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Lectin and antibody labelling of developing rat photoreceptor cells: an electron microscope immunocytochemical study

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Summary

Lectin and rhodopsin antibody binding sites were studied in developing and adult rat photoreceptors in order to compare changes in the total carbohydrate pool with the movement of a known glycoprotein rhodopsin. Electron microscope immunocytochemical techniques utilizing modified colloidal gold methods were used. At birth, all three lectins – Concanavalin A (ConA), Ricinus communis agglutinin II (RCA II) and wheat germ agglutinin (WGA) – showed heavy labelling of the photoreceptor surface scleral to the outer limiting membrane. At the same age, a monoclonal antibody against rhodopsin, RET-P1, revealed sparse labelling of only occasional immature photoreceptor surfaces. At postnatal day 4(P4), all three lectins showed variable binding to the inner segment and along the length of the newly forming connecting cilium. There was generally a region of more intense label at the base of the cilium. RET-P1 binding to P4 retina showed a discontinuous distribution, with heavily labelled inner segments being adjacent to unlabelled inner segments. This pattern indicates that the initial expression of rhodopsin is not a coordinate event but occurs in discrete cells, possibly related to the end of mitosis. RET-P1 binding at this age was reduced or absent from the proximal connecting cilium. AT P7, when the outer segments are beginning to develop, all the lectins and RET-P1 showed reduced binding to the inner segment plasma membrane and heavy labelling of the outer segment surface. In favourable sections, heavy labelling of the photoreceptor cell body plasma membrane by ConA and RCA II was also observed, terminating abruptly at the outer limiting membrane. The variation in ligand binding between different cellular compartments which are all formed from a continuous plasma membrane may indicate the presence of special barriers to diffusion of membrane components. This labelling pattern persisted into maturity. RET-P1 and lectin binding did not always correspond in developing retina, indicating that at least part of the observed lectin label must be due to other glycoproteins or glycolipids. Post-embedding thin section labelling of adult rat retina revealed a uniform binding pattern across the outer segment for ConA, WGA and RET-P1. However, RCA II exhibited labelling only along the basal edge of outer segments. Labelling of isolated, opened discs from bovine rod outer segments revealed binding to a single surface for ConA, WGA and RET-P1, but RCA II only labelled a small amount of membrane. Hence RCA II seems to recognize a determinant present only on the outer segment plasma membrane.

Introduction

The mature mammalian retina is a highly ordered structure specialized for the detection and transduction of light energy into electrical signals. This is achieved in the photoreceptor cells, which have become highly polarized both structurally and biochemically to serve this function. In species such as the rat, photoreceptors are predominantly of the rod form, with only 1.5% cones (LaVail, 1976). The compartmentalized structure of rod cells is largely established postnatally: at birth, the scleral-most immature photoreceptor and Müller cells are connected at their apical surfaces by an elaborate system of gap junctions, the outer limiting membrane (OLM) (Dowling, 1970). During the first postnatal week, rod inner segments (RIS) extend from the level of the OLM into the sub-retinal space. These structures comprise a proximal myoid region containing Golgi bodies and rough endoplasmic reticulum responsible for protein synthesis (Young & Droz, 1968), and a more distally located ellipsoid composed of many mitochondria (Sjöstrand, 1953). Connecting cilia begin to appear in abundance at postnatal day 3–4 (P3–4), and finally at P7–8 the rod outer segments (ROS), large saccular structures containing a large number of membranous discs, are formed (Weidman & Kuwabara, 1968).

The visual pigment in vertebrate rods, responsible for the absorption of light quanta, is rhodopsin. Rhodopsin comprises >95% of the ROS intrinsic membrane protein (Kuhn, 1982) and is a glycopro-

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tein, possessing two asparagine-linked oligosaccharide groups at amino acid residues 2 and 15 of the N terminus (Hargrave, 1977). The structure of these carbohydrate units has been determined as predominantly short hexasaccharides of mannose and N-acetylglucosamine (Fukuda *et al.*, 1979).

Carbohydrate moieties in general have been implicated in events such as protein packaging and transport, cellular adhesion, recognition and differentiation (Barondes & Rosen, 1976; Horowitz & Pigman, 1977; Robbins, 1982). Within the photoreceptor cells, sugar groups may additionally be involved in pigmented epithelial ingestion of ROS debris (O'Brien, 1976), and ROS membrane assembly (Fliesler *et al.*, 1985).

There have been numerous studies investigating the distribution of specific sugar groups within developing and mature retinae. Using lectins as molecular probes, carbohydrate distributions have been described at the level of the fluorescence microscope (Bridges & Fong, 1979, 1980a,b; Uehara *et al.*, 1983; Blanks & Johnson, 1984), scanning electron microscope (Molday, 1976) and transmission electron microscope (TEM) (Nir & Hall, 1979; McLaughlin & Wood, 1980; Hicks & Molday, 1985). These studies have concentrated on the binding patterns of several lectins on mature retina of various species. Developmental changes have been described mainly at the level of the light microscope (Bee, 1980, 1982; Blanks & Johnson, 1983). The present study is a detailed ultrastructural analysis of the developmental changes in total sugar distribution between the cellular compartments, and a comparison of these with the movements of a known glycoprotein, rhodopsin. To achieve this, three lectins – Concanavalin A (ConA), Ricinus communis agglutinin II (RCA II) and wheat germ agglutinin (WGA) – were used. To compare the distribution of their binding sites with a known polypeptide determinant, these studies have utilized a monoclonal antibody against the N terminus of rhodopsin, RET-P1 (Barnstable, 1980; Fekete & Barnstable, 1983). Colloidal gold–dextran reagents have been employed to visualize the binding patterns of these ligands (Hicks & Molday, 1984a, 1985).

Materials and methods

Use of ligand–dextran–gold conjugates

Gold–dextran (Au–Dex) electron-dense markers have been previously used to examine the fine subcellular distribution of antibody (Hicks & Molday, 1984a,b, 1985) and lectin binding (Hicks & Molday, 1985) in adult retina. These reagents possess favourable characteristics such as stability, minimal tendency to aggregate, independent control of ligand concentration and the stable production of low molecular weight ligand conjugates. Protein ligands below a certain molecular weight, such as WGA, cannot be stably adsorbed to colloidal gold by conventional methods (Horisberger & Vonlanthen, 1979). The high electron density facilitates visualization of label on tissue surfaces and intracellular compartments of thin-sectioned material.

