

Opsin Distribution and Protein Incorporation in Photoreceptors After Experimental Retinal Detachment

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The distribution of opsin was examined immunocytochemically after experimental retinal detachment in adult cats. Retinal detachments were produced by injecting fluid between the retinal pigment epithelium and neural retina. One to 60 days later the animals were killed. Tissue areas from detached and attached retinal regions from the eye with the detached retina, as well as normal (control) retinas, were processed for post-embedding light and electron microscopic immunocytochemistry. In normal and attached retinal regions, anti-opsin labeled the outer segments and Golgi apparatus most heavily, although the entire photoreceptor plasma membrane was labeled at a low level. Beginning at 2 days after retinal detachment, immunolabeling increased in the photoreceptor inner segment, cell body and synaptic terminal plasma membranes. This pattern of anti-opsin labeling continued at all intervals up through the 60-day detachment time-point. Injection of radiolabeled amino acid in detachments from 1 to 30 days show that radiolabeled protein is still transported to the truncated outer segments of the photoreceptor cells. In addition, these outer segment disks label with anti-opsin. These data imply that opsin continues to be transported and incorporated into the outer segments of photoreceptors showing severe degeneration as a result of long-term detachment from the RPE.

Key words: retinal detachment; opsin; photoreceptors; degeneration.

1. Introduction

Autoradiographic and biochemical studies have shown that opsin, the principal membrane protein of the photoreceptor outer segment, is synthesized in the inner segment and transported to the base of the outer segment where it is incorporated into newly assembled disks (Young, 1967; Young and Droz, 1968; Young and Bok, 1969; Hall, Bok and Bacharach, 1969; Heitzmann, 1972). Immunocytochemical studies have shown that most opsin is restricted to the outer segment (Dewey et al., 1969; Papermaster et al., 1978; Papermaster, Schneider and Besharse, 1985; Defoe and Besharse, 1985). Other studies, however, have also identified low levels of opsin immunoreactivity in the inner segment and cell body plasma membranes in normal adult photoreceptors (Jan and Revel, 1974; Fekete and Barnstable, 1983; Hicks and Barnstable, 1987; Hicks, Sparrow and Barnstable, 1989; Rohlich et al., 1989; Nir et al., 1989). The mechanism(s) by which such strict structural and biochemical polarity is established and maintained by photoreceptor cells remains unknown.

When the neural retina is separated from the retinal pigment epithelium (RPE) a number of morphological (Kroll and Machemer, 1968; Anderson et al., 1981, 1983; Erickson et al., 1983) and biochemical (Erickson et al., 1987; Lewis et al., 1989) changes occur in the retina. Significant outer segment de-

generation is accompanied by vacuolization within photoreceptor cell bodies and synaptic terminals after detachments of only a few days duration. Eventually this degeneration leads to cell death and thinning of the outer nuclear layer (Erickson et al., 1983). Despite this degeneration, there is evidence for continued outer segment protein biosynthesis and membrane assembly after the retina has been separated from the RPE. This has been shown both in vitro, with isolated retinas in short-term cultures, (O'Brien, Mullenberg and de Jong, 1972; Basinger and Hall, 1973; Bok, Basinger and Hall, 1974; Hale, Matsumoto and Fisher, 1990) and in vivo after experimental detachment in the owl monkey (Machemer and Kroll, 1971) and in the cave salamander where retinal detachment is common as a result of outer segment degeneration (Besharse and Hollyfield, 1976).

In this study, we examined the effect of retinal detachment on opsin distribution in the photoreceptors. The results show that newly synthesized protein continues to be delivered to the opsin-immunoreactive outer segments after long-term detachment. However, as outer segment degeneration proceeds, opsin becomes increasingly evident in the plasma membrane associated with the inner segments, cell bodies and synaptic terminals.

2. Materials and Methods

Retinal Detachment

Cat retinas were experimentally detached from the

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retinal pigment epithelium for 1–7 days, 14, 30, or 60 days. The retinal detachment procedure has been described previously (Anderson et al., 1986). Briefly, the lens was removed and the eye allowed to heal for at least 2 weeks at which time the vitreous was removed and a solution of 0.25% Healon (sodium hyaluronate; Pharmacia, Piscataway, NJ) was infused between the retina and RPE using a micropipette. Cats were entrained to a 12 hr/12 hr light/dark schedule and killed 4 hr after light onset. Several different areas from the normal and experimental eyes were processed for immunocytochemical and autoradiographic analyses.

Tissue Preparation for Immunocytochemistry

After removal of the anterior segment, the eyes were cut into quadrants. For LR White embedment, quadrants were fixed for 1 hr and 1% paraformaldehyde and 1% glutaraldehyde in 0.086 M sodium phosphate buffer, pH 7.2. The tissue was dehydrated in increasing concentrations of methanol, with uranyl acetate (2%) added during the 70% step, and embedded in LR White resin (Erickson et al., 1987; Polysciences, Warrington, PA). Alternatively, quadrants were fixed in 4% paraformaldehyde and 2% picric acid for 1 hr in sodium phosphate buffer, pH 7.2, and the tissue dehydrated in increasing concentrations of ethanol and embedded in Paraplast X-TRA (Monoject Scientific, St Louis, MO).

Antisera

Rabbit anti-bovine-opsin (from Drs D. Bok and B. Matsumoto) was used at a dilution of 1:500. Mouse anti-bovine-opsin (monoclonal rho 4D2 from Dr R. Molday) was used at a dilution of 1:100. These antibody concentrations were chosen from an initial study in which a range of concentrations were applied to the tissue sections. The monoclonal anti-opsin binds along the N-terminal two-thirds of rhodopsin (Hicks and Molday, 1986). The rabbit-anti-bovine opsin was characterized previously by Matsumoto and Bok (1984). Goat-anti-rabbit IgG or goat-anti-mouse IgG conjugated to 15-nm gold spheres was used as the secondary label for electron microscopic immunocytochemistry (both antibodies purchased from Janssen Life Sciences, Olen, Belgium). IgG conjugated to 5-nm gold (Janssen) was amplified with a silver-enhancing solution (Janssen) for the light microscopic immunocytochemistry on LR White sections. Affinity purified goat-anti-mouse IgG-fluorescein (Cappel, Durham, NC) was used for the paraplast sections. All antibodies were diluted with phosphate-buffered saline (PBS) containing 0.5% bovine serum albumin (BSA; Sigma, St Louis, MO). All secondary antibodies were used at a dilution of 1:40. Normal goat serum (NGS; Vector Labs, Burlingame, CA; diluted 1:40) was used as a blocking agent on each section.

