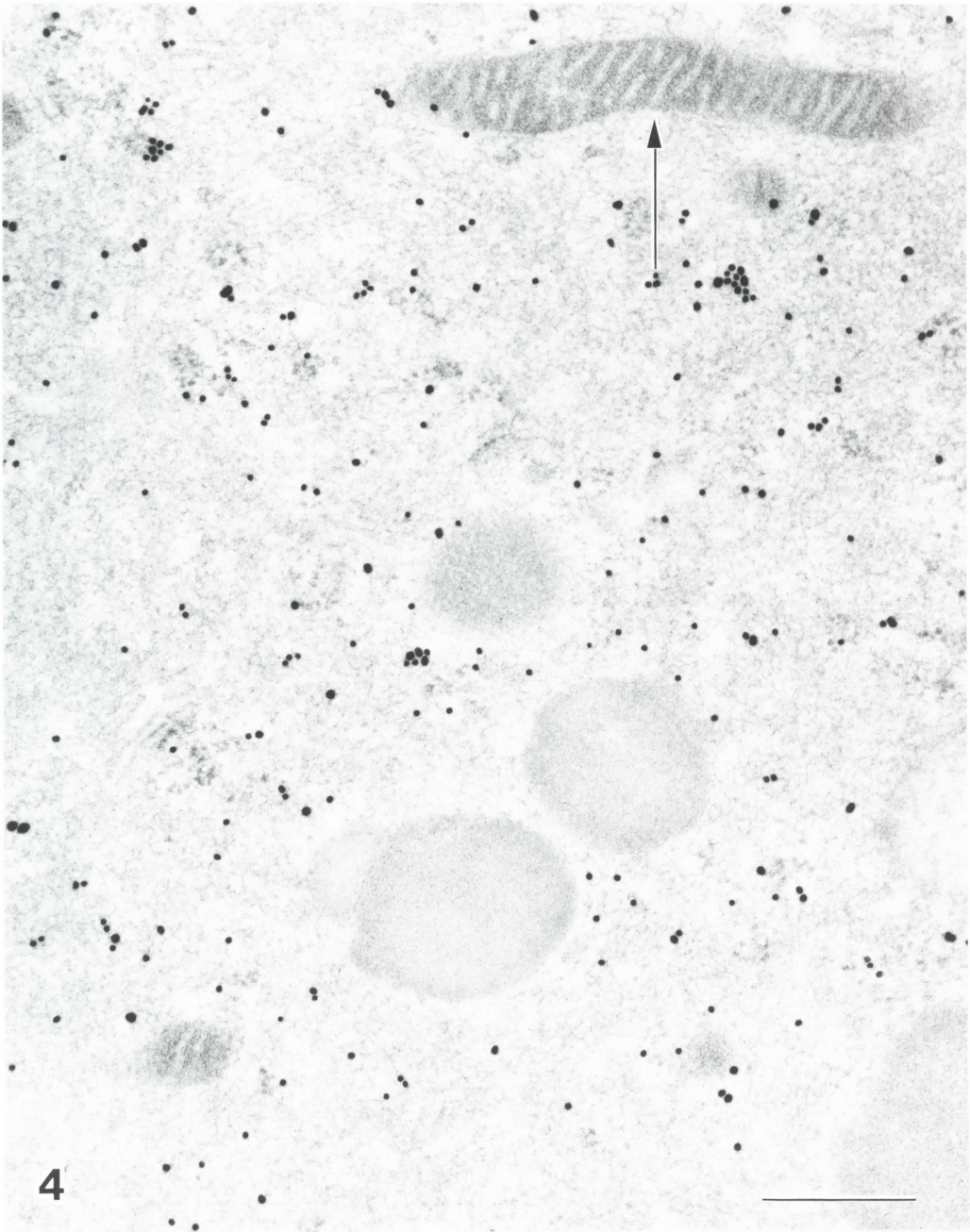


Fig. 4. Immunoelectron micrograph of CRALBP localized to the cytoplasm of a squirrel retinal pigment epithelial cell (tissue fixed only with aldehydes; LR White embedment; tissue section exposed to OsO_4 vapors).

Compare the fixation quality of the mitochondrial membranes (arrow) and the immunolabeling density of the cytoplasm with Figure 5; also see Table 1. $\times 50,000$; bar = $0.5 \mu\text{m}$.