The procedure for the production of Au–Dex conjugates and for attaching various ligands has been described previously (Hicks & Molday, 1984a, 1985). Briefly, gold particles of various sizes from 5 to 17 nm were produced by reduction of gold chloride (Frens, 1973). Diaminoethane-derivatized dextran was then dissolved in the gold sol; this synthetic polymer is stably adsorbed to the gold particles and enables covalent attachment of proteins (Hicks & Molday, 1984a). Protein ligands used in the present study were Avidin (Av) (Vector Labs), goat anti-mouse immunoglobulin (gαm Ig) (Research Products Inc.) and the three lectins ConA (Sigma), RCA II and WGA (Boehringer–Mannheim). After stirring overnight, ligand–Au–Dex conjugates were washed and concentrated to give an absorbance reading at 520 nm of between 0.15 and 0.25 at a 1:100 dilution. Conjugates were stored at 4° C in 0.1 M Tris-buffered saline (TBS), pH 7.2, containing 0.1% bovine serum albumin (BSA) and 0.1% sodium azide. Colloidal markers prepared in this way displayed good stability with minimal loss of activity or tendency to aggregate, even in high salt conditions. The numerical subscript of conjugates, e.g. WGA–Au_{12}–Dex, denotes the average diameter of the gold core for each preparation.

Labelling of intact tissue

Long-Evans rat pups were obtained at birth (defined as P1) until eye opening at P13. In addition, juvenile and adult animals were also sampled. In all cases, animals were decapitated and the eyes rapidly excised and placed in 0.1 M cacodylate buffer, pH 7.2. The retinae were carefully dissected free and fixed in the same buffer containing 0.25% glutaraldehyde for 1 h at 4° C. Tissue was rinsed in the same buffer containing 0.1 M glycine for 1 h, and cut into small blocks. Sample tissue was taken from central retinal areas and incubated as follows. (1) To visualize ConA binding, tissue was incubated in free ConA (50 μg ml^{-1}) in 50 mM Tris, pH 7.4, 5 mM CaCl and 5 mM MnCl overnight at 4° C. Following extensive washing, tissue was incubated in Av–Au–Dex diluted 1:10 to 1:20 overnight at 4° C. (2) Both RCA II and WGA were used directly coupled to Au–Dex, tissue being incubated in a dilution of 1:5 to 1:20 overnight at 4° C. Controls for all three lectins were performed using the free hapten sugar, i.e. 0.1 M α-methyl-mannoside for ConA, 0.1 M D(+)galactose for RCA II and 0.01 M *NN'*-diacetylchitobiose for WGA. (3) To visualize rhodopsin, retinal tissue was incubated in RET-P1 culture fluid diluted 1:20 overnight at 4° C and then washed and incubated in gαm–Au–Dex overnight at 4° C. Controls were performed by either omitting the primary antibody or by using a monoclonal antibody recognizing a determinant on the rhodopsin C terminus, rho 1D4 (Molday & MacKenzie, 1983). All samples were washed, osmicated, dehydrated and embedded in Epon–Araldite. Ultrathin sections were taken and examined using a Jeol 35S TEM.

Post-embedding labelling of retina

The procedure for post-embedding staining of ultrathin sections has been previously described (Roth, 1983, Hicks

& Molday, 1985). Briefly, fixed unosmicated adult rat retinal tissue was dehydrated and embedded in Lowicryl K4M (Polysciences) at −20°C. After UV polymerization, ultrathin sections were cut and mounted on grids. Grids were incubated in the different lectin or antibody solutions for 30 min at 21°C. Following extensive washing and a second staining step in ligand–Au–Dex solutions if necessary, grids were counter stained and examined by TEM.

Labelling of isolated discs

Bovine ROS were prepared from freshly obtained slaughterhouse material using the method of Papermaster (1982). ROS preparations were treated in a number of ways to ensure thorough opening of the disc membranes permitting access to both the interdiscal and intradiscal surfaces. A combination of hyposmotic shock, trypsinization, freezing and thawing and homogenization was generally used. Disc samples were spun down onto polylysine-treated multiwell microtitre plates and fixed in 0.25% glutaraldehyde. Wells were then treated with lectin or antibody solutions as previously described.

Results

Binding of Concanavalin A

At P1, heavy label was visible on the scleral surface of the developing photoreceptor cells (Fig. 1). By P4 there seemed to be a general reduction in the amount of label present on the RIS (Figs 2, 3). A heavy label was frequently observed on the connecting cilia (Fig. 2). There is a great deal of variation in the degree of label found on adjacent RIS with all the lectins. As ROS formation commences around P7–8, there was heavy label apparent on the ROS plasma membrane, with low levels of binding still visible on the cilia and RIS (Fig. 4). With some sections, it was possible to study ligand binding below the level of the OLM, where photoreceptor cell bodies remained intact along the edge of the tissue block. It can be seen that ConA binding is much more intense below the OLM, which forms an abrupt barrier (Fig. 5). This discontinuous pattern argues against non-specific binding due to edge artefacts. The general pattern of heavy ROS labelling and slight RIS labelling is maintained through adulthood. Inclusion of the competing sugar hapten α-methyl-mannoside greatly reduced binding at all developmental ages examined (Fig. 6). Labelling of thin sections of adult rat retina revealed a uniform distribution of gold particles across the ROS (Fig. 7). Labelling of isolated bovine discs demonstrated heavy label on a single surface of much membranous material, with virtually no label on either surface of some membranes (Fig. 8).

Binding of Ricinus communis agglutinin II

At P1, RCA II showed moderate to heavy labelling of the developing photoreceptor surface (Fig. 9). Labelling intensity remained high over the following days and by P4, with the appearance of the connecting cilia, most exposed surfaces were heavily labelled (Fig. 10). There was again evidence of heavier binding in the region of the proximal cilium (Fig. 11). By P8, label was generally not visible on the RIS or proximal cilium, but was intense on the newly forming ROS (Fig. 12). Again, this pattern of heavy ROS and faint RIS label persisted through adulthood (Fig 13). Inclusion of the sugar hapten D(+)galactose reduced binding at all stages examined (Fig. 14).

Adult rat retina was also labelled using post-embedding thin section techniques in order to study the intracellular sugar distribution. The lectin was seen to have a highly localized binding pattern, visible only within the area of approximately the ten most basal discs (Fig. 15). To verify the absence of RCA II binding sites on the majority of discs, isolated and broken discs were incubated with the lectin. There was very little labelling of any of the disc material (Fig. 16).

Binding of wheat germ agglutinin

At P1, WGA was bound strongly to developing photoreceptor surfaces and Müller cell microvilli (Fig. 17). By P4 there was still heavy labelling of these surfaces, with an increase in labelling intensity often visible on the proximal connecting cilia (Fig. 18). By P7–8, heavy label was apparent on the ROS plasma membrane and Müller cell microvilli, with lower levels on the RIS surface (Fig. 19). This pattern persisted into adulthood with heavily labelled ROS (Fig. 20) and very low levels of RIS binding (Fig. 21). Presence of the inhibitor *NN'*-diacetylchitobiose reduced labelling in every case studied (Fig. 22).

Thin-section labelling of adult rat retina with WGA showed a general distribution of gold particles across the ROS (Fig. 23). Labelling of broken disc material exhibited label on only some surfaces of membrane (Fig. 24).