Immunocytochemical Procedures

Light microscopic immunocytochemistry with LR White embedded tissue was performed at room temperature using 1- μ m thick sections taken from normal, detached and attached retinas from experimental eyes. The sections were placed on formvar-coated glass slides, covered with NGS for 30 min, blotted, then incubated overnight with the primary antibody. The following day, the sections were rinsed with PBS-BSA and the secondary antibody added for 1 hr. Sections were rinsed with PBS and distilled water, and then the gold spheres were enlarged using the silver-enhancing procedure. When the enhancement was complete (determined by monitoring with light microscopy), the sections were rinsed in distilled water and air-dried. Sections of attached and detached retinas from each time-point were always processed together and enhanced for the same time period to ensure equal treatment. Light microscopic immunocytochemistry with Paraplast was performed at room temperature using 4- μ m thick sections. The Paraplast was first removed from the sections by dipping in xylene, three times for 1 min each, at which time the sections were rehydrated in increasing concentrations of water in ethanol. The immunocytochemistry was then performed identically to the LR White immunocytochemistry except that here the secondary antibody was conjugated to FITC.

Thin sections (70–80 nm) for electron microscopic immunocytochemistry were cut from the same blocks of tissue that were used for light microscopy. Sections were placed on nickel grids, incubated on drops of NGS for 30 min, blotted and incubated overnight on drops of the primary antibody. The next day the grids were rinsed with PBS-BSA and transferred to drops of secondary antibody for 1 hr. After rinsing with PBS-BSA and distilled water, the grids were stained with uranyl acetate for 10 min and lead citrate for 5 min, exposed to osmium tetroxide vapors (2% osmium solution) for 1 hr and carbon-coated in a vacuum evaporator.

Light Microscopic Autoradiography

Cats were labeled intravitreally with 1 mCi of [³H]leucine 4 hr after light onset and killed 24 hr later. One-micrometer thick sections were placed on glass slides, dipped in NTB-2 emulsion (Kodak) and exposed for 10 days. Autoradiograms were developed in D-19 at 20°C for 1 min, fixed in Kodak rapid fix for 3 min and counterstained with 1% Toluidine blue.

Electron Microscopic Autoradiography—Immunocytochemistry

Thin sections (70–80 nm) from the 30-day time-point were first processed for post-embedding electron microscopic immunocytochemistry and subsequently

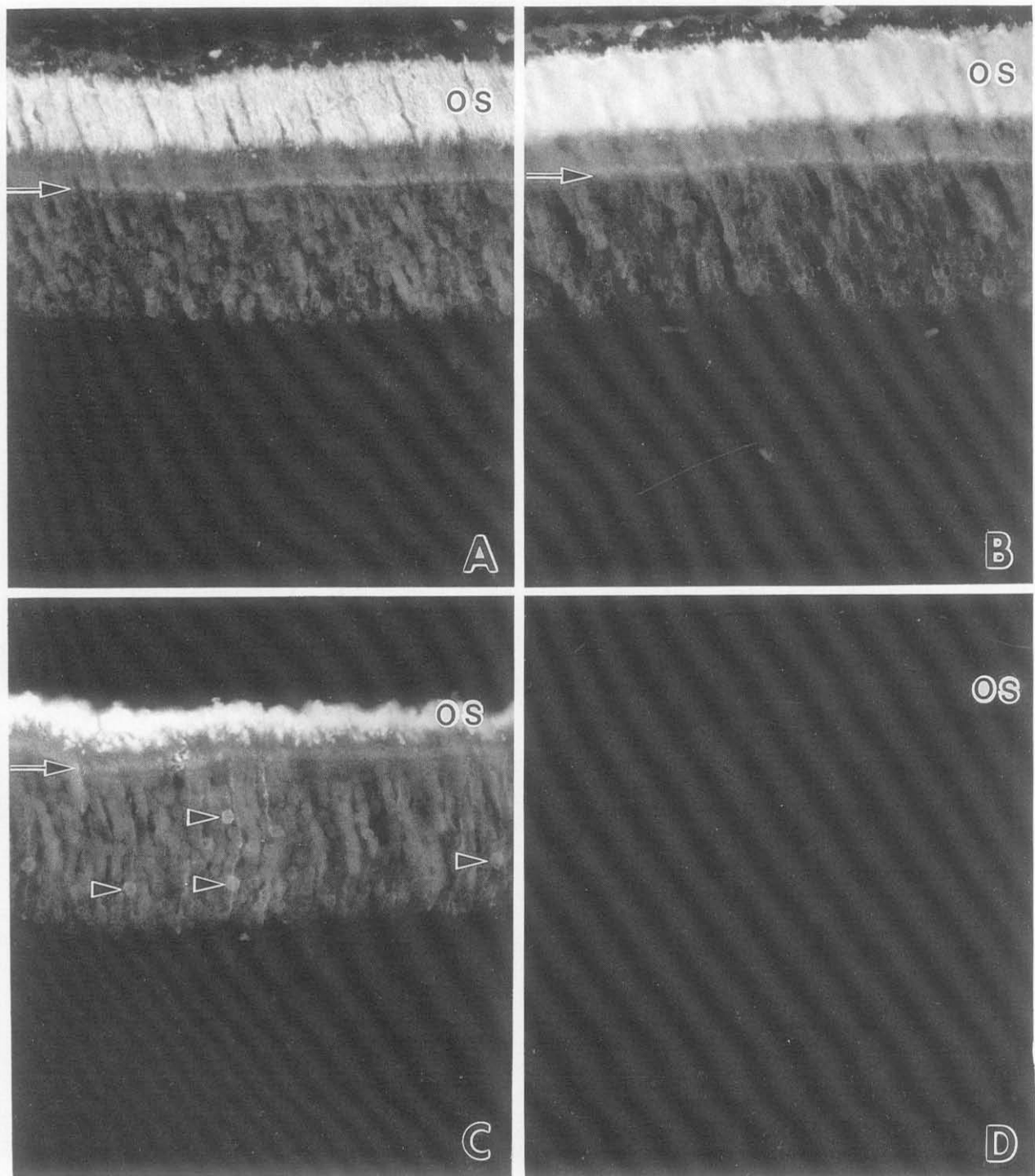


FIG. 1. Light microscopic immunocytochemistry using anti-opsin on 4- μ m thick Paraplast embedded sections. In normal (A) and 7-day attached (B) retinas, labeling is heaviest over the outer segments and Golgi apparatus (arrows) in the inner segments. Diffuse fluorescence is also present over the inner segments and ONL. In 7-day detached retinas (C), there is an increased fluorescence in the ONL where it is often possible to follow the labeling of an entire photoreceptor cell across this layer (arrowheads). No labeling was observed in control sections (D). OS, Outer segments. $\times 463$.

