July 1986 Vol. 27/7
Investigative Ophthalmology
& Visual Science
A Journal of Basic and Clinical Research

Articles

Retinoid-Binding Proteins in Cone-Dominant Retinas

Don H. Anderson, Jay Neitz, John C. Saari,* Deborah D. Kaska, John Fenwick, Gerald H. Jacobs, and Steven K. Fisher

We identified and localized interphotoreceptor (or interstitial) retinoid-binding protein (IRBP) and cellular retinaldehyde-binding protein (CRALBP) in the cone-dominant retinas of diurnal squirrels. Western blots were prepared from sodium dodecyl sulfate polyacrylamide gels (SDS-PAGE) from whole retina, and from retina proximal and distal to the photoreceptor nuclei. Blots were incubated with purified rabbit IgG's specific for the bovine retinal antigens, and the labeled components were visualized using immunoperoxidase techniques. Anti-bovine IRBP and anti-bovine CRALBP recognized single components on gels of retinal supernatants that corresponded to the electrophoretic migration of the bovine antigens. The component recognized by anti-bovine IRBP on blots of outer retinal proteins (M_r 146,000) was absent on blots of inner retinal proteins. Twelve and 24 hr after intravitreal injection of ^{3}H-L-fucose, electropherograms showed one major peak of radioactivity that coincided with the component recognized by anti-bovine IRBP. By immunoelectron microscopy, anti-bovine CRALBP labeling was restricted to the cytoplasm of both RPE and Muller cells, with light labeling of nuclear euchromatin in both cell types. In contrast, anti-bovine IRBP recognized antigenic sites primarily in the interphotoreceptor space (IPS). Intracellular labeling was limited to occasional granules in the photoreceptor myoids and the apical RPE cytoplasm. Extracellular labeling with anti-bovine IRBP was strongly associated with patches or small clumps of amorphous, electron opaque material distributed throughout the IPS. This material was particularly prominent near the cone outer segment plasma membranes, and was tentatively identified as the residual interphotoreceptor matrix that remained after exposure to the solvents used during tissue processing. In general, the results are consistent with those obtained in rod-dominant species. In addition, they imply that cones as well as rods are responsible for IRBP synthesis in the ground squirrel. Invest Ophthalmol Vis Sci 27:1015–1026, 1986

The extracellular space between the apical surface of the retinal pigment epithelium (RPE) and the external limiting membrane (ELM) of the neural retina defines a compartment known as the interphotoreceptor space (IPS). The IPS contains a matrix that consists mainly of protein[1] and a smaller amount of glycosaminoglycans as solutes.[2] In composition, it is distinct from fluid that accumulates in the subretinal space after retinal detachment. The main protein of the IPS, comprising about 60% of the total in monkey,[3] is interphotoreceptor (or interstitial) retinoid-binding protein (IRBP).[4–6] IRBP is a soluble, high molecular weight glycoprotein† that has been localized to the IPS in several mammalian species.[6–11] Its functional role in the visual process is unknown. However, by virtue of its strategic location within the IPS, its endogenous binding of retinol, and the fluctuations in the amount of retinol bound in response to light-dark transitions, it has been proposed to act as a transport protein–shuttling retinol back and forth between the retina and pigment epithelium as part of the visual cycle.[4–5,7]

Recovery and analysis of the culture medium after in vitro incubation indicate that IRBP is synthesized and secreted by the neural retina.[8,12–13] Using ^{3}H-fucose as a probe for glycoprotein synthesis, rods have been proposed as the primary cell type responsible for IRBP synthesis in the human retina.[10] The foveal region of

From the IES Neuroscience Research Program, and the Departments of Biological Sciences and Psychology, University of California, Santa Barbara, California, and the *Department of Ophthalmology, School of Medicine, University of Washington, Seattle, Washington.

Supported by Research Grants EY-02082 (DHA), EY-00105 (GHJ), EY-00343 (JCS), and EY-00888 (SKF) from the National Eye Institute, National Institutes of Health, Bethesda, MD.

Submitted for publication: August 29, 1985.

Reprint requests: Don H. Anderson, IES, Neuroscience Research Program, University of California, Santa Barbara, CA 93106.

† The molecular mass of bovine IRBP is 132,000 g/mol;[14–15] however, its relative molecular weight (M_r 146,000 in this study), measured by SDS-PAGE, is considerably higher.

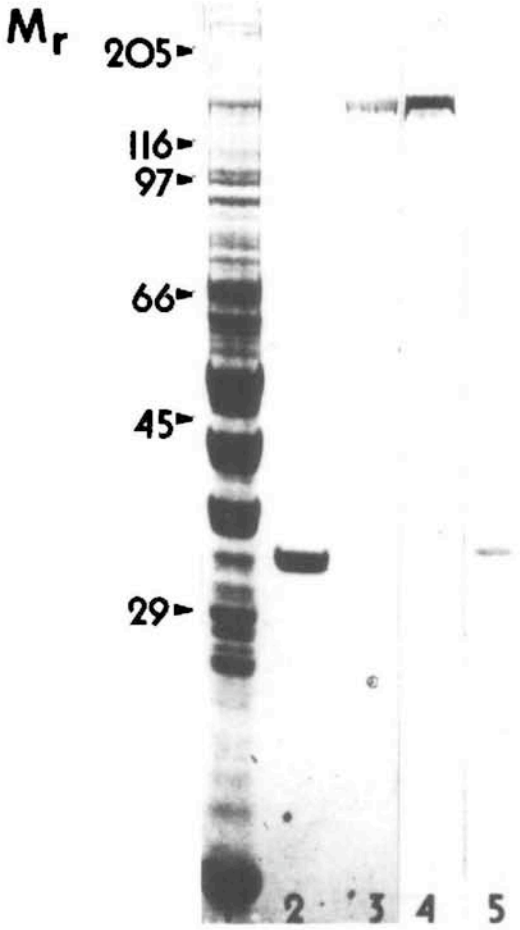


Fig. 1. Specificity of anti-bovine IRBP and CRALBP with ground squirrel retinal antigens. Ground squirrel retinal supernatants (100 μg protein/lane) were analyzed by SDS-PAGE and Western blotting. Lanes 1–3, SDS-PAGE Coomassie blue-stained 10% gels: Ground squirrel retinal supernatant (lane 1). Purified bovine CRALBP (lane 2). Purified bovine IRBP (lane 3). Lanes 4–5, Western blots of squirrel retinal supernatants reacted with anti-bovine IRBP diluted 1:200 (lane 4) or anti-bovine CRALBP (lane 5) and stained using the PAP method.[6] Both antibodies cross-react with single molecular species that correspond with the migration of the bovine antigens (146,000 and 33,000 M_r respectively).

rhesus monkey retinas is reported to show less IRBP labeling than extrafoveal regions by immunofluorescence microscopy.[11]

In addition to IRBP, three other retinoid-binding proteins have been isolated from extracts of retina and RPE. One of these, cellular retinaldehyde-binding protein (CRALBP), has been localized to the cytoplasm of both RPE and Müller cells,[6] thereby raising the possibility that Müller cells play a role in retinal Vitamin A metabolism.

Thus far, these binding proteins have been identified and localized only in rod-dominant species. Bovine and rat retinas contain mostly rods, while the retinas of diurnal primates, including the human retina, contain more cones than rods only within the parafovea and fovea, an area measuring less than 2 mm in diameter.[16] In order to examine the properties and distribution of IRBP and CRALBP without significant interference from rods, we used one of the few mammalian species whose retina is strongly cone-dominant—the ground squirrel. The retinas from this group of diurnal rodents contain at least 90% cones[17] and, therefore, provide an excellent model system for the study of cone photoreceptor metabolism.

Materials and Methods

Animals

The California ground squirrel (*Spermophilus beecheyi*) was used for all the biochemical procedures. In addition to this species, several Eastern Grey squirrels (*Sciurus carolinenesis*) were included in the immunolocalization experiments; their cone:rod ratio is about 3:2.[18] The ground squirrels were trapped locally in Santa Barbara County, California. Grey squirrels were purchased from Starling Squirrel Ranch, Noches, Texas. All animals were treated in accordance with the ARVO Resolution on the Use of Animals in Research.

