PHOTORECEPTOR–PIGMENT EPITHELIAL CELL RELATIONSHIPS IN RATS WITH INHERITED RETINAL DEGENERATION

Radioautographic and Electron Microscope Evidence for a Dual Source of Extra Lamellar Material

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ABSTRACT

Protein synthesis and displacement in photoreceptor and pigment epithelial cells of inbred normal (Fisher) and mutant (RCS) rats with inherited retinal degeneration has been studied by light and electron microscope radioautography. Groups of animals 14, 15, 17, 19, 27, 33, and 50 days of age were injected with amino acids–H3 and killed at subsequent time intervals. In normal rats, radioactive protein synthesized in the rod inner segments was incorporated into outer segment saccules and displaced outward; the total renewal time of outer segments at all ages was approximately 9 days. In RCS photoreceptors, outer segment displacement was slowed from the normal rate before day 17 and at all subsequent stages. Most of the newly synthesized protein appeared to migrate only into the basal third of the outer segments. Labeling of pigment epithelial cells in RCS rats was always heavier than in controls. Labeled protein was displaced as early as 1 hr postinjection from pigment epithelial cell somas into the apical processes, and by 2 hr postinjection was located in the adjacent lamellar whorls characteristic of the mutant rat retina. After 1 day, radioactivity was present in the 14, 15, 17, and 19 day series of RCS rats in the apical third of the outer segment layer (occupied mainly by extra lamellar material) while there were few silver grains in the middle third of the layer (occupied mainly by distal parts of outer segments). The RCS pigment epithelial cells thus have an unusual synthetic role and appear to be a source of the extra lamellar material. Electron microscope examination revealed that many intact pigment epithelial cell processes were incorporated into the large whorls of extra lamellae. In addition, many disorganized outer segment saccules were observed in continuity with longer membranous lamellae and large lamellar whorls. The extra lamellar material therefore appears to be derived from both rod outer segments and pigment epithelial cells.
A disorder of the pigment epithelial cell in the rat disease was demonstrated recently by the radioautographic method that had led earlier to the “escalator” concept of the normal rod disc renewal. Herron et al. (22) discovered that pigment epithelial cells in the mutant animals do not incorporate the membranes of the broken-off tips of the rods. Bok and Hall (3, 4) reported similar observations and added electron microscope evidence that pigment epithelial cells in the mutant lack the cytoplasmic lamellar bodies (phagosomes) which in normal animals represent the phagocytosed rod disc membranes. An interpretation of these observations has been that the mutation affects primarily the pigment epithelial cell, that failure of these cells to ingest the rod discs as they are shed leads to accumulation of extracellular lamellar debris, and this debris forms a barrier between the photoreceptor cells and their blood supply in the choroid such that cell death “is the result of poor nutritional supply to these cells” (22).

Meanwhile, stimulated by the evidence that normal rod discs are continuously renewed (15, 60), we also have reexplored the pathogenesis of the rat disease by radioautography. Our preliminary findings (44) indicated a marked slowing in the rate of renewal of rod discs, and suggested that the extra lamellar material might be generated, at least in part, by the pigment epithelial cells. The present report provides a more complete documentation of the temporal course of the renewal process as well as electron microscope radioautographic data on the locations and sources of the newly synthesized protein. The results have led us to a somewhat different interpretation of the behavior of the pigment epithelial cells in the diseased rats.

MATERIALS AND METHODS

Animals

The Royal College of Surgeons (RCS) strain of rats, homozygous for the rd mutation, was compared with inbred Caesarian-Derived Fisher (CDF) control rats (Charles River Breeding Laboratories, Inc., Wilmington, Mass.). The history of the RCS strain has been given by Sidman and Pearlstein (56), and the phenotype was described by Dowling and Sidman (14). Animals at the F19 generation of brother by sister matings were used in the 1966 radioautographic series described by O’Neil (44), F23 animals in the 1967–68 radioautographic series, and
F29 animals in the electron microscope radioautographic study, and F29-F31 animals for conventional light and electron microscope examination. The pace and character of the disease have remained unchanged by this continued inbreeding over several additional years.

RCS animals are homozygous for the recessive mutant gene, pink-eyed dilution (p) and their eyes are almost free of pigment. Control animals are homozygous for the recessive albinism (c) mutation. Both affected and normal rats were maintained in a 12 hr light-12 hr dark environment at an approximate illumination of 20 ft-candles. All experimental manipulations were carried out in ordinary laboratory illumination.

**Light Microscope Radioautography**

Animals were injected once each intraperitoneally with L-methionine-methyl-³H (specific activity 0.3 Ci/mmole; 15 µCi/g body weight; New England Nuclear Corp., Boston, Mass.) at 14-50 days of age and were killed at times ranging from 1 hr to 23 days later (Table I, column 1). In the 1966 series the eyes were fixed in pairs, one eye by freeze-substitution in propane-isopentane at -175°C followed by 1% picric acid in absolute ethanol at -75°C (16), the other eye by immersion in 2.5% glutaraldehyde in 0.1 M phosphate buffer at pH 7.1. Specimens of both sets were dehydrated at 4°C in methanol: methyl Cellosolve (Fisher Scientific Co., Pittsburgh, Pa.) (1:1) and the posterior halves of the eyes were embedded either in polyester wax (55) or in glycol methacrylate (1). Wax sections were cut at 5 µm and glycol methacrylate sections at 1-2 µm. Sections were either stained by the periodic acid-Schiff method before coating them with emulsion for radioautography, or were stained with toluidine blue in sodium benzoate-benzoic acid buffer (55), after development of the radioautograms. Radioautograms were prepared by the dipping method (32) with Kodak NTB-2 bulk emulsion and were exposed for 3-12 wk.