for electron microscopic autoradiography using a modification of the flat substrate method of Young and Droz (1968). Briefly, sections were placed on parlodian-coated glass slides, blocked in NGS, blotted and incubated overnight in the primary antibody. The following day, they were rinsed with PBS/BSA and the secondary antibody conjugated to 15-nm gold spheres added for 1 hr. They were then rinsed with PBS and distilled water, air-dried, stained with uranyl acetate

and lead citrate and carbon coated. For autoradiography, the slides were dipped in Ilford L4 emulsion, exposed for 10 weeks at 4°C, developed in Phenidon (Lauder Chemicals, San Mateo, CA) for 1 min at 15°C, and fixed in Kodak rapid fix for 5 min. Dipping and development of the autoradiograms was done under sodium vapor illumination. Finally, the parlodian film was floated off the glass slides and copper electron microscope grids were placed over the sections and

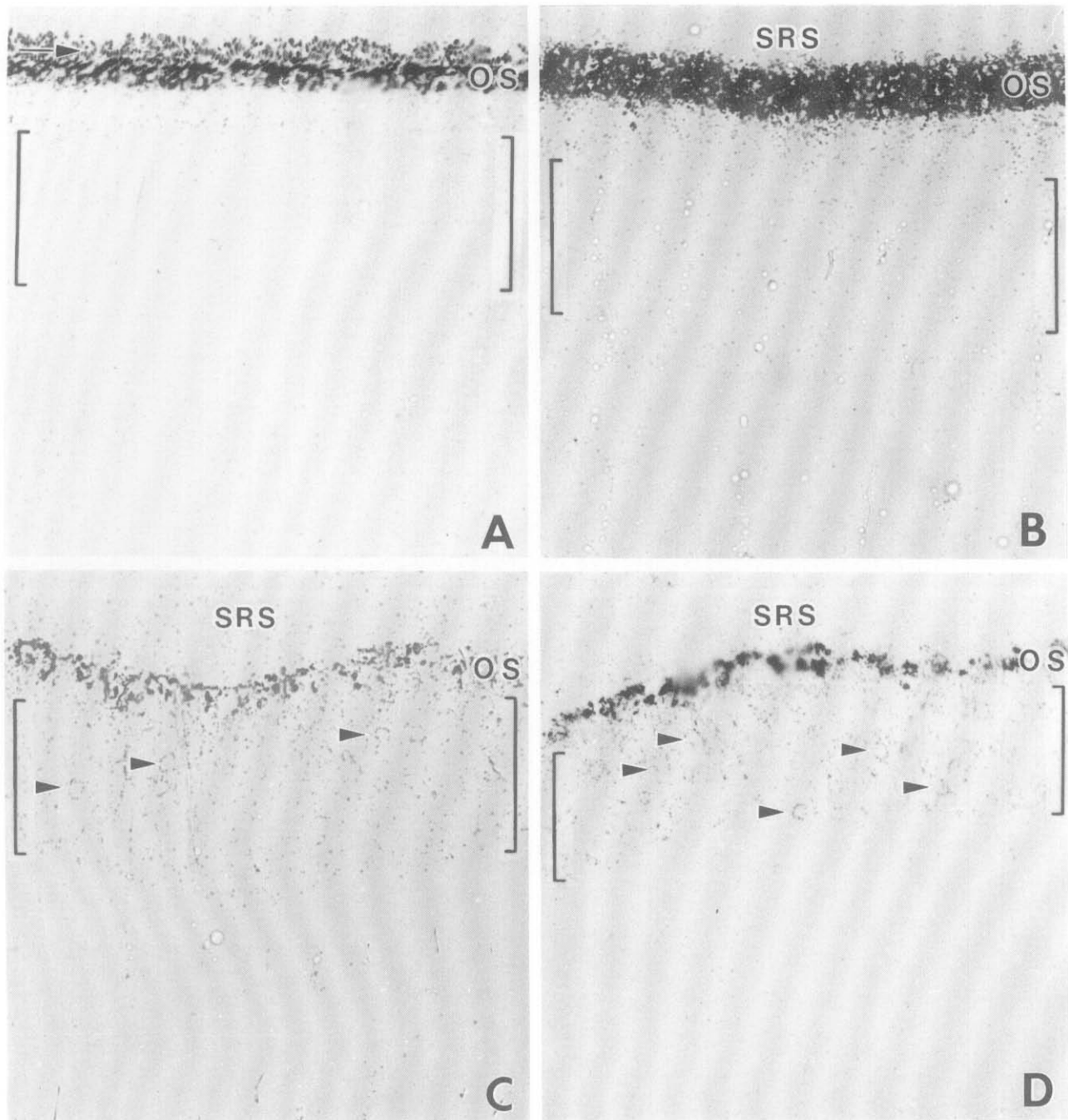


FIG. 2. Light microscopic silver enhanced immunocytochemistry using anti-opsin on LR White embedded sections of normal retina and, 2, 5, and 7-day detached retinas. In the normal retina (A), labeling can be detected primarily over the outer segments. By day 2 (B), labeling increases over the inner segments. At days 5 (C) and 7 (D), the labeling of the ONL and photoreceptor cell bodies (arrowheads) becomes visible. The section in (A) was taken from an inferior retinal region, where the outer segments are known to be shorter, as compared to those in Figs 1(A) or 2(B) which are from superior regions. The sections are unstained to allow the fine detail of the labeling pattern to be revealed. Arrow, RPE; brackets, ONL; SRS, sub-retinal space. $\times 463$.

picked up on parafilm strips. Sections were viewed in a Philips CM-10.

