During the mid 1960’s, Richard Young initiated a series of experiments that ultimately led to the concept of photoreceptor renewal. I was fortunate to have joined his laboratory as a graduate student at that time, and had the distinct pleasure of not only sharing his excitement as the story of photoreceptor renewal unfolded, but in participating as well in some of the renewal experiments.

General Principles of Rod Photoreceptor Renewal

Historical Aspects

The general principles of photoreceptor renewal are now known to vision scientist and clinician alike because of their importance to our current understanding of retinal function and in light of their profound clinical implications. For those who may have recently gained an interest in the retina or for readers in fields outside of vision, I will briefly describe the photoreceptor renewal process and some of the historical events involved in its discovery, because it serves as an essential backdrop for research that followed in the two decades thereafter.

The rod and cone photoreceptors (Fig. 1) are highly polarized cells consisting of the light sensitive outer segment, a robustly metabolic inner segment which is connected to the outer segment by a connecting cilium, a cell body with its nucleus, and a highly variable region, sometimes referred to as the fiber (if it is long), which connects the cell body to the synaptic terminal. Young and co-workers discovered by tissue autoradiography that rods and cones continually renew their components throughout life, thereby maintaining themselves in a perpetual state of youth. The most dramatic example of this process involves the renewal of the outer segment, a stack of light-sensitive, double membranes that are derived through constant growth of the plasma membrane by assembly of components that are, for the most part, synthesized in the inner segment.

Following systemic injection of radioactive amino acids into rats and frogs, Young observed that the majority of them were incorporated into proteins that are synthesized in the myoid region of the inner segment. Thereafter, the radioactive proteins scattered to various parts of the cell, but the majority made their way to the outer segment. The ensuing pattern of radioactive protein distribution in the rod outer segment was most striking. In frog rods, approximately 2 hr after injection of the animals with radioactive amino acids, many of the proteins rendered radioactive by that injection lined up at the base of the outer segment in a pattern perpendicular to its long axis. Over time (about 8 wk at 21°C, and in temperature-dependent fashion) this contingent of radioactive proteins moved from the base of the outer segment to its apex, all the while maintaining its concentration of radioactivity. Following their arrival at the interface between rod outer segment apex and the retinal pigment epithelium (RPE), the radioactive proteins disappeared from the outer segment. Young could not clearly discern the ultimate fate of these proteins in light microscopic autoradiograms of paraffin embedded tissue sections at that time, although he predicted that they were taken up by the RPE. He correctly interpreted the phenomenon of basal to apical movement of radioactive protein in rod outer segments as a process whereby the entire organelle is renewed, including its disc membranes. The basis of this interpretation will be further explained when outer segment morphology is discussed in more detail.

After making his initial observations on protein renewal in rods, Young took a sabbatical leave in the laboratory of Dr. Bernard Droz in France with the intent of learning the relatively new technique of electron microscopic autoradiography. This was a particularly appropriate collaboration because Droz himself had recently reported his own findings on protein turnover...
in rat photoreceptors, although he had not made the correlation between protein turnover in the outer segment and membrane renewal. With aid of the higher resolution afforded by autoradiography at the electron microscopic level, Young and Droz were able to document with great precision the organelles involved in the renewal process. Within the inner segment's myoid region, an area rich in endoplasmic reticulum and Golgi apparatus, radioactive proteins synthesized from their amino acid precursors first appeared in the rough endoplasmic reticulum (Fig. 2a) and subsequently many of them passed through the Golgi apparatus prior to their dispersal to other parts of the cell (Fig. 2b and 2c). Following their departure from the Golgi apparatus, the majority of them made their way to the outer segment, threading their way among the numerous mitochondria that occupy the ellipsoid region of the inner segment (Fig. 2c). The radioactive proteins ap-
peared to pause briefly at the base of the connecting cilium that links the inner segment with the light sensitive outer segment. Thereafter, they traversed the connecting cilium, entered the outer segment, and became incorporated into outer segment discs (Fig. 2d).

The process whereby these proteins were incorporated into disc structure was also accurately portrayed by Young and Droz. Nilsson had suggested a few years earlier that, during differentiation of the rod outer segment, discs are formed by successive basal invaginations of the outer segment plasma membrane such that the discs become stacked upon one another. (It has been subsequently demonstrated through a reexamination by Steinberg et al that the process is actually one of evagination rather than invagination, but the original concept of disc growth from basal membrane assembly is still correct, nonetheless.) As these immature discs are displaced apically by even newer ones, they mature by growing to their final diameter and by gradually losing their attachment to the plasma membrane. Young and Droz, armed with their autoradiographic observations, suggested that this process continues unabated even in the fully differentiated photoreceptor. Cohen and Nilsson had independently observed the persistence of immature discs in the mature rod outer segment and had suggested that some disc formation might persist into adulthood. Young recognized however that, should this be the case, one would have to account for the ultimate fate of these new discs, since the outer segments clearly reach a steady-state length.

Bairati and Orzalesi, reporting their study of the normal human retina a year before Nilsson's definitive work on frog photoreceptor differentiation, also provided early, incisive observations that held great significance for outer segment renewal. Their vantage point was, however, from the opposite end of the outer segment, namely at its interface with the RPE. These authors observed structures in the cytoplasm of the human RPE that resembled outer segment discs. They suggested that discs might be lost from the outer segment ends throughout life and added insightfully that, should this be true, a balanced addition of new discs would have to take place at the base of the outer segment in order to insure a constant outer segment length. Six years passed, however, before this perceptive observation was supported by experimental evidence. Others, both before and after Bairati and Orzalesi's publication, noticed lamellar structures in the RPE, but, without autoradiographic methods, it was difficult, if not impossible, to arrive at a conclusion regarding their origin.

Kuhne described structures in the frog RPE which he called myeloid bodies, and Porter later described their ultrastructure. However, prominent myeloid bodies are not observed in all species and, until recently, were thought not to be present in mammals. Thus, when the RPE was examined in mammals by electron microscopy, confusion arose with respect to the identification of myeloid bodies and other lamellar structures in the cytoplasm. Porter and Yamada clarified the issue by showing that there is a definite distinction between myeloid bodies, which they showed to be part of the smooth endoplasmic reticulum, and the other structures, which they called lamellated lipid bodies. Also, Dowling and Gibbons noted that the "myeloid bodies" of the rat RPE were different from myeloid bodies of lower vertebrates and that the former were enveloped by membrane. In addition, these inclusion bodies, as Dowling and Gibbons correctly called them, showed gradations in their ultrastructure from ordered to amorphous and disappeared during severe vitamin A deficiency but reappeared when deficient animals were returned to a diet containing vitamin A. Like Bairati and Orzalesi, Feeney et al also reported the sighting of structures in the human RPE resembling outer segment fragments. Finally, Kroll and Machemer observed that these lamellar inclusion bodies disappeared during experimental retinal detachment but reappeared when the retina was reattatched. Thus, given the impetus of Young's autoradiographic results, the stage was set for the collection of definitive experimental evidence that would finally bring together a cohesive and convincing case for the incredibly dynamic nature of photoreceptors heretofore thought to be rather static cells, and to include the RPE as another important actor in the story. It was at this point that I became involved in the subject of photoreceptor-pigment epithelium interactions.

Disc Shedding and the Phagocytic Role of the RPE in the Renewal Process

With the powerful technique of electron microscopic autoradiography in hand in Dr. Young's laboratory, we set out to examine in detail the disposal phase of the outer segment renewal process, namely events that occur at the outer segment RPE interface. Autoradiography demonstrated convincingly that frog rod outer segment discs, rendered radioactive by H-amino acids reach the apex of the outer segment, are detached and rapidly phagocytized by the RPE. We were able to verify this process directly because the radioactive discs could be followed as they moved from the rod outer segment into the RPE. They were marked in autoradiograms by a pattern of silver grains that followed the contour of the discs themselves (Fig. 3). Thus the process of "disc shedding," as it came to be called, and their internalization by the RPE could be followed with ease. Because the outer segment fragments within the
Fig. 2. Electron microscopic autoradiograms of frog rod inner and outer segments at selected intervals after the animals received $^{3}$H amino acid injections. All magnifications are $\times 13,000$. a, Fifteen minutes after injection. Polypeptide synthesis has begun in the rough endoplasmic reticulum as evidenced by the presence of silver grains. The Golgi apparatus (G) is not radioactive at this time. b, Thirty minutes after injection. The amount of radioactive protein in the rough endoplasmic reticulum has increased and the protein has begun its migration to the Golgi apparatus (G) which is now slightly radioactive. c, One hour after injection, the Golgi apparatus (G) is the most radioactive organelle in the inner segment. Transport of radioactive protein to the outer segment has now begun as indicated by silver grains in the ellipsoidal region (E). d, Six hours after injection. Most of the radioactive protein destined for the rod outer segment (ROS) has now been assembled into basal discs (arrows). At this time, the specific activity of rhodopsin reaches its maximum. (See Fig. 6.)

RPE were of extracellular origin and because they had been phagocytized by that cell layer, we called them phagosomes. More will be said about the phagocytic process later.

**Rhythmic Shedding in Rods**

A most exciting aspect of the disc shedding story came to light when LaVail$^{21}$ reported the rhythmic nature of disc shedding in rat rods whereby the majority of discs are detached at light onset. Indeed, it was shown that the event followed a light-entrained, free-running circadian rhythm, occurring even in continuous darkness at subjective light onset. The phenomenon of rhythmic shedding in rods is a feature of all vertebrate retinas examined thus far, although the event appears to be purely light-triggered in the leopard frog, *Rana pipiens* as shown by Basinger et al.$^{22}$ Conversely, the rod shedding event in the clawed frog *Xenopus laevis* has features common to *R. pipiens* and the rat.$^{23}$

Having established the fact that packets of rod outer segment discs detach and are phagocytized by the RPE, two important issues concerning disc detachment arose at that time: (1) What is the mechanism by which discs

Fig. 3. Electron microscopic autoradiogram of frog rods 8.5 wk after injection of $^{3}$H-amino acids. A packet of discs has detached from the rod outer segment (ROS) on the right and has been phagocytized by the retinal pigment epithelium (RPE). The origin of this phagosome (Ph) from this rod outer segment can be directly determined because it contains the pulse of radioactivity administered 8.5 wk earlier. The presence of the radioactive pulse is illustrated by the intense band of silver grains (long arrows in the phagosome). The rod outer segment on the left has not yet shed its most radioactive discs (short arrows) ($\times 13,000$).
detach; and (2) does a similar process occur in cones? The answer to the second question was quickly provided by several laboratories; the first question is still under exploration, although, as you will see, progress has been made.