A semiquantitative assessment of the distribution and concentration of silver grains was made on the glycol methacrylate sections. Background radioactivity was scored 0; activity just greater than background was scored 1; and progressively heavier labeling was graded up to a maximum of 4.

**Electron Microscope Radioautography**

Six 15-day old RCS littermate rats were injected once each subcutaneously with a mixture of equal parts of L-leucine-4,5,6-³H and L-phenylalanine-³H (specific activities 36.2 Ci/mmole and 7.4 Ci/mmole, respectively; 65 µCi/g body weight; New England Nuclear Corp.). These animals were killed 1, 2, 3, 8, 24, or 48 hr later by vascular perfusion with 4% methanol-free formaldehyde in 0.1 M phosphate buffer at pH 7.4, 38°C. Formaldehyde was used to render proteins insoluble while allowing labeled free amino acids to be washed out of the tissue during the histological procedures (46). After approximately 1 hr, the eyes were dissected out, bisected through the posterior pole and cornea, and then immersed in the same fixative for one additional hour. They were then postfixed in 1% osmium tetroxide in the same buffer for 1-2 hours at room temperature, rapidly dehydrated in ethanol, rinsed in propylene oxide, and embedded in an Epon-Araldite mixture.

In 1-2 µ plastic sections of hemispheres of the eyes, areas of retina were selected which were located in the posterior two-thirds of the eye but at least 150 µm from the optic disc and which did not show separation of retina from pigment epithelium. Thin sections from two to four of these areas in each animal were cut with diamond knives on an LKB Ulrotome I (LKB Instruments, Inc., Rockville, Md.). Sections approximately 700 A thickness as judged by interference colors were picked up on bare copper grids.

The grids were placed on a clear glass microscope slide and coated with a thin film of Ilford L4 emulsion (Ilford Ltd., Ilford, England) diluted with equal parts of water according to the loop method of Caro and van Tubergen (7). After exposure in a low humidity atmosphere at 4°C for 12 wk, the radioautograms were developed in Kodak D-19 for 1 min at 18°C, rinsed briefly in distilled water, fixed in Kodak Hypo, and rinsed again in distilled water. Without drying, the sections were stained for 5 min with lead citrate (32).

The sections were examined with a Zeiss EM 9-S2 electron microscope. Areas of pigment epithelium and adjacent neural retina were systematically photographed at a direct magnification of × 5000. Prints were prepared at a magnification of × 15,000, and photomontages were assembled which displayed the region between Bruch's membrane and the outer limiting membrane. Four photomontages were prepared from each animal.

The outer segment layer was cut into basal, middle, and apical thirds. The individual components (Table II, column 1) were then cut from the photomontages, their surface areas were estimated by weighing (Table II, column 2), and the silver grains overlying each component were counted. Data from the montages from each animal were pooled, and the radioactivity in each cell component was expressed as a percentage of the total number of silver grains for each postinjection interval (Table II).

Large lamellar whorls in the apical third of the outer segment layer were studied in the photomontages described above as well as in other electron
microscope radioautographs. Within whorls showing five or more silver grains, the grains were scored as central or peripheral (inside or outside a circle drawn concentric to the margin of the whorl and through a bisected radius). These results are presented in Table III.

**Light and Electron Microscopy**

Retinas from 25 RCS and 4 control rats ranging in age from postnatal days 6 to 22 (Table IV, column 1) were studied by light and electron microscopy. Retinas from 4 additional RCS rats at 30-34 days of age were examined by light microscopy.

The animals were anesthetized with ether and killed by vascular perfusion for 2-5 min with a mixture of 1% formaldehyde, 1.25% glutaraldehyde, and 0.2% 2,4,6-trinitro-m-cresol (26) in 0.1M phosphate buffer, pH 7.4, 38°C, followed by 2-5 min with a mixture of 2% formaldehyde, 2.5% glutaraldehyde, and 0.2% 2,4,6-trinitro-m-cresol in the same buffer. On occasion, 0.1 M sodium cacodylate was used in place of the phosphate buffer. The corneas were slit and the heads of the animals were submerged in the more dilute aldehyde fixative for variable periods (1 hr to overnight at 4°C). The eyes were then treated as described above for plastic-embedded tissues, except that, in addition, the hemispheres were stained en bloc with uranyl acetate (29), and the thin sections were also stained with uranyl acetate before lead citrate.

**OBSERVATIONS**

**Description of the Phenotype**

A detailed morphological, chemical, and physiological description of the over-all time course

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**Figures 1-9** Light microscope radioautographs of retinas from rats injected with methionine$^3$H on postnatal day 14 and killed at subsequent time intervals. 1-4 μ glycol methacrylate. Toluidine blue. X 640.

**Figures 1-5** Control (Fisher) rats.

**Figure 1** 1 day after injection. Silver grains are present over the inner segments (i), over the junction of the inner and outer segments (o), and over the basal third of the outer segments. m, photoreceptor nuclei; pe, pigment epithelial cell.

**Figure 2** 3 days after injection. A band of silver grains lies between the basal and middle thirds of the outer segment layer, distinctly beyond the inner segment–outer segment junction (j).

**Figure 3** 5 days after injection. The band of labeling is slightly distal to the middle of the outer segments. Some label above background is seen more proximally.

**Figure 4** 7 days after injection. The major band of silver grains is present over the apical third of the outer segment.

**Figure 5** 11 days after injection. A concentrated band of labeling is no longer present.

**Figures 6-9** Dystrophic (RCS) rats.

**Figure 6** 1 day after injection. Labeling is present over the inner segments and the junction of inner and outer segments. A substantial number of silver grains also are located adjacent to the pigment epithelial cells in the apical third of the outer segment layer; these are either isolated, oriented in vertical rows perpendicular to the pigment epithelium, or in clumps of three to seven grains over rounded lamellar whorls (arrows). Few silver grains are present in the middle third of the outer segment layer.

