Some Cytological and Initial Biochemical Observations
on Photoreceptors in Retinas of rds Mice

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As a control for biochemical studies in progress, an ultrastructural study has been carried out on the
deteriorating, 21-day photoreceptors of the 020/Cpb strain of mice, homozygous for retinal degener-
ation, slow (rds). At 21 postnatal days, outer segments were essentially lacking, but cilia erupting
from the inner segments were common. A low percentage of cilia bore small cytoplasmic masses
containing a few layered membranes, and rare inner segments possessed spherical aggregations of
multilayered membranes. Pigment epithelial cells also possessed membranous aggregations in pre-
sumed phagosomes. While other parts of photoreceptors possessed the usual organelles of normal
rods, inner segments were reduced in volume, and the layer of photoreceptor synaptic terminals was
thinner. Mutant 21-day retinas possessed about two-thirds of the protein of normal 21-day retinas
but 50% more protein than "rodless" (rd/rd) 21-day retinas. Surprisingly, while dark-adapted rds
retinas possessed markedly lower levels of cyclic GMP as compared to controls, light-adaptation
significantly reduced cyclic GMP and cyclic AMP levels, and biochemical data point to persistent
light-modulated cyclic nucleotide levels in the photoreceptors. Invest Ophthalmol Vis Sci 24:832-
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In 1978, a new retinal mutant, retinal degeneration, slow (rds), was reported by Van Nie et al1 in the
inbred strain 020/A. Over the last few years, Sanyal and various associates have published a number of
papers,2-4 further describing the retina of this mutant. The photoreceptor layer was reported as persistently
rudimentary during the first 2 weeks of postnatal de-
velopment. Cytologically, an absence of outer seg-
ments was noted throughout the postnatal period,
while initially other parts of photoreceptor cells were
relatively unaffected. Consistently, they noted an ab-
sence of a rhodopsin peak in difference spectroscopy
of extracts of these retinas. Following the first 2 post-
natal weeks, there was a slow and progressive loss of
photoreceptors, so that by a year, they had vanished
in all retinal regions. On the other hand, the devel-
opment of other retinal layers appears to proceed
normally.

In normal mice, the eyes are open and functional
at the third postnatal week. However, in rds/rds mice,
the early studies2-4 indicate the existence of a sub-
stantial interval, beginning with the third week, where
photoreceptor outer segments were lacking, while all
other photoreceptor regions persist. This appeared to
offer an opportunity to obtain clues not only to the
basis of the dystrophy, but also clues to chemical ac-
tivities unique to outer segments and other regions
of these cells and/or how materials synthesized else-
where in the cell for insertion in outer segments be-
have when this entity is lacking. This might be ac-
complished by comparing retinas of the same devel-
opmental age from rds/rds mutants, from rd/rd
mutants, and from normal animals. The 21st post-
natal day seemed most appropriate for the biochem-
ical studies, since it represents a compromise between
one of the first days after eye opening, when control
photoreceptors may be presumed to be fully func-
tional, and a time when, apart from outer segments,
photoreceptors of rds/rds mice reportedly show min-
imal structural loss and are only slightly fewer than
control photoreceptors. Unlike rds/rds, at 21 days,
rd/rd retinas exhibit but a single remaining layer of
photoreceptor cell bodies, no outer segments, some
inner segments, sometimes with cilia, and sparse syn-
apses.1

Demant et al6 have reported the rds mutation to
be located on the 17th chromosome of the mouse, in
contrast to the rd mutation on the 5th chromo-
some, and Sanyal and Hawkins4 have described some
interactive effects of these genes in mice homozygous
for both.

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More detail was sought on the morphology of the degeneration as background for the biochemistry, and this brief report provides more detailed ultrastructural information on the rds photoreceptor during the first postnatal month but centered on the 21st postnatal day. Initial biochemical comparisons of 21-day rds, rd, and control retinas centering on light sensitivity and on cyclic nucleotide levels are provided. This metabolism is especially pertinent because it is normally both light influenced and involves interactions of outer segment and inner segment components. The main body of biochemical data will be reported after these studies are completed.