Preparation of Antibodies to IRBP and CRALBP

Antibodies to purified bovine IRBP and CRALBP were prepared in rabbits, and IgG fractions were isolated using protein A Sepharose, as described previously.[6] Before use in the immunocytochemical procedures, their specificities with respect to ground squirrel retina were examined by immunoblot analysis (Western blots) of squirrel retinal antigens separated by SDS-PAGE. Detailed procedures for these techniques have been published previously.[6]

Microseparation of Ground Squirrel Retina

Light-adapted squirrels were anesthetized with an overdose of sodium pentobarbital (80 mg/ml), and the eyes were enucleated. After excising the anterior segment the posterior pole was cut into four quadrants. The retina-RPE was peeled away from the choroid-sclera and the vitreous gel was teased away from the retinal surface. Quadrants of squirrel retina and attached RPE were placed RPE side down on a piece of dry nylon-66 filter paper, and then covered by a second piece of filter paper. The surfaces of the tissue adhered to the filter papers. The papers were then carefully separated, shearing the retina uniformly just distal to the photoreceptor nuclei. The two retinal samples, one consisting of the RPE and the distal part of the photoreceptors and the other containing the rest of the retina, were analyzed separately. At this stage, the tissue was either: 1) dissolved from the filter paper with sample application buffer (0.125 M Tris-HCl, pH 6.8; 4%

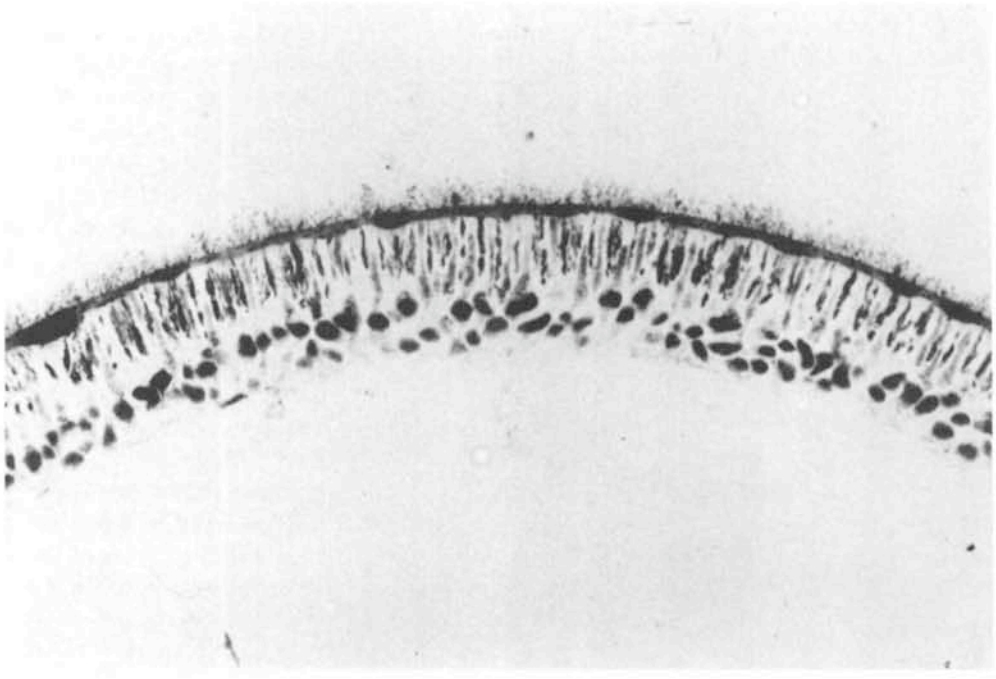


Fig. 2. Light micrograph of the photoreceptor-RPE preparation used in the biochemical analysis. The photoreceptor-RPE layers were separated from the rest of the retina using the filter paper technique described in the Materials and Methods section. The microseparation yielded an outer retinal fraction (as shown) that included the RPE cells, outer segments, inner segments, photoreceptor nuclei, and interphotoreceptor matrix. The outer fraction included all retinal layers proximal to the photoreceptor cell bodies. ×600.

SDS (wt/vol); 20% glycerol; 10% 2-mercaptoethanol) for analysis by SDS-PAGE; 2) fixed for light microscopy; or 3) prepared as follows.

Preparation of Photoreceptor Membranes

Filter papers with attached RPE and proximal portions of photoreceptors were transferred to chilled (0°C) 40% sucrose (wt/vol), 0.01 M Tris-HCl, pH 7.4, 1 mM $MgCl_2$, 1 mM dithiothreitol (0.25 ml/retina), and shaken vigorously until all the tissue was washed from the filter paper and suspended in sucrose solution. The suspension was drawn off, overlayed with chilled buffer, and centrifuged at 50,000 × g for 90 min. After centrifugation, the membranes were collected from the sucrose/buffer interface, washed with chilled isotonic buffer, and pelleted by centrifugation at 50,000 × g for an additional 30 min. Pellets were either transferred directly to fixative for EM or dissolved in sample application buffer for SDS-PAGE analysis.

Measurement of Fucose Radioactivity Profile

At 12 hr and 24 hr after intraocular ^{3}H-fucose injection, proteins from outer retina were separated by SDS-PAGE and transferred to nitrocellulose.[19] Each lane was cut into 1 mm slices that were dissolved in 100 μl of DMSO, added to 5 ml of hydrofluor (National Diagnostics, Somerville, NJ), and counted in a liquid scintillation spectrometer. The efficiency of transfer, as judged by Coomassie blue staining of the gel after transfer, was nearly quantitative.

Immunocytochemistry

After intracardiac perfusion of 1% glutaraldehyde, 1% paraformaldehyde in 0.1 M phosphate buffer (pH 7.1), retinal specimens for immunocytochemistry were immersed for an additional 1–2 hr in fixative. After several brief rinses in buffer, the specimens were dehydrated in a graded dimethylformamide (DMF)-H_2O series (50, 75, 90% at 20 min/change), and then rotated overnight in a 1:1 solution of DMF and Lowicryl K4M. In the morning the tissue was cut into small wedge-shaped specimens, transferred to 1.5 ml microfuge tubes filled with 100% Lowicryl, and capped. Larger specimens were placed in capped polypropylene pans and treated similarly. The plastic was polymerized by uniform exposure to long wavelength ultraviolet radiation in the cold (4°C). Other retinae were processed similarly, but dehydrated in ethanol or methanol and transferred to LR White resin (2 changes). After overnight rotation at room temperature, the plastic was polymerized at 52–55°C for 24 hr.

Light microscopy: For light microscopic immunocytochemistry, an indirect immunoperoxidase technique was employed. One micron Lowicryl or LR White sections were placed on cleaned glass slides and dried on a slide warmer. The sections were exposed to drops of blocking serum (1% goat serum) for 20 min, and then incubated overnight in a 1:200 or 1:400 dilution of primary antibody (anti-bovine IRBP or anti-bovine CRALBP). After washing, drops of diluted biotinylated anti-IgG were placed on the sections for 30

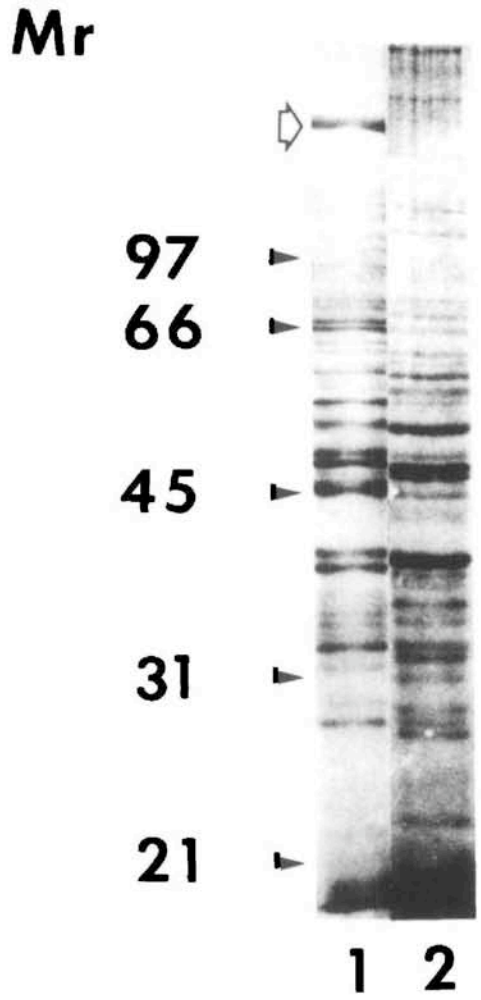


Fig. 3. Comparison of outer and inner ground squirrel retina by SDS-PAGE (10% gels stained with Coomassie blue). Lane 1, outer retinal fraction as described in Figure 2. Lane 2, inner retinal fraction. Note the absence of the darkly stained M_r 146,000 component (IRBP) (arrow) from the inner retinal fraction.