**Figure 7** 3 days after injection. Heavy labeling is present over the inner segments and the junction of inner and outer segments. Newly synthesized protein has not migrated as far from the inner segments into the outer segment layer as in control retinas at the same interval (compare with Fig. 9). Conspicuous clumps of silver grains over lamellar whorls are present adjacent to the pigment epithelium (arrows).

**Figure 8** 5 days after injection. Labeling is primarily in the basal third of the outer segment layer and adjacent to the pigment epithelium.

**Figure 9** 13 days after injection. Most of the radioactivity presumably derived from the inner segments is still present in the basal third of the outer segment layer. Some silver grains are located adjacent to the pigment epithelial cells.
of this inherited retinal dystrophy has been presented by Dowling and Sidman (14); only the immediately pertinent morphological features will be summarized here.

In the diseased rat retina, extra lamellar material between the pigment epithelium and the apical part of the rod outer segment first appears on postnatal day 12 or 13. During the next week, the over-all width of the outer segment layer gradually increases above normal because of the accumulation of extra lamellae. They are most abundant in the apical third of the outer segment layer, but some of the large membranous whorls extend into the middle third (e.g., Fig. 10). Subsequently, rod nuclei begin to degenerate so that by day 60, only a few pyknotic photoreceptor nuclei remain. At the same time the outer segment layer becomes disorganized and reduced in width progressively, until most of the residual rod fragments and extra lamellae ("debris") in the outer segment layer are absent by 90 days of age. This process of selective cell death unaccompanied by signs of inflammation is conventionally referred to as "dystrophy."

Light Microscope Radioautography

The results of the light microscope radioautographic analysis of normal and mutant rat retinas are presented in Table I.

**NORMAL PHOTORECEPTORS:** The sequence of outer segment renewal in normal rats was similar whether they were injected on postnatal day 14 or 27. The 14-day series is illustrated in Figs. 1–5. 1 hr after injection of the tritiated amino acids, label appeared over the inner segments. 1 day after injection, silver grains were located over the inner segments, the junction between inner and outer segments, and over the basal third of the outer segments (Fig. 1). At 3 days a band of radioactivity was present between the basal and middle thirds of the outer segment layer (Fig. 2).

After longer time intervals, the silver grains appeared in progressively more scleral positions, but remained essentially unaltered in concentration. At 5 days postinjection, the intense band of labeling was located slightly past the middle of the outer segment (Fig. 3); at 7 days it had reached the apical third of the outer segments (Fig. 4), and by 11 days it was no longer present (Fig. 5). Likewise, 9 days after injection into 27-day old rats the band of radioactivity was missing.

The outer segment renewal time in these normal rats is therefore approximately 9 days. This confirms earlier findings of others (4, 60) and indicates that approximately 10% of the outer segment protein is produced each day. Much of this continuously synthesized protein has been shown to be opsin, the protein constituent of rod visual pigment (2, 20, 21, 41).

**NORMAL PIGMENT EPITHELIAL CELLS:** The pigment epithelium displayed moderate labeling 1 hr after isotope injection in 27-day old rats and light labeling at all time intervals from 1 day onward in both 14- and 27-day old series of normal rats. Occasionally silver grains were seen between the apices of outer segments, probably over pigment epithelial cell processes. In comparable experiments in the frog, Young and Bok (62) have demonstrated that macromolecules synthesized in the pigment epithelial cell soma enter the apical processes between 1 and 6 hr after the injection of radioactive amino acids.

**DYSTROPHIC PHOTORECEPTORS:** Protein displacement in the dystrophic rat retina differed at later time points from that in the normal. 1 day after injection of labeled amino acids into 14-day old RCS rats, radioactivity presumably representing protein produced in the rod inner segments was located, as in the controls, mainly over the inner segments and over the junction of the inner and outer segments (Fig. 6). However, at 17 days of age (3 days after injection) heavy labeling still occurred over the inner segments and the junctional region (Fig. 7, compared with Fig. 2). Protein displacement into the outer segment layer is thus slowed before day 17 in the RCS rats.

In additional animals of the 14-day series, labeled protein was found primarily in the basal third of the outer segment zone by 5 days after injection (Fig. 8), and it had not shifted in position by 9 or 13 days (Fig. 9).

Much less radioactivity was localized over the inner segments and basal parts of the outer segments in the 27-day series, and labeling was only slightly greater than background over the inner segments in the 35- and 50-day series. This matches reasonably well the recent evidence of Herron et al. (24) that photoreceptor outer segment renewal in the RCS rat ceases by approximately 36 days of age.

**DYSTROPHIC PIGMENT EPITHELIAL CELLS:** The concentration of radioactivity over the nucleus and cytoplasm of the pigment epithelial
TABLE I

Distribution of Radioactivity* in Different Components of Photoreceptors and Pigment Epithelium at Various Intervals after the Injection of Methionine-3H into RCS and Control Rats of Different Ages

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<th>IS-3 of OS</th>
<th>IS-3 of OS</th>
<th>Middle 3 of OS</th>
<th>Apical 3 of OS</th>
<th>Distinct lamellar whorls</th>
<th>PE nuclei and cytoplasm</th>
<th>PE apical processes (and nondistinct whorls)</th>
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<td>0-1</td>
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<td></td>
</tr>
</tbody>
</table>
cells was in all cases greater in the RCS rats than in control animals (Table I). In the 14-, 17-, and 19-day RCS series, moderate labeling was still present over the pigment cells 5 days after injection; at later times the labeling decreased to just above background levels. In the 27- and 35-day RCS series light to moderate labeling was found over the pigment cells for only 3 days before dropping to a lower level, and in the 50-day series, the heavier labeling was apparent for only 1 day.