Binding of anti-rhodopsin monoclonal antibody RET-P1

At birth, it was difficult to distinguish structurally between immature RIS and Müller cell surfaces Antibody binding was seen on the surface of only 7% of structures present on the scleral retinal surface ($n = 100$), the majority of the tissue surface remaining unlabelled (Fig. 25). By P4, the RIS were clearly distinguishable from Müller cells and, of these, the proportion of labelled RIS had risen to 20–30%, with single (or occasionally two or three) strongly labelled structures separated by wider areas devoid of antibody labelling (Fig. 26). Ciliary binding usually appeared intense on the distal portion and faint to absent on the proximal section of the connecting cilia (Fig. 27). Concurrent with the emergence of the cilia

Fig. 1. P1 rat retina incubated in ConA followed by Av–Au_{14}–Dex. The developing photoreceptor surface is strongly labelled, but the particles do not penetrate past the outer limiting membrane (OLM). × 19400.

Fig. 2. P4 rat incubated in ConA followed by Av–Au_{14}–Dex. The RIS protrude from the photoreceptor cell bodies, and their plasma membranes are still strongly labelled. The connecting cilia (CC) have also emerged, and show heavy label on their surface. Remnants of the overlying pigmented epithelial cell microvilli (PE) are also labelled. × 19400.

Fig. 3. P4 rat retina incubated in ConA followed by Av–Au_{14}–Dex. In the majority of areas of the photoreceptor surface, the degree of labelling has already started to decrease. Labelling of the pigmented epithelial microvilli (PE) remains high. M = mitochondria; OLM = outer limiting membrane. × 23000.

Fig. 4. P7 rat retina incubated in ConA followed by Av–Au_{14}–Dex. The emerging ROS are heavily labelled, as are the distal portions of the connecting cilia (arrows). The RIS surface is faintly labelled. × 14600.

Fig. 5. P7 rat retina incubated in ConA followed by Av–Au_{14}–Dex. On favourable sections, the edge of the tissue remains undamaged and exhibits intense labelling of the cell body (CB) until the level of the OLM. An overlying Müller cell microvillus (MM) is strongly labelled, whereas the RIS arising from the labelled cell body is only faintly labelled. × 38000.

Fig. 6. Adult rat retina embedded in Lowicryl and processed for post-embedding thin section labelling. The section was incubated in ConA and α-methyl-mannoside followed by Av–Au_{14}–Dex. The ROS and RIS are both virtually free of gold particles. × 15000.

Fig. 7. Adult rat retina embedded in Lowicryl and processed for post-embedding thin section labelling. The section was incubated in ConA followed by Av–Au_{14}–Dex. Gold particles are visible scattered across the ROS but not the RIS. × 15000.

Fig. 8. Bovine disc membrane incubated with ConA followed by Av–Au_{14}–Dex. Label is visible on one surface of some of the membranous material (arrow). Unlabelled membrane presumably corresponds to discs folded with their interdiscal surface outermost, while the carbohydrate-bearing intradiscal surface is innermost and therefore inaccessible to the lectin. × 34000.

Fig. 9. P1 rat retina incubated in RCA II-Au_{12}–Dex. The developing photoreceptor surface is moderately labelled. M = mitochondria. × 16200.

Fig. 10. P4 rat retina incubated in RCA II–Au_{15}–Dex. The RIS surface is still substantially labelled. A connecting cilium (CC) cut in cross-section shows heavy label. A Müller cell microvillus (MM) also displays decoration by gold particles. G = Golgi body. × 24000.

Fig. 11. P6 rat retina incubated in RCA II–Au_5–Dex. The proximal connecting cilium exhibits strong label (arrow), whereas the more distal regions and the RIS surface express less binding. × 42500.

Fig. 12. P8 rat retina incubated in RCA II–Au_{12}–Dex. The heavy proximal connecting cilium label is no longer detectable, whereas the distal cilium (arrow) and newly formed ROS are strongly labelled. The RIS is weakly labelled. M = mitochondria. × 18000.

Fig. 13. Adult rat retina incubated in RCA II–Au_{15}–Dex. The ROS surface possesses a strong degree of label, whilst the RIS is virtually unlabelled. M = mitochondira; V = vesicles. × 15200.

Fig. 14. P9 rat retina incubated in RCA II–Au_{12}–Dex and D(+)galactose. The ROS, cilium and RIS are practically unlabelled. × 19500.

Fig. 15. Adult rat retina embedded in Lowicryl and processed for post-embedding thin section labelling. The section was incubated in RCA II–Au_{15}–Dex, and exhibits binding only in the vicinity of the basal ROS (arrowhead). The more sclerally positioned discs and the RIS are unlabelled. × 48000.

Fig. 16. Bovine disc membrane preparation incubated with RCA II–Au_5–Dex. There is no evidence of heavy labelling of any of the exposed disc membrane. The discs were treated exactly the same way as for Fig. 8. × 42500.

Fig. 17. P1 rat retina incubated in WGA–Au_{17}–Dex. The surface of the newly emerging RIS and Müller cell microvilli (MM) are both heavily labelled. × 14500.

Fig. 18. P4 rat retina incubated in WGA–Au_{17}–Dex. The proximal area of the connecting cilium (arrow) is more heavily labelled with gold particles than either the more distal cilium or the RIS from which it arises. Neighbouring RIS surfaces (arrowheads) express a higher degree of binding than the ciliated one. Pigmented epithelial microvilli (PE) are also strongly labelled. × 20000.

Fig. 19. P7 rat retina incubated in WGA–Au_{17}–Dex. RIS surfaces are only slightly labelled, whereas Müller cell microvilli (MM) and ROS are more heavily decorated with gold particles. A less advanced RIS expresses a high degree of labelling (arrow). × 17500.