**Cone Photoreceptor Disc Renewal**

For several years, there was uncertainty over the issue of cone disc shedding. When Young made his initial observations, he used rats and frogs in his experiments. Rats have very few cones (fewer than 5%), and the cone outer segments of frogs are very small. Thus, by light microscopy, patterns of silver grain distribution that might have given some clue regarding outer segment renewal in that cell type were impossible to interpret. When Young and Droz studied frog cone outer segments in electron microscopic autoradiograms, however, no discernible moving front of radioactive protein was present. Instead, the pattern of labeling appeared to be random once radioactive proteins reached the outer segment (Fig. 4). Furthermore, the half-life of the labeled proteins that did reach the cone outer segments was quite short (about 24 hr). The initial interpretation of this phenomenon was that cone discs ceased de novo assembly when the outer segment reached the fully differentiated state, and that their discs did not shed. It was thought that cone outer segment disc renewal might occur by a process in which individual molecules are replaced rather than entire disc membranes. However, when salamander larvae were injected with H-leucine, it was observed that differentiating rod and cone outer segments displayed the same autoradiographic patterns as their fully differentiated counterparts. A solution to this dilemma was soon to come through the observation by Poo and Cone, and Liebman and Entine that, due to their low viscosity, photopigments are highly mobile within the disc membrane lipid bilayer. Cone discs, through their mutual continuity (they do not pinch off from the outer segment plasma membrane during development and therefore do not become isolated from the extracellular space like rod discs), could provide an unbroken diffusion pathway from the basal to the apical discs, and thereby allow a random distribution of radioactive membrane proteins.

As far as the issue of disc shedding was concerned, Hogan et al and Anderson and Fisher provided convincing electron microscopic evidence that cones shed their discs just as rods do. Young and O'Day then made the exciting observation that the majority of cone disc shedding in several species
(Western Fence Lizard, goldfish and chicken) takes place soon after the onset of darkness, 180° out of phase with the major rod shedding event. In the tree squirrel most cone disc shedding occurs during the middle of the dark period. These observations suggested that there may be a general rule for rod shedding during the light period and a rule for cone shedding in the dark. Unfortunately, exceptions to these observations are increasing in number. Rod and cone shedding in the Rhesus monkey have been reported for both periods. In the cat, rod and cone shedding are concurrent with light onset. The cones of the tree shrew retina shed mostly during the light period. Nonetheless, as has been emphasized by both Young and La Vail, the time of day must be considered a variable along with other factors when photoreceptor metabolism is studied. In addition, the cell biologist is challenged to reveal mechanisms whereby cone outer segments remodel themselves after each shedding event; Since the most apical cone outer segment discs have a constant but smaller diameter than the basal ones in most species, how is this configuration re-established once the terminal discs are shed? Disc fluidity could be an important factor in this remodeling process, but cytoskeletal elements may play a role as well.

Proposed Disc Shedding Mechanisms

As implied earlier, mechanisms for photoreceptor disc shedding have been more difficult to document and understand than aspects relating to the physical phenomenon of shedding, and therefore research in this area, which requires a solution at the molecular level, has not made the progress that we may have hoped for. Nonetheless the first tentative steps have recently been taken.

What cell type is in control? Before discussing disc shedding mechanisms, it should be pointed out that we have been using the term disc shedding in an operational sense. In doing so, it has perhaps been implied that shedding is a process initiated purely by the photoreceptor. This, however is a subject which will require considerably more experimentation before such a treatment can be made with confidence. To state the problem succinctly, we don’t know whether the photoreceptors shed their discs independently while the RPE remains passive or whether the RPE “bites off” pieces of the outer segment. Spitznas and Hogan were the first to address this problem. The former authors who examined this process in the human retina felt that the RPE was the active partner. Their electron micrographs suggested that broad raptur-like cytoplasmic sheets from the apical surface of the human RPE protruded into the substance of the outer segment from all sides, thereby separating part of that organelle as a prelude to phagocytosis. Young, in his studies of the monkey photoreceptor-RPE interface, observed that the plasma membrane of the rod outer segment protruded inward, dissecting away groups of apical discs from their partners that remained behind, without apparent involvement of the apical RPE processes. Young’s data suggested that the incipient phagosome was well on its way toward being separated from the outer segment before the RPE became actively involved. Currie et al. while studying disc detachment in Rana pipiens noted that in infrequent cases, prolonged exposure to constant light (6 days) followed by a brief dark period (16 min) and then another 2 hr of light, caused disc membrane vesiculation at the point of disc detachment, as though the cell was creating a “perforated line” across the scission point of the outer segment. Thus, one could invoke evidence in favor of either the photoreceptor or RPE as the initiator of the response. As will become evident later, the truth may lie somewhere in between.

Control of Disc Shedding by Circulating and Central Factors

In his original experiments on circadian disc shedding in the rat, LaVail observed that the response was inhibited by reserpine, a drug that interferes with pineal function. It was initially thought therefore that shedding might be controlled by circulating humoral factors from the pineal. However, removal of the pineal complex or hypophysis or hypophysis, or thyroid-parathyroid in rats did not interfere with shedding. This strongly implied that circulating factors were not necessary for the response. Furthermore, transection of the optic nerve in frogs and in rats also left the shedding response intact, although it abolished phase shifting of the event under altered lighting schedules.

Effect of Altering the Light Period

A number of ingenious experiments were conducted to illustrate the striking effects of constant light or dark treatment on rod shedding. Basinger et al. were the first to show that shedding was suppressed in prolonged darkness (5 complete diurnal cycles), although some unsynchronized shedding and phagocytosis occurred as evidenced by phagosomes in various stages of digestion. Animals exposed to 2 hr of light after this prolonged dark period exhibited a large burst of shedding which involved 54% of the rods in Rana pipiens (the normal response in the standard light-dark cycle is 25%). Currie et al. utilizing the same species, observed that 10 days of constant light produced an inhibition of shedding even greater than constant darkness. During that period, the rod outer segments increased in
length by 58.7%. A 1-hr period in the dark, however, followed by re-exposure to light, produced a massive shedding response in which every rod outer segment shed packets of discs, some of them in multiples, so that the normal outer segment length was quickly restored. There followed a host of experiments from multiple laboratories in which animals of various sorts were exposed to unusual lighting regimens in order to manipulate the shedding response (for an excellent review, see Besharse). However, the definitive experiments by LaVail, Basinger et al, and Currie et al made the essential point, namely that, depending on the species, light is necessary for entrainment or triggering of the response, but darkness is required for disc shedding as well.

**Disc Shedding in the Isolated Eye**

A series of important experiments by Hollyfield and Basinger on *Rana pipiens* and Tierstein et al on *Rana esculenta* demonstrated that the control of disc shedding is an intrinsic feature of the eye. Eyes that were patched (occluded with an opaque substance) acted independently of those that were allowed to receive light. For example, a patched frog eye in an animal exposed to prolonged light did not show a massive shedding response when put through the dark-light sequence, whereas its unpatched mate did. The ultimate experiments demonstrating intrinsic ocular control of shedding was performed by Flannery and Fisher on the excised *Xenopus laevis* eye. These specimens were not only capable of a light-triggered shedding response but, like their in situ counterparts, displayed an endogenous rhythmicity when maintained in prolonged darkness, with bursts of shedding corresponding to the time of subjective light onset.

**Intraocular Factors that Control Disc Shedding**

Clearly independent of the need for circulating humoral factors for the control of disc shedding, the eye must therefore synthesize its own molecular triggers for this event. What might these triggers be, given the evidence that the eye is independent of the pineal gland, the traditional generator of rhythms in vertebrates? Investigators knowledgeable in the field of rhythm and melatonin have argued that this independence does not eliminate the possibility that substances of the type secreted by the pineal gland might be involved. Indeed, it has been known since 1965 that enzymes involved in the synthesis of the pineal hormone, melatonin, are present in the eye. The melatonin-synthesizing enzymes, N-acetyltransferase (NAT) and hydroxyindole-O-methyltransferase (HIOMT) undergo diurnal fluctuations of activity in the retina as do their respective products, N-acetylserotonin and melatonin. Thus, the stage was set for studies involving the effects of melatonin on disc shedding. What was needed very much at this point, however, was an in vitro system whereby the retina could be probed for the effects of hormones without systemic influences. This essential research tool was provided by Besharse et al in the form of a *X. laevis* eyecup preparation that supports the normal disc shedding response in terms of magnitude, phagosome size and time course. The eyecup, which lacks the anterior segment (cornea, iris and lens), can be readily exposed to and manipulated with experimental reagents. The key element in the development of this preparation was the use of a culture medium containing amino acids and a relatively high (30 mM) bicarbonate concentration. Interestingly, Besharse and Dunis found that the amino acids in the medium are responsible for maintaining good adhesion between the photoreceptor outer segments and the RPE. The high bicarbonate concentration may have some relationship to the fact that this ion hyperpolarizes the apical plasma membrane of amphibian RPE cells. How these factors combine to promote normal disc detachment is not known, but the required close association between photoreceptors and RPE implies that the RPE plays an active role in this process.

Besharse and Dunis discovered that melatonin, added to eyecups that were incubated in medium containing amino acids plus bicarbonate levels (25 mM) non-permissive for shedding, would support a normal, light-triggered shedding response. In addition, when incubated in medium containing permissive concentrations of bicarbonate ion (35 mM), eyecups from animals maintained in the light for 4 days were stimulated by exogenous melatonin to produce a shedding response equivalent to what one would see if those same eyecups were simply placed in the dark. Thus, melatonin is, in some undetermined fashion, capable of priming the shedding response. In light of earlier evidence that *X. laevis* rods exhibit a circadian shedding rhythm and inspired by earlier evidence that indoleamine-synthesizing enzymes undergo diurnal fluctuations in the retina, Besharse and Iuvone looked for circadian fluctuations of NAT in *X. laevis* eyecups. NAT activity not only displayed a circadian rhythm in eyecups from entrained animals, but could be rapidly phase reversed as well.