Shortly after injection of the tritiated amino acids, a substantial number of silver grains was found adjacent to the pigment epithelium in the apical third of the outer segment zone. Figs. 6 and 7 illustrate that the silver grains in this region are found either isolated, oriented in vertical rows perpendicular to the pigment epithelium, or in clumps of three to seven grains over large lamellar whorls. Electron microscope radioautography has confirmed that the clumps of silver grains in this region are localized over whorls of lamellae and suggests that the labeled protein migrates into these whorls via the pigment cell processes which actually are a component of the whorls (described below).

<table>
<thead>
<tr>
<th>Age (at injection - at sacrifice)</th>
<th>Component</th>
</tr>
</thead>
<tbody>
<tr>
<td>P35-P35, 1 hr RCS</td>
<td>P35-P35, 5 hr RCS</td>
</tr>
<tr>
<td>P35-P35, 1 hr RCS</td>
<td>P35-P35, 5 hr RCS</td>
</tr>
<tr>
<td>P33-P36 RCS</td>
<td>P33-P36 RCS</td>
</tr>
<tr>
<td>P33-P38 RCS</td>
<td>P33-P38 RCS</td>
</tr>
<tr>
<td>P33-P40 RCS</td>
<td>P33-P40 RCS</td>
</tr>
<tr>
<td>P50-P50, 1 hr RCS</td>
<td>P50-P50, 1 hr RCS</td>
</tr>
<tr>
<td>P50-P50, 5 hr RCS</td>
<td>P50-P50, 5 hr RCS</td>
</tr>
<tr>
<td>P50-P51 RCS</td>
<td>P50-P51 RCS</td>
</tr>
<tr>
<td>P50-P53 RCS</td>
<td>P50-P53 RCS</td>
</tr>
<tr>
<td>P50-P55 RCS</td>
<td>P50-P55 RCS</td>
</tr>
<tr>
<td>P50-P60 RCS</td>
<td>P50-P60 RCS</td>
</tr>
</tbody>
</table>

* Silver grain concentration: 0 or -- = not greater than background labeling; 1 = just greater than background labeling; 4 = maximal labeling.

**P**, postnatal day; **IS**, inner segment of photoreceptor cell; **OS**, outer segment of photoreceptor cell; **PE**, pigment epithelial cell.

§ Silver grains here could not be distinguished from grains localized over PE processes and extra lamellae not in distinct whorls.

**FIGURES 10** and **11** Photomontages of electron microscope radioautographs of the retinas from dystrophic (RCS) rats injected with amino acids-3H on postnatal day 15 and killed at subsequent time intervals. X 7500.

**Figure 10** 1 hr after injection. Silver grains are localized mostly over the pigment epithelial cells and the rod inner segments (is). **bm**, Bruch's membrane; **pen**, pigment epithelial cell nucleus; **lw**, lamellar whorl; **os**, rod outer segments.

**Figure 11** 2 hr after injection. Labeling in the lamellar whorls (lw) adjacent to the pigment epithelial cells is considerably heavier than at 1 hr postinjection. **l**, lipid droplets; **s**, extracellular space between adjacent pigment epithelial cells, distended by perfusion pressure.
Moderate to heavy labeling of distinctly rounded, lamellar whorls was seen after isotope injection in the 14-, 17-, and 19-day RCS rat series (Table I). The concentration of silver grains was somewhat greater in the 14-day than in the 19-day series, but the time of maximum rate of synthesis was not determined precisely. The labeling of the whorls and other lamellar material in the apical third of the outer segment layer in the 27-day dystrophic rat series was considerably less. Almost no labeling of distinct whorls or other lamellae was observed in the 35-day series and none in the 50-day series.

A conspicuous radioautographic feature in every series was the paucity of silver grains over the middle third of the outer segment layer. This, coupled with the fact that newly labeled outer segment lamellae moved distally at an even slower than normal rate, supports the notion that the labeled protein in the apical third of the outer segment layer is produced by the pigment epithelium. An alternate possibility, however, is that protein is synthesized by the inner segments and migrates very rapidly through the matrix between photoreceptors to the apical region. This problem will be considered now.

**Electron Microscope Radioautography**

Electron microscope radioautographic data were obtained in an attempt to define more closely the sources of new protein and its localization within the complex outer segment layer of the dystrophic retina. The distribution of radioactivity in the different components of the photoreceptor layer and pigment epithelium at several time intervals up to 48 hr after injection on day 15 is presented in Table II.

1 hr after injection of the tritiated amino acids, the heaviest labeling occurred over the pigment epithelium and over the inner segments of the photoreceptors, with light to moderate labeling over the pigment cell processes, over the extra lamellae adjacent to the pigment epithelium, and over the basal third of the outer segments (Fig. 10).

From 2 hr (Fig. 11) to 48 hr (Fig. 14) after injection, several changes in the distribution of radioactivity were evident among the several tissue components.

**Dystrophic Photoreceptors:** The inner segments were heavily labeled initially and then showed progressively less radioactivity at each interval studied. Conversely, the adjacent basal parts of the outer segments showed a concomitant increase in radioactivity. By 48 hr some labeled outer segment saccules had been displaced outward as far as the middle third of the layer, but most of the radioactivity was still in the basal third. Little, if any, radioactivity was present in the outermost third of the outer segments at any time through 48 hr postinjection.

At the 24- and 48-hr intervals, some labeled outer segment saccules in the middle third of the outer segment layer were oriented vertically and were in continuity with large lamellar whorls (Fig. 13). This indicates that some of the radioactivity in the extra lamellar material in the RCS retinas probably was derived from disorganized outer segments. In addition, many radioactive, vertically oriented membranes were observed in...
the basal third of the outer segment layer (Fig. 14), but their relationship to the disease process is uncertain because vertically oriented outer segment saccules are often observed in normally developing retinas (13, 57).