3. Results

Both opsin antibodies gave identical immunocytochemical results and these results, therefore, will be referred to collectively. In the 4- μm thick Paraplast sections of normal retina and attached regions from

the detached eye, the heaviest anti-opsin labeling was observed over the outer segments and the Golgi apparatus of the inner segment. In addition, there was diffuse, faint fluorescence associated with the plasma membrane of the inner segment and outer nuclear layer regions [Figs 1(A) and (B)]. The results from a region detached for 7 days are shown in Fig. 1(C). Outer segment fluorescence is still apparent although the outer segments show significant shortening by

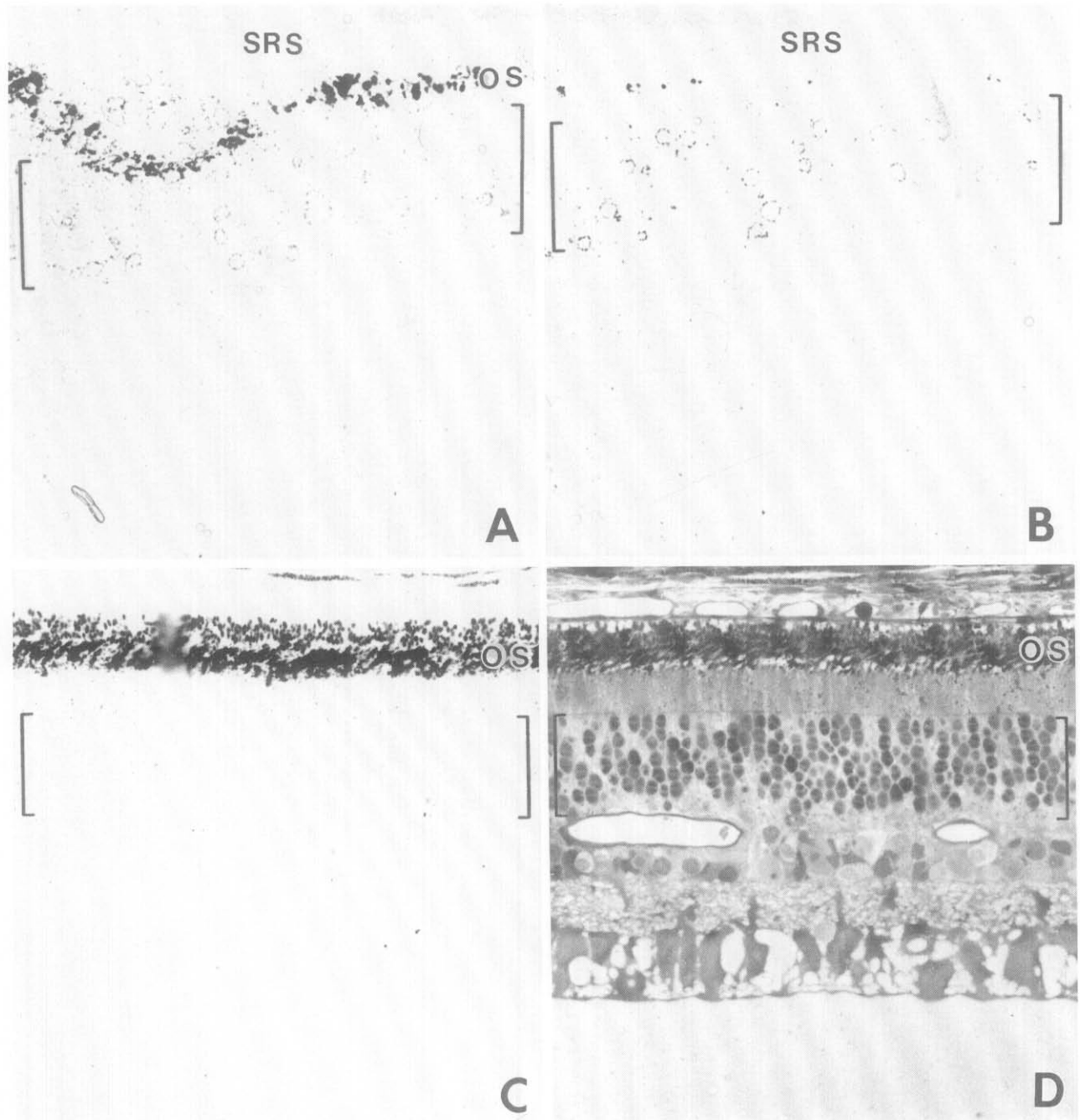


FIG. 3. Light microscopic silver enhanced immunocytochemistry using anti-opsin on LR White embedded sections of 60-day detached (A and B) and adjacent attached retinas (C and D). Labeling is present in the photoreceptor outer segments and the plasma membrane of cell bodies both in the presence (A) and absence (B) of outer segments. Attached retinas from the detached eye show normal labeling patterns at this time-point (C and D). The section in (D) was counterstained to provide orientation for the previous retinal sections. Brackets, ONL; SRS, sub-retinal space. $\times 463$.

comparison to those in Figs 1(A) and (B). The most prominent change is seen as increased fluorescence associated with some cells in the outer nuclear layer (ONL). Indeed, it is sometimes possible to follow the staining of the plasma membrane of an entire photoreceptor cell across this layer. No labeling was present in control sections where the primary antibody was omitted [Fig. 1(D)].

Using LR White embedded tissue, where only the surface of the tissue sections is labeled by the antibodies, the increase in anti-opsin labeling in the

ONL is also detected and can be localized to the plasma membranes of the photoreceptor cells. Using this surface-labeling technique, there is very little labeling over the ONL of normal or attached retina, probably reflecting the fact that only the surface of the section is labeled [Fig. 2(A)].

