THE EFFECTS OF MONENSIN ON TRANSPORT OF MEMBRANE COMPONENTS IN THE FROG RETINAL PHOTORECEPTOR

I. Light Microscopic Autoradiography and Biochemical Analysis¹

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Abstract

We have explored the use of the Na⁺-H⁺ ionophore monensin as a potential tool for the investigation of membrane assembly and transport in retinal photoreceptors. Autoradiographic analyses of frog retinas incubated with [³H]leucine in the presence of monensin revealed a lack of concentrated silver grains ("bands") at the base of the rod outer segments, in contrast to controls. This is indicative of a pronounced monensin-induced decrease in disc membrane assembly. Biochemical analyses of whole retinas and isolated rod outer segment membranes showed that protein synthesis (including opsin synthesis) was not significantly inhibited under these conditions, whereas passage of membrane protein to the rod outer segment was blocked. Glycerolipid synthesis was not significantly affected by monensin. The results suggest that membrane proteins (e.g., opsin) destined for incorporation into the rod outer segment must pass through the Golgi apparatus and demonstrate the potential utility of monensin for inhibiting aspects of macromolecule transport in photoreceptors.

Membrane transport in neurons is not a well understood process (Pfenninger, 1979; Grafstein and Forman, 1980; Holtzman and Mercurio, 1980; Hammerschlag et al., 1982). Several major questions remain unanswered: How are lipids and proteins assembled into the membranes? Is participation of the Golgi apparatus an obligatory step in the formation of membranes or the assembly and transport of membrane components? How are different membrane constituents sorted and shipped to their final destinations?

We have attempted to resolve some of these questions, using as our experimental system the frog retinal rod photoreceptor cell. The photoreceptor is an extensively compartmentalized and highly polarized neuron which transports a variety of membrane components to its two poles from the myoid region of the cell body, where the rough endoplasmic reticulum and Golgi apparatus are located. For example, opsin (the apoprotein of the visual

pigment rhodopsin) is synthesized in the rod inner segment and transported to the base of the rod outer segment where it is incorporated into the disc membranes and participates in visual excitation (reviewed in Papermaster and Schneider, 1982). The components of synaptic vesicles and other materials destined for the rod cells' presynaptic terminals are transported in the opposite direction, down the short axon that connects the cell body to the terminal (reviewed in Mercurio and Holtzman, 1982a, b).

The synthesis, transport, and turnover of opsin have been extensively studied. Following its synthesis on the rough endoplasmic reticulum, opsin passes through the Golgi apparatus (Young and Droz, 1968; Young, 1974; Matheke and Holtzman, 1984). The routes of opsin into and through the Golgi apparatus have been little studied, but once it leaves the apparatus, opsin probably is transported through the ellipsoid region of the cell via vesicles (Kinney and Fisher, 1978; Papermaster et al., 1979; Besharse and Pfenninger, 1980). These vesicles are thought to fuse, eventually, with a region of specialized plasma membrane at the tip of the ellipsoid known as the periciliary ridge complex (Andrews, 1982; Peters et al., 1983). The opsin-rich membrane is believed to pass through or along the cilium to the base of the outer segment (Young, 1968; Holtzman and Mercurio, 1980).

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To evaluate further the role of the Golgi apparatus with regard to the assembly and transport of membrane components in photoreceptors, we have made use of the carboxylic ionophore monensin. This agent is known to inhibit intracellular transit of secretory proteins (Tartakoff and Vassalli, 1977, 1978, 1979) and viral membrane proteins (Johnson and Schlesinger, 1980; Tartakoff et al., 1981) within various cell types and has been shown to affect axonal transport, probably at a Golgi-dependent step prior to the initial stages of "loading" of material into the transport system (Hammerschlag et al., 1982). While the molecular basis of monensin's effects is unclear (Tartakoff and Vassalli, 1979; Tartakoff, 1983), the locus of the effects appears to be at the level of the Golgi stack (Griffiths et al., 1983; Quinn et al., 1983; Tartakoff, 1983).

In the present paper we provide evidence which demonstrates that monensin can effectively block intracellular transport of protein to the rod outer segments under conditions that do not appreciably affect protein synthesis or glycerolipid synthesis. The companion paper (Matheke and Holtzman, 1984) analyzes this blockage by quantitative, electron microscopic autoradiography, evaluates the morphological effects of monensin, compares the effects of monensin on protein transport in the two principal directions within the cell, and investigates the impact of monensin on the distribution of newly synthesized glycerolipids in the photoreceptor cell.