Materials and Methods
Breeding pairs of mice of the 020/Cpb strain, homozygous for rds, were obtained from Centraal Proefdierenbedrijf TNO (Zeist, The Netherlands); breeding pairs of mice of C57BL/6J origin, homozygous for rdle (the rd mutation plus a linked marker gene, le) or C3H mice that are also rd/rd were obtained from the R.P. Jackson Memorial Laboratory (Bar Harbor, ME) as were C57BL/6J and BALB/c control stocks. Each stock was kept in isolation from other mice. The 020/Cpb and BALB/c stocks are albino; the others are pigmented. At the recommendation of Sanyal, breeding pairs of 020/A mice were ordered from the Dutch breeding facility but the rds/rds mice received were marked 020/Cpb and are from a separate breeding colony with rds derived from 020/A. Only limited quantities of rdle/rdle retinas were available for these comparative studies. All animals were kept on 12-hr dark/light cycles, with the illumination of both control and mutant albino animals held to less than 1 ft cd at their cages, although in pilot experiments
albino controls exposed to the same illumination as pigmented controls gave similar cyclic nucleotide levels.

For microscopy, isolated retinas or hemi-eyecups with or without hemi-retinas in place, were fixed in cold 2.5% glutaraldehyde in 0.16 M cacodylate buffer for 1 hr, rinsed 3X (10 min) in 0.2 M cacodylate buffer and then post-fixed in 1% Os in 0.2 M cacodylate buffer for 1 hr. For transmission microscopy, these were dehydrated in alcohols and embedded in Araldite. For scanning electron microscopy, glutaraldehyde-fixed retinas were dehydrated in acetone prior to critical point drying, and gold sputtering.

For the chemical studies, dark or light-adapted animals of 21 days of age were killed by decapitations and retinas removed under dim red light or white light, as described earlier.9 Retinas were either frozen at once or retinas from dark-adapted mice were incubated in total darkness or in darkness followed by bright light, and then frozen (within tubes plunged into liquid nitrogen). Isolations of retinas were in Earle's medium (with a 3 mM K+ level) buffered with either 2.6 mM bicarbonate-5% CO₂ or 5 mM HEPES.

The HEPES-buffered version also lacked phosphate and sulfate ions and was used in experiments where divalent cations employed for synaptic blocking action were present in incubation media. The frozen retinas were ground in ice-cold 10% trichloracetic acid. Protein was measured by the method of Lowry,9 and cyclic nucleotide levels by radioimmunoassay.10-11

Within each variety of mice randomized for sex, retinas from 21-day animals were incubated or not,
and light exposed or not. Data from the groups to be compared were analyzed by a one-tailed Student's "t" test for the significance of differences.

Results

Microscopy

A survey by days of isolated postnatal rds/rds retinas from 8 to 28 days confirmed the light microscopic study of Sanyal et al,2 and a brief, low power electron microscopic study of Sanyal and Jansen,3 as to the essential absence of outer segments of photoreceptors in the rds/rds retina (Figs. 1A-B). In 21-day retinas, both of the control retinas possessed about 9-deep cell nuclei in the thickest part of the photoreceptor layer, and columns of 7 to 9 photoreceptor cell nuclei could be found in this region of the rds retinas. Many (perhaps most) of the inner segments of rds retinas possessed a cilium at their apex. In preparations where
In retinae not isolated, the cilia were seen to interdigitate with the microvillous processes from the apical face of the pigment cells (Fig. 2). Examined by electron microscopy, cross-sections of the cilia of rdS photoreceptors revealed no obvious deviation in microtubule arrangements or numbers, as compared to controls (Figs. 3, 4), nor were there apparent differences in basal bodies. However, the ring of microtubule pairs in rdS cilia often had an increased diameter similar to that normally seen in the ciliary region at the base of normal mouse outer segments. In addition, among the cilia, occasional non-circular cross-sections of similar area were found that contained up to 25 microtubule singlets (Fig. 5, arrow). These appear to be sections through constricted regions of inner segments. They were seen rarely in controls.

Fig. 5 illustrates a portion of a section tangential to the rdS retina at a level that lies just above or below the junction of individual cilia with the inner segments from which they erupt. The full low-power field of which this is a part contained 40 cilia, 15 inner segments with basal bodies or centrioles and 48 inner segment cross-sections with neither (total = 103).
A nearby field, cutting inner segments well below their apices, contained 113 cross-sections in the same area. Considering the variability in the levels of ciliary junctions with inner segments, the above result is consistent with the hypothesis that most rds inner segments bear cilia. Scanning electron microscopy of isolated retinas (Fig. 6) confirmed the high frequency of cilia, although, partly due to distortions related to isolating the retinas, these were not always erect and discerned at first glance.