By 2 days post-detachment, plasma membrane labeling in the inner segment became evident [Fig. 2(b)]. As the detachment interval progressed from days 2 to 7, plasma membrane labeling in the inner segment and ONL increased [Figs 2(C) and (D)]. This

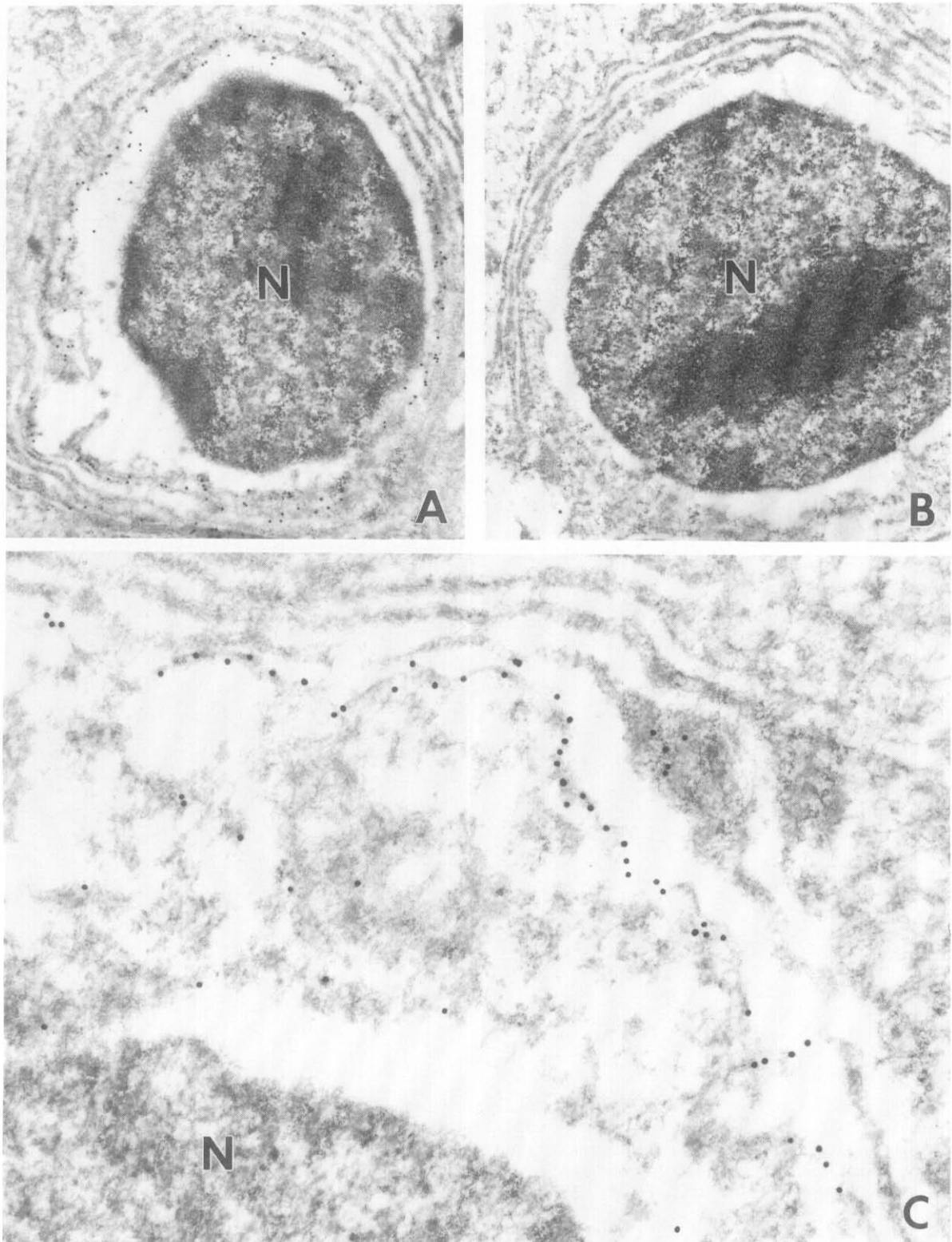


FIG. 4. Electron microscopic immunocytochemistry using anti-opsin on sections of 60-day detached (A and C) and adjacent attached retinas (B). The labeling (15 nm gold particles) occurs on the plasma membrane of the photoreceptor cell bodies in the detached but not the attached retinal regions, N, Photoreceptor nucleus. (A) $\times 17250$; (B) $\times 18445$; (C) $\times 57000$.

pattern persisted in detachments up to 60 days duration. Labeling was present around photoreceptor cell bodies both in the presence and absence of photoreceptor outer segments, and around photoreceptor cell bodies that had been displaced into the subretinal space [Figs 3(A) and (B)]. Adjacent attached

regions from eyes detached for 60 days continued to show normal anti-opsin labeling patterns [Figs 3(C) and (D)]. Figure 3(D) was counterstained with Toluidine blue to provide orientation in the previous figures.

Electron microscopic immunocytochemistry of de-

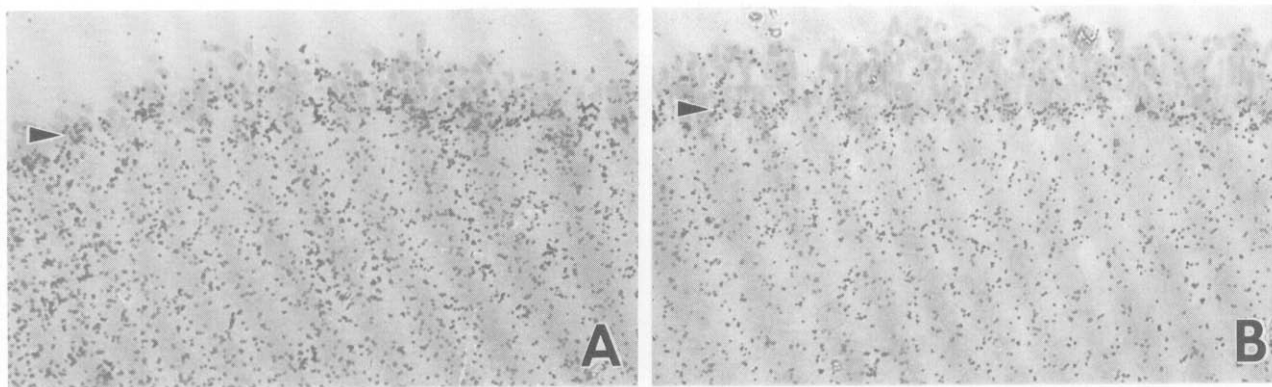


FIG. 5. Light microscopic autoradiographs of 30-day detached (A) and attached (B) retinas. A band of [^3H]leucine incorporation (arrowheads) is present near the base of the outer segments in both the detached and attached retinas. The attached region in (B) was peeled from the retinal pigment epithelium just prior to fixation and therefore appears detached. $\times 563$.

tached retinas revealed that the inner segment and ONL labeling was indeed attributable to plasma membrane labeling of the photoreceptor cells in these areas [Figs 4(A) and (C)]. In adjacent attached retina, no labeling was observed in the plasma membrane proximal to the outer segment [Fig. 4(B)].