min, sections were washed in buffer once again, and then incubated for 60 min with an avidin DH-biotinylated horseradish peroxidase complex (Vector, Burlingame, CA). After washing for 10 min in buffer, the sections were incubated for 30 min in either 3-amino-9-ethylcarbazole (AEC) or diaminobenzidine (DAB) substrate solution. After a final wash, the slides were dried and then viewed using phase contrast optics. All incubations were performed at room temperature. Controls consisted of eliminating the primary antibody from the incubation or replacing it with pre-immune IgG.

Electron microscopy: Parlodian-coated nickel grids (200 mesh) were coated by vacuum evaporation with a thin carbon layer. Thin sections of Lowicryl(K4M)-embedded tissue were transferred from the boat by bringing the grids into contact with the sections on the water surface, inverting the grid, and then wicking away the drop of water. Thin sections of LR White-embedded material were placed directly onto grids without film. Grids were then incubated face down on drops of diluted goat serum for 5–10 min. After wick drying, grids were immediately transferred to drops of diluted primary antibody (1:200 or 1:400 in 0.1 M phosphate buffer + 0.08% NaN_3) and incubated overnight in a humidified enclosure. After a series of brief washings, grids were placed on drops of goat anti-rabbit IgG-gold complex (Janssen GAR-40; GAR-15, Piscataway, NJ) diluted 1:5 or 1:20 for 60 min. The grids were immersed and then jet washed in buffer several times. All the incubations took place at room temperature. Finally, the grids were stained with uranyl acetate (5–7 min) and lead citrate (1–2 min). The controls were the same as those employed for light microscopy. A thin coat of carbon was evaporated over the LR White grids at the very end of the procedure to increase thermal stability in the electron microscope.

Results

Antibodies to IRBP and CRALBP Recognize Single Proteins in Squirrel Retinal Homogenates

The antibodies used in this study have been found to be highly specific with respect to bovine retinal antigens.[6] Similar specificity studies were conducted with

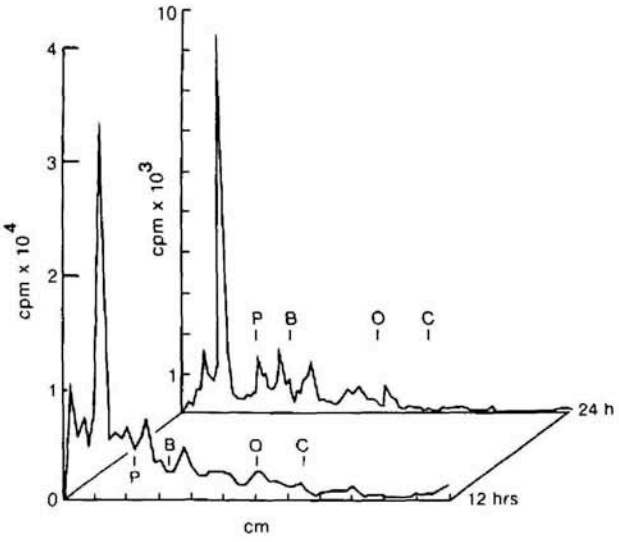


Fig. 4. ^{3}H-Fucose labeled proteins in the RPE-photoreceptor layers of ground squirrel retina. Ground squirrels received an intravitreal injection of ^{3}H-fucose 12 hr or 24 hr prior to sacrifice. After solubilizing in sample application buffer, the RPE-photoreceptor proteins were separated by SDS-PAGE and transferred to nitrocellulose. Each lane was cut into 1 mm slices, dissolved in DMSO, and counted by liquid scintillation spectrometry. At both timepoints (z-axis), a sharply defined peak of radioactivity is present that corresponds to the M_r 146,000 component recognized by anti-IRBP on western blots. M_r markers: Phosphorylase b (P) = 97,400; Bovine serum albumin (B) = 66,000; Ovalbumin (O) = 45,000; Carbonic anhydrase (C) = 29,000.

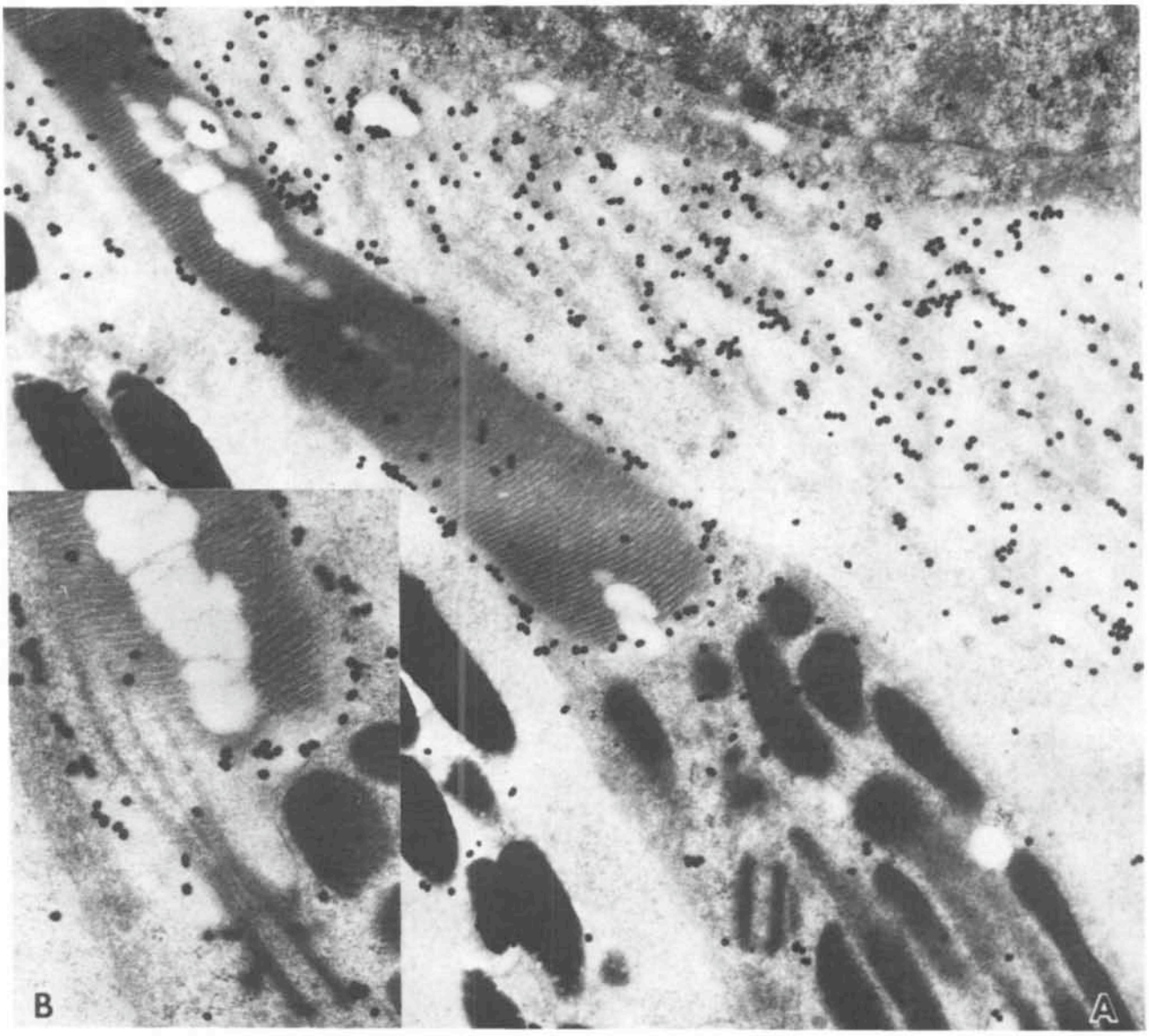


Fig. 5. Localization of IRBP in ground squirrel retina using the immunogold method (Lowicryl K4M). **(A)** Low power electron micrograph at the photoreceptor-RPE interface. Heavy labeling is present at the RPE apical surface. It is unclear whether the gold particles overlie extracellular matrix, RPE microvilli, or both. The region bordering the cone outer segment plasma membrane is also labeled; but the cone outer segment interior, cone inner segments, and RPE cytoplasm show only scattered background labeling. ×30,250. **(B)** The connecting cilium at the base of the cone outer segment is shown. Labeling occurs in the cleft between inner and outer segments, in the periciliary space, and between the calycal processes and the outer segment plasma membranes. Gaps in outer segment disc structure may be due to lipid extraction during the processing and embedding procedure. Such artifacts are characteristic of retinal tissue embedded in Lowicryl K4M. ×34,200.