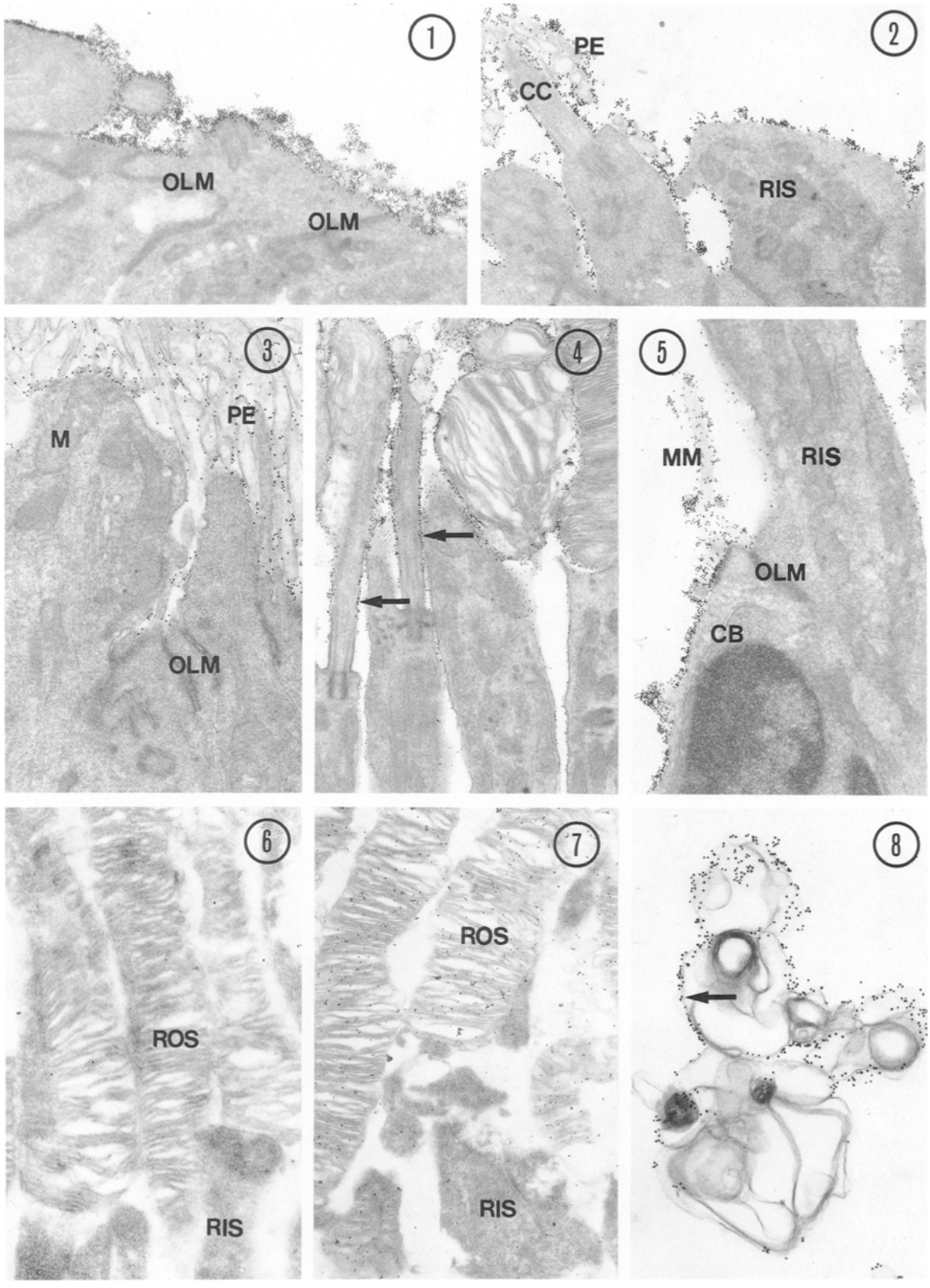
Fig. 20. Adult rat retina incubated in WGA–Au_{17}–Dex. The ROS are almost uniformly coated with gold particles. × 28000.

Fig. 21. Adult rat retina incubated in WGA–Au_{17}–Dex. The surface of the RIS is essentially unlabelled. × 28000.

Fig. 22. P9 rat retina incubated in WGA–Au_{17}–Dex and *NN'*-diacetylchitobiose. This control retinal tissue is unlabelled. × 16000.

Fig. 23. Adult rat retina embedded in Lowicryl and processed for post-embedding thin section labeling. The section was incubated in WGA–Au_{17}–Dex, and exhibits scattered label across the ROS but not the RIS. × 19000.