Preliminary facets of some of this work have been reported (Matheke and Holtzman, 1981; Matheke et al., 1983a, b).

Materials and Methods

Experimental animals

For the autoradiographic experiments, *Rana pipiens* (Lake Champlain Frog Farm, Alberg, VT, and West Jersey Biological Supply, Winona, NJ) were kept on a light-dark cycle (12-hr light/12-hr dark) for at least 4 weeks prior to experimentation. They were fed regularly on crickets and/or mealworms, and the tank water was changed daily to prevent redleg.

Frogs for the biochemical experiments were kept on a light-dark cycle (13-hr light/11-hr dark) for at least 6 months. The night before the experiment, the frogs were placed in an incubator with the lights on all night. The light was turned off 3 hr prior to the dissections.

Reagents and solutions

Ringer's solution used for incubations contained 120 mM NaCl, 5.1 mM KCl, 2.75 mM CaCl₂, 1.25 mM MgSO₄, 1.25 mM KH₂PO₄, 25 mM NaHCO₃, and either 10 mM D-glucose (RBG) or 18 mM sodium pyruvate (RBP) (Basinger and Hall, 1973). Monensin (Calbiochem-Behring Corp., La Jolla, CA) was added, and the chilled Ringer's was gassed with 95% O₂/5% CO₂ for at least 30 min while allowing the solution to equilibrate to room temperature prior to the experiment. The pH was adjusted to 7.2 as necessary.

[³H]Leucine (L-[4,5-³H(N)]leucine, specific activity 55.0 Ci/mmol, New England Nuclear, Boston, MA) was dried under nitrogen in a glass test tube and stored under nitrogen atmosphere at 0 to 4°C overnight. Gassed Ring-

er's was added to give a final [³H]leucine concentration of 40 μCi/ml (autoradiographic experiments) or 100 μCi/ml (biochemical experiments).

Experimental procedure

Incubations for autoradiography. In the hour prior to the onset of the light phase of the light/dark cycle, frogs were decapitated under dim red light, and their retinas were dissected free of the pigment epithelium and saved in ice-chilled Ringer's solution.

All incubations were done in a chamber with a steady flow of 95% O₂/5% CO₂ in a water bath maintained at 20 to 22°C, with gentle agitation. Incubations were carried out either in multiwell Corning plates containing 0.5 ml of solution or in 50-ml Fernbach flasks containing 10 ml of solution. In one of the autoradiographic experiments and in the biochemical experiment, retinas were pre-incubated for 30 min in 10 ml of solution in the presence or absence of monensin prior to the pulse. Comparisons of experiments with and without pre-incubation showed no difference in grain counts over the rod outer segments.

To pulse, retinas were incubated for 1 hr in solutions containing [³H]leucine with or without 10⁻⁵ M monensin. At the end of the pulse period, the retinas were gently rinsed in fresh RBG to remove extracellular isotope. Retinas from experiments without a "chase" were then fixed in 4% formaldehyde in 0.1 M phosphate buffer for 2 hr at 0 to 4°C. Retinas from experiments with a "chase" were placed in 10 ml of RBG containing 10 μg/ml of casamino acids (Difco Laboratories, Detroit, MI) with or without monensin. This solution was replenished once, after 2.5 hr, during the 5-hr "chase".

Retinas for pulse-chase autoradiographic experiments were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, for 2 hr at 0 to 4°C, followed by thorough rinsing in three changes of cold 0.1 M cacodylate buffer. (Although both fixatives give similar grain distributions (cf. Mercurio and Holtzman, 1982b), glutaraldehyde preserves the tissue, especially its inner layers, markedly better than formaldehyde and, thus, was the fixative of choice, especially for the electron microscope pulse-chase experiments that are the heart of the companion paper. We used formaldehyde for the pulse only preparations to avoid artifactual binding of [³H]leucine due to the fixative, a problem that no longer exists after a prolonged chase of the type we used.)