After intravitreal injection of [^3H]leucine, light microscopic autoradiographs of detached and attached retinal regions from 1 through 7, 14, and 30 days invariably showed evidence of radiolabeled protein incorporation near the base of the outer segment [Figs 5(A) and (B); 30 days]. The attached region in Fig. 5(B) was peeled from the retinal pigment epithelium just prior to fixation and therefore appears detached. This allowed biochemical analysis to be performed on adjacent retinal tissue. Figures 6 and 7 show both radiolabeling and anti-opsin immunolabeling at the electron-microscopic level in the outer segments of attached retina (Fig. 6) and the adjacent retina that had been detached for 30 days (Fig. 7). In some cases, these [^3H]leucine and opsin positive outer segments can be observed being phagocytosed by macrophages present in the sub-retinal space (Fig. 8).

4. Discussion

Retinal detachment induces a number of morphological changes in the retina (Kroll and Machemer, 1968; Anderson et al., 1981, 1983; Erickson et al., 1983). One of the most prominent of these is progressive degeneration of the photoreceptor outer segments (Machemer, 1968; Kroll and Machemer, 1968; Anderson et al., 1983). The autoradiographic results in this study strongly suggest that the degenerating photoreceptors continue to transport and incorporate newly-synthesized protein into their respective outer segments, even after detachments of relatively long duration, i.e. 30 days. The immunocytochemical data indicate that degenerating outer segments, while highly disorganized, still retain opsin immunoreactivity. Taken together, these data imply that, even in the detached state, opsin continues to be

synthesized, transported, and inserted into newly-assembled disc membranes. Furthermore, the identification of phagosomes that are both radiolabeled and opsin-immunoreactive (see Fig. 8) in the cytoplasm of macrophages within the sub-retinal space suggests that the newly-synthesized disc membrane material is phagocytosed by these cells. Thus, while the photoreceptors are incapable of assembling normally configured outer segments and often show concurrent signs of inner segment degeneration after retinal detachment, they apparently retain at least some capacity to carry on the processes of disc morphogenesis and disposal.

There is, however, a change in the distribution of the major outer segment membrane protein opsin. After detachment, an increase in opsin labeling can be detected on the plasma membrane of the photoreceptor cell inner segment, cell body, axon, and synaptic terminal. This pattern became well-established by 1 week after detachment and continued for at least 60 days, in parallel with the progressive degeneration of the outer segments.

These results also suggest that opsin is not absolutely restricted to the outer segment even in normal adult photoreceptors. This finding is in agreement with several previous studies (see Introduction). Our data indicate that the opsin in other plasma membrane compartments is demonstrable using volume labeling (Paraplast) but not surface labeling (LR White) techniques. These data, therefore, could explain inconsistencies reported in the literature where plasma membrane labeling at the level of the inner segment or cell body may or may not be present depending upon the antibodies used and/or the immunochemical procedures employed. Hicks et al. (1989) suggested that opsin found in the inner parts of the cell may differ in some way from that in the outer segment, which would explain why it has been detected with some antibodies but not with others. In our case, detection was not antibody-dependent, but instead depended upon the tissue embedding medium. The presence of opsin at other proximal plasma membrane

