CORRELATION OF RHODOPSIN BIOGENESIS WITH ULTRASTRUCTURAL MORPHOGENESIS IN THE CHICK RETINA

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Previous attempts to correlate structure and function in developing retinal tissues have centered largely around the frog (Nilsson and Crescitelli, 1970), although one study has appeared dealing with the onset of electrical response and rhodopsin content in the chick (Witkovsky, 1973). In addition to the lack of definitive ultrastructural investigation by electron microscopy in conjunction with the biochemistry, data is lacking concerning the ontogeny in photoreceptor cells of the visual pigment rhodopsin, a substance necessary for a functional light-induced electrical response in the retina.

Witkovsky, in an earlier paper (1963), had found that the first appearance of the a1 and a2 components in the electroretinogram (ERG) of the embryonic chick was not directly related to rhodopsin development, and had postulated that it was due instead to a cone receptor potential. However, the relatively small retina sample size used (n = 8) and the diminutive amount of visual pigment present made it seem likely to the present investigators that rhodopsin, in the small concentrations to be expected in developing retinas, would not have been detected by his techniques. Although light microscopy was also performed in this study, the inherently limited resolution failed to demonstrate unequivocally the relationship between the ERG, the morphogenesis of rod outer segments, and the presence of light-sensitive visual pigment.

In this paper, high resolution electron microscopy and sensitive biochemical techniques were utilized to study the correlation between retinal development and the ontogeny of rhodopsin associated with the rod outer segments. This study utilizes in vivo incubation of the retinas as opposed to the in vitro incubation used in some earlier studies by other workers on retina. By means of these studies it was sought to establish unequivocally the time course and mechanism of membrane assembly in the rod outer segment.

Through their in vivo study of protein renewal in the frog retina, Droz and Young (Droz, 1963; Young, 1967) have found that rhodopsin is synthesized in the inner segment and assembled into the disk membranes in the outer segment region. It was hoped that by studying the rhodopsin content of the retina over the same period of time that outer segment differentiation occurs, additional support for the postulates of Droz, Young, and others (Young and Droz, 1968; Young, 1968; Young and Bok, 1969) could be obtained by a somewhat different approach. The correlation of such biochemical data with electron microscope evidence might thus serve as further substantiation of this hypothesis.

MATERIALS AND METHODS

All experiments were carried out on embryonic chicks incubated at 37°C for periods of time ranging from 15 days (stage 40) to 21 days (stage 45). At a given day of incubation, the embryos were removed from their shells and decapitated. The eyes were then carefully excised and used for one of the following purposes.

Electron Microscopy

For high resolution studies of differentiation in the chick retina by electron microscopy, the eye was gently slit open by perpendicular incisions through the corneal region and immersed in 4% glutaraldehyde (Polysciences...
Inc., Warrington, Pa.) in Millonig's buffer (pH 7.2). After 3 h of fixation at room temperature (23°C), the eyes were removed, rinsed three times in buffer, and cut into small pieces (1 x 3 mm). These pieces were then immersed in 2% buffered osmium tetroxide (pH 7.2) for 2 h. After three more buffer washes the retinas were carried through a graded series of acetone dehydrations (30%, 50%, 70%, 90%) of 10 min each, ending in 100% acetone, followed by infiltration for 4 h in Epon-acetone (1:3, vol/vol). The retinas were then placed in Epon-acetone (3:1) for 4 h, and finally in 100% Epon overnight. The Epon resin was hardened by addition of catalyst and heating at 45°C for 12 h, and at 60°C for 24 h. Sections were cut from both the fundus and peripheral regions by use of a diamond knife and an LKB Ultrotome, and were positioned on uncoated copper grids (200 mesh). Staining was carried out with 1% uranyl acetate followed by 1% lead citrate. The stained sections were then carbon coated and examined in a Hitachi IIUA electron microscope.

**Extraction of Rhodopsin**

In embryos to be examined for rhodopsin content, the eggs were wrapped in several thicknesses of aluminum foil and dark adapted. Further manipulations were carried out under dim red light. The retinas from 18 to 20 chicks were then floated free from pigment epithelium in 0.9% saline (pH 7.2). They were washed once in saline to eliminate any whole blood and adhering material. The pellets were weighed, then extracted at 4°C in 2.0 ml of 2% digitonin in 0.15 M hydroxylamine (pH 6.5) by being stirred into two successive changes of detergent (for intervals of 3 h each), and the samples were pooled. A full difference spectra measurement between fully dark- and light-adapted samples (i.e., samples bleached in white light for 1 min) was taken on a Cary 14 double beam scanning spectrophotometer (Cary Instruments, Monrovia, Calif.) with the 0.1-0.2 OD slide wire. A measurement of absorbance difference at 500 nm was checked by comparison with the retinal oxime complex formed upon bleaching, and the molarity of the rhodopsin present was calculated with an average extinction coefficient of ε = 41,000 cm⁻¹ and the ratio of rhodopsin per weight of retina calculated accordingly. In samples where iodopsin levels were examined, hydroxylamine-free digitonin extracts were used, followed by addition of the amine before bleaching.