Fig. 24. Bovine disc membrane preparation incubated with WGA–Au_{15}–Dex. Some membranous material exhibits gold label on one surface only. Unlabelled membrane represents discs still oriented with their interdiscal faces outwards while the carbohydrate-bearing intradiscal face is innermost and hence inaccessible to the lectin. × 42500.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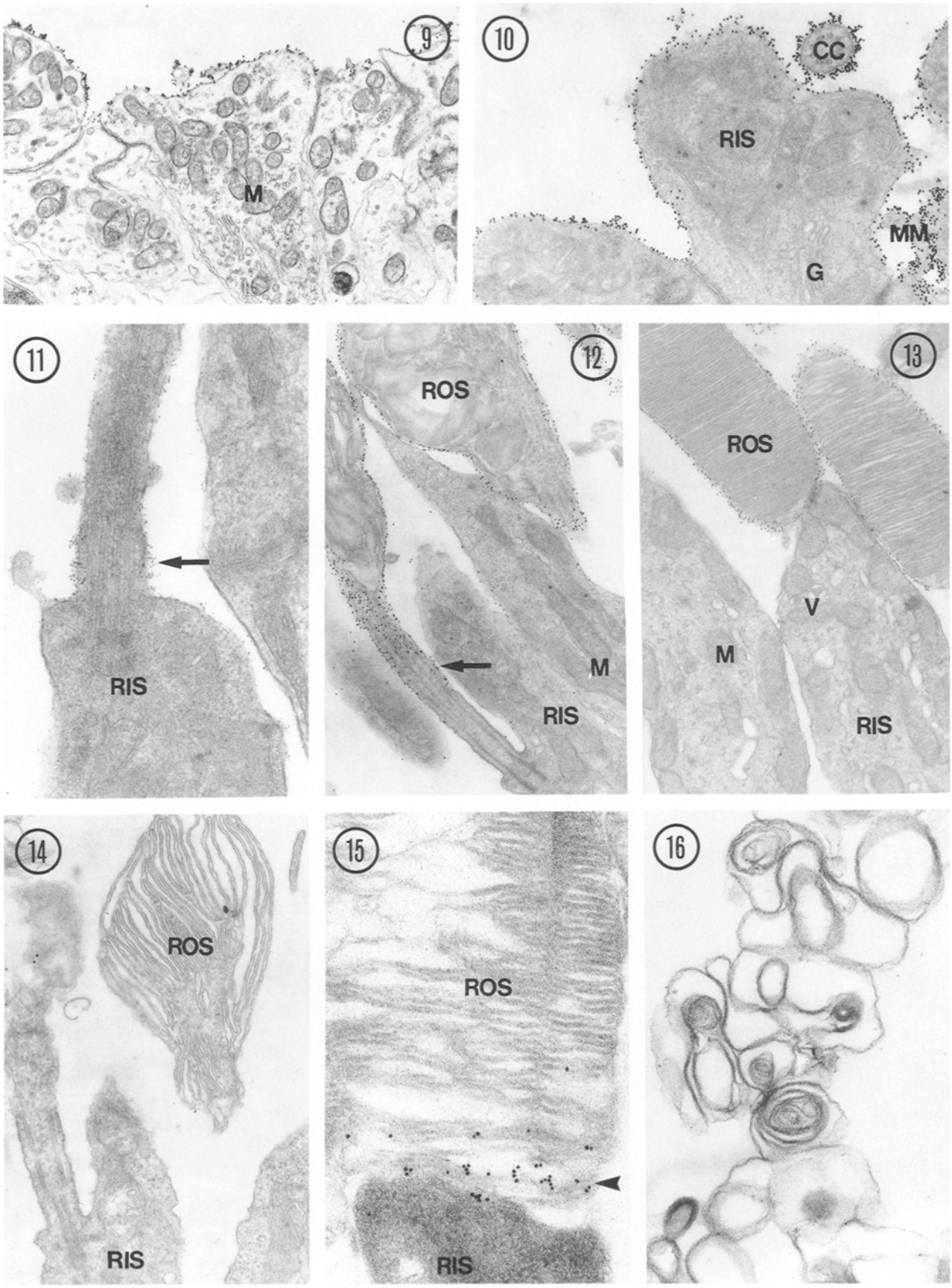
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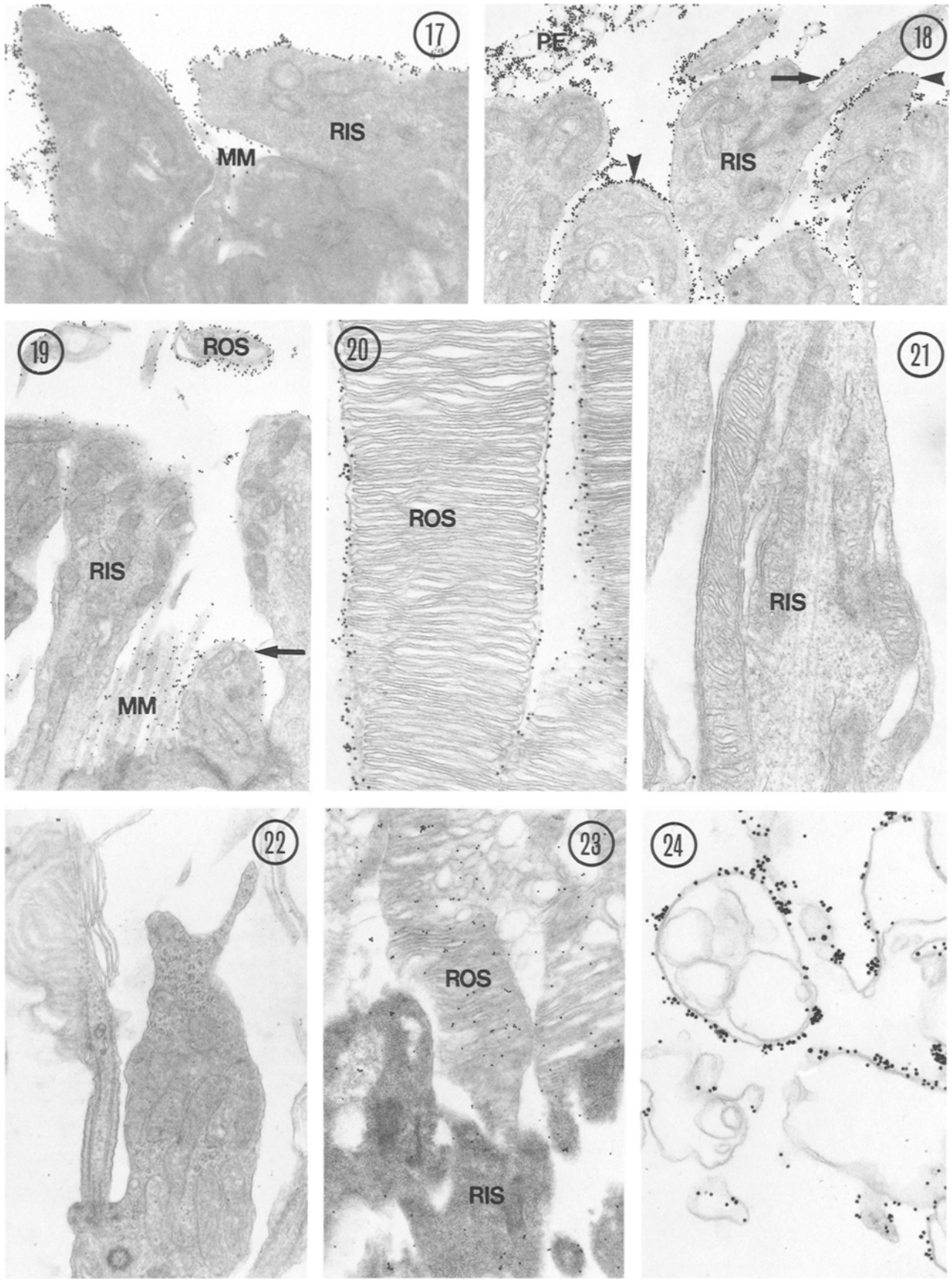
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the amount of RIS label was sometimes observed to decrease although this was not always the case.

By P5–6, the proportion of labelled RIS had risen to 60–70%. As ROS began to appear at P7–8, they exhibited intense label on their surfaces (Fig. 28). In the majority of cases the labelling of the cilia and RIS decreased to near background levels (Fig. 28); however, in some areas there was still substantial label on the RIS surface (Fig. 29). The adult pattern always showed a heavy ROS label and a very low level of binding to the RIS (Fig. 30). When retinal tissue was incubated in Tris buffer without RET-P1, or RET-P1 was replaced with rho-1D4 rhodopsin antibody, whose binding site is hidden on the cytoplasmic surface of the photoreceptor cell (Molday & MacKenzie, 1983), negligible labelling was observed. As expected, since most rhodopsin is sequestered to the ROS, indirect labelling of thin sections of adult rat retina with RET-P1 culture fluid followed by gαm Ig–Au–Dex revealed heavy uniform binding across the ROS domain (Fig. 31).

Discussion

The present paper compares the developmental appearance and distribution of a known membrane glycoprotein, rhodopsin, with the binding patterns of three lectins at equivalent ages. Although the developmental binding profiles of the three lectins and RET-P1 are similar in gross respects, they clearly differ upon fine comparison. Each of the lectins binds more heavily than RET-P1 at birth; the lectin binding is more continuous between adjacent RIS than RET-P1 at ages prior to ROS formation. The lectins have all been observed to exhibit more intense labelling of the proximal cilium whilst RET-P1 binding is normally absent from this region. As rhodopsin binds both ConA (Steinemann & Stryer, 1973) and WGA (Nicolson, 1974), and it forms > 95% of the ROS intrinsic membrane protein (Kuhn, 1982), one would expect a large subset of the lectin binding pattern to be due to rhodopsin. However, these results indicate that much of the observed lectin binding, particularly at birth and around the proximal cilium, must be due to alternative glycoproteins and glycolipids.

The developmental changes in rhodopsin distribution in rat retinal photoreceptor cells has been described previously (Nir *et al.*, 1984). The present study largely confirms and extends their observations on the initial presence of rhodopsin in the RIS plasma membrane followed by its preferential sequestration within the ROS upon the appearance of the latter. In the present study, rhodopsin was detected using RET-P1, a monoclonal antibody recognizing the N terminal region (Fekete & Barnstable, 1983). The discontinuous distribution of rhodopsin in neonatal rat retina indicated by antibody binding in the present study and previous investigations (Barnstable, 1982; Nir *et al.*, 1984) points to asynchronous initial expression of this protein. Birth dating experiments with mouse retinal cells show cessation of cell division within the photoreceptor cells during the first postnatal week (Sidman, 1961). This correlates with the observed scattered label and its increase towards P7, indicating that the appearance of rhodopsin may be related to the end of mitosis.