ground squirrel retinas to insure that the antibodies would have the requisite specificity for studies in this species. After homogenization and centrifugation of squirrel retina-RPE, the supernatant was analyzed by SDS-PAGE (Fig. 1; lane 1) along with purified bovine CRALBP and IRBP in the gel as reference proteins (lanes 2–3). The gel was stained either with Coomassie Blue (lanes 1–3), or the proteins were transferred to nitrocellulose and reacted with either anti-bovine IRBP (lane 4) or anti-bovine CRALBP (lane 5) using the PAP method. The two antibodies appeared to be highly specific in that each recognized single molecular species that corresponded in molecular weight to that of the bovine antigens.

Microscopy of the Photoreceptor-RPE Preparation

When the neural retina was peeled away from the RPE in the squirrel, some of the cone outer segments adhered to the apical RPE surface; others broke off at the ciliary stalk and floated free in the dissecting me-

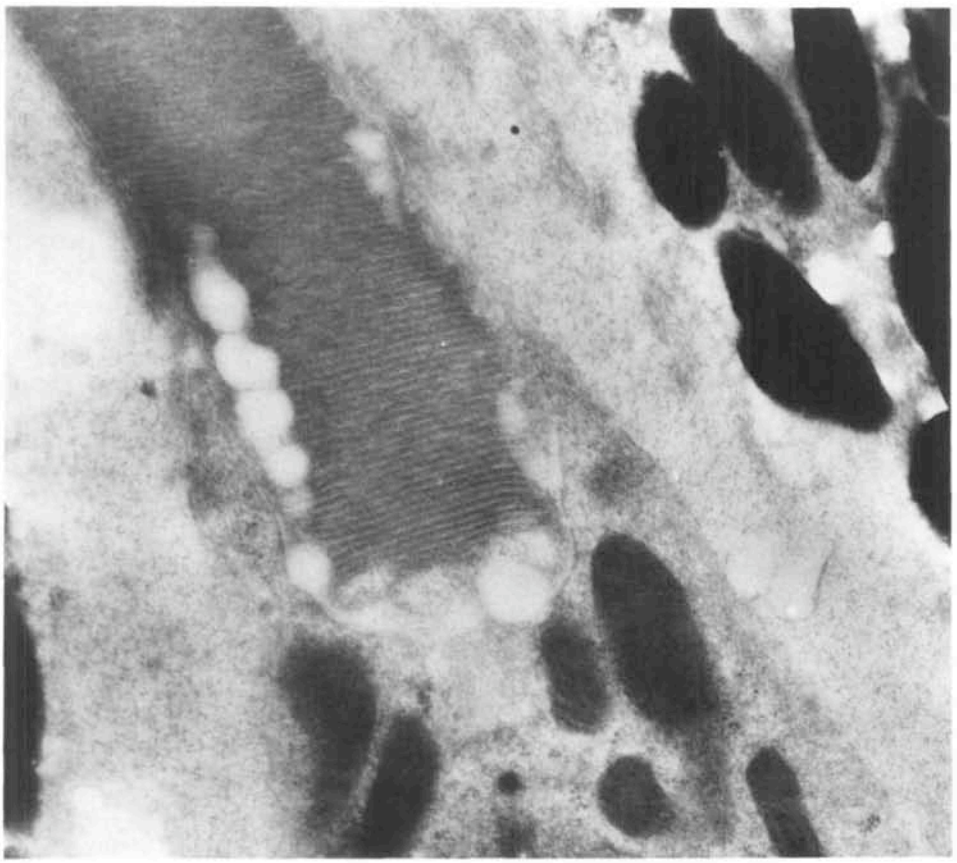
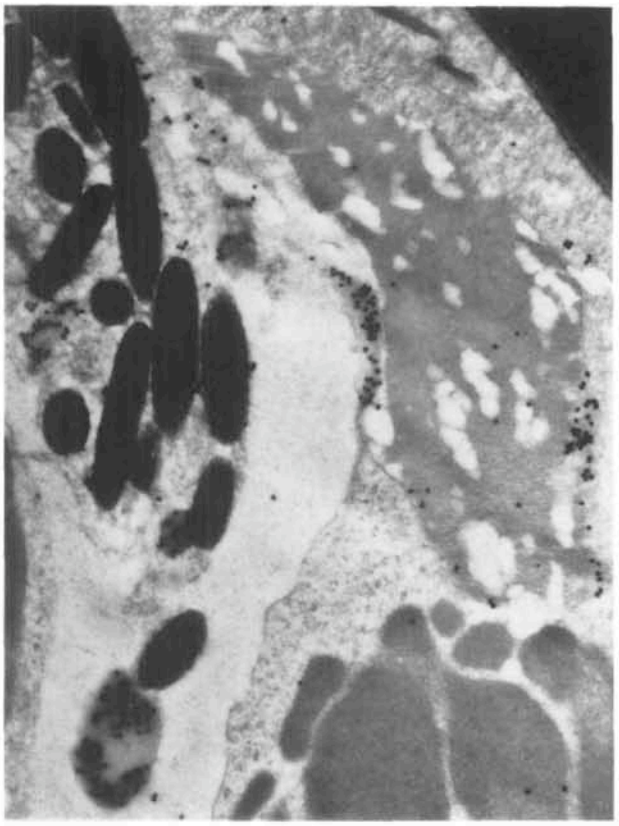


Fig. 6. Control micrograph of ground squirrel cone outer segment base. This control grid was treated with pre-immune IgG (1:400 in PBS) instead of anti-bovine IRBP. All other steps in the localization procedure were the same as in Figure 5. Only a few gold particles appeared over the tissue; no specific labeling was identified. Lowicryl K4M; ×44,000.



dium. Only a few remained intact and attached to their inner segments. In order to obtain a retinal preparation that included all of the photoreceptor outer segments, we developed a technique for shearing the retina-RPE into two parts. Using this technique, the squirrel retina tore uniformly at the level of outer nuclear layer (ONL). The RPE cells, interphotoreceptor matrix (IPM), photoreceptor cell outer segments, inner segments, and somata comprised one fraction (Fig. 2). The rest of the retina proximal to the photoreceptor cell bodies constituted the other. Electron micrographs of membranes isolated from the RPE-photoreceptor fraction showed small stacks of outer segment lamellae interspersed with numerous vesicular profiles and occasional dense granules.

IRBP is Present Only in the Photoreceptor-RPE Layer

Protein profiles of the proximal and distal retina were significantly different when compared on Coomassie

Fig. 7. Localization of IRBP in the tree squirrel retina. Labeling was heavy over circular profiles of electron opaque material adjacent to, or, perhaps, within, the melanin-containing apical processes of the RPE (left). Antigenic sites are also present on the lateral surface of the cone outer segment. Lowicryl K4M; ×15,300.