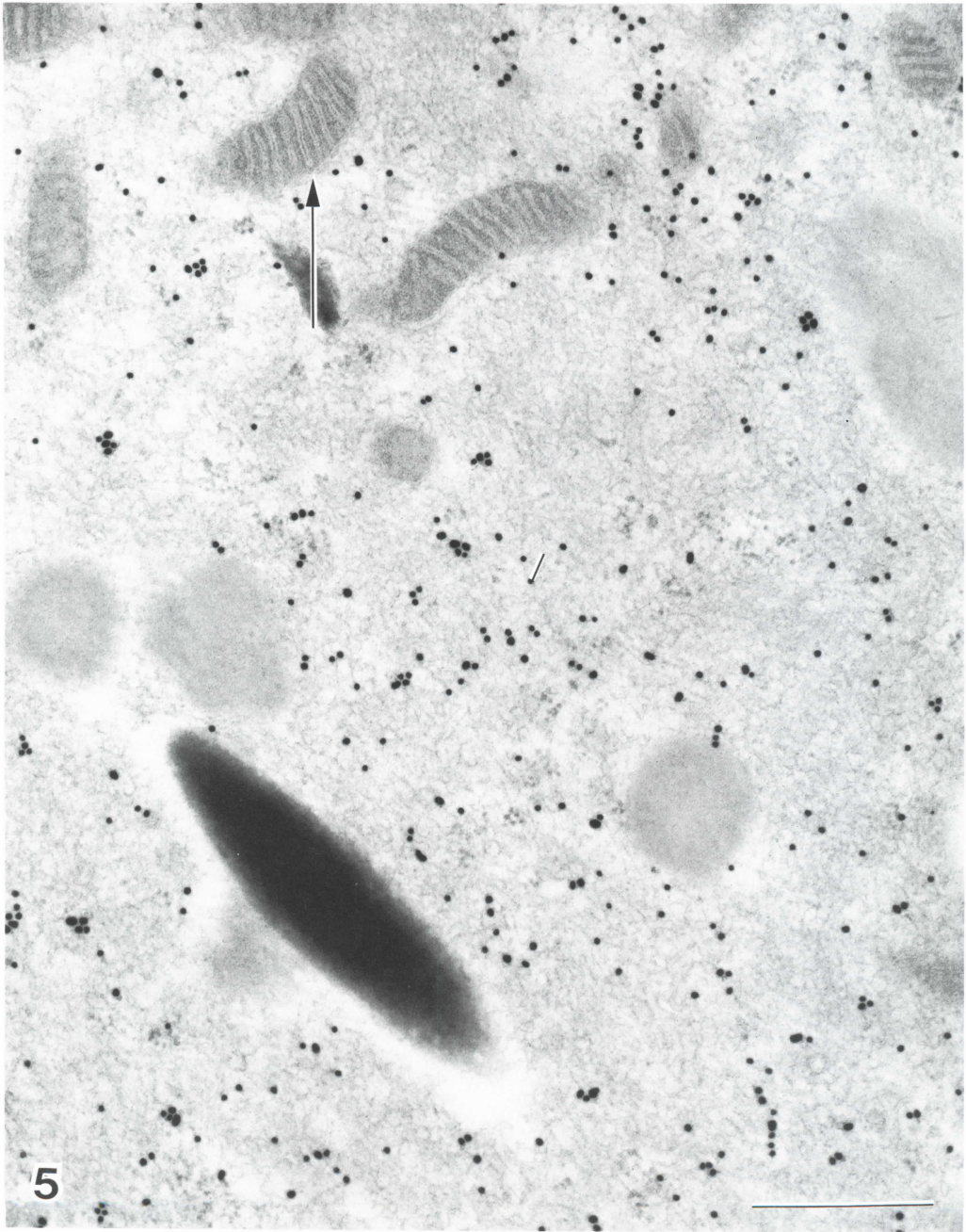


Fig. 5. Immunoelectron micrograph of CRALBP localized to the cytoplasm of a squirrel retinal pigment epithelial cell (tissue fixed with aldehydes and postfixed with uranyl acetate; LR White embedment; tissue sec-

tion exposed to OsO_4 vapors). Compare the fixation quality of the mitochondrial membranes (arrow) and the immunolabeling density of the cytoplasm with Figure 4; also see Table 1. $\times 50,000$; bar = $0.5 \mu\text{m}$.