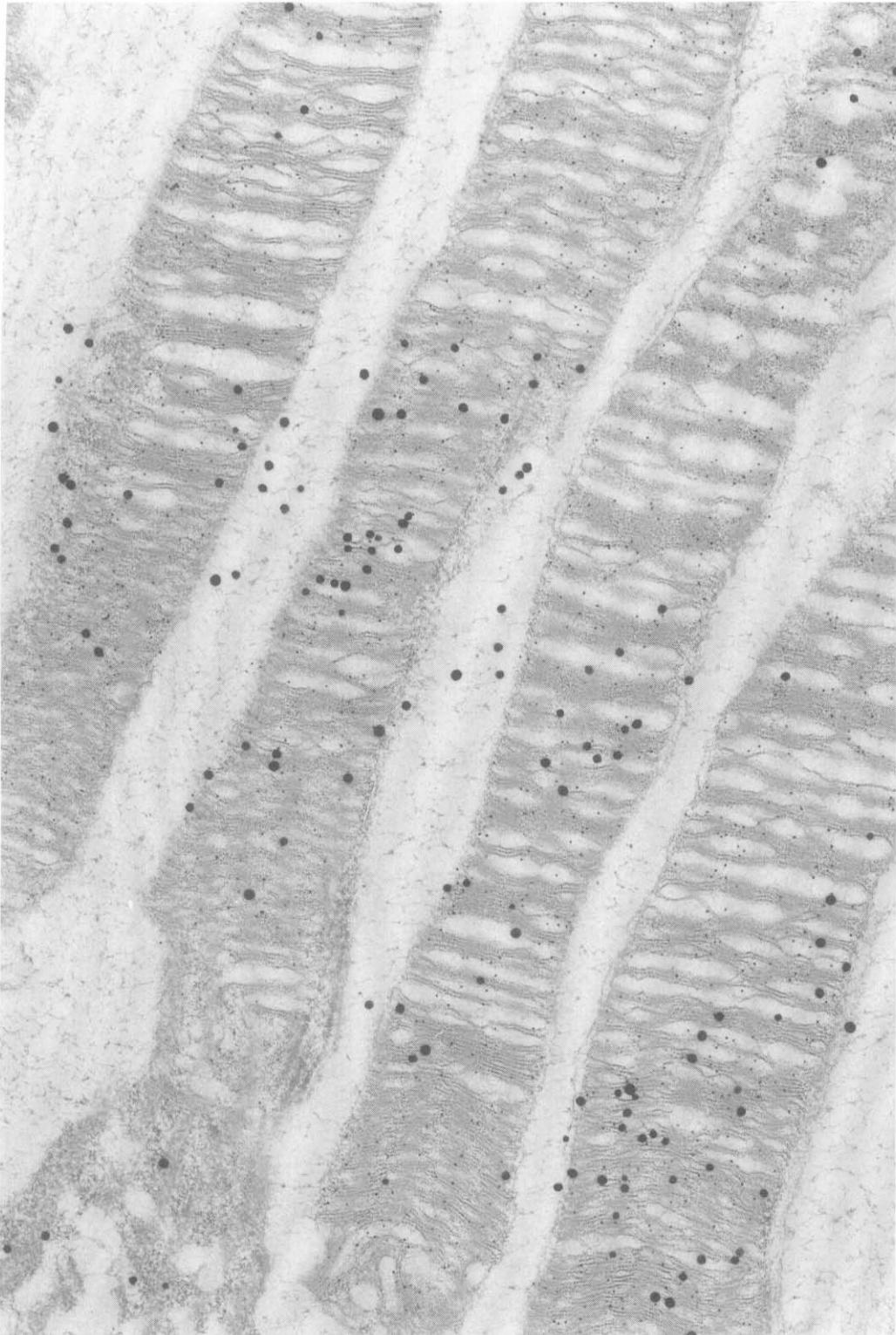


FIG. 6. Combined electron microscopic autoradiography and anti-opsin cytochemistry of an attached retinal region from an eye that had been detached for 30 days. [³H]leucine (large spheres) and opsin (small spheres) are both present in the attached retinas. $\times 25\,588$.

locations may not be surprising since the mechanism for opsin delivery to the outer segments may involve fusion of inner segment opsin-bearing vesicles to the inner segment plasma membrane (Papermaster et al., 1986). Any imperfection in the partitioning of opsin into newly forming disks could lead to 'leakage' of opsin into proximal regions of the cell.

Although there is some disagreement in the literature about plasma membrane labeling in proximal regions of the photoreceptor cell, the consensus is that anti-opsin labeling is prevalent in these regions during photoreceptor development (Nir, Cohen and Papermaster, 1984; Besharse, Forestner and Defoe, 1985; Usukura and Bok, 1987; Hicks and Barnstable, 1987;

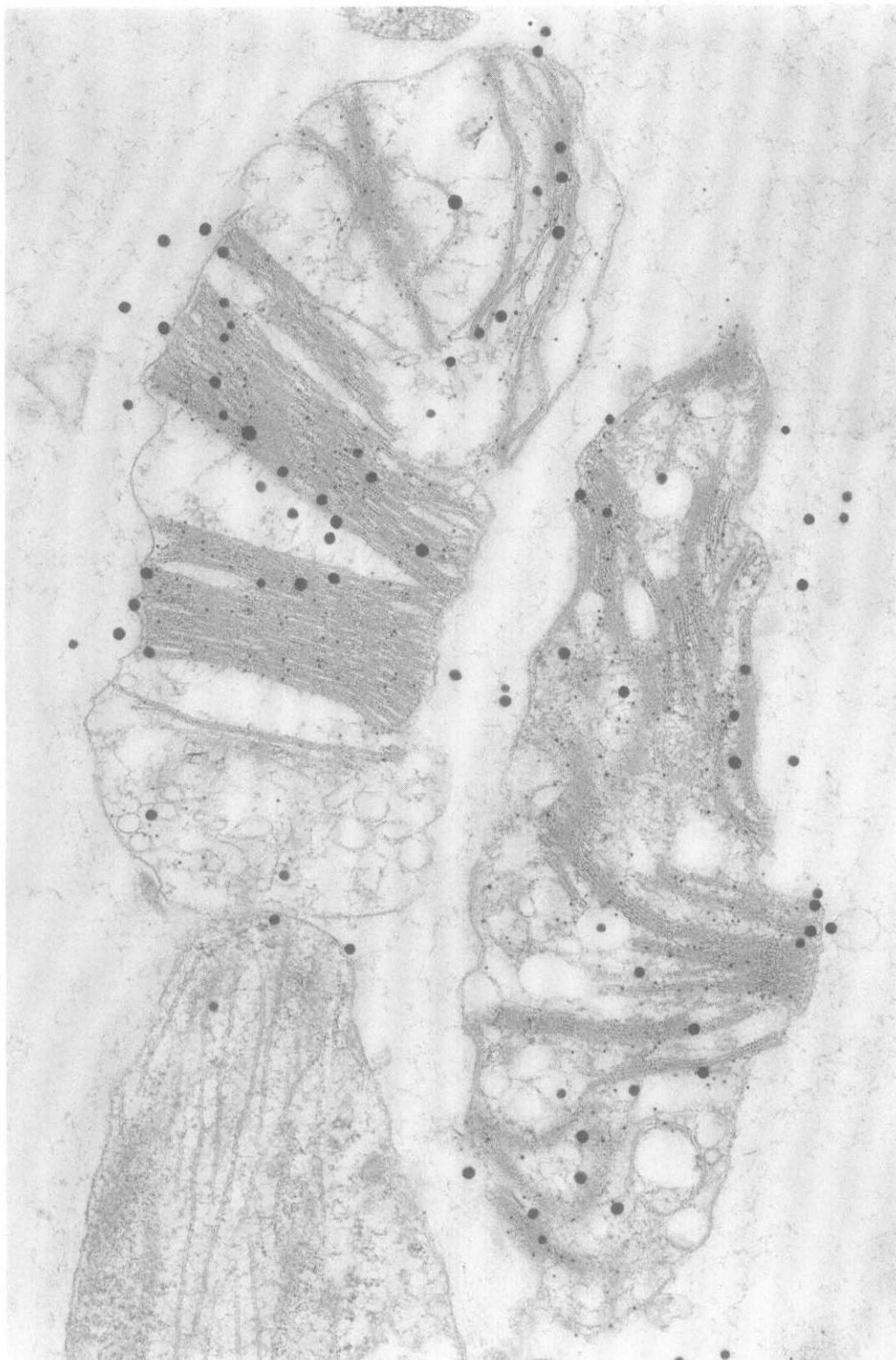


FIG. 7. Combined electron microscopic autoradiography and anti-opsin cytochemistry of a 30-day detached retina. [^3H]leucine (large spheres) and opsin (small spheres) are both present in the truncated outer segments. $\times 33063$.