Although low levels of rhodopsin persist in the adult RIS plasma membrane (Jan & Revel, 1974; Fekete & Barnstable, 1983; Papermaster *et al.*, 1985),

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Fig. 25. P1 rat retina incubated in RET-P1 followed by gαm–Au_5–Dex. The surface is almost unlabelled except for a few gold particles. OLM = outer limiting membrane. × 21 000.

Fig. 26. P4 rat retina incubated in RET-P1 followed by gαm–Au_{12}–Dex. Two newly emergent RIS are heavily decorated with gold particles, whereas the other visible RIS surfaces are practically unlabelled. The cilium visible on one RIS is also unlabelled (arrow). M = mitochondria. × 19 400.

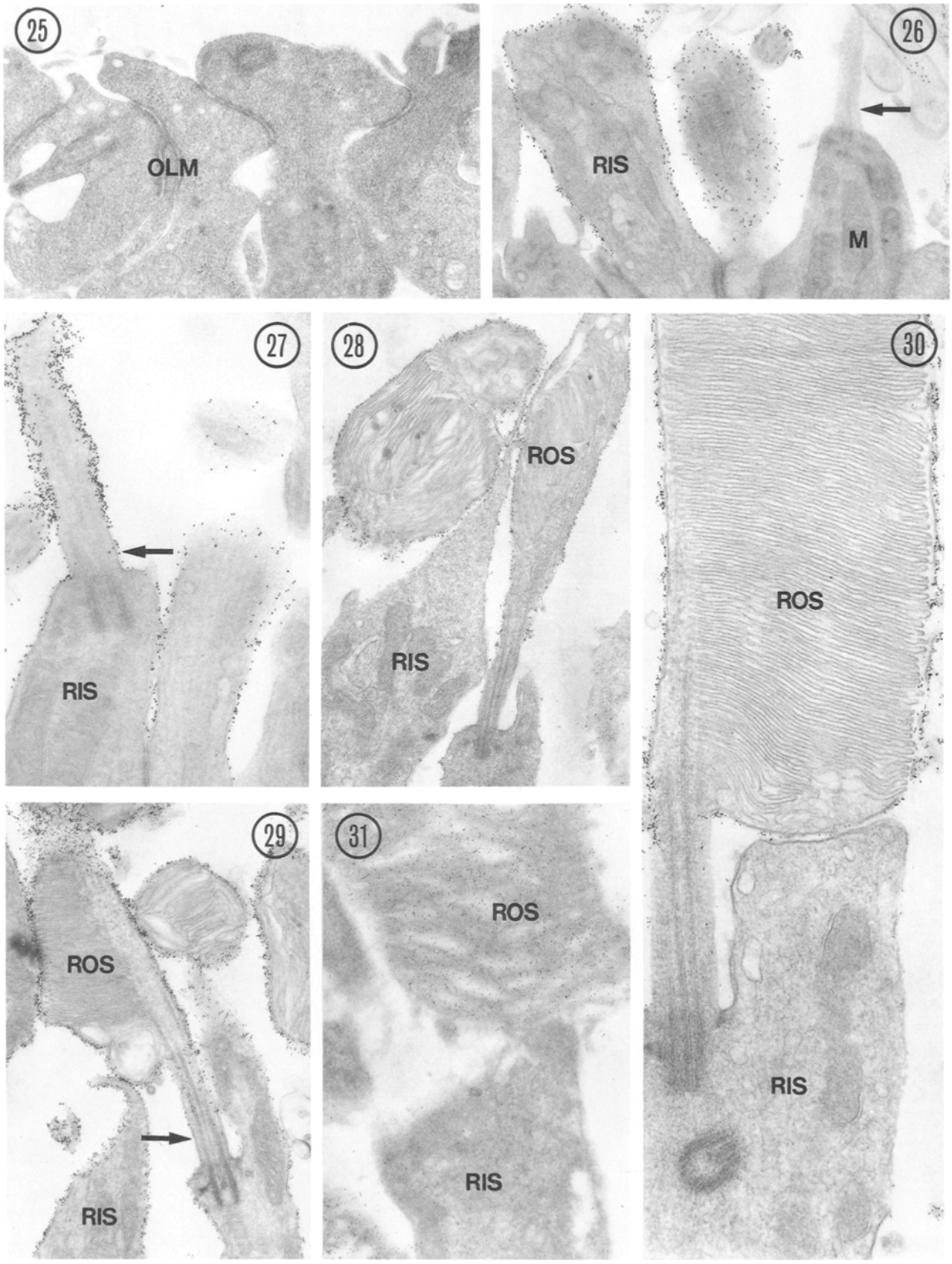
Fig. 27. P4 rat retina incubated in RET-P1 followed by gαm–Au_{12}–Dex. In this section, gold particles are faintly bound over the proximal connecting cilium (arrow), whereas the distal length is heavily coated with label. The RIS surface is slightly labelled, whereas the adjacent RIS is more heavily labelled. A third RIS is unlabelled. × 25 000.

Fig. 28. P7 rat retina incubated in RET-P1 followed by gαm–Au_5–Dex. The RIS surface is only faintly labelled whilst the newly formed ROS possess numerous gold particles on their surface. The proximal cilium is unlabelled. × 14 500.

Fig. 29. P7 rat retina incubated in RET-P1 followed by gαm–Au_5–Dex. In this area of the retina, there is still substantial label present on the surface of the RIS although the proximal cilium is unlabelled (arrow). The ROS expresses strong labelling. × 20 000.

Fig. 30. Adult rat retina incubated in RET-P1 followed by gαm–Au_5–Dex. The ROS plasma membrane is uniformly labelled down to the level of the cilium, which possesses hardly any label. The RIS is slightly labelled. × 70 000.

Fig. 31. Adult rat retina embedded in Lowicryl and processed for post-embedding thin section labelling. The section was treated with RET-P1 followed by gαm–Au_5–Dex, and exhibits uniform labelling across the ROS with very little label present in the RIS. × 30 000.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the large concentration difference of this protein between the ROS and RIS indicate the existence of a barrier to free diffusion. The possibility of such barriers between membrane domains has been postulated previously for photoreceptors (Besharse *et al.*, 1985; Papermaster *et al*, 1985) and other polarized cells such as spermatozoa (Myles *et al.*, 1984). The structural presence of this barrier may be indicated by the prominent glycocalyx at the base of the cilium demonstrated in the present work and previous studies (Besharse *et al.*, 1985; Hicks & Molday, 1985). The heavily glycosylated region at the base of the cilium has been described previously for rats (Besharse *et al.*, 1985) and cows (Hicks & Molday, 1985). Furthermore, the lectin labelling seen in the present study also partitions unequally between the ROS and RIS, and other membrane components segregated to RIS or ROS domains include a number of ion channels (Bader *et al.*, 1982) and Na^+K^+ ATPase (Stahl & Baskin, 1984). These data indicate the barrier may be relatively non-specific, preventing movement of large membrane constituents.