**Localization of Retinal Indoleamines**

Bubnen et al were the first to attempt to localize melatonin in the retina by immunocytochemical means. Both N-acetylserotonin, the precursor of mel-
atonin, and melatonin itself were localized in the outer nuclear layer of the rat retina by immunofluorescence. Bubenik et al. also reported that the amount of melatonin immunoreactivity increased in the dark. Vivian-Roels et al. extended these observations to the outer nuclear layer of the fish, tortoise, and hamster retina. These data, although encouraging, could not be used as proof that melatonin was in fact synthesized by the photoreceptors. It could be argued that photoreceptors contain melatonin receptors that mediate its uptake from some other cellular source. Furthermore, it had been shown earlier by Cardinali et al. that HIOMT, the terminal enzyme in melatonin synthesis, is retained and even enhanced in rat retinas exposed to green light (65 uwatts/cm²) for 17 days, a treatment that was assumed to cause destruction of the photoreceptor layer (although histological proof of this was not provided). It therefore became apparent that immunohistochemical localization of HIOMT would be most useful in clarifying the issue of indoleamine synthesis by photoreceptors.

Dr. Allan Wiechmann, then a graduate student working jointly in my laboratory and that of my colleague, Dr. Joseph Horwitz, raised an antibody to bovine pineal HIOMT in rabbits, and localized the enzyme immunohistochemically in the retinas of human, cow, rat, and Western Fence Lizard (Sceloporus occidentalis). In all of these species, including the Fence lizard with its cone-dominant retina, the photoreceptor inner segments, cell bodies, and synaptic terminals were positive for HIOMT. There was no outer segment labeling. Although photoreceptors were the most prominently-stained cells, there was, in addition, variable labeling of cells in the inner nuclear layer and in strata of the inner plexiform layer. This is consistent with reports of indoleamine-accumulating cells in the inner retina. Interestingly, although the photoreceptors and some of the inner retinal neurons were positive for pineal HIOMT antibody, the retinal protein responsible for this reactivity had an apparent subunit molecular weight of 25 kd as assessed by SDS-PAGE electrophoresis and staining of electrophoretically-transferred replicas of retinal extracts ("western blot analysis") whereas that of the pineal protein was a mixture of 25 and 39 kd subunits. One-dimensional peptide mapping showed, however, that the 25 kd protein is identical to a portion of the 39 kd protein. Currently, we do not know whether the 25 kd protein is a proteolysis product of the larger species or whether the 39 kd protein in the pineal is a precursor of the active form. Nonetheless, by virtue of their HIOMT content, it would appear that photoreceptors, both rods and cones, have the ability to synthesize indoleamines, as do some of the inner retinal neurons. We cannot state unequivocally that the product would necessarily be melatonin, since HIOMT is the terminal enzyme in two separate indoleamine biosynthetic pathways, that of melatonin and 5-methoxytryptophol, both of which are ultimately derived from serotonin. In addition, Besharse and Dunis have shown that 5-methoxytryptophol, like melatonin, is capable of activating disc shedding in X. laevis eyecups. On other hand, the demonstration of circadian fluctuations in activity for NAT, the rate limiting enzyme in the melatonin biosynthetic pathway, suggests that the HIOMT in the retina is involved in melatonin synthesis. Also, the retina is capable of synthesizing radiolabeled melatonin from radiolabeled serotonin.

If melatonin is synthesized by photoreceptors, what is the target cell for this hormone? Wiechmann et al. attempted to determine this by autoradiographic localization of 3H-melatonin binding sites. When isolated retinas of R. pipiens were incubated in 3H-melatonin, freeze-dried, and analyzed by autoradiography, specific binding was observed in the outer plexiform layer and the melanin granules of the retinal pigment epithelium. Parallel binding analysis of tissue homogenates indicated binding of 3H-melatonin to the membrane fraction of the neural retina and RPE melanosomes with dissociation constants in the mid-10⁻⁷ range. Unfortunately, 3H-melatonin binding was not high enough in the outer plexiform layer for localization by electron microscopic autoradiography, therefore we could not resolve the neuronal elements in the outer plexiform layer that bound the hormone.

Knowing that photoreceptors have the potential for synthesizing melatonin and having some notion concerning the sites for high affinity melatonin uptake has brought us a few steps closer to understanding the role of melatonin in photoreceptor function, but we still are lacking a clear picture regarding its role, if any, in disc shedding. Certainly, the action could be indirect. Dubocovich has found that melatonin inhibits dopamine release in the retina. Greenberger and Besharse have shown that uncoupling of photoreceptors from second order neurons with L-glutamate and L-aspartate causes massive disc shedding in X. laevis eyecups. The same authors observed that omission of Ca²⁺ from the eyecup medium inhibited shedding. Could melatonin action on photoreceptors be effected through some neuronal feedback pathway? This remains to be seen. Clearly, the higher levels of melatonin in the dark and the presence of melatonin receptors on RPE melanosomes are consistent with pigmentary migration into the RPE cell body in the dark. Also, Pierce and Besharse have reported that melatonin stimulates cone elongation in the light; in other words, it mimicks the normal dark response. Whether or not the cone
contraction that takes place in the light in some species has anything to do with disc shedding in this cell type also needs clarification. In fact, we’ve said virtually nothing about cone shedding because very little work has been done on shedding mechanisms in these cells.

Recent Evidence for the Role of Photoreceptors and RPE in Disc Detachment

At this point it would be appropriate to take up the issue once again of disc detachment mechanisms. As before, most of our information on this subject pertains to rods. It was suggested earlier in this discussion that both the RPE and photoreceptors may be required for detachment. Let us recall that Currie et al. observed occasional vesiculation of outer segment discs at the point of separation in R. pipiens when the animals were given prolonged, constant light exposure, followed by a very brief period in the dark (16 min) prior to their return to the light. It was as though some inherent instability in this part of the outer segment was displayed but, due to the brief light exposure, the dark processes required for shedding were not allowed sufficient time for the event to proceed to completion. Matsumoto and Besharse have observed that rod outer segments of the freshly isolated X. laevis retina become stained with fluorescent dyes at the point of incipient disc separation. These dyes, which do not cross the plasma membrane of the living cell, apparently enter some of the discs, causing single or multiple parallel bands of fluorescence near the apex of the outer segment. Since the stain did not diffuse within the outer segment, it was concluded that the stained discs must have become temporarily confluent with the extracellular space by fusing with the plasma membrane. Staining was never observed in more than 25% of the rods, but frequency could be manipulated with altered lighting schedules. The effect could not be produced in retinas that were isolated and incubated separately in culture medium prior to light exposure, suggesting that the RPE was necessary for induction of transient disc opening to the extracellular space. This again implicates the RPE as an active partner in priming of the detachment process.

Besharse et al. have shown that activation of shedding in X. laevis eyecups leads to the formation of large pigment granule-free pseudopods containing 7–8-nm microfilaments. These filaments probably contain actin, since they stain with rhodamine-labeled phalloidin, a toxin that is specific for filamentous actin. Disc detachment takes place as the pseudopods envelope the outer segment apex, but does not occur if cytochalasin D, an actin inhibitor, is present. However, the photoreceptor appears to contribute to the detachment process as well, because the cytochalasin treatment leads to disc vesiculation within the outer segment at the point of incipient separation, a phenomenon similar to that observed by Currie et al. Thus shedding appears to involve active processes in both the distal outer segment and the RPE cell.

Phagocytic Defects in the RPE

We have known for some time about a strain of mutant rats, the so-called RCS strain, in which the phagocytic process is defective. Herron et al. and Bok and Hall were the first to realize that the debris observed between photoreceptors and RPE of these rats, which carry the gene for retinal dystrophy (rdy), is due to a buildup of membranes derived from outer segment discs that are not phagocytized by the RPE (Fig. 5). With the use of rat chimeras, it was convincingly demonstrated by Mullen and LaVail that the cellular site of gene expression in this disease is the RPE. That is to say, phagocytosis fails, not because the outer segments lack some component that is required for their interaction with the RPE, but because the RPE is incapable of completing the phagocytic process. Mullen and LaVail performed in vitro fusion of the blastulae of rdy animals with blastulae of the wild type and reimplanted them into surrogate mothers. The RPE of the rdy parents that provided the blastulae is nearly devoid of melanin due to a pigment dilution gene whereas that of the wild type is heavily pigmented. Therefore, RPE containing the rdy gene could always be distinguished from RPE carrying the normal gene in chimeras. Without exception, lightly pigmented RPE had outer segment debris associated with it whereas pigmented RPE did not. It should be noted, incidently, that a small amount of phagocytosis does occur in rdy rats. This is observed both in cultured cells from rdy animals and in intact animals where a small burst of shedding and phagocytosis is observed at light onset.

It was assumed for some time that phagocytosis failed in rdy rats because of a lack of recognition of incipient phagosomes by the RPE. However, it has recently become apparent from the work of Chaitin and Hall on cultured normal and rdy RPE cells that recognition and attachment do take place, but that some undefined, crucial step is lacking between the processes of attachment and internalization. In the normal, cultured rat RPE, attachment of outer segment fragments is quickly followed by the assembly of filamentous actin “feltworks” on the cytoplasmic side of the RPE plasma membrane opposite the attached particle. In the rdy RPE, although outer segment fragments fed to the cultures attract in numbers equivalent to controls, feltworks rarely appear. On those occasions where they do appear, normal internalization follows. Thus, the contractile apparatus necessary for phagocytosis appears to be intact in these mutants, but it is rarely ac-
Phagosome Ingestion Processes

Incipient Phagosome Recognition

What is the signal that causes the RPE cell to begin its process of ingestion? This is a question that has remained unanswered. Although it is apparent that actin mediates the internalization of attached particles as long as the appropriate transmembrane signaling mechanism is in place, we still do not understand how the incipient phagosome is actually recognized by the RPE. O'Brien was the first to suggest that sugars on the surface of the outer segment plasma membrane, perhaps those bound to opsin, might serve as ligands that could be recognized by receptors on the apical surface of the RPE. An analogy might be drawn from the hepatocyte membrane receptor which recognizes and endocytoses blood glycoproteins that have lost their terminal sialic acid residues. This receptor recognizes galactose that has been exposed on the asialoglycoproteins and endocytosis follows. Similarly, the oligosaccharides of opsin in the outer segment plasma membrane could be altered in some way as a prelude to recognition. Although this remains an attractive hypothesis, evidence for receptor-ligand interactions between apical RPE and outer segments remains scarce. Several lectins show an affinity for outer segment plasma membranes, but differential binding to the apical outer segment in response to lighting conditions has not been observed. It has been shown, however, that phagocytosis in eyecups is inhibited by certain sugars that are known to be present in the outer segment glyocalyx, and it has been postulated that a mannose-sensitive rhodopsin binding protein is pres-

Fig. 5. Retinal pigment epithelium-photoreceptor interface in the dystrophic rat retina. a. Retina from an 18 day-old rat. Outer segment debris (D) has begun to accumulate between apical microvilli (arrows) of the retinal pigment epithelium (RPE) and intact rod outer segments (ROS) (X 13,000). b. Electron microscopic autoradiogram of a 16 day-old dystrophic rat retina 5 days after the animal received an injection of 3H-amino acids. Radioactive debris (D) of outer segment origin has begun to accumulate between retinal pigment epithelium (RPE) and intact rod outer segments (ROS). Some outer segments still contain their radioactive discs (arrows). (Modified, from Bok and Hall: J Cell Biol 49: 664, 1971 with permission of the authors and Rockefeller University Press) (X4,800).
ent on the apical RPE plasma membrane. Also, the RPE does show specificity for phagocytic targets. Polystyrene spheres are preferred over pasteurized bacteria. Damaged outer segment fragments injected into the subretinal space are frequently ignored by the RPE, perhaps due to the absence of a plasma membrane, whereas more intact fragments fed to cultures are avidly phagocytized. Indeed, outer segment fragments are the preferred target when a variety of particles are tested on cultured cells. Recent emphasis on the development of RPE cell cultures for the study of phagocytosis should aid considerably in future studies regarding recognition mechanisms.