The ventricular space (sub-retinal space) also contained vesicular elements (Fig. 7) of 200- to 400-nm diameter that may, in part, derive from the breakdown of outer segment membrane, but could also derive from the breakdown of microvillous processes of pigment or Müller cells.

Examination of the cells of the albinotic pigment epithelium from rds eyes of 10 to 28 days found them highly similar to those from the albino control eyes at these ages. There were membranous accumulations in their cytoplasm (Fig. 8). Although usually somewhat smaller in rds cells, they were similar to those seen in the control pigment cells (not illustrated).

Suggestions of forming outer segment membrane could be found at the apices of some cilia in eyecups of 10-day animals. In addition, at this age, in a few localized areas of the ventricle, masses were evident, consisting of fibrils of about 6.5 nm with particles of 10 to 20 nm (Fig. 9). These likely result from the
Fig. 9. An electron micrograph of the ventricular (sub-retinal) space of a 10-day rds/rds eyecup preparation. At the upper left are processes of pigment cells containing amelanotic melasomes (M). Below are inner segments, from one of which erupts a cilium (C) at whose apex are some disorganized membranes. This particular region of ventricular space contains a mass of fine fibrils (F) of approximately 6.5 nm diameter with particles ranging from 10 to 20 nm. Bar equals 1.5 μm.

Fig. 10. An electron micrograph of the inner segments of several photoreceptors from a 21-day rds/rds retina. Note the lamellar object (L) in one of the cells, basal bodies, rootlets with adjacent vacuoles (arrow), mitochondria, ribosomes, and SER vacuoles. Bar equals 1.25 μm.

Fig. 11. An electron micrograph of the apex of an inner segment of a 21-day rds/rds retina. Note cilium with attached cytoplasmic mass with lamellar object and numerous small vesicles. Also note some small vesicles in the cilium. Bar equals 0.75 μm.
However, the lengths of the inner segments were clearly reduced without obvious enlargement in their diameters. The mass of certain organelles (e.g., mitochondria) may be less per cell. The photoreceptor nuclei appeared normal, as did most synaptic terminals (Figs. 12, 13). The latter possessed synaptic vesicles, ribbons, etc., but the synaptic terminal layer was thinner. Despite the qualitatively normal appearance of the terminals, the loss or developmental failure of some terminals is likely, and others may be reduced in size and quantitatively modified with respect to vesicles, ribbons, etc.

Raising rds/rds litters in either constant light or total darkness from birth did not obviously affect the retinal condition at 21 days.

One problem with a potential for influencing the biochemical data in this study was the tendency on detaching both normal and rds/rds retinas of this age for the apical (ventrical-facing) portion of the pigment cells (with the usual microvilli on this surface) to tear from these cells and to form flattened, membrane-bound plaques. These were adherent to a presumed deterioration of a cell, but they may be a transient extracellular element. Although most ciliary shafts or tips seemed free of cytoplasmic masses with membranes, a diligent survey of 21-day retinas revealed rare membrane-containing cytoplasmic masses either in the apical regions of inner segments (Fig. 10) or on ciliary shafts (Fig. 11). In Fig. 11, note the crisp notch at the base of the cytoplasmic mass appended to the ciliary shaft. In addition, a few small vesicles are present within the ciliary shaft and may occur in the mass at the ciliary tip. It appears possible that this illustrates an abortive attempt to construct an outer segment and its membranes. Occasional vesicles were seen within normal ciliary processes, but too few cilia, such as those of Fig. 11, were encountered to permit the generalization that vesicles are somewhat more frequent within rds cilia bearing membranous accumulations.

The remainder of the 21-day photoreceptor possessed a normal appearance, by comparison, to 21-day controls or retinas of older control mice (not illustrated). Examination of rds/rds inner segments revealed basal bodies, ciliary rootlets, mitochondria, ribosomes, SER, microtubules, and a Golgi region.
ventricular gel that is transparent (Figs. 14, 15). A systematic study of this tendency as a function of age has not been carried out, but such plaques were rare in isolated mouse retinas used in previous studies from this laboratory. These were from normal animals of 60 days or older. However, isolated rdle/rdle
Table 1. Protein content of 21-day isolated mouse retinas