DYSTROPHIC PIGMENT EPITHELIAL CELLS AND EXTRA LAMELLAR MATERIAL: The initial heavy labeling of the pigment epithelial cell somas at 1 hr after injection was reduced by about 33% at 2 hr. The lower level of radioactivity was maintained through the 8-hr interval, but was reduced by about another 33% at 24 hr. At 48 hr even less label was evident in the pigment epithelial cells. No phagosomes, labeled or unlabeled, were observed in the pigment epithelial cells (see also 4).

The initial moderate labeling of the pigment epithelial cell processes at 1 hr postinjection was maintained throughout 48 hr. The relative grain counts in this cellular compartment (as well as in the apical third of the outer segments, in the connecting cilia, and in the rare extra lamellae in the basal third of the outer segment layer) have little significance since its surface area comprised such a small percentage of the total area in the montages (Table II, column 2).

The label in the extra lamellar material in the apical and middle thirds of the outer segment layer increased almost threefold between 1 and 2 hr after injection. Extra lamellae adjacent to the pigment epithelium were more heavily labeled at 4 hr (Fig. 12) and 8 hr than at 2 hr. Radioactivity increased progressively by 24 and 48 hr, with most of the increase in the extra lamellae nearest to the pigment epithelial cells. These data indicate that protein synthesized in the pigment epithelial cell somas was promptly displaced into the apical processes and then into the lamellar whorls.
TABLE III

Distribution of Radioactivity in Large Lamellar Whorls* in the Apical Third of the Outer Segment Layer at Various Intervals after the Injection of Amino Acids-3H into 15-Day Old RCS Rats

<table>
<thead>
<tr>
<th>Time after injection</th>
<th>2 hr</th>
<th>4 hr</th>
<th>8 hr</th>
<th>24 hr</th>
<th>48 hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of whorls analyzed</td>
<td>14</td>
<td>13</td>
<td>18</td>
<td>12</td>
<td>20</td>
</tr>
<tr>
<td>Silver grains in center of whorls†</td>
<td>20</td>
<td>20</td>
<td>69</td>
<td>34</td>
<td>71</td>
</tr>
<tr>
<td>Silver grains in periphery of whorls†</td>
<td>101</td>
<td>86</td>
<td>116</td>
<td>71</td>
<td>115</td>
</tr>
<tr>
<td>Total silver grains</td>
<td>121</td>
<td>106</td>
<td>185</td>
<td>105</td>
<td>186</td>
</tr>
<tr>
<td>% of silver grains in center of whorls</td>
<td>17</td>
<td>19</td>
<td>37</td>
<td>32</td>
<td>38</td>
</tr>
<tr>
<td>% of silver grains in periphery of whorls</td>
<td>83</td>
<td>81</td>
<td>63</td>
<td>68</td>
<td>62</td>
</tr>
</tbody>
</table>

* Counts made only on lamellar whorls with five or more silver grains.
† Grains either inside or outside the circumference of a circle drawn concentric to the margin of the whorl and through a bisected radius.

Large rounded whorls in the apical thirds of the outer segment layers which displayed five or more silver grains were examined at each of the post-injection intervals from 2 to 48 hr to determine the distribution of radioactivity within them (Table III). At 2 and 4 hr, 17–19% of the silver grains were localized in the central half of the lamellar whorls, and 81–83% in the periphery. At 8–48 hr postinjection, the percentages of silver grains in the centers had doubled. In the 24- and 48-hr samples, silver grains were often observed over compacted, myelin-like whorls of membranes (Fig. 14).

Light and Electron Microscopy

FORMATION OF EXTRA LAMELLAR MATERIAL: Morphological configurations which could represent extra lamellar membrane formation were sought in the outer segment layers of retinas of RCS rats 14–20 days of age, i.e., the time of maximal accumulation of the extra lamellae (14).

Rod outer segment saccules were observed in various states of disarray in the dystrophic eyes. Near the apices of the outer segments, 5–20 saccules often were oriented vertically, parallel with the long axis of the photoreceptor cells (Fig. 15). This configuration is similar to that seen in the normal shedding of apical outer segment saccules (61). However, such lamellar arrangements also were observed in the middle third of the outer segment layer, a site where they are not found in normal specimens. Pigment epithelial cell processes were usually found next to and surrounding the groups of saccules.

Figs. 16–18 illustrate abnormal arrays of outer segment saccules which appeared independent of surrounding pigment cell processes. These saccules seemed to have lost their orderly, horizontal stacking, coalesced into elongated membranous lamellae, and formed either vertically oriented groups of membranes (Fig. 16) or distinctly rounded whorls of lamellae (Fig. 17). Lamellar whorls sometimes appeared to surround portions of outer segment saccules (Fig. 18). Thus, one component of the rhodopsin-containing extra lamellar material in the dystrophic rat retina is broken-down rod outer segments.

Large lamellar whorls measuring up to several microns in diameter were found most frequently in the apical third of the outer segment layer adjacent to the pigment epithelium. Pigment cell processes were traced into these large masses of lamellae, usually at the periphery of the whorls (Figs. 19 and 21). Less frequently, pigment cell processes were observed in the inner portions of the whorls (Fig. 20). Thus, pigment epithelial cell processes also contributed to the extra lamellar whorls in the dystrophic rat retina.

Some rod outer segment saccules could be identified in even the largest lamellar whorls, especially at points where the saccules had not yet fused end to end (Fig. 20). In most cases, however, lamellae from outer segment saccules and pigment cell processes could not be distinguished. The cytoplasm was often excluded from the attenuated processes of the pigment epithelial cells (Fig. 21), so that the processes closely resembled the abnormally oriented rod outer segment saccules. In addition, membranes in the whorls commonly fused to pro-
duce myelin-like membranes which precluded ready identification of their sources.