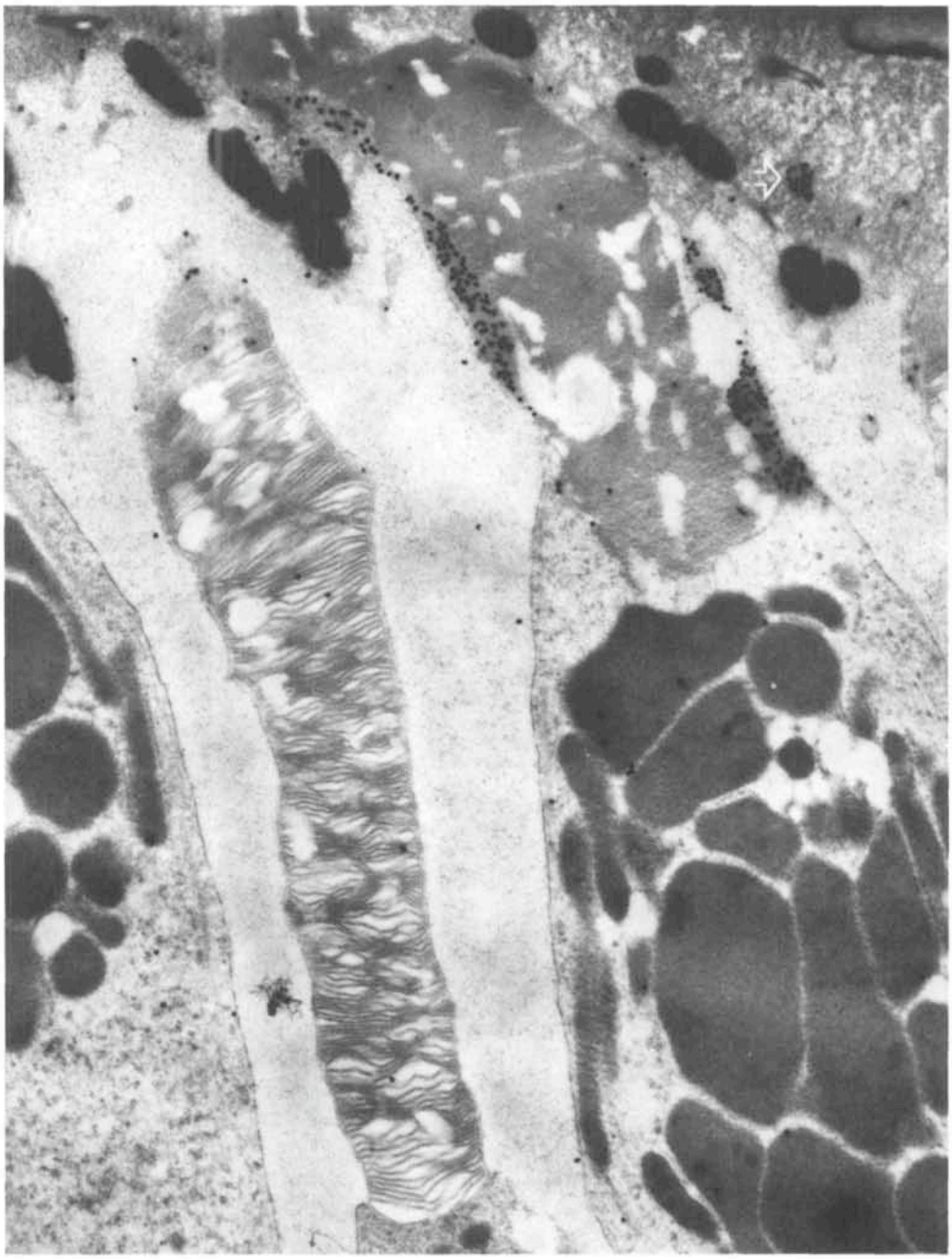


Fig. 8. IRBP localization bordering tree squirrel rod and cone outer segments. Heavy labeling occurs over an electron opaque zone of amorphous matrix that ensheaths most cone outer segments (right). This amorphous material is not associated with the rod outer segment on the left; however, smaller patches or clumps of matrix are found adjacent to some rod outer segment plasma membranes. Note the labeling over a small granule that appears to be within the apical RPE cytoplasm (arrow). Lowicryl K4M; ×13,300.

blue stained gels (Fig. 3). One of the most prominent components in the photoreceptor-RPE preparations (lane 2 M_r 146,000) was absent from the inner retina, the region proximal to the photoreceptor cell bodies (lane 1). Following transfer of the proteins to nitrocellulose, this component was identified as squirrel retina IRBP by its reaction with rabbit anti-bovine IRBP (Fig. 3). No protein components of inner retina were recognized by the antibody.

Radioactivity Profile of Fucosylated Retinal Proteins

Quantification of the radioactivity of photoreceptor-RPE proteins following injection of ^{3}H-fucose revealed that most of the counts were associated with one component on the gel (Fig. 4), a result also observed in bovine retina by Fong et al.[12] At both 12 and 24 hr after intraocular injection of ^{3}H-fucose, there was one sharply defined peak of radioactivity (M_r 146,000) that accounted for over 50% of the total counts. The major peak of radioactivity coincided with the band recognized by anti-bovine IRBP on SDS gels of outer retinal proteins.

Immunocytochemical Localization of IRBP

Using the immunogold technique, IRBP was localized to the extracellular compartment bounded by the

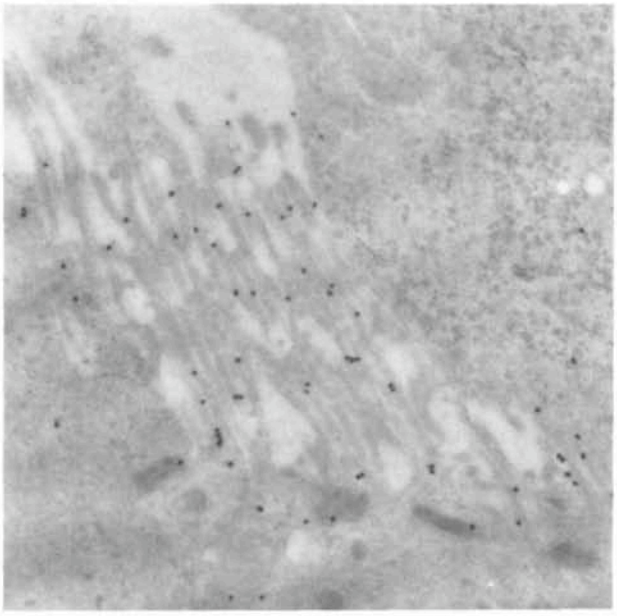


Fig. 9. Localization of IRBP near the external limiting membrane. Moderate labeling is found in the region of the Muller cell microvilli. It was not possible to determine whether the microvilli were labeled due to the size of the gold particles and the lack of membrane contrast. The cone myoid on the right shows no evidence of labeling. Lowicryl K4M; ×17,000.

apical RPE surface and Müller cell microvilli. No indication of labeling within the inner retina was found, a result in agreement with the immunoblots of SDS gels (Fig. 3). There was intense labeling adjacent to the outer segments, and at the apical border of the RPE (Fig. 5). The 40 nm gold particles tended to overlie the extracellular space between individual microvilli. However, due to the lack of membrane definition that accompanies embedment in Lowicryl or LR White, the presence of some microvillar labeling cannot be ruled out as a possibility. In sections treated with preimmune IgG instead of the primary antibody or in sections processed with PBS in place of the primary antibody, only a few scattered gold particles were present in the IPS (Fig. 6).

The extracellular space between the photoreceptor outer and inner segments was lightly labeled at some locations. At other locations, especially in the grey squirrel retinas, we identified numerous patches or clumps of intensely labeled, electron opaque material scattered throughout the IPS. This material was particularly evident: 1) adjacent to the melanin containing processes that interdigitate with the outer segments (Fig. 7); 2) bordering cone outer segment and, to a lesser extent, rod outer segment plasma membranes (Fig. 8), and 3) within the narrow cleft between the photoreceptor inner and outer segments next to the connecting cilium (Fig. 5B). Aldehyde-fixed squirrel and primate[20-21] retinas post-fixed in osmium tetroxide and embedded in Araldite or Epon also show patches of this opaque matrix in the IPS. It consistently appears as a thick, electron opaque coat that ensheaths the cone outer segments, including their tips and the connecting cilium.