The method of incubating intact tissue in lectin or antibody solutions generally limits detection of label to the level of the OLM. However, with favourable sections the exposed tissue edge sometimes exhibits preserved tissue integrity. In these cases, at least two lectins (ConA and RCA II) showed heavier labelling of the cell body plasma membrane as compared to the RIS membrane distal to the OLM. It is possible that the complex array of gap junctions forming the OLM represent an additional barrier to movement of plasma membrane components, perhaps analogous to the tight junctional restrictions to plasma membrane diffusion seen in epithelial cells (Pisam & Ripoche, 1976). Preliminary electron microscopic observations suggest rhodopsin itself is present at greater concentrations in the mature cell body plasma membrane than within the RIS plasma membrane. Also, light microscope studies indicate its presence throughout the mature photoreceptor cell body layer both *in vivo* and *in vitro* (Barnstable, 1982; Barnstable *et al.*, 1983).

An interesting feature of RCA II binding is revealed by thin section labelling techniques which expose intracellular sites. Labelling is restricted to the basal area of the ROS, i.e. the region of new disc formation (Steinberg *et al.*, 1980). This highly localized distribution has been observed previously in other species, including monkey (Uehara, *et al.*, 1983) and cow (Hicks & Molday, 1985). In our hands at least, the thin section labelling method suffers from limited sensitivity since the RCA II binding to the ROS surface is not detected. Hence, to ensure there is not a lower level of labelling of more sclerally located discs, bovine ROS were disrupted and disc membrane labelled directly. Bovine sources were chosen in preference to rat due to the ready availability of large quantities of slaughterhouse material and the similarity of the two species at the cytochemical level. As very little RCA II binding was observed, in contrast to heavy ConA labelling, galactose-bearing determinants in the ROS must be restricted to or accessible only in the plasma membrane and basal area. As the disc membrane and plasma membrane are continuous at the basal foldings and the present study and previous biochemical analyses indicate differences between these two membranes (Kamps *et al.*, 1982), a mechanism for differentially sorting components of the two membranes can be envisaged. Although in many species cones bind galactose-specific lectins more strongly than rods (Uehara *et al.*, 1983; Blanks & Johnson, 1984; Hicks & Molday, 1985), at least in the rat this predominant RCA II binding of rod plasma membrane as opposed to disc plasma membrane may present a convenient method for purifying the former by affinity chromatographic techniques. The isolation of ROS plasma membrane is seen as a crucial step towards understanding the mechanism of photoactivation. Previous studies have suggested that a sub-population of rhodopsin may possess galactose residues (O'Brien, 1976; Papermaster, 1982). Biochemical studies are currently in progress to examine the nature of the galatose-bearing determinant in the retina.

The monoclonal antibodies and lectins used in this study have defined some of the developmental events in mammalian rod photoreceptors in intact tissue. The overall picture which emerges is the early neonatal presence of several glycoprotreins and/or glycolipids within the RIS plasma membrane, and their subsequent disappearance from or reduction within this domain concurrent with their appearance within the newly forming ROS. The mature photoreceptor is seen as a highly polarized cell with its plasma membrane divided into at least three (possibly more) domains. The use of tissue culture techniques, combined with monoclonal antibody markers to each class of retinal cell (Barnstable *et al.*, 1983), will extend this analysis to allow discrimination of those features of rod photoreceptor topography that are intrinsic to the cells from those that depend upon retinal integrity and cell interactions. Such studies are currently in progress.

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References

BADER, C. R., BERTRAND, D. & SCHWARTZ, E. A. (1982) Voltage-activated and calcium-activated currents studied in solitary rod inner segments from the salamander retina. *Journal of Physiology* **331**, 253–84.

BARNSTABLE, C. J. (1980) Monoclonal antibodies which recognise different cell types in the rat retina *Nature* **286**, 231–5.

BARNSTABLE, C. J. (1982) Analysis of retina and other neural tissues using cell-specific antibodies. In *Molecular Genetic Neuroscience* (edited by SCHMITT, F. O., BLOOM, F. E. & BIRD, S.), pp. 137–47. New York: Raven Press.

BARNSTABLE, C. J., AKAGAWA, K., HOFSTEIN, R. & HORN, J. P. (1983) Monoclonal antibodies that label discrete cell types in the mammalian nervous system. *Cold Spring Harbor Symposia on Quantitative Biology* **48**, 863–75.

BARONDES, S. H. & ROSEN, S. D. (1976) Cell surface carbohydrate-binding proteins: role in recognition. In *Neuronal Specificity* (edited by BARONDES, S. H.), pp. 331–51. New York: Plenum Press.

BEE, J. (1980) A cytochemical study of lectin receptors on chick neural retina neurons *in vitro*. *Journal of Cell Biology* **87**, 89a.

BEE, J. (1982) A cytochemical study of lectin receptors on isolated chick neural retina neurons *in vitro*. *Journal of Cell Science* **53**, 1–20.

BESHARSE, J. C., FORESTNER, D. M. & DEFOE, D. M. (1985) Membrane assembly in retinal photoreceptors. III. Distinct membrane domains of the connecting cilium of developing rods. *Journal of Neuroscience* **5**, 1035–48.

BLANKS, J. C. & JOHNSON, L. V. (1983) Selective lectin binding of the developing mouse retina. *Journal of Comparative Neurology* **221**, 31–41.

BLANKS, J. C. & JOHNSON, L. V. (1984) Specific binding of peanut lectin to a class of retinal photoreceptor cells. *Investigative Ophthalmology and Visual Science* **25**, 546–57.

BRIDGES, C. D. B. & FONG, S. L. (1979) Different distribution of receptors for peanut and ricin agglutinins between inner and outer segments of rod cells. *Nature* **282**, 513–15.

BRIDGES, C. D. B. & FONG, S. L. (1980a) Lectin receptors on rod and cone membranes. *Photochemistry and Photobiology* **32**, 481–6.

BRIDGES, C. D. B. & FONG, S. L. (1980b) Lectins as probes of glycoproteins and glycolipid oligosaccharides in rods and cones. *Neurochemistry* **1**, 255–67.

DOWLING, J. E. (1970) Organization of vertebrate retinas. *Investigative Ophthalmology* **9**, 655–80.

FEKETE, D. M. & BARNSTABLE, C. J. (1983) The subcellular localization of rat photoreceptor-specific antigens. *Journal of Neurocytology* **12**, 785–803.