Role of the Cytoskeleton in Phagosome Internalization and Transport

Internalization of incipient phagosomes occurs quickly and, as has been stated earlier, is apparently triggered by a poorly defined, transmembrane signalling process. This signal is required for marshalling of the contractile machinery that provides the motive force for uptake. Chaitin and Hall have suggested that subplasmalemmal actin is the contractile protein responsible for internalization, and Chaitin has supported this with evidence in cultured cells by inhibiting uptake with cytochalasin. Likewise, Besharse and Dunis have observed the same effect in X. laevis eyecups. Once inside the RPE, the phagosomes are transported into the interior of the cytoplasm where they interact with lysosomes and are degraded. This degradation must be quite rapid because the phagosomes are quickly reduced in size and cleared from the cytoplasm. It is thought that movement of phagosomes basally within the RPE cell is performed by microtubules. In support of this, Besharse and Dunis and Herman and Steinberg have observed that phagosomes are retained in the apical cytoplasm in the presence of colchicine, a microtubule inhibitor. Finally, primary lysosome formation and function appear to be impaired by this toxin as well.

Digestive Capacity of the RPE

Because of its formidable digestive capability, the RPE is able to handle a large phagocytic burden on a daily basis for the lifetime of its host. It has been estimated that a single rat RPE cell is in contact with and maintains the outer segments of 250–300 rods at a constant length. Approximately 25% of these rods shed roughly 100 discs per day which, added together, amount to about 7,500 discs per day for each RPE cell. This, combined with the burden contributed by the process of autophagy may explain why RPE cells suffer from the accumulation of lipofuscin in old age. The accumulation of lipofuscin is exacerbated by processes that go on in the outer segment. This organelle, by virtue of its high, unsaturated lipid content and high oxygen environment, is at considerable risk regarding lipid peroxidation and cross-linking. In fact, the removal of antioxidants such as Vitamin E from the diet of experimental animals is known to result in the accumulation of outer segment-derived lipofuscin in the RPE of monkeys and rats. To counteract these destructive processes, the outer segment is endowed with antioxidants that prevent the formation of free radicals which, if left to accumulate, would participate in the formation of peroxides such as malonaldehyde. Unsaturated fatty acids in disc phospholipids are cross-linked by these peroxides and rendered indigestible by RPE lysosomal enzymes. The rapid exchange of fatty acids in outer segment discs may protect against this process.

Inner Segment Events in the Biosynthesis of Rhodopsin

Having discussed the general subject of outer segment turnover and what is currently known about the process of disc shedding and phagocytosis, let us now return to the photoreceptor inner segment and the basal portion of the outer segment and describe what we have learned concerning rhodopsin biosynthesis and disc assembly in rods. Shortly before Dr. Young and I began our experiments on disc shedding, the late Dr. Michael Hall, a colleague of Dr. Heller's, to serve on my doctoral committee. This was a most timely opportunity for me. During the course of our association, as we discussed the exciting developments in the relatively new field of retinal cell biology, it became apparent that all of the tools for combining morphological and biochemical techniques for the study of rhodopsin biosynthesis and its intracellular transport were within our grasp. We therefore began our longer-term studies of these processes using autoradiographic and radiobiochemical methods.

Polypeptide Synthesis

Our initial strategy was to monitor the specific activity of rhodopsin as a function of time after a single systemic injection of H-amino acids into frogs. Thus, using Heller's column gel filtration methods, we followed changes in the specific activity of rhodopsin at selected intervals ranging from 15 min to 10.5 wk after injection. The specific activity curves (Fig. 6) followed...
faithfully, the autoradiographic patterns of intracellular protein migration in the inner and outer segments.\textsuperscript{1-3,124} The result indicated a highly atypical half-life curve for opsin.\textsuperscript{124} At early intervals between 15 and 60 min, there was very little radioactivity detected in outer segment opsin because, as was indicated by autoradiographic studies,\textsuperscript{1-3,124} it takes approximately 120 min for radioactive disc membrane proteins to travel from their site of synthesis on inner segment ribosomes to their site of assembly at the base of the outer segment (Fig. 2). At the same time that these proteins appeared in basal outer segment discs, there was a striking increase in opsin radioactivity (Fig. 6). Beyond that point, the rhodopsin half-life curve took on its most atypical appearance. By 6 hr after injection, rhodopsin specific activity achieved its maximum from the radioactive pulse of \textsuperscript{3}H-amino acids, and it maintained that maximum for 8.5 wk thereafter. At 8.5 wk, just as the rod outer segment discs were shed, there was a precipitous drop in rhodopsin specific activity (Fig. 6). Thus, it was clear that the stable, migrating front of radioactive opsin observed in autoradiograms included radioactive opsin. It also became apparent that opsin, once inserted into the disc membrane bilayer, was very stable. Degradation only began in conjunction with disc shedding and subsequent digestion by the retinal pigment epithelium.

### Diurnal Variations in Opsin Synthesis

Combined morphological, immunocytochemical and biochemical evidence suggest that the rate at which opsin is utilized in the assembly of new disc membranes varies with the lighting cycle in amphibians.\textsuperscript{23,125,126} With Dr. Brian Matsumoto, formerly a graduate student in my laboratory, we examined the rates at which \textsuperscript{3}H-amino acids were incorporated into nascent inner segment opsin and outer segment opsin. The specific activity of nascent opsin was analyzed by rocket immunoelectrophoresis of retinal microsomes and that of outer segment opsin by gel filtration chromatography. Inner segment opsin analyzed just after light onset was 13 times more radioactive than that analyzed during the late night hours.\textsuperscript{126} Opsin from the outer segment showed only a twofold increase in specific activity when equivalent time points were compared. Taken together, these experiments suggest that opsin synthesis proceeds at a relatively steady pace during the light-dark cycle, but that opsin accumulates within the inner segment during the night. When a radioactive amino acid pulse is incorporated into this relatively large inner segment pool of opsin at night, the rise in specific activity is quite low. Following light onset, however, the pool size is rapidly reduced due to accelerated disc assembly.\textsuperscript{23} When the radioactive pulse of amino acids is incorporated under these circumstances, the resulting specific activity is much higher. Significant pooling of opsin within the inner segment should be readily detectable by quantitative immunocytochemical methods. With Dr. Alan Bird, of the University of London, we are currently undertaking such experiments.

### Glycosylation of Opsin

Heller, in his initial purification and characterization of rhodopsin\textsuperscript{123} observed that this visual pigment is a
Fig. 7. Electron microscopic autoradiograms of frog rods from retinas incubated in $^3$H-glucosamine. a, Fifteen minute incubation. $^3$H-glucosamine is simultaneously incorporated into rough endoplasmic reticulum and Golgi apparatus (G). Linkage of preassembled oligosaccharides to growing polypeptides occurs in the RER. Oligosaccharide chain modification and completion takes place in the Golgi apparatus (X 13,000). b, Approximately 6 hr after incubation in $^3$H-glucosamine has commenced, most of the radioactive, glycosylated opsin is assembled into basal outer segment discs (arrows) (X13,000).

Glycoprotein. Heller and Lawrence$^{127}$ reported that all of the sugar residues are represented by glucosamine and mannose. Knowing therefore what sugars to use as precursors, we sought to follow the glycosylation of opsin with the methods that were used for analysis of opsin polypeptide synthesis. The general scheme that was developing at that time from studies of other cellular systems was that the rough endoplasmic reticulum and the Golgi apparatus are the glycosylation sites for proteins.$^{128-130}$ Due to the relatively small number of sugar residues reported for rhodopsin,$^{127}$ we used an in vitro labeling technique modified from O'Brien et al.$^{131}$ in an attempt to study glycosylation. Isolated frog retinas were incubated in $^3$H-glucosamine$^{132,133}$ for selected periods ranging from 5 min to 6 hr. Thereafter, tissue autoradiography was performed on some of the samples while rhodopsin was purified and analyzed for radioactivity in parallel samples. In confirmation of results reported for isolated bovine retinas by O'Brien and Muellenberg,$^{134}$ rhodopsin was readily labeled by this method. These authors, using biochemical methods, had shown a lag in rhodopsin radioactivity, when labeling due to $^3$H-leucine and $^3$H-glucosamine were compared. Opsin labeled with $^3$H-glucosamine appeared in outer segment discs shortly ahead of $^3$H-leucine-labeled opsin, consistent with the idea that events involving amino acid incorporation were at more proximal biosynthetic sites than glycosylation. Our electron microscopic evidence$^{132}$ suggested simultaneous labeling of the rough endoplasmic reticulum and Golgi apparatus in frog inner segment myoid cells, but we were initially perplexed by the fact that these organelles required about 20 min before exhibiting significant levels of $^3$H-glucosamine incorporation. Since nascent opsin was presumably present at both sites, glycosylation should have occurred in both organelles as soon as the precursor became available. The answer to this question rested in the fact that frog photoreceptors, no doubt like many cells, contain pools of UDP-N-acetylglucosamine (the activated form of the sugar) and other glycosylation intermediates that compete with the exogenous $^3$H-glucosamine. We were able to demonstrate this$^{133}$ by preincubating the retinas in 6-diazo-5-oxo-L-norleucine (DON), which blocks the synthesis of glucosamine-6-phosphate and all intermediates leading to and including UDP-N-acetylglucosamine by inhibiting the enzyme L-glutamine D-fructose-6-phosphate amidotransferase.$^{135}$ Exogenous $^3$H-glucosamine is able to bypass this enzyme blockage because glucosamine is phosphorylated to glucosamine-6-phosphate by a hexokinase. In the presence of this inhibitor, we achieved rapid and simultaneous incorporation of glucosamine in both the rough endoplasmic reticulum and Golgi apparatus (Fig. 7a).$^{133}$ Labeling of basal outer segment discs (Fig. 7b) was not observed until 2 hr of incubation in $^3$H-glucosamine had occurred in keeping
with earlier observations that this amount of time is required for transport of opsin from its sites of synthesis to its site of assembly into membranes.\(^{1,2,124}\) It was thus suggested by this study that opsin glycosylation begins in the rough endoplasmic reticulum but continues in the Golgi apparatus.