At the border of the ELM there was moderate labeling in the region of the Müller microvilli (Fig. 9). Due to the size of the gold particles and the lack of membrane definition, we could not determine whether the labeling was strictly associated with the extracellular space or with the microvilli.

There was little intracellular labeling in any of the cell types bordering the IPS. In the RPE cells, a few small labeled profiles near the apical RPE surface were present that appeared to be within the RPE cytoplasm (Fig. 8). However, no enclosing membrane could be identified surrounding these granules, and they were not evident at more basal locations in the RPE cytoplasm. There was no evidence of labeling within the cone outer segment interior (Fig. 10). Both rod and cone ellipsoids were also unlabeled. Occasionally, labeled membrane-bound granules were observed within the cone myoids near the ELM. Their lumen contained patches of electron opaque material similar in appearance to that found in the IPS.

Immunocytochemical Localization of CRALBP

The Müller cell and RPE cell cytoplasms labeled uniformly with antibodies to CRALBP. By light microscopy, using an indirect avidin-biotin peroxidase technique, reaction product was found throughout individual Müller cells including: the end feet, cell bodies, radial processes, and apical microvilli (Fig. 11A). Similarly, RPE cytoplasm labeled uniformly except for the nuclei. Using phase contrast optics, control sections showed only faint outlines of the Müller processes (Fig. 11B).

Using the immunogold procedure, electron micrographs revealed light labeling of nuclear euchromatin in both cell types. In the RPE cells, gold particles appeared throughout the cytoplasm extending into both the basal infoldings and the apical processes (Fig. 12). Mitochondria, phagolysosomes, and other membrane-bound organelles were not labeled.

Discussion

In the cone-dominant squirrel retina, antibodies to bovine IRBP and CRALBP recognized single proteins in retinal homogenates and supernatants that corresponded in molecular weight to those of the bovine antigens, thereby indicating their utility as specific probes for immunolocalization in these species. A

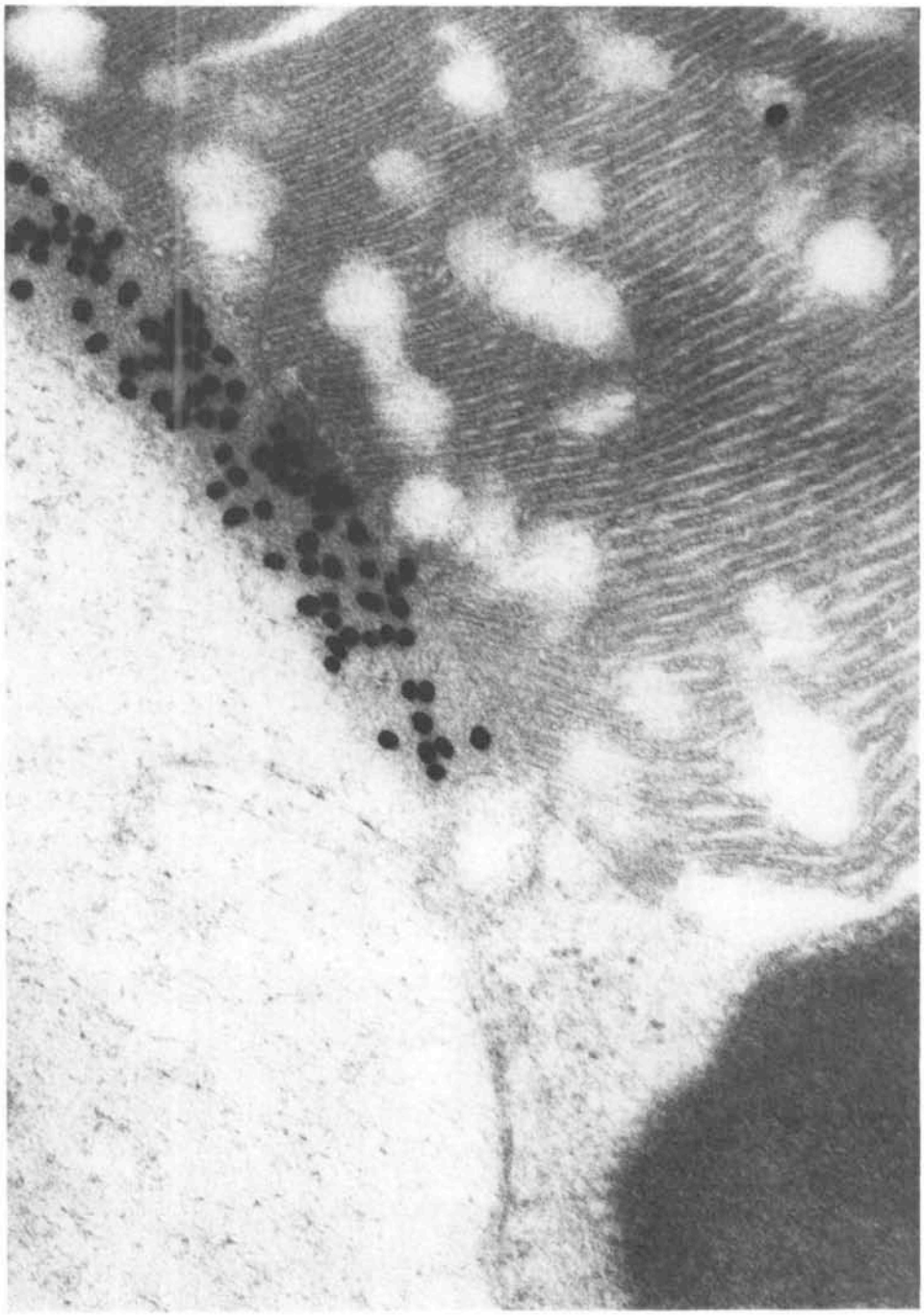


Fig. 10. High magnification of matrix bordering cone outer segments. The labeled matrix material is not present within the cone outer segment interior. There is no evidence of labeling associated with the lamellar rims, or in the intra- or interdisc spaces. Lowicryl K4M; ×55,000.

comparison of RPE-photoreceptor proteins to those of the inner retina by SDS-PAGE showed that a major component (M_r 146,000), reactive with anti-bovine IRBP, was present in the photoreceptor-RPE layer, but absent from the inner retina. At 12 and 24 hr after intravitreal injection of ^{3}H-fucose, over 50% of the total radioactivity was associated with this component. These results strongly suggest that IRBP is present in the RPE-photoreceptor fraction of the squirrel retina, but absent from the inner retina vitreal to the photoreceptor nuclei. In addition, its rate of synthesis appeared to be substantially higher than any other fucosylated proteins under the conditions employed.

In accordance with the results obtained in other species,[6,22] immunoelectron microscopy of squirrel retinas showed that anti-CRALBP labeled antigens throughout the RPE and Müller cell cytoplasms, with light labeling of nuclear euchromatin in both cell types. In contrast, anti-bovine IRBP recognized antigenic sites primarily in the IPS. Intracellular labeling was limited to occasional small granules in the apical RPE cytoplasm and in the cone myoids just distal to the ELM. The most