Figs. 22-25 illustrate a possible sequence of formation of the large lamellar whorls. Vertically oriented arrays of outer segment saccules were often observed next to the pigment epithelium and surrounded by vertically oriented pigment cell processes (Fig. 22). Generally, the outer membrane of the rod outer segment was at least partially intact. In some profiles the distal tips of pigment cell processes appeared to wrap around small bundles of rearranged outer segment saccules (Fig. 23), resembling an early step of phagocytosis of shed outer segment saccules by pigment epithelial cells in the normal retina (61). The pigment cell processes in the dystrophic retina often branched at proximal and/or distal regions of the processes (Figs. 19 and 22-24) and were oriented horizontally (Figs. 24 and 25) and interdigitated with one another. This horizontal positioning of the pigment cell processes apparently entraps vertically oriented bundles of outer segment saccules of different sizes (Fig. 25), resulting in whorls of membranes from two sources.

Large, well-formed whorls in the apical third of the outer segment zone may gain additional membranes at their periphery both by the direct apposition of outer segment saccules and by the entrapment of vertically oriented bundles of saccules by pigment cell processes (Fig. 19). Some lamellar whorls possibly were composed solely of pigment cell processes.

Membranes were fused in many of the lamellar whorls. Intralamellar spaces were lost and the fusion of the inner leaflets of the unit membranes produced pentalaminar membranes with very osmiophilic, dense, middle periods. With further fusion, many of the whorls became composed partially or almost entirely of myelin-like membranes. Shortly after the injection of tritiated amino acids, radioactive protein apparently passed from pigment epithelial cell somas into the processes, and became located in compacted lamellar whorls (Fig. 14). Therefore, within the whorls it seems likely that pigment cell membranes may fuse with outer segment membranes (or with other pigment cell membranes).

LAMELLAR WHORLS BETWEEN ADJACENT PIGMENT EPITHELIAL CELLS: As early as day 6, lamellar whorls of different sizes and with different degrees of membrane fusion were observed within the pigment cell layer in the spaces between adjacent pigment epithelial cells in the RCS retina (Figs. 26-29). Some of the whorls mistakenly appeared to reside within the pigment cells (Fig. 27), but closer examination revealed their intercellular location (Figs. 28 and 29). This observation was facilitated by a perfusion pressure sufficiently high to distend the extracellular spaces between the basal foot processes and between the sides of the pigment epithelial cells external to the junctional complex (Fig. 29). The lamellar whorls often were separated from Bruch's membrane only by thin-foot processes (Fig. 29); in some instances there was no apparent separation, but whorls have not yet been observed actually crossing the complex basal lamina.

Although some of the whorls between the pigment cells appeared to be formed, at least in part, by pigment cell processes (Fig. 29), the source of most could not be determined. Occasionally, lateral pigment cell processes appeared to invaginate...
into adjacent pigment cell bodies, which suggested the initial stages of lamellar whorl formation between pigment epithelial cells.

The whorls in the pigment epithelial layer often contained one or more droplets of nonpolar lipid (Fig. 29). Although this suggests a degenerative process, it is inconclusive because lipid droplets were present in the cytoplasm of pigment epithelial cells of both RCS and control rats. No typical phagosomes were seen in the RCS pigment epithelial cells; however, some focal condensations in pigment cell processes did suggest ongoing intracellular lysis.

The frequency and distribution of lamellar whorls between pigment epithelial cells were estimated in 1–2 μ sections of retinas from RCS and control rats of various ages. The number of basophilic lamellar whorls anterior and posterior to a point midway between the optic disc and ora serrata was counted (Table IV). Since less condensed whorls (Fig. 26 and 29) were not conspicuous with the light microscope, the number is probably an underestimate. The total number of whorls varied within each age group, but a consistent trend with age was noted. Between days 6 and 15, the number of lamellar whorls in the anterior halves of the RCS retinas was three times that in the posterior half. The difference became progressively less in older animals, and by days 30–34, the numbers in each half were equal. These observations must reflect some temporal and spatial difference of as yet unknown significance in activity among RCS pigment epithelial cells.

Except for a few unidentified elements between pigment epithelial cells (Table IV), most basophilic structures in the control eyes were clearly within pigment epithelial cells; these may have been phagosomes. While no large lamellar whorls were observed in control eyes, many small lipid droplets were noted in the pigment cells at all ages, and these were more numerous in the older animals.

**DISCUSSION**

One of the most intriguing features of this inherited disease in rats is the excess net content of rhodopsin in the eye (14, 43). Dowling and Sidman (14) recognized the increase beginning at 14 or 15 days of age. By 20 days the rhodopsin content of the RCS eyes was twice that of the controls. They suggested that there must be a marked increase in the synthesis of outer segment protein in the RCS retina. Subsequent radioautographic studies (22, 44), however, have demonstrated a marked slowing of the rate of production of rod outer segments. In the present study we have demonstrated that the slowing begins before day 17. Herron et al (22) have indicated with similar experiments that the rate of outer segment production in RCS rats is slowed beginning on day 18. The apparent discrepancy probably results from the greater resolution we obtained in 1–2 μ glycol methacrylate radioautographs than in our own (or Herron’s) 5 μ paraffin sections.

Herron et al. (22, 24) and Bok and Hall (4) have shown that in RCS rat retinas, newly synthesized protein is displaced from the inner segments either to the apical third of the outer segment layer, or more often, to about halfway into the outer segment layer. Also, no typical phagosomes are observed in RCS pigment epithelial cells (4). These features led to the interpretation that the abundant, rhodopsin-containing debris in RCS rat retinas forms from degenerated outer segments (3, 4, 22). Although this may be correct, simple quantitative considerations indicate that it does not fully account for the extra lamellar material.