Heller and Lawrence\(^{127}\) had shown that rhodopsin oligosaccharide is bound to the polypeptide through an asparagine-glucoasamine linkage, but that additional glucosamine is more peripherally situated in the oligosaccharide chain. Their analysis indicated three glucosamine and three mannose residues. Plantner and Kean\(^{136}\) reinvestigated this subject and reported 9 mol of mannose and 5 mol of glucosamine per mol of opsin. Hargrave helped to clarify this issue by detecting a second glycosylation site on opsin.\(^{137}\) Finally, Fukuda et al\(^{138}\) determined the structure of the 2 oligosaccharides and showed that the composition at the two sites is comparable. The predominant form is (Man)\(^{3}\)- (GLcNAc)\(^{2}\), although microheterogeneity has been reported.\(^{139}\) The structure of the major oligosaccharide was shown by Fukuda et al\(^{138}\) to be:

\[
\begin{align*}
\text{GLcNAc} \\
M & M \\
M & \text{GLcNAc} \\
\text{GLcNAc} & \text{GLcNAc} \\
\text{Asn}
\end{align*}
\]

It was believed at one time that all protein glycosylations involved the sequential addition of sugars to the growing polysaccharide chain in the cisternae of the rough endoplasmic reticulum. However, this is not true for N-linked sugars. N-linked (asparagine-linked) glycosylation begins with the transfer of a preassembled oligosaccharide unit containing N-acetylglucosamine, mannose and glucose [(GLc)\(^{3}\)(Man)\(^{9}\)(GLcNAc)\(^{2}\)] by a lipid carrier.\(^{140}\) In the case of opsin glycosylation, the lipid carrier is the polyphenol lipid, dolichyl phosphate.\(^{141}\) Following the transfer of this oligosaccharide to the appropriate asparagine, the oligosaccharide is "processed" by specific glycosidases that remove the glucose and some of the mannose, and then add an additional N-acetylglucosamine in the terminal position. This is consistent with our observation that initial additions of glucosamine begin in the rough endoplasmic reticulum while the polypeptide is still being translated on ribosomes. Addition of the terminal glucosamine would then occur in the cisternae of the Golgi apparatus.

It should be noted that all of the evidence up to this time suggesting that rhodopsin moves through the Golgi apparatus was indirect, although perfectly logical and consistent with what was known for glycoprotein processing in other systems. Papermaster et al\(^{142}\) provided the final essential evidence by ferritin immunocytochemical localization of opsin in the Golgi membranes of frog photoreceptor cells. Initial localizations were performed in tissue that was embedded in glutaraldehyde cross-linked bovine serum albumin. Recent improvements brought on by hydrophilic cross-linked embedding resins (Lowicryl K4M and LR White, Polysciences, Inc., Warrington, PA) have allowed the choice of a variety of media for studies of this type (Fig. 8).

At this point, it is necessary to relate one more bit of important evidence regarding synthesis of the opsin polypeptide. In the mid 1970's, Blobel and Dobberstein\(^{143}\) reported that immunoglobulin G light chains are initially synthesized as precursor molecules which contain cleavable, hydrophobic peptides that precede the NH\(_2\) terminus of the mature protein. This phenomenon was observed in other secretory proteins as well. It was suggested that the hydrophobic peptide might direct polyribosomes actively synthesizing the protein to the membranes of the endoplasmic reticulum. The peptide, by virtue of its affinity for putative membrane receptors on the cytoplasmic surface of the RER and its hydrophobicity, was thought to enhance penetration of the nascent polypeptide through the hydrophobic interior of the RER membrane. Once the process of polypeptide penetration through the membrane was properly initiated, the hydrophobic peptide was shown to be cleaved inside the RER cisternae by specific peptidases.\(^{144}\) This scheme was termed the "signal hypothesis" by Blobel and Dobberstein,\(^{145}\) and the hydrophobic peptides, which vary among protein species (15–29 amino acid residues) but exhibit sequence homology, were called signal peptides. Schechter et al\(^{145}\) made the important observation that opsin does not contain such a signal peptide. Analysis of other proteins such as ovalbumin\(^{146}\) and the main intrinsic polypeptide (MIP) of lens fiber cells\(^{147}\) has shown that these diverse proteins lack signal peptides as well, so we are left without a unifying hypothesis regarding the manner in which nascent proteins are directed across the lipid bilayer of the RER. Blobel\(^{148}\) has recently modified the signal hypothesis to suggest that proteins lacking N-terminal signal sequences have comparable internal sequences that accomplish the same purpose. However, one is still confronted with the challenging problem of explaining how proteins such as opsin arrive at their final topology within the membrane. The complete amino acid sequences for bovine\(^{149–150}\) and human\(^{151}\) rhodopsin have now been produced. Based on its primary and secondary structure and by virtue of selected analogies with bacteriorhodopsin,\(^{152}\) it is popularly held that the opsin polypeptide traverses the
lipid bilayer seven times, each trans-bilayer portion being represented by a segment of alpha helix (Fig. 9). In situ proteolytic analysis and the use of lectin probes for the N-terminal oligosaccharides provide convincing evidence that the COOH terminus plus a considerable mass of the polypeptide protrude from the cytoplasmic (extradiscal) surface and that the N-terminal region with its two oligosaccharides protrude into the intradiscal (lumenal) space. The signal hypothesis does not provide an adequate explanation regarding the manner in which opsin and other proteins like it are threaded back and forth across the lipid bilayer during translation. More recently, other hypotheses have been offered suggesting methods of protein insertion into membranes that do not require signal peptides. Wickner has proposed that the folding patterns of proteins are triggered within the membrane by interactions between the hydrophobic interior and the primary structure of the polypeptide in question. Although this “trigger hypothesis” does not explain how a relatively hydrophilic N-terminus such as that of opsin is able to traverse the hydrophobic region of the lipid bilayer, it does offer a rather more satisfying mechanism for folding of other domains of the protein. The reader is referred to this author and others for further information on the subject of alternate mechanisms for protein insertion into membranes.

**Transport of Glycosylated Opsin from Inner to Outer Segment**

**Transport from Golgi Apparatus to Apical Inner Segment**

Our insights into mechanisms involving the transport of glycosylated opsin from the Golgi apparatus to
Fig. 9. Model of the rhodopsin molecule and its membrane topology as deduced from its amino acid sequence, its amount of alpha helical structure, the length of the polypeptide, and in situ proteolytic cleavage sites. Amino acids are indicated by their one-letter symbols. The rhodopsin molecule is believed to thread its way back and forth seven times across the lipid bilayer of the disc membrane. The carboxyl terminus (COOH) protrudes from the cytoplasmic side of the membrane whereas the acetylated amino terminus protrudes into the disc lumen. Glycosylation sites (y-shaped arrays of dots) near the amino terminus are shown in amino acid positions 2 and 15. The seven portions of the polypeptide that span the lipid bilayer are thought to be alpha helical. The lysine residue that binds 11-cis retinal is shown as an open circle in the alpha helix on the extreme right. (Modified from PA Baldwin, Doctoral Dissertation, University of California, Berkeley, 1983, with permission of the author and her graduate advisor, Dr. Wayne Hubbell).

the outer segment have relied heavily upon electron microscopic immunocytochemistry. The contributions of Papermaster and Schneider and their collaborators have been indispensable in this regard. To date, nearly all of the data on this subject has been gathered from amphibian rods and it is that story that will be presented here. It is not currently known whether the general principles of the process differ in mammals, but there is no compelling reason to believe that they do.

Papermaster et al. were the first to demonstrate that opsin remains in a water-insoluble membrane fraction throughout its transport from inner to outer segment. This biochemical evidence was supported by subsequent immunocytochemical studies employing ferritin antibody complexes as electron microscopic markers for opsin. Opsi-rich membranous vesicles bud off from the Golgi cisternae and make their way toward the apical inner segment by moving between inner segment mitochondria. Some planes of section suggest that they move in groups through cytoplasmic lanes (Fig. 10). They are transported in the general direction of the connecting cilium and then fuse with the plasma membrane in a specialized region consisting of a complex array of ridges and grooves called the periciliary ridge complex. The complex exhibits a ninefold symmetry which is in harmony with the 9 + 0 microtubule arrays of the connecting cilium. Freeze fracture analysis of this region reveals membrane features in common with the neuromuscular junction, and Andrews has suggested that this may be related to the fact that both the periciliary ridge complex and neuromuscular junction are sites of membrane fusion.

Transport across the Connecting Cilium

Once the opsin-laden vesicles fuse with the apical inner segment plasma membrane, the opsin is prevented from diffusing throughout the inner segment plasma membrane by some undetermined process. Instead, the opsin apparently diffuses in the plane of the connecting cilium's plasma membrane and from there to the distal cilium where disc elaboration takes place. Surprisingly, immunocytochemical studies show an abundance of opsin in the plasma membrane of the distal cilium with virtually none in the proximal region. Three possible explanations of this paradox have been offered. (1) The proximal ciliary region is the site of stable membrane components including parallel strands of intramembranous particles known collectively as the ciliary necklace. These intramembranous components occupy a significant amount of membrane volume and may thereby render the opsin density low in this domain. (2) Opsin antigenic sites in the plasma membrane of the proximal cilium could be masked by other membrane components, thereby giving only the appearance of a lower opsin density. (3) Finally, an alternate mechanism involving exocytosis of membrane vesicles from the apical inner segment plasma membrane, followed by vesicle fusion with the distal cilium cannot be discounted at this time. Whatever the mechanism, it is clear that opsin molecules must ultimately move against a concentra-
Fig. 10. Electron microscopic immunocytochemistry of opsin in the region of the connecting cilium of a frog rod. Sections of LR White-embedded tissue were stained as in Figure 8. Transport vesicles (arrows) rich in opsin are lined up below the connecting cilium (CC). They will ultimately fuse with a specialized region of the apical inner segment plasma membrane called the periciliary ridge complex. Rod outer segment (ROS) discs are heavily labeled (×50,000); courtesy of Dr. Alan Bird.