In experiments testing for the uptake of [³H]leucine at the termination of the "chase" period, retinas were pre-incubated, given a mock "pulse" without the isotope, "chased" as described above, rinsed, and then given a "pulse" of [³H]leucine (120 μCi/ml) for 1 hr. After the pulse period, the retinas were rinsed again and "chased" in RBG containing casamino acids for 2 hr.

Tissue processing. Fixed tissue was stored overnight at 0 to 4°C in 0.1 M cacodylate buffer with 2.5% sucrose. On the subsequent day the tissue was cut into pieces approximately 1 mm² and postfixed for 2 hr at 0 to 4°C in 1% OsO₄ in 0.1 M cacodylate buffer. Serial dehydration was performed in cold acetone-water mixtures followed by infiltration and embedment in Epon or LX-112 (Ladd Research Industries, Inc., Burlington, VT).

For autoradiography, 1- μ sections were coated with NTB3 emulsion (Eastman Kodak Co., Rochester, NY) diluted with an equal amount of water. After exposures of 2 to 4 weeks, the slides were developed in D-19 (Eastman Kodak Co., Rochester, NY) for 4 min at 17°C, rinsed, fixed in Kodak fixer for 10 min at 20°C, and subsequently rinsed several times in water. The sections were stained for 5 min at 40°C with 1% toluidine blue in 1% sodium borate.

The slides were examined and photographed on a Zeiss Photomicroscope II employing a Zeiss Planapo 40 $\times$ oil immersion objective and a green-yellow filter.

Biochemical analysis of retinas. Incubations were carried out similarly to the autoradiographic experiments except that all incubations were done in 50-ml Fernbach flasks at 24°C at a density of 17 retinas/10 ml. Pre-incubation and "pulse" were done in RBP; the pulse contained 100 μ Ci/ml of [3 H]leucine. The "chase" was done in RBG containing 10 μ g/ml of casamino acids and 2.5 mM nonradioactive leucine. (RBP was used for the pulse since it enhances uptake of the labeled amino acid. See Basinger and Hoffmann (1982) for additional details.) Samples of the preparations were examined by light microscope autoradiography to ensure that their labeling patterns resembled those in the other, autoradiographic experiments. Retinas for determination of glycerolipid were incubated in a manner similar to the pulse-chase leucine preparations, using an initial pulse in RBP containing 50 μ Ci/ml of [3 H]glycerol (New England Nuclear).

To determine the total incorporation of [3 H]leucine into protein, the retinas were cut in half, and half of each retina was placed in a microfuge tube containing 1.0 ml of ice-cold 10% trichloroacetic acid (TCA) and 0.5% phosphotungstic acid (PTA). After standing overnight at 0 to 4°C, the tissue was disrupted by sonication and then centrifuged (5 min at 13,000 $\times$ g, 0 to 4°C). The resultant precipitate was washed twice with 1.0 ml of TCA-PTA solution and then solubilized in 1 ml of 1 N sodium hydroxide overnight at 65°C. Aliquots were taken for protein assay (Lowry et al., 1951) (in triplicate) and for scintillation counting (in duplicate). For evaluating glycerol labeling, quarter to half retinas were precipitated in 10% TCA and then sonicated in 20% SDS in 0.2 M sodium borate buffer (pH 9.2); the sonicate was heated for 2 hr at 75°C before counting. In the glycerol experiments, the fluorescamine method of Udenfriend et al. (1972) was used for protein assays.

To determine the protein species into which the [3 H]leucine has been incorporated, the remaining half retinas were analyzed by SDS polyacrylamide gel electrophoresis (SDS-PAGE) using a discontinuous system (Laemmli, 1970). Retinal tissue was placed into microfuge tubes containing 0.1 ml of distilled water and frozen overnight; 0.1 ml of a 2-fold concentrated SDS sample buffer (Laemmli, 1970) was then added, and the tissue was dispersed by sonication and heated for 30 min at 65°C in the sealed tubes. After centrifugation (5 min at 13,000 $\times$ g), the solubilized tissue was applied to two different gel systems: (1) a 13-cm 10%T/2.7%C separating gel with a 1-cm 4%T/2.7%C stacking gel; and (2) a 21-cm separating gel consisting of a linear acrylamide gradient from 7.5%T/0.8%C to 15%T/0.8%C with a 1.5-cm 4%T/

0.8%C stacking gel. The following molecular weight standards were employed (Sigma Chemical Co., St. Louis, MO): myosin (M_r = 205,000, rabbit muscle); β -galactosidase (M_r = 116,000, *Escherichia coli*); phosphorylase b (M_r = 97,000, rabbit muscle); bovine serum albumin (M_r = 68,000); hen ovalbumin (M_r = 45,000); and carbonic anhydrase (M_r = 30,000, bovine erythrocyte). Following electrophoresis, gels were stained with 0.125% Coomassie blue R-250 in 50% methanol/10% acetic acid (2 hr) and then destained overnight in 5% methanol/7% acetic acid with gentle reciprocal agitation.