<table>
<thead>
<tr>
<th>Stock</th>
<th>Protein</th>
<th>Number retinas</th>
</tr>
</thead>
<tbody>
<tr>
<td>C57BL/6</td>
<td>330 ± 3.9</td>
<td>55</td>
</tr>
<tr>
<td>BALB/c</td>
<td>311 ± 3.9</td>
<td>61</td>
</tr>
<tr>
<td>rds (020/Cpb)</td>
<td>214 ± 4.0</td>
<td>57</td>
</tr>
<tr>
<td>rdle (C57BL/6)</td>
<td>144 ± 2.4</td>
<td>57</td>
</tr>
<tr>
<td>rd (C3H)</td>
<td>176 ± 2.7</td>
<td>59</td>
</tr>
</tbody>
</table>

Protein: mgm/retina ± SEM.

Protein at 21 days were generally free of these plaques. This does not seem to be an effect of the rd/rd condition, since isolated 21-day retinas of C3H mice that are also homozygous for rd/rd possessed a heavy contamination with such plaques. The individual plaques in rds retinas seemed to have lost lateral contact with neighboring plaques and thus far, membranous accumulations of potential photoreceptor origin have not been encountered within rds pigment cell plaques or, externally, adherent to their microvilli. Scanning electron microscopy revealed that areas with such plaques in some rds/rds retinas could cover as much as one-third of an isolated retina. In terms of complicating the interpretation of biochemical data, it was fortunate that many of these plaques (but not all) came off the retinas during (6 mn, 37 C) incubations. It is also of interest that the "empty," presumptively gel-filled ventricular space between the plaques and the isolated rds/rds retinas is largely maintained, despite the dehydration related to processing for microscopy.

At 21 days, macrophages were not infrequent in the ventricular space (Fig. 16), confirming Sanyal et al.2 A macrophage in this position was seen as early as the eighth postnatal day, suggesting early awareness by the immune system of some signal. Most, if not all, ventricular macrophages were lost during incubations of isolated retinas. Some macrophages are present within the retinas, and these remain in the incubated retinas. The latter might affect biochemical data.

Biochemistry

Table 1 reveals the significant differences in the comparative post-incubation protein contents of the types of isolated retinas used in the biochemical studies and used as controls for morphology in the current study. The 21-day rds retinas possessed about two-thirds of the protein of the two normal lines (C57 and BALB) but about 50% more protein than the rd/rd stocks. The protein difference between BALB and C57, while small, is significant (<0.01), as is that between rdle and C3H (<0.001).

Table 2 shows the result of cyclic nucleotide assays on retinas removed from dark-adapted mice under dim red light or from light-adapted mice under white light and immediately frozen. The surprising observation was that significant (<0.01) light effects were evident on cyclic nucleotides of the rds retinas—even those (a single study) from animals of 163 days of age, where the layer of photoreceptor nuclei was but 4-deep compared to 7 to 9 at 21 days.

Table 3 presents evidence that divalent ions commonly employed as blockers of calcium-dependent synaptic mechanisms fail to block the ability of light to reduce cyclic GMP. The levels of cyclic GMP

Table 2. Cyclic nucleotide levels of freshly isolated retinas

<table>
<thead>
<tr>
<th>Stock</th>
<th>Number</th>
<th>Adaptation</th>
<th>cGMP</th>
<th>Signif. Level</th>
<th>cAMP</th>
<th>Signif. Level</th>
</tr>
</thead>
<tbody>
<tr>
<td>C57</td>
<td>6</td>
<td>DK</td>
<td>100.1 ± 7.6</td>
<td>&lt;0.001</td>
<td>13.6 ± 1.6</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>21 days</td>
<td>6</td>
<td>LT</td>
<td>25.4 ± 1.7</td>
<td></td>
<td>7.5 ± 0.77</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>BALB/c</td>
<td>6</td>
<td>DK</td>
<td>70.6 ± 1.8</td>
<td>&lt;0.01</td>
<td>13.1 ± 1.0</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>21 days</td>
<td>6</td>
<td>LT</td>
<td>38.4 ± 2.2</td>
<td>&lt;0.001</td>
<td>10.6 ± 1.6</td>
<td>N.S.</td>
</tr>
<tr>
<td>rds</td>
<td>8</td>
<td>DK</td>
<td>18.0 ± 2.4</td>
<td></td>
<td>17.9 ± 1.76</td>
<td></td>
</tr>
<tr>
<td>21 days</td>
<td>10</td>
<td>LT</td>
<td>9.2 ± 1.3</td>
<td>&lt;0.01</td>
<td>11.0 ± 0.67</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>rds</td>
<td>6</td>
<td>DK</td>
<td>8.7 ± 1.1</td>
<td></td>
<td>12.5 ± 1.1</td>
<td></td>
</tr>
<tr>
<td>163 days</td>
<td>6</td>
<td>LT</td>
<td>4.7 ± 0.3</td>
<td>&lt;0.01</td>
<td>9.9 ± 0.34</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