Armed with considerable knowledge concerning opsin biosynthetic sites within the inner segment, we can now draw a fairly accurate picture of the sequence of events that take place in the RER and Golgi cisternae with respect to opsin. The scheme that emerges is illustrated in Figure 11. Opsin, the rhodopsin apoprotein, is synthesized on polyribosomes that are attached to cisternae of the inner segment RER. Concomitant with this synthesis, glycosylation is initiated in the RER by the transfer of preassembled precursor oligosaccharides from dolichyl phosphate to asparagine residues 2 and 15 near the NH₂ terminus of the nascent opsin polypeptide. Trimming of some of the oligosaccharide residues and addition of other sugars takes place in the Golgi apparatus. The fully glycosylated opsin is then transported to the apical plasma membrane of the inner segment within the lipid bilayer of vesicles that fuse at special sites on the membrane. The opsin molecules probably make their way through the plasma membrane of the connecting cilium by translational diffusion and, thereafter, become structural components of developing and mature outer segment discs.

Thus far, we have said nothing about the site of addition of 11-cis retinaldehyde, the opsin chromophore. The reason, as will be explained in the following section, is that chromophore addition during rhodopsin biosynthesis is not an event that takes place in the RER or Golgi apparatus of the inner segment.

Chromophore Addition during Rhodopsin Biosynthesis

The issue of chromophore addition during rhodopsin biosynthesis was one that began to occupy our attention in the early 1970's, but took a great deal of time to resolve. When Dr. Michael Hall and I initially undertook these experiments, we were faced with two technical problems. We wanted to combine radiobiochemical analysis of ³H-retinol uptake with autoradiography as we had done for amino acids and sugars. What was lacking was a source of high specific activity ³H-retinol for injection into experimental animals. Furthermore, we were without a means whereby the ³H-11-cis retinal produced by the retina from this precursor might be retained in its natural binding site during the histological procedure, which employs organic solvents and other reagents that cause hydrolysis of the labile Schiff base linkage between retinal and opsin. Low specific activity ³H-retinyl acetate was available from F. Hoffman La Roche in Switzerland, however, and while initiating some biochemical experiments with the ester,
Fig. 11. Scheme for opsin biosynthesis, glycosylation and transport from the rough endoplasmic reticulum to the outer segment. Polypeptide synthesis begins on the rough endoplasmic reticulum (RER). At that point, preassembled oligosaccharides are N-linked through glucosamine and asparagine on amino acid positions 2 and 15. Opsin is illustrated as a series of alpha helices with the carboxyl terminus on the cytoplasmic side of the respective membranous organelles that carry it and the glycosylated amino terminus on the lumenal side. Only three alpha helices are shown for convenience. The rhodopsin icons are modified from Fung and Hubbell: (Biochemistry 17:4403, 1978, with permission of the authors and the American Chemical Society). Vesicles (CV) pinch off from the RER and fuse with cis-ternae of the Golgi apparatus where the oligosaccharides are first trimmed and then edited to their final form. Transport vesicles (TV) carry opsin to a special region near the connecting cilium where they fuse with the plasma membrane. Presumably, the opsin then diffuses in the plane of the connecting cilium and thereafter becomes an integral part of a growing outer segment discs.

we asked F. Hoffmann La Roche to prepare high specific activity retinol for us. They graciously agreed to provide us with $^3$H-retinoic acid (~1 Ci/mM) which is less susceptible to autoradiolysis than retinol during storage and which was internally labeled on the 11th carbon atom to avoid tritium exchange. We converted this $^3$H-retinoic acid to $^3$H-retinol by reduction prior to each experiment and were able to use it for autoradiographic studies which I will describe shortly.

Kinetics of Rhodopsin Labeling with $^3$H-Retinyl Ester

Our biomedical experiments with $^3$H-retinyl acetate provided surprising information that was difficult to interpret in the absence of autoradiographic studies. Rhodopsin labeled by systemic injection of $^3$H-retinyl acetate and purified by column chromatography exhibited labeling kinetics that were quite similar in both dark and light-adapted frogs killed at selected periods up to 8 wk after injection. For both lighting conditions, rhodopsin specific activity rose gradually and became asymptotic at about 7 wk. We had expected dramatic differences in specific activity between the rhodopsins from light and dark adapted animals since we assumed at that time that incorporation of 11-cis retinal in the dark occurred only through de novo biosynthesis of rhodopsin. We reasoned that rhodopsin from light-adapted rods at some steady state of bleaching and regeneration (~50% under our lighting con-
they are hydrolyzed to retinol and secreted as a complex with a reducing agent with several important properties. (1) It had to work on unbleached opsin; (2) the reducing agent had to be stable and functional at low pH (1.5) in order to prevent transiminization of retinal among neighboring amino groups, including those of amino phospholipids; and (3) it had to be amphipathic in order to allow simultaneous solubility in aqueous media and the hydrophobic lipid bilayer of the outer segment disc membrane. Borane-dimethylamine (BDMA) fulfilled all of these requirements.172

High specific activity \(^3\)H-retinol was injected systemically into dark and light adapted \textit{R. pipiens}, then tissues were exposed to BDMA under red light and subsequently processed for autoradiographic analysis.133 As expected, light adapted rods had a random distribution of silver grains over their outer segments due to the steady state bleaching conditions under which they were maintained. However, dark adapted rods from animals killed at selected periods from 30 min to 3 wk after injection also had a heavy distribution of silver grains over discs that were assembled prior to injection. Discs assembled after injection appeared to be somewhat more heavily labeled than the others. The biochemical and autoradiographic data now began to make sense. We began to suspect that 11-cis retinal is capable of exchanging among opsin molecules in the dark. Given the proper exchange rate, this could account for the earlier observation that the specific activity of rhodopsin rose nearly as rapidly in dark adapted rods as it did in light adapted ones.168 Another significant observation was made from our autoradiograms. \(^3\)H-retinal was not incorporated into opsin in the inner segment (Fig. 12).

**Rhodopsin Chromophore Exchange in the Dark**

We had to further assure ourselves that every precaution had been taken to avoid artifacts in these experiments. With Dr. Dennis Defoe, a graduate student in my laboratory at that time, the studies were extended along with several important refinements. First, the frogs were injected intravenously with \(^3\)H-retinol bound to its natural carrier protein, plasma retinol binding protein (RBP). In earlier experiments we had used a mixture of the detergent Tween 80, ethanol and bovine serum albumin as a vehicle for retinol injection.173 RBP is secreted by the liver as a complex with retinol174,175 and, as will be discussed later, the RPE has, on its basolateral plasma membrane, specific membrane receptors for RBP that mediate the uptake of retinol from the blood.176 Thus, we presented the retinol in physiological form to its target cells. In addition, all handling of dark adapted animals and processing of tissues were performed under infrared illumination rather than red safelights in order to avoid all chances of bleaching rhodopsin and translocation of the label. Finally, the experiments included mice as well as frogs in an attempt to document chromophore-exchange in another species. We were also interested in obtaining an approximation of the exchange rate and felt that the mouse retina would allow the introduction of a sharper pulse of radioactive retinol since, unlike the frog, the mouse RPE does not contain large storage droplets of retinyl ester within its cytoplasm. The results from these experiments clearly indicated that the rhodopsin chromophore exchanges among opsin molecules in the dark.177 As expected, the rate of incorporation of \(^3\)H-retinal into mouse rhodopsin was more rapid than in the frog. Incorporation into mouse rhodopsin in the dark was observed less than 30 min after injection (it takes over 2 hr for synthesis of opsin and its transport to the outer segment in the mouse), and we were able to compute an exchange rate of 1.77 \(\times\) 10\(^3\) molecules/min/rod. This is without question a lower limit value since we learned later that the mouse RPE, in spite of its lack of lipid droplets, does in fact contain significant stores of retinyl ester (Dr. CD Bridges, personal communication) and this nonradioactive pool of retinoids would serve to dampen the pulse of exogenous \(^3\)H-retinol. Nonetheless, we concluded that the observed exchange is probably not due to thermal isomerizations...
Fig. 12. Electron microscopic autoradiograms of dark adapted frog rods following injection of the animal with \(^3\)H-retinol complexed with plasma retinol binding protein. a, Due to dark exchange of \(^3\)H-11-cis retinal, labeling of the rod outer segment (ROS) is diffuse, unlike the labeling pattern observed when \(^3\)H-amino acids or \(^3\)H-glucosamine are used as precursors. The Schiff's base linkage between 11-cis retinal and opsin was reduced to a single bond in order to prevent translocation of the label by organic solvents during tissue processing (×13,000). b, Rod photoreceptor from the same retina as that shown in Figure 12a, except that exhaustive extraction of lipids was performed following reduction of the Schiff's base. Although rod outer segment (ROS) structure is severely damaged due to the extraction, the stability of the \(^3\)H-retinal label is apparent from the silver grain density. The absence of label in the ellipsoid (E) and myoid (M) regions of the inner segment indicates that there is no covalently-bound retinal in this portion of the cell. In other words, all chromophore incorporation takes place in the outer segment (×13,700). Courtesy of Dr. Dennis Defoe.

of 11-cis retinal. Baylor et al.\(^{178}\) experimentally determined dark noise in toad rod outer segments (which they believe is caused by thermal isomerizations) to be only one event per 50 sec at 20°C. Thus, even if these values are corrected for the higher body temperature of the mouse, the exchange rate exceeds that of...
that similar mechanisms are operative in the retina. It is mediated by exchange proteins in other tissues, and are particularly prone to damage by oxygen radicals. Present in high amount in outer segment phospholipids are particularly prone to damage by oxygen radicals. Bibb and Young found that H-fatty acids are replaced individually, independent of whole phospholipid exchange. The exchange of phospholipids is mediated by exchange proteins in other tissues, and Dudley and Anderson have recently demonstrated that similar mechanisms are operative in the retina. It may very well be that the active processes of retinal and fatty acid exchange are also mediated by exchange proteins.

Retinoid Transport from the Blood to the Retina

The final aspect of this lecture deals with the subject of the transport of retinol and its derivatives, the retinoids, into and within the retina. We will focus specifically on the subject of retinoid binding proteins and their proposed role in the delivery of their ligands from the blood to the retina. This is currently a rather active field, and many of the details regarding purification and other aspects of the characterization of these proteins can be obtained from recent reviews. I will deal primarily with immunocytochemical and autoradiographic evidence for the compartmentalization of retinoid binding proteins within the retina and will then speculate on the proposed function of the retinoid binding proteins in this tissue.