**RESULTS**

**Rhodopsin Concentration**

As can be seen in Fig. 1, the concentration of rhodopsin in the chick retina, as measured by the absorbance difference between dark- and light-adapted samples at 500 nm, increases hyperbolically from day 14 and continues to increase past day 21. These data are somewhat in conflict with the previous results of Witkovsky (1963), who initially detected rhodopsin on day 18. However, the large sample used here (approximately 40 retinas) probably facilitated detection of significant concentrations of visual pigment. Of further interest is the detection of small but significant concentrations of bleached rhodopsin (all trans-retinal) with absorbances at 365 nm (metarhodopsin III). Morphologically, little contact of the receptor cells with processes of the pigment epithelium exists at the 14-16-day stage, and thus a complete mechanism for isomerization of retinal rhodopsin may not be in existence, thereby providing a rationale for the presence of all trans-retinal rhodopsin.

An observation of primary interest is the correlation obtained between rhodopsin content of the...
chick retina and the growth of rod outer segments. In no case were rod outer segments observed to differentiate before the 15th day of incubation, and thus rhodopsin in the unbleached, native form (11 cis-retinal), present by day 14, exists some time biogenically before disk membranes and rod outer segments are formed. It seems probable that this rhodopsin resides in the developed inner segment.

**Electron Microscopy**

The chick embryo of 15-days' incubation time is characterized by a distinct lack of outer segment material and little synaptic organization at the receptor base, as well as few mitochondria in the inner segments of both rods and cones, i.e., two to eight mitochondria per receptor cell inner segment (Fig. 2). At this stage of development (stage 40 by the criteria of Hamburger and Hamilton, 1951), a "bud" of the inner segment has formed as a result of cytoplasmic invasion through the region of the external limiting membrane. The process of inner segment formation is accompanied by a large number of microfilamentous processes streaming intracellularly from the vitreal to the scleral region of the photoreceptor cell (Fig. 2 and 3). A precursor of the mature connecting cilium is observed in both rods and cones, as well as a highly organized basal body consisting of nine triplet microtubules. However, as of day 15 the connecting cilium is only beginning to organize what will eventually become the outer segment. No vesicle or disk formation of any sort is observed.

Outer segment differentiation is in its rudimentary stages on the 15th day of incubation, and small vesicles of membrane (10-30 nm in diameter) are observed at the base of the outer segment shortly after this stage. These vesicles grow in size and number, and by day 19 (stage 45) have formed a more flattened sacculae and are contained by an extensive plasma membrane. At intermediate developmental stages, this plasma membrane, surrounds a cytoplasmic cavity devoid of other cell structure: the entire assembly appears to be the precursor of the mature rod or cone outer segment.

In all cases the rod outer segment types are more highly developed at stage 45, and exhibit a stack of 10-50 flattened disk membranes ordered along the connecting cilium perpendicular to the axis of vitreal-scleral differentiation. With respect to rods, outer segments at day 19 show a greater degree of development in the central region than in the peripheral region, peripheral outer segments containing only 10-20 laminar disks, and central rods some 100-200 disk membranes (Fig. 4). Cone outer segments exhibit the same developmental pattern, as peripheral cones are highly disorganized and vesiculated with at most 5-10 disk membranes. On the average, cones in the central retinal region contain 20-40 disks, the single cones of any retinal region containing the most, double cones fewer still, and the accessory cone outer segment none at all.