FLIESLER, S. J., RAYBORN, M. E. & HOLLYFIELD, J. G. (1985) Membrane morphogenesis in retinal rod outer segments: Inhibition by tunicamycin. *Journal of Cell Biology* **100**, 574–87.

FRENS, G. (1973) Controlled nucleation for the regulation of the particle size in monodisperse gold suspensions. *Nature* **241**, 20–2.

FUKUDA, M. N., PAPERMASTER, D. S. & HARGRAVE, P. A. (1979) Rhodopsin carbohydrate: structure of small oligosaccharides attached at two sites near the NH2 terminus. *Journal of Biological Chemistry* **254**, 8201–7.

HARGRAVE, P. A. (1977) The amino terminal tryptic peptide of rhodopsin: a glycopeptide containing two sites of oligosaccharide attachment. *Biochimica et Biophysica Acta* **492**, 83–94.

HICKS, D. & MOLDAY, R. S. (1984a) Analysis of cell labeling for scanning and transmission electron microscopy. In *Science of Biological Specimen Preparation* (edited by JOHARI, O.), pp. 203–19. Chicago: SEM Inc.

HICKS, D. & MOLDAY, R. S. (1984b) Monoclonal antibody labeling of the retina. *Investigative Ophthalmology and Visual Science* (Suppl.) **23**, 56.

HICKS, D. & MOLDAY, R. S. (1985) Localization of lectin receptors on bovine photoreceptor cells using dextran–gold markers. *Investigative Ophthalmology and Visual Science* **26**, 1002–13.

HORISBERGER, M. & VONLANTHEN, M. (1979) Multiple marking of cell surface receptors by gold granules: simultaneous localization of three lectin receptors on human erythrocytes. *Journal of Microscopy* **115**, 97–102.

HOROWITZ, M. I. & PIGMAN, W. (1977) *The Glycoconjugates*, Vol. 1. New York: Academic Press.

JAN, L. Y. & REVEL, J. P. (1974) Ultrastructural localization of rhodopsin in the vertebrate retina. *Journal of Cell Biology* **62**, 257–73.

KAMPS, K. M. P., DE GRIP, W. J. & DAEMEN, F. J. M. (1982) Use of a density modification technique for isolation of the plasma membrane of rod outer segments. *Biochimica et Biophysica Acta* **687**, 296–302.

KUHN, H. (1982) Light-regulated binding of proteins to photoreceptor membranes and its use for the purification of several proteins. In *Methods in Enzymology* (edited by PACKER, L.), Vol. **81**, part H, pp. 556–64. New York: Academic Press.

LaVAIL, M. M. (1976) Survival of some photoreceptor cells in albino rats following long-term exposure to continuous light. *Investigative Ophthalmology* **15**, 64–70.

MCLAUGHLIN, B. J. & WOOD, J. G. (1980) The localization of lectin binding sites on photoreceptor outer segments and pigment epithelium of dystrophic retinas. *Investigative Ophthalmology and Visual Science* **19**, 728–42.

MOLDAY, R. S. (1976) A scanning electron microscope study of Concanavalin A receptors on retinal rod cells labeled with latex microspheres. *Journal of Supramolecular Structure* **4**, 549–57.

MOLDAY, R. S. & MACKENZIE, D. (1983) Monoclonal antibodies to rhodopsin: characterization, cross-reactivity and application as structural probes. *Biochemistry* **22**, 653–60.

MYLES, D. G., PRIMAKOFF, P. & KOPPEL, D. E. (1984) A localised surface protein of guinea pig sperm exhibits free diffusion in its domain. *Journal of Cell Biology* **98**, 1905–9.

NICOLSON, G. L. (1974) The interactions of lectins with animal surfaces. *International Review of Cytology* **39**, 89–190.

NIR, I., COHEN, D. & PAPERMASTER, D. S. (1984) Immunocytochemical localization of opsin in the cell membrane of developing rat photoreceptors. *Journal of Cell Biology* **98**, 1788–95.

NIR, I. & HALL, M. O. (1979) Ultrastructural localization of lectin binding sites on the surfaces of retinal photoreceptors and pigment epithelium. *Experimental Eye Research* **29**, 181–94.

O'BRIEN, P. J. (1976) Rhodopsin as a glycoprotein: a possible role for the oligosaccharide in phagocytosis. *Experimental Eye Research* **23**, 127–37.

PAPERMASTER, D. S. (1982) Preparation of retinal rod outer segments. In *Methods in Enzymology* (edited by PACKER, L.) Vol. **81**, part H, pp. 48–52. New York: Academic Press,

PAPERMASTER, D. S., SCHNEIDER, B. G. & BESHARSE, J. C. (1985) Vesicular transport of newly synthesized opsin from the golgi apparatus toward the rod outer segment. *Investigative Ophthalmology and Visual Science* **26**, 1386–404.

PISAM, M. & RIPOCHE, P. (1976) Redistribution of surface macromolecules in dissociated epithelial cells. *Journal of Cell Biology* **71**, 907–20.

ROBBINS, P. W. (1982) The processing of cell-surface glycoproteins. In *Molecular Genetic Neuroscience* (edited by SCHMITT, F. O., BLOOM, F. E. & BIRD, S.), pp. 161–9. New York: Raven Press.

ROTH, J. (1983) Application of lectin–gold complexes for electron microscopic localization of glycoconjugates on thin sections. *Journal of Histochemistry and Cytochemistry* **31**, 987–99.

SIDMAN, R. L. (1961) Histogenesis of mouse retina studied with thymidine-^{3}H. In *The Structure of the Eye* (edited by SMELSER, G. K.), pp. 487–506. New York: Academic Press.

SJÖSTRAND, F. S. (1953) The ultrastructure of the outer segments of rods and cones as revealed by the electron microscope. *Journal of Cellular and Comparative Physiology* **42**, 15–44.

STAHL, W. L. & BASKIN, D. G. (1984) Immunocytochemical localization of $Na^{+}K^{+}$ Adenosine Triphosphatase in the rat retina. *Journal of Histochemistry and Cytochemistry* **32**, 248–50.

STEINBERG, R. H., FISHER, S. K. & ANDERSON, D. H. (1980) Disc morphogenesis in vertebrate photoreceptors. *Journal of Comparative Neurology* **190**, 501–18.

STEINEMANN, A. & STRYER, L. (1973) Accessibility of the carbohydrate moiety of rhodopsin. *Biochemistry* **12**, 1499–502.

UEHARA, F., SAMESHIMA, M., MURAMATSU, T. & OHBA, N. (1983) Localization of fluorescence-labeled lectin binding sites on photoreceptor cells of the monkey retina. *Experimental Eye Research* **36**, 113–23.

WEIDMAN, T. A. & KUWABARA, T. (1968) Postnatal development of the rat retina: An electron microscopic study. *Archives of Ophthalmology* **79**, 470–84.

YOUNG, R. W. & DROZ, B. (1968) The renewal of protein in retinal rods and cones. *Journal of Cell Biology* **39**, 169–84.