In the normal rat, the band of labeled protein maintains a uniform intensity during the 9 day escalator excursion proximodistally along the outer segment (60). This and the lack of ribosomes in the outer segment imply that the pro-

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**FIGURE 19** Large lamellar whorl (lw) adjacent to a pigment epithelial cell (pe). Several pigment cell processes can be traced into the periphery of this whorl (arrows), one of which branches (b). Additional outer segment sacules appear to be adding to the whorl by directly coalescing with the membranes (oss, lower left) or apparently by becoming oriented vertically and alternating with or being entrapped by pigment cell processes (oss, upper right). X 20,925.

**FIGURE 20** Large extra lamellar whorl adjacent to a pigment epithelial cell (pe). A pigment cell process (arrows) is present in the central region of the whorl. Some rearranged outer segment sacules (oss) can be identified where sacules have not yet fused end to end. X 28,300.
tein moves the length of the outer segment without metabolic turnover. Rod protein is synthesized in the inner segment, incorporated into the innermost (youngest) outer segment discs, and is degraded after these discs attain the outermost position and are shed from the outer segment (61, 62). Thus, about 10% of the rod protein is renewed per day. During the second postnatal week, when the rod outer segments are first lengthening, the degradation rate must be less than 10%, and thereafter, when outer segment length is stable, it must equal the synthesis rate. If the degradation mechanism were to become inactive in RCS rats during the third week, as suggested by Herron et al. (22, 24) and by Bok and Hall (3, 4), 5 days later one would expect approximately 50% more outer segment material to be present. However, between days 15 and 20 the rhodopsin content in the mutant eye actually increases to 100% more than the control value (14). This indicates that even if degradation were zero, the rate of synthesis would have to be increased in order to account for the net accumulation of rhodopsin. The site of this increased synthesis could not be the RCS photoreceptor cells, for their rate of outer segment production is slowed before day 17 and at all stages thereafter. Therefore, the extra lamellar material must come, at least in part, from some other source. Furthermore, the degradation rate is not zero, at least at later stages of the disease, since the rod outer segments and the extra lamellae do not persist indefinitely. If a degradation mechanism is operative at all during the third postnatal week, an even greater synthesis of rhodopsin-containing material would be necessary to account for the net increment in 20-day old rats.

Perhaps the lamellar whorls we observed between pigment epithelial cells represent an aberrant disposal mechanism of extra lamellar membranes. Removal of lamellae from the outer segment layer by this route, however, would require material to pass through the junctional complexes at the apices of adjacent pigment epithelial cells. Also, no whorls were seen penetrating Bruch's membrane, presumably another requisite for such a disposal mechanism.

Our radioautographic data suggest that the pigment epithelial cells are a source of extra lamellar material. In RCS animals injected with tritiated amino acids on days 14, 17, or 19, radioactive protein is localized by light microscope radioautography in lamellar whorls adjacent to the pigment epithelium as early as 5 hr postinjection. With electron microscope radioautography, labeled protein may be traced from pigment epithelial cell somas into the pigment cell processes within 1 hr and into the lamellar whorls.
within 2 hr. The possibility exists that radioactive protein might have been released rapidly from the inner segments into the interphotoreceptor matrix, bypassing the outer segments to accumulate in the apical region adjacent to the pigment epithelium. This seems unlikely, however, since the electron microscope radioautographic study includes time intervals before the appearance of label in the lamellar whorls, and virtually no silver grains were observed in the matrix between the outer segments.

The pigment epithelial cells in the RCS rat retina displayed greater than normal radioactivity at all ages examined after the injection of labeled amino acids. This may reflect differences in pool sizes of amino acids, in uptake or transport of the protein precursors, in rates of protein synthesis, or in local reutilization. At present we cannot distinguish among these possibilities.

The quantity of labeled protein displaced into pigment cell processes and lamellar whorls was maximal at precisely the time (days 14–20) when the accumulation rate of the rhodopsin-containing lamellar material was greatest (14). Likewise, the displacement of labeled protein from the pigment epithelium into the outer segment layer was progressively diminished in the 27, 35, and 50 day series, concomitant with the decrease in the rate of excess rhodopsin accumulation in the RCS rat eye (14). We should stress, however, that we have no evidence that the RCS pigment cells produce specifically opsin as opposed to other proteins. Similarly, we cannot assume that the labeling patterns in the mutant eye necessarily represent ongoing metabolic mechanisms identical to those in normal eyes.

Bok and Hall (4) also noted some early accumulation (1 day postinjection) of labeled membranes next to the pigment epithelium. They injected 11-day old rats, however, an age at which the outer segments are still so short that the pigment epithelium and the basal parts of the outer segments lie too close together for the resolution of separate sources of radioactivity. Fig. 7 of Herron et al. (22) also supports our interpretation of an unusual synthetic activity by the RCS pigment epithelial cells.

In the present ultrastructural study, the lamellar whorls in the retinas of RCS rats were observed to be composed of membranes from two sources—pigment epithelial cell processes and rod outer segment saccules. In the original description of the abnormal lamellar product (14), the material was referred to as "debris" or "extracellular lamellae." In light of the current observations, it is suggested that the abundant membranes be termed extra lamellae or extra lamellar material, since the whorls are neither entirely extracellular nor entirely "dead" material.