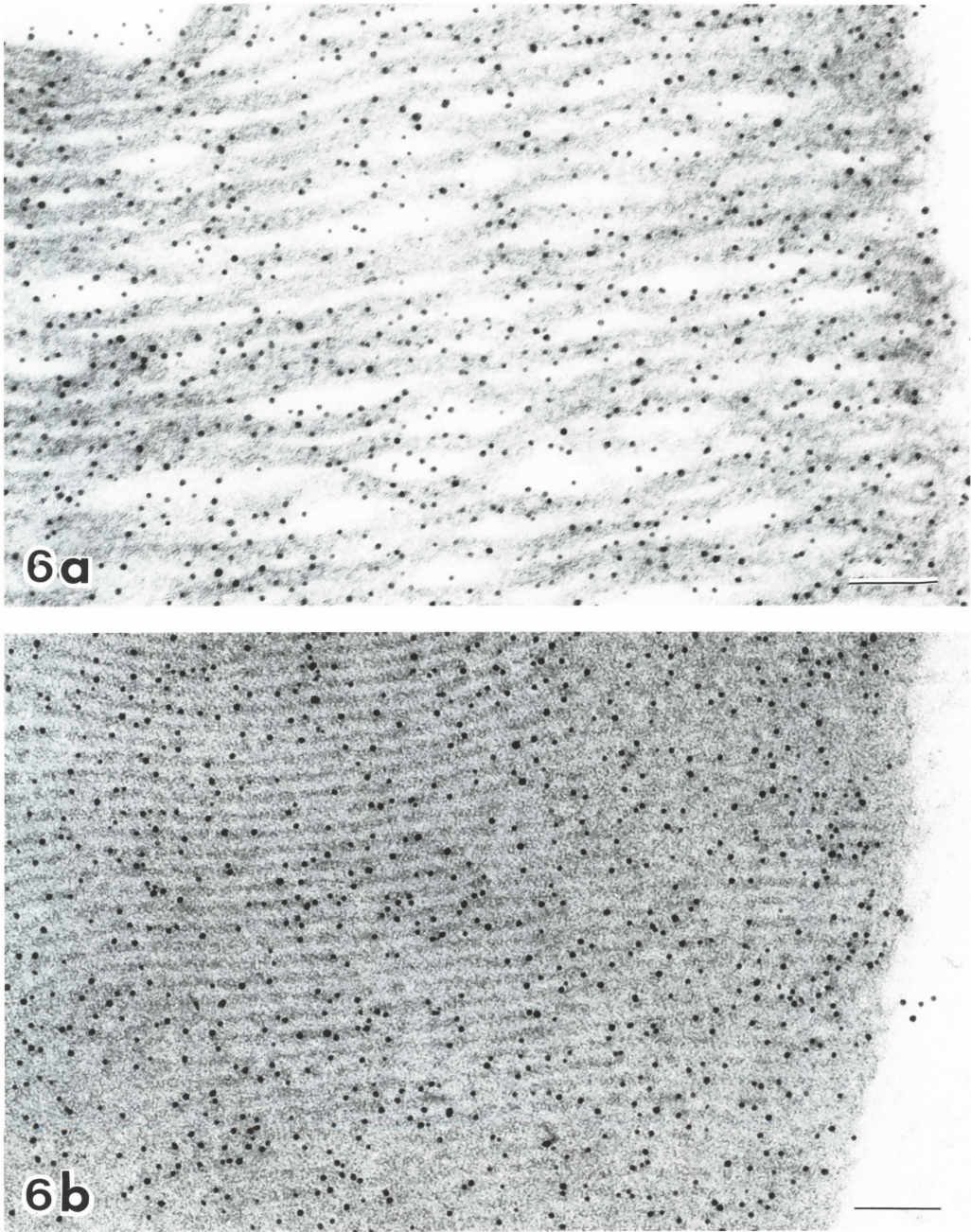


Fig. 6. Immunoelectron micrographs of opsin localized in squirrel rod outer segment membranes (LR White embedment; tissue sections exposed to OsO_4 vapors). a: Tissue fixed only with aldehydes. b: Tissue fixed with aldehydes and postfixed with uranyl acetate. The disc

membranes are better preserved and more closely apposed in the uranyl acetate treated tissue. This enhanced preservation correlates with an increased immunolabeling density; also see Table 1. Both = $\times 120,000$; bar = $0.1 \mu\text{m}$.