Fluorography was performed as follows. The destained gels were rinsed for 30 min in two changes of distilled water and then infiltrated with Autofluor (National Diagnostics, Somerville, NJ) for 2 hr with gentle reciprocal agitation. Gels were then dried directly onto Whatman 3MM paper using a Bio-Rad slab gel dryer (Bio-Rad Laboratories, Richmond, CA) attached to a lyophilizer. The dried gels were placed in x-ray cassettes containing Cronex Lightning-Plus intensifier screens (Gilman X-Ray, Dallas, TX), exposed for 1 to 4 weeks to Kodak X-OMAT XAR-5 film, and the resulting fluorograms were developed with a Kodak X-OMAT film processor.

Biochemical analysis of isolated rod outer segments. Rod outer segment membranes were prepared from incubated retinas by a modification of the method of Papermaster and Dreyer (1974) as follows. Retinas (four per condition) were added to 4.0 ml of ice-chilled 42% (W/W) sucrose (density = 1.19 gm/cm 3) in a 17-ml capacity cellulose nitrate centrifuge tube. After homogenization with a loose-fitting Teflon pestle (1/2-inch diameter pestle in a 9/16-inch diameter tube; motor driven at 1200 rpm for 2 min), the homogenate was overlaid with sucrose solutions of the following densities (gm/cm 3): 3.0 ml of 1.15, 4.0 ml of 1.13, and 3.0 ml of 1.11. The resulting discontinuous sucrose gradient was centrifuged at 100,000 $\times$ g for 1 hr at 4°C. The rod outer segment membrane fraction was removed by pipette from the 1.11/1.13 gm/cm 3 interface, diluted with 0.1 M Tris acetate buffer, pH 7.4, and centrifuged (30 min at 40,000 $\times$ g, 4°C). The membrane pellet was then washed twice with buffer and collected by centrifugation as described above.

The washed membrane preparation was resuspended in distilled water, and aliquots were taken from protein assay, scintillation counting, and SDS-PAGE analysis as described above.

Results

Light microscopic autoradiography of [3 H]leucine-labeled tissue

Time course and concentration dependence. The primary consideration in these studies was: Does monensin have an effect on the incorporation of labeled proteins into the rod outer segment (ROS), and, if so, what concentration is necessary to effect this change? Most work with monensin has been on cultured cells or comparable systems, and we were concerned that the extensive membrane systems of the outer segments and the barriers that restrict access of extracellular materials to the spaces between cells in the retina would impede or, worse, prevent penetration of monensin to the sites of

Figure 1. Light microscope autoradiograms of tissue incubated for 1 hr in [^3H]leucine and chased for 5 hr in isotope-free solution. One-micron sections were coated with Kodak NTB-3 emulsion, developed in D-19, and stained with toluidine blue. The scale bar corresponds to 10 μm . *a*, Control retina (incubated without monensin). Note the concentrations of silver grains (bands) evident at the base of the ROS (arrows). The prominent spheres are oil droplets in the cone cells. *M* indicates the retinal layer where the myoid regions are located. *b*, Monensin-treated retina. Grains are concentrated in the myoids (at the level of *M*); no bands are evident at the base of the ROS.

interest to us, as has proved true with some other membrane-associating agents we have employed.

The duration of the chase was chosen to give the shortest period in which clear, unequivocal "banding" of silver grains could be seen in control autoradiograms. The "bands" (Fig. 1) are clusters of grains indicative of the formation of new outer segment discs (Hall et al., 1969). Although bands could be seen with as little as 3 hr of chase, a chase of 5 hr was necessary to give sections

that showed unequivocal banding in virtually all of the cells.