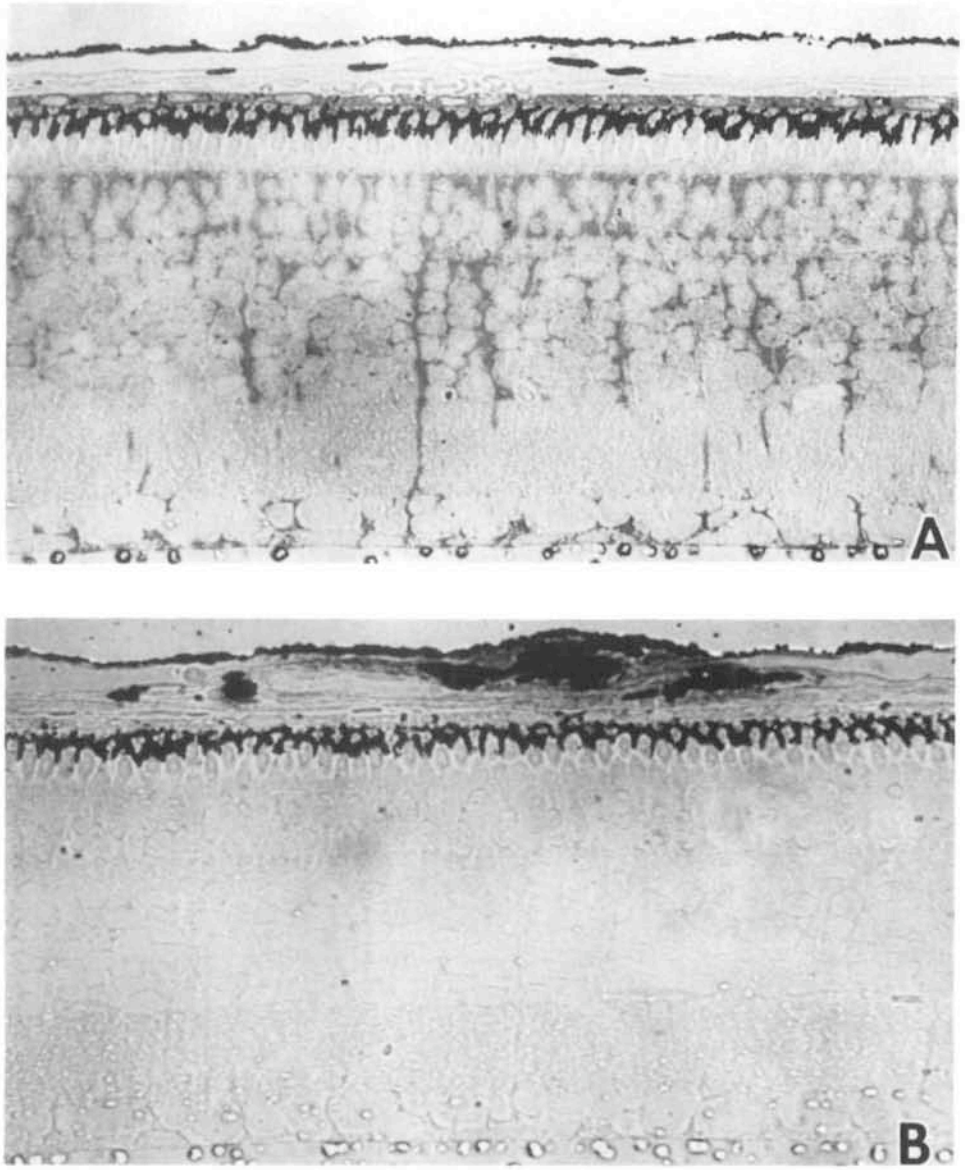


Fig. 11. Immunoperoxidase localization of CRALBP in the ground squirrel retina. **A**, Reaction product is visible throughout the RPE and Muller cell cytoplasms in this phase contrast light micrograph. No nuclear labeling can be detected with this technique. ×550. **B**, Control section treated with pre-immune IgG. There is virtually no retinal or RPE staining. The darkly stained structures are melanin-laden RPE processes. Lowicryl K4M; ×550.

intense anti-IRBP labeling occurred over amorphous electron opaque material scattered throughout the IPS. Small clumps or patches of this material were sequestered between the apical RPE and Müller cell microvilli, and in the periciliary region between photoreceptor inner and outer segments. It was most easily identified as a thick coat that enveloped the cone outer segments and, to a lesser extent, the rod outer segments.

An amorphous matrix of this description was first noted by Fine and Zimmerman[23] in the human retina, and later identified in several other species by Rohlich,[20] who coined the term interphotoreceptor matrix (IPM). Feeney[24] showed by autoradiography that ^{3}H-fucose labeled the "cell-coat" surrounding mouse rod outer segments. By electron microscopy, a sheath of IPM is reported to envelop cone outer segments in the cynomolgus, rhesus monkey, and human retinas.[20-21] Our observations in rhesus monkey indicate that antibodies to bovine IRBP intensely label the IPM surrounding foveal cone outer segments,‡ just as they do in squirrel cones. It is highly likely that this IPM adherent to cone outer segments is composed of IRBP and perhaps other, unidentified components.

The patches and clumps observed by electron microscopy probably represent the matrix that is left after exposure to solvents used during tissue processing. However, it is unclear why the matrix is particularly adherent to specific membrane surfaces, such as the cone outer segment plasma membranes. Cell-matrix adhesion in the IPS may vary with the surface characteristics of the RPE, photoreceptor, or Müller cell plasma membranes. Light microscopic lectin-binding studies suggest that there is a distinct extracellular do-

‡ Anderson DH, unpublished results.

main surrounding cone outer and inner segments[26-27] in primate and porcine retinas that binds peanut agglutinin (PNA), a lectin specific for galactose-galactosamine disaccharides. This matrix may, in fact, coincide with the IRBP-containing matrix that we have identified by electron microscopy adjacent to squirrel and monkey cones.

Although IRBP is known to be secreted by the neural retina,[8,10,12] the cell type/s that synthesize it remain unknown. Evidence from in vitro studies suggests that the photoreceptors are the site of synthesis. IRBP is not secreted by the isolated RCS (Royal College of Surgeons) rat retina when photoreceptors are actively degenerating.[9] In human retinal explants, Hollyfield et al[10] found a correlation between reduced levels of ^{3}H-fucose labeling in human rod inner segments (and, to a lesser extent, in cone inner segments) and the appearance of ^{3}H-fucose labeled IRBP in the incubation medium. In addition, IRBP was not detected in an eye with advanced retinitis pigmentosa in which virtually all non-foveal photoreceptors were probably degenerate.[28] Bunt-Milam et al[29] have recently identified vesicles in bovine rod and cone inner segments that label positively with IRBP antibodies. Similar vesicles were not apparent in Müller cells; however, it is uncertain whether such vesicles indicate the source of IRBP biosynthesis or whether they define a degradative pathway for the turnover of extracellular matrix.[30] In the squirrel retina, the ^{3}H-fucose-labeling experiments strongly suggest that newly-synthesized IRBP is present only in the retinal fraction distal to the photoreceptor nuclei. This fraction includes RPE cells, photoreceptors, and the distal portion of Müller cells. Since the ELM has recently been shown to be a diffusion barrier to molecules much smaller than IRBP,[31] it is unlikely to be secreted into the IPS from sites proximal to the ELM. Rather, it must originate from cells that border the IPS. IRBP is not found in RPE homogenates and it is not synthesized by RPE cells in vitro.[13,22] Therefore, the photoreceptors and Müller cells are the only remaining candidates for IRBP synthesis. Thus far, there is no evidence implicating Müller cells. All available evidence points toward the photoreceptors as the site of IRBP synthesis. But it remains to be determined whether both photoreceptor classes, or only one type, are involved. Hollyfield et al[10] concluded that, in the human retina, rods are primarily responsible. Although we cannot rule out the possibility that most or all IRBP in the ground squirrel retina is synthesized by the 5–10% rod population, the simplest assumption at the present time is that both rods and cones synthesize IRBP in this species.

The absence of IRBP from the IPS in cone-dominant retinas or in cone-rich retinal regions such as the primate fovea would be inconsistent with the suggestion that it is the primary vehicle for retinoid transport in the eye. The results from this study corroborate those already obtained in rod-dominant species, and also extend the occurrence of IRBP to cone-dominant retinas. The function of CRALBP remains uncertain, but the demonstration of its presence in Müller cells of a cone-dominant retina strengthens the contention that these cells may be the site of one or more reactions of the visual cycle.