RPE Membrane Receptors for Plasma Retinol Binding Protein

In 1975, Dr. Joran Heller and I began collaborations on the subject of retinol delivery from the blood to the retina. We turned our attention at that time to plasma retinol binding protein (RBP) which, as stated earlier, is secreted by the liver and circulates as a tight 1:1 complex with transthyretin. The 1:1 retinol-RBP complex is, in turn, bound in 1:1 ratio to transthyretin (TTR), formerly known as prealbumin. This protein-protein interaction probably promotes the conservation of circulating RBP, which has a molecular weight of only 21,000. The relatively low molecular weight of RBP renders it readily filterable by the kidney glomerulus. The TTR association gives the complex an effective molecular weight of about 75,000, which is far above the cutoff size for significant glomerular filtration. We suspected that RBP might interact in a very specific manner with target tissues that have a high requirement for retinol. We labeled RBP with iodine and then exposed RPE cells to it by incubating detached sheets of them in the labeled binding protein by injecting the radioactive complex intravenously into rats or by injecting the complex into frogs. Taken together, autoradiograms of these preparations clearly suggested specific membrane receptors for RBP on the basolateral membrane of the RPE cells (Fig. 13). Data from isolated cells also indicated that the affinity of holo RBP (that which contains its ligand) for its receptor was higher than that of apo RBP (that which lacks its ligand). This variation in affinities probably provides a mechanism whereby the RBP dissociates from its receptor after retinol is released into the cell, since the higher affinity holo RBP would have the capability of displacing apo RBP. On the basis of autoradiographic analysis and displacement studies, RBP does not appear to enter the cell during its delivery of retinol. However, in light of our current knowledge about other membrane receptor systems, this process should be further explored. Serum glycoproteins such as orosomucoid and transferrin that have had their terminal sialic residues removed (asialoglycoproteins) bind to hepatocyte membrane receptors. This receptor-ligand complex is rapidly internalized and then dissociated into separate intracellular compartments. The receptor is recycled back to the surface. Autoradiography lacks the resolution for the detection of a process of this type. Therefore electron microscopic immunocytochemistry of RBP or its receptor using ferritin or colloidal gold as markers would provide the necessary resolution, and these studies should be performed in the near future.

Having entered the RPE cell, trans retinol is ready to begin its passage through the visual cycle. Following the discovery of an intracellular retinol binding protein by Bashor et al in other tissues, it became apparent that retinoid binding proteins could play a role in the visual cycle as well. Because of the very low solubility of retinol in water (about 1 nmol) and the susceptibility of retinoids to oxidation, participation of specific binding proteins in the visual cycle was almost a foregone conclusion. In fact, Hubbard and Coleman, in 1959, stated the necessity for such transport vehicles in the retina well before that, Baumann et al postulated a role for binding proteins in the liver.

Ocular Retinoid Binding Proteins-Historical Aspects

Before beginning a description of the immunocytochemical localization of each of the retinoid binding proteins and their putative roles, I would like to briefly
describe each of them in the historical context of their discovery in the retina. For the reader's convenience Table I provides a list of the binding proteins and some of their properties. The first laboratories to begin an exploration of ocular retinoid binding proteins were those of Chader and Wiggert,191-197 and Saari and Futtermann.198 199 Chader, Wiggert and collaborators incubated cytosol from the neural retina and RPE-choroid with 3H-retinoids and subjected these cytosols to sucrose density centrifugation. By liquid scintillation counting of gradient fractions, these authors detected two binding proteins in the neural retina. One was small, with a sedimentation coefficient of 2S191 and the other a rather large 7–8S protein.194 The 2S protein was similar to a retinoid binding protein from testis and liver called cellular retinol binding protein (CRBP),188,200 which is specific for trans retinol. The 7–8S species bound trans retinol as well, but was far less specific. Subsequent studies have shown that the 2S proteins from retina, testis, and liver are identical.199,200 The molecular weights are about 16,600, and the proteins are distinct from plasma RBP. The 7–8S binding protein from neural retina has turned out to be a large glycoprotein which was recently purified from interphotoreceptor matrix by Adler and associates.201,202 Lai et al203 have named this protein interphotoreceptor retinol binding protein, whereas Liou et al204 have called it interstitial retinol binding protein (IRBP). However, this protein contains many endogenous hydrophobic ligands,204 206 as you can discern from Table I. Indeed, it has some of the same nonspecific properties as serum albumin, a protein that binds and transports many hydrophobic molecules such as fatty acids and retinoic acid. In addition to its large number of ligands, this binding protein is unusual in light of its relatively high molecular weight of approximately 130,000,207,208 because it is a glycoprotein and because it carries 2 mol of retinol per mol of protein.209 Wiggert and co-workers191,192,196 found only the retinol-specific 2S (CRBP) species in the RPE. Futtermann, Saari, and collaborators, however, purified two additional retinoid-binding proteins from the retina, one exclusively from neural retina and one from both neural retina and RPE-choroid. Cellular retinoic acid binding protein (CRABP), which was first described by Ong and Chytil in rat testis and other tissues210 was purified from bovine neural retina by Saari et al.199 Its molecular weight was estimated at 16,300, very similar to that of CRBP. These two proteins, although distinct in terms of their endogenous ligands, have considerable
Table 1. Properties of Retinoid Binding Proteins

<table>
<thead>
<tr>
<th>Retinoid binding protein</th>
<th>Molecular weight</th>
<th>Endogenous ligand</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma retinol Binding protein (RBP)</td>
<td>21,000</td>
<td>t-retinol</td>
</tr>
<tr>
<td>Cellular retinol Binding protein (CRBP)</td>
<td>16,600</td>
<td>t-retinol</td>
</tr>
<tr>
<td>Cellular retinoic acid Binding protein (CRABP)</td>
<td>16,300</td>
<td>t-retinoic acid</td>
</tr>
<tr>
<td>Cellular retinaldehyde Binding protein (CRALBP)</td>
<td>33,000</td>
<td>11-cis retinol</td>
</tr>
<tr>
<td>Interphotoreceptor retinol Binding protein or interstitial retinol binding protein, (IRBP)</td>
<td>126,000</td>
<td>t-retinol, 11-cis retinol, fatty acids</td>
</tr>
</tbody>
</table>

niques, IRBP was shown to be extracellular as predicted by its behavior during extraction from the intact retina. It was sharply confined to a region between the outer limiting membrane and the apical RPE surface (Fig. 14a and b). This distribution is consistent with its suggested role in the extracellular shuttling of retinoids and other hydrophobic ligands in the interphotoreceptor matrix.182,183 My colleague, Dr. Joseph Horwitz, and I have also studied the localization of IRBP in the cow and monkey retina. In addition to using the PAP method (data illustrated in Fig. 14), we stained ultrathin sections of tissue with primary antibodies followed by secondary antibodies conjugated to ferritin. By this means, we were able to examine the distribution of IRBP in the extracellular space and to look for it in intracellular compartments as well. We confirmed the observation of Bunt-Milam and Saari195 regarding its extracellular distribution (Fig. 15a-d) with one exception. Their ultrastructural PAP studies suggested that IRBP might be present between cone outer segment discs. It was suggested that this might be possible due to their patency with the extracellular space and the interphotoreceptor matrix. We observed no staining of cone outer segment discs, however (Fig. 15b), and because of the large size of IRBP207,208 feel that this molecule would probably not be able to diffuse into the limited space between cone discs. It has been suggested that IRBP is secreted by the neural retina209 and that, based on autoradiographic studies employing fucose as a precursor, this protein is secreted by the rods of the human retina.216 In further support of this, it has recently been demonstrated that rodless retinas do not secrete IRBP.217 There is, however, still no direct evidence for the secretory site of IRBP. We have searched immunoferitin-labeled sections for evidence of IRBP antigen in the RER, Golgi apparatus, and cytoplasmic vesicles and have found none. Bunt-Milam and Saari,218 however, using an immunogold label and a different embedding medium (LR white), have reported IRBP-positive vesicles in bovine inner segments, near the plasmalemma and in association with the Golgi apparatus although not within it. These could in fact be secretory vesicles or, alternatively, they could function in the uptake of IRBP from the interphotoreceptor.

Immunocytochemistry of Ocular Retinoid Binding Proteins

Immunocytochemistry has given us much valuable information concerning the cellular localization of retinoid binding proteins in the retina. Bunt-Milam and Saari218 were the first to apply this approach to the localization of retinoid binding proteins in their studies of cow and monkey retinas. Using immunofluorescence and the peroxidase antiperoxidase (PAP) technique, IRBP was shown to be extracellular as predicted by its behavior during extraction from the intact retina. It was sharply confined to a region between the outer limiting membrane and the apical RPE surface (Fig. 14a and b). This distribution is consistent with its suggested role in the extracellular shuttling of retinoids and other hydrophobic ligands in the interphotoreceptor matrix.182,183 My colleague, Dr. Joseph Horwitz, and I have also studied the localization of IRBP in the cow and monkey retina. In addition to using the PAP method (data illustrated in Fig. 14), we stained ultrathin sections of tissue with primary antibodies followed by secondary antibodies conjugated to ferritin. By this means, we were able to examine the distribution of IRBP in the extracellular space and to look for it in intracellular compartments as well. We confirmed the observation of Bunt-Milam and Saari195 regarding its extracellular distribution (Fig. 15a-d) with one exception. Their ultrastructural PAP studies suggested that IRBP might be present between cone outer segment discs. It was suggested that this might be possible due to their patency with the extracellular space and the interphotoreceptor matrix. We observed no staining of cone outer segment discs, however (Fig. 15b), and because of the large size of IRBP207,208 feel that this molecule would probably not be able to diffuse into the limited space between cone discs. It has been suggested that IRBP is secreted by the neural retina209 and that, based on autoradiographic studies employing fucose as a precursor, this protein is secreted by the rods of the human retina.216 In further support of this, it has recently been demonstrated that rodless retinas do not secrete IRBP.217 There is, however, still no direct evidence for the secretory site of IRBP. We have searched immunoferitin-labeled sections for evidence of IRBP antigen in the RER, Golgi apparatus, and cytoplasmic vesicles and have found none. Bunt-Milam and Saari,218 however, using an immunogold label and a different embedding medium (LR white), have reported IRBP-positive vesicles in bovine inner segments, near the plasmalemma and in association with the Golgi apparatus although not within it. These could in fact be secretory vesicles or, alternatively, they could function in the uptake of IRBP from the interphotoreceptor.