Hicks et al., 1989), during the time of outer segment degeneration in a variety of hereditary retinal degenerations (Nir and Papermaster, 1986; Usukura and Bok, 1987; Jansen et al., 1987; Nir, Sagie and Papermaster, 1987; Nir et al., 1989) and in mechanically-dissociated photoreceptors (Spencer, Detwiler and Bunt-Milam, 1986; Hicks et al., 1989). Our data are

consistent with these studies in showing that induced, as well as hereditary outer segment degeneration leads to a similar situation. Nir et al. (1984) hypothesized that several possible mechanisms could produce the shift in opsin localization during development: (1) sites of opsin insertion into the plasma membrane may change; (2) the adult outer segment may act as a

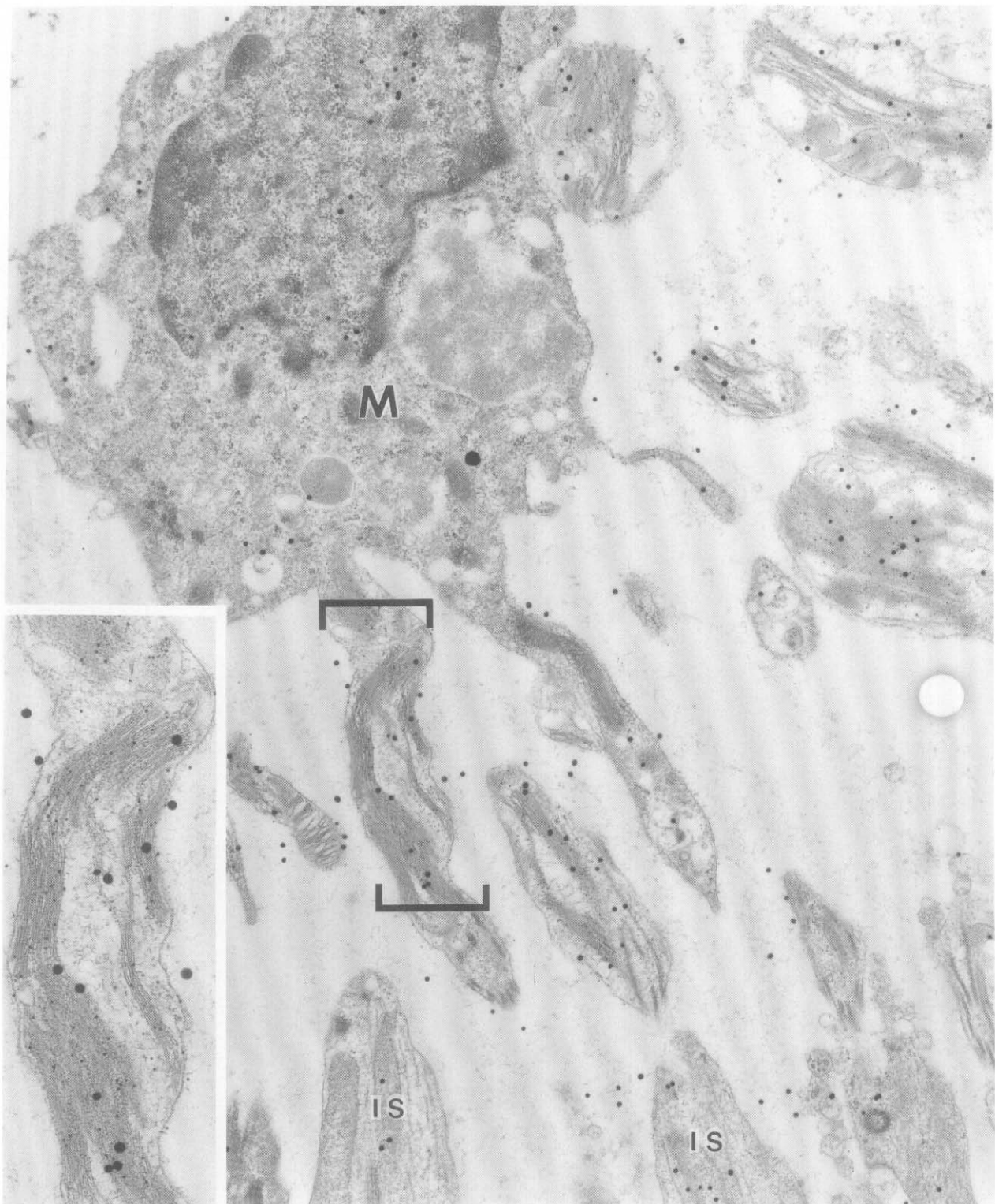


FIG. 8. Combined electron microscopic autoradiography and anti-opsin cytochemistry of [^3H]leucine (large spheres) and opsin (small spheres) positive outer segments being phagocytosed by a macrophage present in the subretinal space of a 30-day detached retina. Inset is a higher magnification of the region in brackets. IS, Inner segment; M, macrophage. $\times 16\,525$; inset $\times 32\,224$.

'sink', rapidly clearing opsin from the remainder of the cell; (3) the adult photoreceptor could possess a barrier preventing the back-diffusion of membrane protein from the outer segment (see also Besharse et al., 1985; Hicks and Barnstable, 1986; Spencer et al., 1986; Besharse and Horst, 1990). A breakdown

of any of these mechanisms during photoreceptor degeneration, or an increase in the amount of opsin inappropriately partitioned into the inner segment plasma membrane, could account for the observed increase in immunolabeling. Indeed, recent evidence suggests that the rate of opsin biosynthesis may not

decrease significantly during the early phase of inherited photoreceptor degeneration when there are no outer segments present (Nir and Papermaster, 1989; Nir, Agarwal and Papermaster, 1990). The increased opsin labeling in the proximal region of the cell may simply reflect the fact that opsin is continuing to be delivered to normal sites of insertion at normal rates while the outer segment, the appropriate 'sink' for opsin, is dwindling in size or absent, thus displacing more protein into the proximal membrane compartment. Alternatively, the abnormal distribution of opsin may be an early sign of degenerative change that may, in some cases, result in cell death. A decrease in photoreceptor cell bodies is known to occur following retinal detachment (Erickson et al., 1983). We do not yet know if opsin synthesis continues at normal rates in detached retinas, or if opsin levels remain elevated in proximal parts of the photoreceptor cell after retinal reattachment when the outer segments regenerate and recover a significant portion of their full length (Anderson et al., 1986; Guerin et al., 1989). Answers to such questions should help us to understand more completely the mechanism(s) underlying the compartmentalization of opsin that occurs during photoreceptor morphogenesis, degeneration, and regeneration.

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