To determine the minimum concentration of monensin necessary to cause an effect, retinas were given a 1-hr "pulse" and a 5-hr "chase" in varying concentrations of monensin. Concentrations used in previously published experiments (e.g., Tartakoff and Vassalli, 1978; Kitson and Widnell, 1981; Hammerschlag et al., 1982) varied from more than 10^{-4} M to 10^{-9} M, so concentra-

tions for this experiment were varied from 10^{-4} M to 10^{-9} M. Banding was always compared to control retinas which had no monensin in the incubation medium. Light microscopic autoradiographs showed almost complete inhibition of banding in 5×10^{-6} M monensin and no banding at 10^{-5} M or greater concentration; therefore, a concentration of 10^{-5} was used for all subsequent experiments.

As far as could be judged from visually evaluating the extent of labeling in the photoreceptors, the monensin-treated retinas showed levels of incorporation of label similar to the controls (in all compartments except the basal ROS discs).

That the photoreceptors were still metabolically active at the end of the incubation periods used in this study was shown by the fact that they were able to incorporate [3 H]leucine after the mock pulse-chase procedure described.

Distribution of grains in the light microscopic autoradiographs. Light microscopic autoradiographs of retinas that were given a "pulse" with no chase showed no significant differences between monensin-treated and monensin-free tissue; as we have found in our prior studies of retinas given short exposures to leucine (Mercurio and Holtzman, 1982b), neither type of preparation showed banding, and both showed light, diffuse label over the ROS. Most of the label was over the cell body.

As in previous reports (Young, 1974; Basinger et al., 1976; Matheke and Holtzman, 1981; Matheke et al., 1983b), the most prominent labeling of the outer segments of the control pulse-chase retinas was the bands at their bases (Fig. 1); labeling of the periphery, corresponding to radioactivity associated with the plasma membrane, was also sometimes seen. There was some light, diffuse labeling of the ROS, not concentrated at the base or the plasma membrane (cf. Basinger et al., 1976). Labeling of the cell body was about of comparable density to the labeling over the outer segment. The region of the photoreceptor synaptic terminals was moderately labeled, although it is difficult to discern pre- and post-synaptic compartments at the level of light microscopy. Labeling of synaptic terminals will be described more fully in the discussion of electron microscopic autoradiography in the companion paper (Matheke and Holtzman, 1984).

Monensin-treated retinas (Fig. 1) showed no bands in the ROS and no concentration of label over the ROS plasma membrane. However, the diffuse ROS labeling, thought to represent rapidly incorporated, diffusible protein (Basinger et al., 1976), was similar to that in control material. There was very heavy labeling over the cell bodies, primarily over the supranuclear region, giving the cell bodies a "congested" appearance. On closer inspection, the myoid regions of the cells were observed to contain large vacuoles, clearly visible at the light microscopic level. (They are not evident in Figure 1 owing to the overlay of autoradiographic grains.) These vacuoles correspond to the distended Golgi elements seen by electron microscopy in the monensin-treated retinas (Matheke and Holtzman, 1984). There seemed to be a slight decrease in the amount of label over the region of the photoreceptor synaptic terminals; however, quantitative electron microscopic autoradiography was necessary to evaluate this observation rigorously.

Biochemical analysis

The biochemical studies we undertook were designed to complement the autoradiographic ones, and particularly to evaluate the effects of monensin on the synthesis and intracellular transport of opsin, the major ROS protein (Hall et al., 1969; Papermaster and Dryer, 1974). As can be seen from Table I, monensin treatment decreased the specific activity of whole retinal TCA-PTA-precipitable material by only 9% relative to controls, indicative of marginal inhibition, if any, of retinal protein synthesis. Therefore, this observation confirmed our impressions from examination of the corresponding autoradiographs. The incorporation of [3 H]leucine into the ROS, TCA-PTA-precipitable material, was decreased 84% in monensin-treated tissue relative to controls, in good agreement with the autoradiographic results.

To identify the species of proteins that were labeled, both whole retinas and isolated ROS membranes were solubilized and analyzed by SDS-PAGE. The results are seen in Figures 2 and 3.

The isolated outer segments of the control tissue, seen in lane c of both fluorograms, showed an intense band corresponding to an apparent $M_r = 36,000$, the approximate molecular weight of opsin. At the same protein loading level, ROS isolated from monensin-treated retinas (lane d on both fluorograms) exhibited virtually no labeling of any protein species.