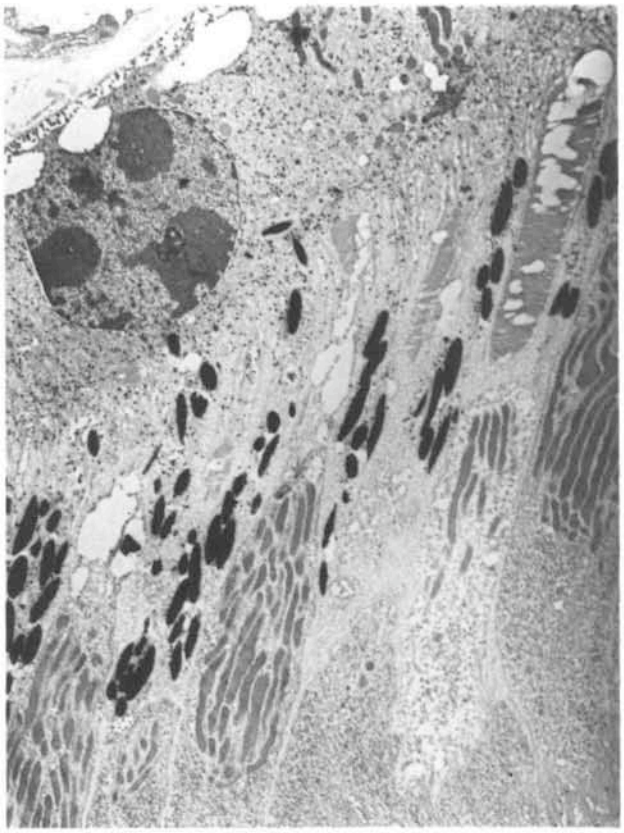


Fig. 12. Immunogold localization of CRALBP in the ground squirrel RPE. Gold particles are present throughout the RPE cytoplasm. The basal infoldings as well as the apical processes are uniformly labeled. There is no labeling of cellular organelles except for light labeling of nuclear euchromatin. LR White; ×4,000.

Key words: retinoid, cone, photoreceptor, extracellular matrix, immunocytochemistry

References

1. Adler A and Severin K: Proteins of the bovine interphotoreceptor matrix: tissues of origin. Exp Eye Res 32:755, 1981.
2. Berman ER and Bach G: Acid mucopolysaccharides of cattle retina. Biochem J 108:75, 1968.
3. Pfeffer BA, Wiggert B, Lee L, Zonnenberg B, Newsome D, and Chader G: The presence of a soluble interphotoreceptor retinol-binding protein (IRBP) in the retinal interphotoreceptor space. J Cell Physiol 117:333, 1983.
4. Lai YLB, Wiggert B, Liu YP, and Chader GJ: Interphotoreceptor retinol-binding proteins: possible transport vehicles between compartments of the retina. Nature (Lond) 298:848, 1982.
5. Liou GI, Bridges CDB, Fong S-L, Alvarez RA, and Gonzalez-Fernandez F: Vitamin A transport between retina and pigment

epithelium. An interstitial protein carrying endogenous retinol (interstitial retinol-binding protein). Vision Res 22:1457, 1982.
6. Bunt-Milam AH and Saari JC: Immunocytochemical localization of two retinoid-binding proteins in vertebrate retina. J Cell Biol 97:703, 1983.
7. Adler A and Martin M: Retinol-binding proteins in bovine interphotoreceptor matrix. Biochem Biophys Res Commun 108: 1601, 1982.
8. Fong S-L, Liou GI, Landers RA, Alvarez RA, Gonzalez-Fernandez F, Glazebrook PA, Lam DMK, and Bridges CDB: The characterization, localization and biosynthesis of an interstitial retinol-binding glycoprotein in the human eye. J Neurochem 42: 1667, 1984.
9. Gonzalez-Fernandez F, Landers RA, Glazebrook PA, Fong S-L, Liou GI, Lam DMK, and Bridges CDB: An extracellular retinol-binding glycoprotein in the eyes of mutant rats with retinal dystrophy: development, localization, and biosynthesis. J Cell Biol 99:2092, 1984.
10. Hollyfield JG, Fliesler SJ, Rayborn ME, Fong S-L, Landers RA, and Bridges CDB: Synthesis and secretion of interstitial retinol-binding protein by the human retina. Invest Ophthalmol Vis Sci 26:58, 1985.
11. Rodrigues M, Gaskins R, Wiggert B, Redmond M, and Chader G: Immunocytochemical localization of interphotoreceptor retinoid-binding protein in the primate retina and pineal gland. ARVO Abstracts. Invest Ophthalmol Vis Sci 26(Suppl):340, 1985.
12. Fong S-L, Liou GI, Landers RA, Alvarez RA, and Bridges CDB: Purification and characterization of a retinol-binding glycoprotein synthesized and secreted by bovine neural retina. J Biol Chem 259:6534, 1984.
13. Wiggert B, Lee L, O'Brien PJ, and Chader GJ: Synthesis of interphotoreceptor retinoid-binding protein (IRBP) by monkey retina in organ culture: Effect of monensin. Biochem Biophys Res Comm 118:789, 1984.
14. Adler AJ, Evans CD, and Stafford WF III: Molecular properties of bovine interphotoreceptor retinol-binding protein. J Biol Chem 260:4850, 1985.
15. Saari JC, Teller DC, Crabbe JW, and Bredberg L: Properties of an interphotoreceptor retinoid-binding protein from bovine retina. J Biol Chem 260:195, 1985.
16. Hogan MJ, Alvarado JA, and Weddell J: *In* Histology of the Human Eye, Philadelphia, WB Saunders, 1971, pp. 492.
17. Long KO and Fisher SK: The distributions of photoreceptors and ganglion cells in the California ground squirrel *Spermophilus beecheyi*. J Comp Neurol 221:329, 1985.
18. Cohen AI: Some observations on the fine structure of the retinal receptors of the American gray squirrel. Invest Ophthalmol 3: 198, 1964.
19. Towbin H, Staehelin T, and Gordon J: Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc Natl Acad Sci USA 76: 4350, 1979.
20. Rohlich P: The interphotoreceptor matrix: Electron microscopic and histochemical observations on the vertebrate retina. Exp Eye Res 10:80, 1970.
21. Feeney-Burns L, Malinow MR, Klein ML, and Neuringer M: Maculopathy in cynomolgus monkeys. Arch Ophthalmol 99: 664, 1981.
22. Saari JC, Bunt-Milam AH, Bredberg D, and Garwin GG: Properties and immunocytochemical localization of three retinoid-binding proteins from bovine retina. Vision Res 24:1595, 1984.
23. Fine BS and Zimmerman LE: Observations on the rod and cone layer of the human retina. Invest Ophthalmol 2:446, 1963.
24. Feeney L: Synthesis of interphotoreceptor matrix: I. Autoradiography of ^{3}H-fucose incorporation. Invest Ophthalmol 12: 739, 1973.
25. Feeney-Burns L: The early years of research. *In* The Interphotoreceptor Matrix in Health and Disease, Bridges CDB and Adler AJ, editors, New York, AR Liss, Inc., 1985, pp. 3.
26. Blanks JC and Johnson LV: Specific binding of peanut lectin to a class of retinal photoreceptor cells. Invest Ophthalmol Vis Sci 25:546, 1984.
27. Johnson LV, Hageman GS, and Blanks JC: Restricted extracellular matrix domains ensheath cone photoreceptors in vertebrate retinae. *In* The Interphotoreceptor Matrix in Health and Disease, Bridges CDB and Adler AJ, editors, New York, AR Liss, Inc., 1985, pp. 33.
28. Bridges CDB, O'Gorman S, Fong S-L, Alvarez RA, and Berson E: Vitamin A and interstitial retinol-binding protein in an eye with recessive retinitis pigmentosa. Invest Ophthalmol Vis Sci 26:684, 1985.
29. Bunt-Milam AH, Saari JC, and Bredberg DL: Characterization of the interstitial space: immunocytochemical and biochemical studies. *In* The Interphotoreceptor Matrix in Health and Disease, Bridges CDB and Adler AS, editors, New York, AR Liss, Inc., 1985, pp. 152.
30. Hollyfield JG, Varner HH, Rayborn ME, Liou GI, and Bridges CDB: Endocytosis and degradation of interstitial retinol-binding protein: differential capabilities of cells that border the interphotoreceptor matrix. J Cell Biol 100:1676, 1985.
31. Bunt-Milam AH, Saari JC, Klock IB, and Garwin GG: Pore size measurement of *zonulae adherentes* in the external limiting membrane of the rabbit retina. Invest Ophthalmol Vis Sci 26: 1377, 1985.
32. Bridges CDB: Retinoids in photosensitive systems. *In* The Retinoids, Vol. 2, Sporn MB, Roberts AB, and Goodman DS, editors, New York, Academic Press, Inc., 1984, pp. 152.