**DISCUSSION**

The electron micrographs obtained in this study and a previous work (Mason and Bighouse, manuscript in preparation) have shown that rod outer segments develop before cone outer segments; the initial development is rapid, i.e. over a period of 4-6 days after initial visual cell outer limb differentiation has commenced at the inner segment level. An observation of primary importance related to the morphological development of outer limbs is the finding that in the embryonic chick retina relatively large molar quantities of the rod-associated visual pigment rhodopsin exists before outer segment formation. The extremely sensitive techniques utilized here revealed that 2-3 days before outer segment differentiation, the rhodopsin content of the retina begins an exponential increase. Earlier work by Witkovsky (1963) had demonstrated the appearance of two cornea-negative waves at the stage where rhodopsin appears in significant quantities, although by his biochemical and spectrophotometric techniques no rhodopsin was detected. He considered the a-wave of the ERG to be a cone receptor potential, although it seems possible that it may have arisen instead from the light response of the visual pigment rhodopsin. If indeed rhodopsin does give rise to such a potential, it seems doubtful that it is a true membrane potential, but may conceivably arise from a change in dipole strength of rhodopsin in response to light. For similar reasons, it seems unlikely that the a-wave Witkovsky identified in the chick retina is attributable to a cone receptor potential, as evidence obtained here shows no apparent iodopsin or cone outer segment formation at this stage. Although the number of cone outer segments exceeds rod outer segments by seven or eight to one in the fundus region, the ratio of the respective visual pigments iodopsin and rhodopsin in the adult retina is nearly one to five,
Figure 2  Electron micrograph of the peripheral retina from a stage 40 chick embryo. Note lack of outer segment at this stage and abundance of cytoplasmic microtubules. Terminal bar structure of the external limiting membrane is clearly defined (ELM). The absence of glial cell processes which project vitreally from the external limiting membrane in the mature retina is also noted. Developing mitochondrial vacuoles are present at the inner segment apex (MI), as well as numerous endoplasmic reticulum. Note also small receptor cell (DR) inner segment, in lower right of micrograph, which has not invaded the external limiting membrane. × 18,700.

Thus precluding the detection of any significant and meaningful levels of cone visual pigment at early developmental stages. The low levels of the visual pigment iodopsin at this time were confirmed, however, by a full spectrum from the infrared to the ultraviolet region taken for each retinal extract with no meaningful detection of cone visual pigment being observed.
The fact that rhodopsin appears in the inner segment several days before outer segment development gives some insight to the nature of membrane biogenesis which transpires in the vertebrate retina. Numerous membrane vesicles are evident at the rod outer segment base at day 19 of development. If one estimates in a rough fashion the amount of surface area available in one of the 15-20-nm vesicles observed, it is apparent that at most, one or two rhodopsin molecules could be contained in a single vesicle. It is then conceivable that a membrane assembly process could occur whereby single vesicles fuse in a stepwise process at the outer segment base to form the large, flattened saccules comprising the disk membranes of the rod. Rhodopsin may in fact migrate through the connecting cilium as either a single protein synthesized in the inner segment, or as a membrane unit formed in a similar region. It is feasible, as well, that if rhodopsin migrates as a single protein

**Figure 3** Electron micrograph of developing receptor cell inner segment in the central area of a stage 40 chick embryo. Note well-oriented connecting cilium (CC), and rudimentary outer segment lacking disk membranes. As well, observe segregated endoplasmic reticulum (ER) and highly developed mitochondria. x 28,100.
through the connecting cilium, then infolding of the outer segment plasma membrane may occur, as many workers have suggested. The final assembly of rhodopsin into the membrane would thus proceed at the outer segment base with the assembled membrane unit forming the flattened disk saccules. The high degree of fluidity associated with vertebrate photoreceptor membranes may well facilitate this assembly. The theory of rhodopsin transport from inner segment to outer segment has been noted by a number of workers including Young and Droz (1968),
Matsubara et al. (1968), and Bargoot et al. (1969), as well as Hall et al. (1968, 1969). It thus seems to be a point of some considerable agreement, and the present work merely serves to underscore the fact that rhodopsin must be formed outside of the outer segment by reason of its presence in developing retinas free of outer limbs.

The mechanism presented here whereby membranes are assembled is an important and crucial one and perhaps best studied at the point in an animal's metabolism when cells are arising and there is turnover, namely during either an embryonic or a regenerative period. It is felt that the data obtained here by biochemical and electron microscope observations warrant such speculation and support previous suggestions for the mechanism of the development and turnover of the conspicuously ordered outer segments.

SUMMARY

The developing chick retina from stages 39-45 has been examined by biochemical and electron microscope techniques. The levels of rhodopsin contained in the maturing chick retina were evaluated by detergent extraction and correlated with rod outer segment formation. It was found that the appearance of rhodopsin in significant levels preceded outer segment formation by at least 2 days, thus implying that rhodopsin is synthesized in the receptor cell inner segment and translocated to the outer limb when disk membrane biogenesis occurs. The level of rhodopsin continues to rise as the rod outer segment develops. Development of both rods and cones originates and proceeds most rapidly in the fundus or central region and proceeds toward the periphery. In general, rod outer segments were noted to develop far more rapidly than cone outer segments.

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