Cyclic nucleotides: pmole/mg protein ± SEM.
were increased in these experiments by including the phosphodiesterase inhibitor 3-isobutylmethylxanthine (IBMX) at 1 mM.

Discussion

The most interesting new morphologic observation on the altered photoreceptors of the rds retina is the common persistence of a cilium extending from the apex of the inner segment. This mimics the conjectural ancestral cell from which vertebrate photoreceptors arose. In terms of biochemistry, inasmuch as Fleischman and Denisevich,12 and Fleischman et al13 have implicated the ciliary axonemes and/or their basal bodies as the main locus of the activity of photoreceptor particulate guanylate cyclase and as a locus of activity of adenylate cyclase, the opportunity exists to look for these activities both in cell-free preparations and in whole, incubated rds retinas.

The surprising persistence of dark-light differences in retinal cyclic nucleotides demonstrates that the failure to detect photopigment by the usual spectroscopic techniques may only mean that the level is very low and not in the range of the sensitivity of these methods. Possibly a radioimmune assay would detect opsin.

The possible locations of persistent photopigment are of interest. It could be in transfer vesicles in inner segment cytoplasm or in the plasma membrane. However, candidate transfer vesicles are not obvious in normal mouse inner segments and were not evident in the mutant inner segments. Some rds ciliary shafts contained vesicles, but these may represent breakdown products of membranes. The photopigment might be confined to the plasma membrane of the cilium or the inner segment. Photopigment may well occur in the membranous aggregations (Fig. 10) found in a low percentage of the apices of photoreceptor inner segments. In preliminary studies utilizing tangential sections of 21-day rds/rds retinas at the apical zone of the inner segments, membranous aggregations were noted in less than 1 in 500 inner segments, but a careful statistical analysis as to their retinal distribution, the volumes of the aggregates, and the membrane surface area they represent has not been carried out. It seems more desirable at first to ascertain how old rds/rds mice may be and still possess light-sensitive pools of cyclic GMP in their retinas, and then to examine their photoreceptors qualitatively and quantitatively for such aggregates. Such studies are in progress as are studies on lightsensitive retinas16 from rd/rd mice. Immunocytologic techniques utilizing “tagged” antibodies to opsin might help resolve the localization issues by surveying plasma membranes and potential sites in pigment cell plaques, cilia, and cytoplasmic membranes.

As light effects were discerned by an ability to reduce cyclic nucleotide levels, the question is raised as to whether this loss occurs in the photoreceptors and, if so, whether it occurs via the usual mechanism of outer segments. This would require that the ingredients of the cyclic GMP nucleotide cascade (the intrinsic membrane protein, rhodopsin, and the peripheral membrane proteins, GTP-binding protein, and cyclic GMP phosphodiesterase) be in reasonable proximity to one another wherever they are. Is the light-induced diminution in cyclic GMP levels intraretinal? This position is supported by the results obtained with incubated rds retinas, where 5 mM Cd++, Co++, or Mn++ failed to block significant light-induced losses of dark cyclic GMP levels obtained in the presence of phosphodiesterase inhibitor 3-isobutylmethylxanthine (IBMX).

There is no clue as yet as to the causal sequence leading to the absence of outer segments. There appears to be some ability to form, but not retain, membranes associated with the photoreceptor cilium. This membrane may be abnormal or, perhaps, the disc shedding mechanism is in constant, uncontrolled activity under some abnormal influence.

Finally, I confirm the interesting observation of Sanyal et al17 that despite the virtual absence of outer segment membrane of all 21-day photoreceptors, the loss of the individual modified photoreceptors is distributed over many months. One suspects that rds/rds mice, like rd/rd mice,18 will prove to possess high threshold vision for months.

Key words: mice, photoreceptors, dystrophy, rds, cilium, retina, cyclic nucleotides, pigment epithelium

Acknowledgment

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