The fluorograms of the lanes containing homogenates of whole retinas (lanes a and b) revealed several interesting features. There were no evident differences in the relative number or intensities of corresponding bands between lanes a and b, in agreement with the evidence described above, that protein synthesis was not significantly disrupted by monensin under the conditions used. Like the band in the ROS samples (lanes c and d), the major band in lanes a and b corresponded to an apparent molecular weight similar to that of opsin. The intensities of the 36,000-dalton band in lanes a and b were quite similar, suggesting that monensin did not significantly inhibit opsin biosynthesis under our incubation conditions.

Monensin had no significant effect on incorporation of [3 H]glycerol into lipids. After a 1-hr exposure to [3 H]glycerol, control retinas showed $2.2 \pm 0.55 \times 10^3$ dpm/ μ g of protein, and monensin-exposed retinas showed $1.99 \pm 0.57 \times 10^3$ dpm/ μ g of protein (mean $\pm$ SD; $n = 4$).

TABLE I

Effect of monensin on incorporation of [3 H]leucine into proteins of whole retinas and isolated ROS membranes

Retinas were incubated in medium containing [3 H]leucine for 1 hr and chased for 5 hr. At the end of the chase period the proteins were precipitated with TCA-PTA and solubilized with 1 N NaOH, and specific activity was determined by scintillation counting and protein assay. (Data are means of two determinations; these agreed to within 4%.)

Conditions	Specific Activity dpm/mg protein	% Control
Retinas		
Control	1.61×10^7	
Monensin	1.46×10^7	90.7
ROS membranes		
Control	9.44×10^6	
Monensin	1.49×10^6	15.8

Figures 2 and 3. Fluorograms of ^3H -labeled retinal proteins resolved, as a function of molecular weight, by SDS-PAGE. Figure 2, 13-cm 10%T-2.7%C separating gel; Figure 3, 21-cm separating gel, using a linear acrylamide gradient from 7.5%T-0.8%C to 15%T-0.8%C. In each case, lane *a* is whole retina, incubated under control conditions; lane *b* is whole retina, incubated with monensin; lane *c* is ROS membranes, control; lane *d* is ROS membranes, monensin. For lanes *a* and *b*, 150 μg of protein were applied to the gel. For Figure 2, *c* and *d*, 25 μg of protein were used. For Figure 3, *c* and *d*, 15 μg of protein were used. The arrow marks the position of opsin (36,000 daltons) on the gel. Exposure time: lanes *a* and *b*, 1 week; lanes *c* and *d*, 3.5 weeks.

Discussion

These results indicate that monensin can block transport of newly synthesized proteins, including the membrane protein opsin, to the outer segment of rod photoreceptors under conditions where protein synthesis and lipid synthesis are not appreciably inhibited. (Opsin, the principal rod outer segment protein, remains membrane-associated throughout its lifetime in the photoreceptor: Papermaster et al., 1975; Papermaster and Schneider, 1982). Therefore, monensin evidently is a tool with which we should be able to dissect further the events of membrane transport on the photoreceptor neurons.

Our results extend to neuronal membrane proteins the previous findings on monensin effects on secretory proteins (Tartakoff and Vassalli, 1977, 1978, 1979) and on virally coded membrane proteins (Johnson and Schlesinger, 1980) in other cell types. Studies on myelin assembly (Townsend and Benjamins, 1983) and on lymphoid surface glycoproteins (Tartakoff et al., 1981) have also suggested that monensin affects membrane transport or assembly. In the various cell type studied, the effects of monensin have been found to be selective for the Golgi apparatus. Transit of proteins through or away from the apparatus is inhibited, with little effect on prior stages of protein synthesis or processing (reviewed in Tartakoff,

1983). There also are effects of the ionophore on lysosomal pH (Tartakoff, 1983), but these seem separable from the effects on protein transport and Golgi functioning. The companion paper (Matheke and Holtzman, 1984) will document our observations that in frog rods, as in the other cells studied, monensin does selectively disrupt the Golgi apparatus. Therefore, it appears that the participation of the Golgi apparatus is an obligatory step in formation or transport of the membrane of the outer segment; in the absence of a functional Golgi apparatus, the cell still makes the membrane components, but it cannot transport them properly.

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