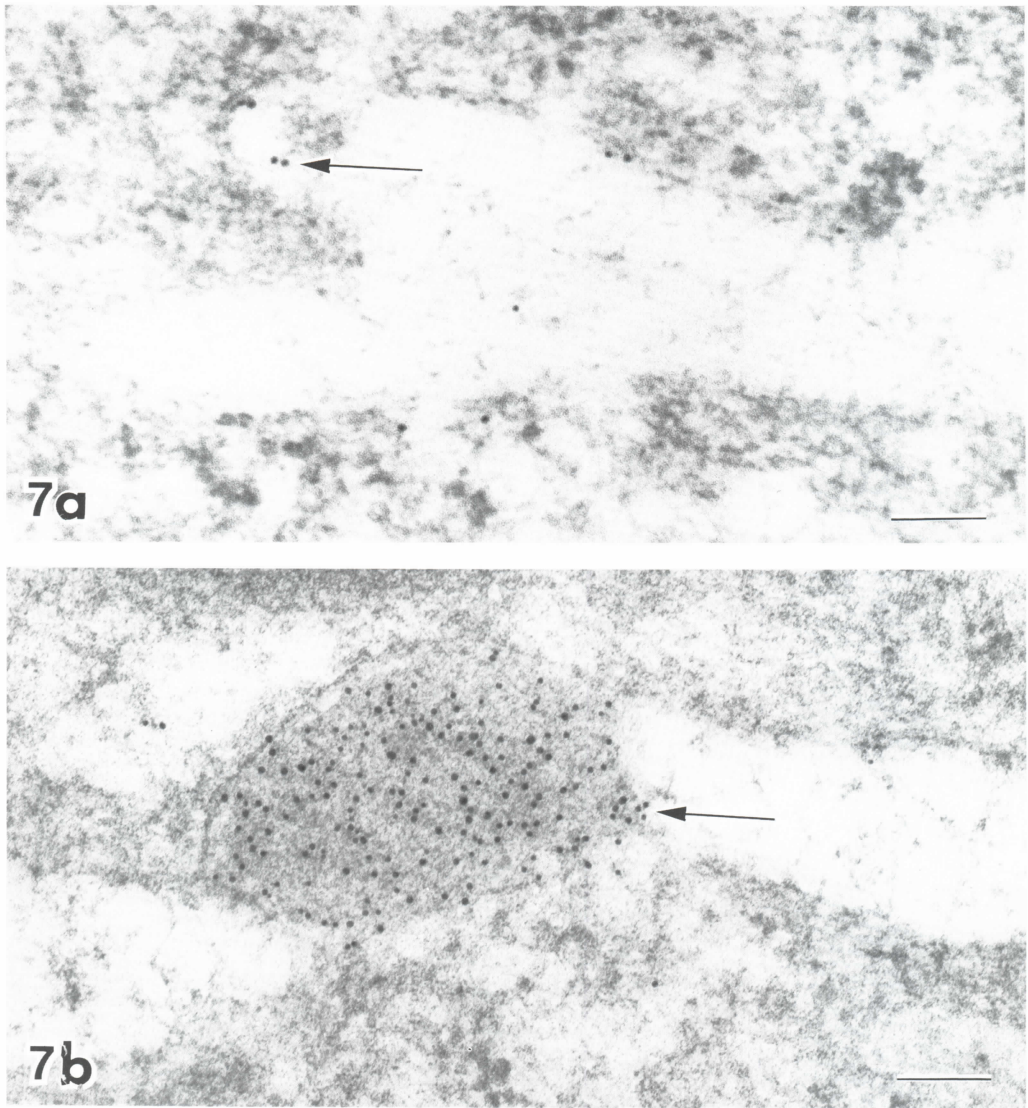


Fig. 7. Immunoelectron micrographs of IRBP localized in the extracellular space between two photoreceptors of squirrel retina (LR White embedding; tissue sections exposed to OsO_4 vapors). **a:** Tissue fixed only with aldehydes; anti-IRBP labeling (arrow) was always

very light in this tissue. **b:** Tissue fixed with aldehydes and postfixed with uranyl acetate. Electron dense areas that label with anti-IRBP (arrow in 7b) were very prominent when the tissue was fixed by this method. Both = $\times 120,000$; bar = $0.1 \mu\text{m}$.

1983; Roth et al., 1981). By comparison to conventional processing methods, however, fixation techniques for post-embedding immunocytochemistry result in inferior tissue preservation and poor contrast in the electron microscope.