In the short time intervals after the injection of tritiated amino acids, the whorls in the apical third of the layer presumably would be composed of radioactive pigment cell processes and non-radioactive outer segment saccules. The dynamic events are reflected also in the change in distribution of radioactivity within the lamellar whorls from predominately peripheral labeling at 2 and 4 hr to more central labeling at 8, 24, and 48.
TABLE IV

Number of Lamellar Whorls between Pigment Epithelial Cells in the Eyes of RCS and Control Rats

<table>
<thead>
<tr>
<th>Age</th>
<th>Number of animals</th>
<th>Number of sections examined</th>
<th>Avg number in posterior half</th>
<th>Avg number in anterior half</th>
<th>Avg number total</th>
<th>Ratio posterior/anterior</th>
</tr>
</thead>
<tbody>
<tr>
<td>RCS</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P6-10</td>
<td>5</td>
<td>14</td>
<td>2.6</td>
<td>6.3</td>
<td>8.9</td>
<td>0.41</td>
</tr>
<tr>
<td>P12-13</td>
<td>3</td>
<td>8</td>
<td>2.9</td>
<td>11.0</td>
<td>13.9</td>
<td>0.26</td>
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<td>P14-15</td>
<td>9</td>
<td>19</td>
<td>2.6</td>
<td>9.4</td>
<td>11.9</td>
<td>0.28</td>
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<td>8</td>
<td>4.8</td>
<td>6.3</td>
<td>11.1</td>
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<tr>
<td>P12-13</td>
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<td>11</td>
<td>0.3</td>
<td>0.1</td>
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<tr>
<td>P20-21</td>
<td>2</td>
<td>9</td>
<td>0.9</td>
<td>0.8</td>
<td>1.7§</td>
<td>—</td>
</tr>
</tbody>
</table>

P, postnatal day.

Counts made from 1–2 μ plastic sections in the sector of retina between the optic disc and ora serrata.

$ Anterior or posterior to a point halfway between the optic disc and ora serrata.

§ Unidentifiable basophilic structures between pigment epithelial cells.

hr. If one assumes that newly synthesized protein does not leave the pigment cell processes, two interpretations of these data are available. First, the rate of protein transfer may be slower in the more narrow pigment cell processes nearer the central part of a whorl. Second, pigment cell processes may surround nonradioactive outer segment membranes and then rotate to form lamellar whorls. The grain counts in this case would indicate the rate of whorl formation and imply that whorls are formed within about 8 hr.

It has been suggested that inherited retinal degeneration in the rat is a pigment epithelial disease even though the outstanding phenotypic expression is loss of photoreceptor cells (22). Consistent with this view, the RCS pigment cell does not phagocytose rod outer segment saccules (3, 4), and displays dedifferentiated (14) and otherwise abnormal configurations (e.g., lamellar whorls produced, at least in part, by lateral processes of pigment cells) and unusual protein synthetic properties as shown in the present study. In addition, there appears to be a fragility of lysosomal membranes in the RCS pigment epithelial cells possibly as early as the first postnatal week (6, 59). On the other hand, the formulation that the accumulation of extra lamellar material results in a separation of the photoreceptors from the pigment epithelium and their nutritional source, the choriocapillaris, thus leading to visual cell death (22) seems unlikely as a complete explanation of the disease process. The pigment epithelial cells through which metabolites must pass are actually not isolated from the photoreceptors. In fact, many pigment cell processes surround lamellar whorls and are actually in intimate contact with rod outer segments. Another objection comes from the report that detachment of neural retina from pigment epithelium in the owl monkey causes degeneration of outer segments, but the visual cells remain viable and capable of regenerating outer segments for at least 12 wk (40). Similarly, immature photoreceptors differentiate and survive for up to 3 wk when isolated from the pigment epithelium and grown in tissue culture (25, 37).

In the RCS rat retinas some of the outer segment saccules become disorganized forming vertically oriented or circular arrays of lamellae. Perhaps some defect in the normal orienting mechanism accounts for the loss of outer segment integrity in the RCS retinas. The pigment epithelium is thought somehow to promote the orderly arrangement of outer segment saccules in normal developing photoreceptor cells (42). When immature rat neural retina is separated from its pigment epithelium and grown in tissue culture, photoreceptors can synthesize some outer segment saccules but do not form appreciable lengths of horizontally oriented stacks of discs (25, 37). Also, when neural retina is mechanically separated from the pigment epithelium, there is a loss in the orderly arrangement of outer segment saccules (33). Conversely, when outer segments
are regenerating after experimental retinal detachment (34, 35) or after experimental vitamin A deficiency (12), or even when they are first forming in normal development (13, 57), newly synthesized outer segment membranes usually go through a vertically oriented stage before their rearrangement into orderly stacks of sacculles. Furthermore, Kuwabara (36) has suggested that during recovery from retinal damage due to light exposure, myelin-like membranes may be formed by pigment epithelial cells.

The RCS photoreceptor cells also exhibit several abnormal features before the first histological signs of the disease which are recognized at about day 12 (4, 14). Some metabolic abnormalities perhaps precede the earliest formation of outer segments which occurs at about day 5 in normal rats (57). For example, the hexosemonophosphate shunt activity in the dystrophic neural retina is considerably higher than that of normal retina as early as postnatal days 6–7 (47). Amino acid uptake and protein synthesis are reduced in whole retinas of RCS rats as early as day 5 (50), and at day 7 (as well as at “mature” ages), the fifth isoenzyme of lactic acid dehydrogenase (“M” isoenzyme) is substantially lower in RCS than in normal retinas (17, 18). Interestingly, this isoenzyme is also deficient in some other inherited diseases, including muscular dystrophy (27, 58).

Light adaptation (bleaching) results in a far greater loss of vitamin A aldehyde (retinal) from the retinas of dystrophic rats than from controls (48). Also, dark adaptation in the dystrophic animals results in the regeneration of less visual pigment than normal (48). Despite these and other biochemical anomalies in the dystrophic retina (for reviews see 19 and 49), the nature and cellular localization of the primary genetic defect is not yet known. Although the problem of defining the primary action of the rd genetic locus is significantly more complex with the interrelationships of two cell types, the morphological relationships and chemical interactions between these two cells are perhaps better understood than any other examples in the nervous system. The photoreceptor-pigment epithelial cell complex serves as an excellent system for further analysis of genetic control of cell interactions.

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LaVail, Sidman, and O'Neil. *Inherited Retinal Degeneration in the Rat* 209