Fig. 14a-d. Peroxidase antiperoxidase immunocytochemistry of interphotoreceptor retinol binding protein (IRBP) and cellular retinol binding protein (CRBP). a, Tapetal region of the cow retina stained with affinity-purified rabbit antirat IRBP antibodies. IRBP, as indicated by the brown reaction product, is restricted to the region between the apical surface of the retinal pigment epithelium (RPE) and the outer nuclear layer (ONL). The fact that this protein is extracellular can be observed in Figure 15 (×1,200). b, Control section stained as in Figure 14a, except that non-immune affinity-purified antibodies were substituted for immune antibodies (×1,200). c, Albino rat retina stained with affinity-purified rabbit antirat CRBP antibodies. The retinal pigment epithelium (RPE) contains CRBP, but the underlying photoreceptors do not. CRBP is also present in Muller cell end feet (long arrows) and in Muller cell processes in the inner plexiform layer (short arrows). The latter has been determined by electron microscopic immunocytochemistry (×750). d, Control section stained as in Figure 14c except that nonimmune anti-CRBP antibodies were substituted for immune antibodies (×750).
matrix. In that light, Hollyfield et al.\textsuperscript{219} have shown that IRBP, adsorbed to colloidal gold, is taken up by both rod and cone inner segments. Whether both rods and cones participate in the secretory process also remains an open question. Cone as well as rod-dominant retinas contain IRBP,\textsuperscript{220} so it is not likely that rods alone possess the capability of synthesis.

Bunt-Milam and Saari have also localized CRALBP by immunocytochemistry.\textsuperscript{215} This retinoid binding protein is present both in RPE and neural retina as predicted from biochemical evidence but, to everyone's surprise, it is exclusively expressed in Müller cells in the neural retina. An 11-cis retinoid binding protein would have been expected in photoreceptors, the only known cells in the body to utilize this retinoid, but not in the Müller cell. Clearly, in light of this striking result, a role for the Müller cell must now be considered in any discussion of the visual cycle.

Antibodies to CRALBP were generously provided to us by Dr. Saari, and we have repeated some of the localization experiments in the cow retina with immunoferritin and colloidal gold labeling methods. We have confirmed the observations of Bunt-Milam and Saari\textsuperscript{215} regarding the general distribution of this protein in the cytoplasm of Müller and RPE cells (Fig. 16). In addition, we have noted that this protein is present in the RPE nucleus as well as the cytoplasm.

With Drs. David Ong and Frank Chytil, we have studied the distribution of CRBP in the rat,\textsuperscript{221} cow, and human retina. Some of these results were as surprising as those for CRALBP. Like CRALBP, and in accordance with earlier biochemical evidence,\textsuperscript{191,199} CRBP is present in the RPE (Fig. 14c). However, as the transition is made from the RPE to the ciliary epithelium, expression of this protein halts abruptly. Within the neural retina, the localization is similar to that of CRALBP. Only the Müller cell cytoplasm is positive. In the rat retina, CRBP is limited primarily to the end feet of Müller cells and to the cell processes that ramify within the inner plexiform layer (Fig. 14c). The Müller cells of bovine and human retina are much richer in CRBP, however, (Bok and Ong, unpublished) and staining is observed along the entire length of the Müller cell. In these species, the staining patterns are identical to that previously shown for CRALBP.\textsuperscript{215}

Immunoferritin labeling indicates that CRBP is uniformly distributed throughout the RPE cytoplasm and, like CRALBP, is present in the nucleus as well. It is not evident at this point why the nucleus would require CRALBP or its ligand. With respect to CRBP, however, Chytil, Ong and co-workers have suggested that trans retinol may be involved in the control of gene expression.\textsuperscript{222} Thus, within the RPE, CRBP could be involved in the dual role of intracellular transport of trans retinol and the maintenance of an appropriate state of cell differentiation. The co-existence of CRALBP and CRBP in the Müller cell implies a major role for this cell in the processing and transport of retinoids for photoreception.

Finally, to round out the constellation of retinoid binding proteins known to exist in the retina, we should make brief mention once again of cellular retinoic acid binding protein (CRABP). As stated earlier, Saari et al.\textsuperscript{199} purified CRABP from the neural retina but did not find it in the RPE. As Wiggert et al. have shown, however, it is present in the choroid.\textsuperscript{196} The purpose of CRABP in the neural retina is not clear, since it has no known involvement in the visual cycle. We have initiated immunocytochemical localization of this binding protein in the rat retina and, in accordance with the earlier biochemical evidence, find it lacking in the RPE. It is present, however, in the inner retina, once again probably in Müller cells and, unlike CRBP, it is found in the ciliary epithelium as well (Bok, Ong and Chytil, unpublished).

The discovery of cellular and intercellular retinoid binding proteins in the retina, their purification and characterization and their localization in specific cell types, has contributed significantly to our understanding of the visual cycle, although as you will see in my summary of this subject, many gaps in our understanding persist. The most astonishing result concerns the photoreceptors themselves. The only binding protein other than rhodopsin that localizes to this cell type is IRBP. This is most surprising, since the photoreceptor ultimately uses 11-cis retinal during photopigment regeneration and releases trans retinol following photobleaching. There is, of course, always the possibility that we have missed a binding protein that is crucial to photoreceptor function. A binding protein or en-
zyme that promotes chromophore exchange could be one example. Another major gap in our information pertains to our lack of firm information regarding binding proteins for retinyl esters in the RPE. Heller\(^{223}\) described a lipoprotein complex from liver that contains retinyl esters and retinol, but it is not known whether this is related to the lipoglycoprotein that he isolated from RPE\(^{224}\) and which binds exogenous retinol.

**Summary of Intraretinal Retinoid Transport**

Utilizing the results described above, I would like to summarize our current understanding of the role played by retinoid binding proteins in the visual cycle. Many aspects of it are speculative however, and must await more extensive experimentation. A cartoon depicting the location of retinoid binding proteins thought to be directly involved in the visual cycle is shown in Figure 17. CRBP and CRALBP in the nucleus of the RPE are probably not involved in any way in the visual cycle but, as stated earlier, may be involved somehow in gene expression.

Plasma RBP binds to receptors on the basolateral plasma membrane of the RPE. During this interaction, trans retinol is delivered into the RPE and plasma RBP remains extracellular. When RBP loses its trans retinol, its affinity for TTR is reduced and the two proteins dissociate. CRBP is the most likely binding protein to receive trans retinol from RBP because the two proteins have identical endogenous ligands. Within the RPE, CRALBP carries only 11-cis retinaldehyde. The source of this could be twofold. It could be scavenged from unbleached rhodopsin in RPE phagosomes, or it could be produced in the RPE from trans retinol. This could take place through the combined action of an alcohol dehydrogenase and an isomerase. An 11-cis isomer-specific dehydrogenase has been detected in the RPE,\(^{225,226}\) and CRALBP serves as a substrate carrier for this enzyme.\(^{227}\) Unfortunately, aside from an early and unconfirmed report,\(^{228}\) the isomerase has remained elusive. The form of retinoid that is transmitted to the photoreceptors is not known. It is probably not a retinyl ester since esters have not been found in the interphotoreceptor matrix. Furthermore, retinoids are not secreted by the RPE bound to CRBP or CRALBP, since immunocytochemistry shows that neither of these binding proteins is present in the interphotoreceptor matrix.\(^{215,221}\) That leaves IRBP as the sole candidate for the shuttling of retinoids in the subretinal space. Trans retinol, 11-cis retinol\(^{204}\) trans retinal and 11-cis retinal\(^{205}\) (J. Saari, personal communication) have now been reported as endogenous IRBP retinoids. Trans retinol is the predominant IRBP ligand in light adapted retinas\(^{229}\) and 11-cis retinal is its predominant counterpart in dark adapted retinas.\(^{205}\) However, examination of the endogenous ligands of IRBP does not aid us directly in determining the form of retinoid that is transported to the photoreceptors because there are so many of them. We can, nonetheless, speculate on the cellular origin of some of these retinoids. As stated earlier, both the RPE and Müller cells contain CRBP and CRALBP, and both are therefore candidates for the isomerization site. Based on the requirement of the RPE for the regeneration of rhodopsin, Bridges et al\(^{230}\) have suggested that cis isomerization of retinol could take place in the Müller cell. Since the photoreceptors (of mammals) lack the cis isomer-specific oxidoreductase,\(^{225,226}\) they suggest that, following isomerization in the mammalian retina, 11-cis retinol leaves the Müller cell and travels to the RPE where it is oxidized to 11-cis retinal. In this scenario, 11-cis retinal would be the species that is provided to the photoreceptor for rhodopsin regeneration. However, in cold-blooded vertebrates, the retinoid that enters the photoreceptor could be 11-cis retinol since this retinoid can be used in opsin regeneration by isolated retinas.\(^{231}\) Some 11-cis retinal on IRBP could also originate from the chromophore exchange that occurs in dark adapted photoreceptors. Trans retinol would flow to the Müller cell from the RPE and back to the RPE from photoreceptors following strong photobleaching. The source of the trans retinal that is found on IRBP is not obvious.

If RBP is shuttling all of these retinoids from cell to cell, how does it know the proper destination for each of them? One mechanism could be through membrane receptors. It is not presently known whether IRBP interacts with specific membrane receptors on the cells that line the subretinal space, but it appears to be both taken up and secreted by photoreceptors.\(^{209,216,219}\) If
receptor-mediated interactions were shown to take place, IRBP might have differing affinities for given cell membranes, depending on the ligand that is bound to it.

The major reason for the ambiguities that persist in our knowledge of the visual cycle is that a key element is still missing, namely information concerning the cellular site of isomerization. If this problem, which has plagued us for decades, could be solved, all other aspects would probably fall into order rather quickly.

I have tried during this lecture to describe what I consider to be the significant developments in the field of photoreceptor-RPE interactions since 1967 when Dr. Richard Young first published his observations on photoreceptor renewal. I hope that I have convinced you that the advances have not been trivial. Most of the contributions that I have described have been made by individuals who are members of the group that honors me by asking me to present this lecture, the Association for Research in Vision and Ophthalmology. Estimated conservatively, at least 30 laboratories, perhaps over 100 investigators, have made important
contributions to the advances that I have outlined today. It’s been a marvelous experience to interact with all of them and in a broader and even more significant sense, to be a part of this wonderful Association of vision scientists. Thank you for this privilege!

**Key words:** photoreceptors, retinal pigment epithelium, rhodopsin, retinoid binding proteins, glycosylation, biosynthesis, chromophore exchange

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