Uranyl acetate was first used en bloc to stabilize viral and bacterial DNA (Kellenberger et al., 1958). Later studies showed that it

had a similar effect on the structure of cell junctions in amphibian skin (Farquhar and Palade, 1965), cardiac and skeletal muscle (Karnovsky, 1967) and vertebrate brain (Brightman and Reese, 1969). This stabilizing effect was attributed to decreased protein and phospholipid extraction (Silva et al., 1968, 1971; Terzakis, 1968) and was interpreted as further fixation of the phospho-

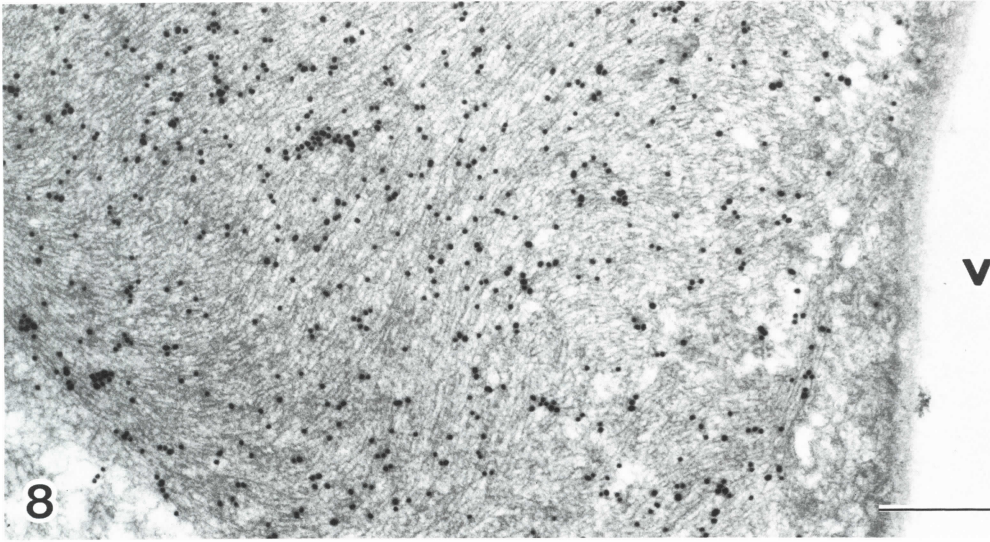


Fig. 8. Immunoelectron micrograph of GFAP localized in cat retina (LR White embedment; tissue section exposed to OsO_4 vapors). The tissue was fixed with alde-

hydes and postfixed with uranyl acetate. These anti-GFAP labeled filaments are in a Muller cell near the vitreous cavity (V). $\times 30,000$; bar = $0.5\mu\text{m}$.

Table 1. Comparison of immunolabeling density:¹

Fixation	Primary Antiserum	ROS ² labeling	RPE ³ labeling	Background labeling
Aldehydes	Anti-opsin	$874 \pm 94^*$		25 ± 20
Aldehydes plus UA	Anti-opsin	$1035 \pm 86^*$		26 ± 16
Aldehydes plus UA	Anti-CRALBP		$28 \pm 5^*$	2 ± 2
Aldehydes plus UA	Anti-CRALBP		$39 \pm 6^*$	3 ± 3

¹Tissues fixed with: 1) aldehydes only, or 2) aldehydes plus secondary fixation with uranyl acetate. Labeling density is expressed as gold spheres/ μm^2 (mean \pm standard deviation; $n=15$).

²ROS = rod outer segment.

³RPE = retinal pigment epithelium.

* = $P < 0.001$ with Student's t-test).

lipids by the uranyl acetate. Thus, en bloc staining (i.e., fixation) in uranyl acetate has become a common procedure in tissue processing for conventional electron microscopy.

In this study, we determined that retinal tissue embedded in either LR White or Lowicryl K4M shows improved preservation and contrast when the tissue is postfixed in uranyl acetate solution (during dehydration) and thin sections are subsequently exposed to osmium tetroxide vapors. In addition, sufficient antigenicity is retained after uranyl acetate fixation to allow antibody labeling of four different classes of proteins (filamentous, cytoplasmic, membrane and extracellular matrix).

In an independent study, Valentino et al. (1985) recently described a similar protocol

that used uranyl acetate in immunoelectron microscopy. Their qualitative data showed that three synaptic vesicle proteins and a post synaptic density protein could be localized in rat brain that was fixed with aldehydes plus aqueous uranyl acetate (before dehydration) and subsequently embedded in Lowicryl K4M. Although our protocol is different from theirs, our results corroborate their conclusions that uranyl acetate fixation improves the preservation of tissue and allows immunolabeling of antigens.

The significantly higher immunolabeling of CRALBP, opsin, and IRBP after uranyl acetate fixation could be attributed to an increased retention of these proteins in a way that does not affect their ability to bind with the antibodies. In addition, the increase la-

beling density of opsin may be due to the better preservation, and thus closer apposition, of the photoreceptor disc membranes. Overall, these results indicate that secondary fixation with uranyl acetate can be a valuable addition to post-embedding immunocytochemistry.

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