Distribution and synthesis of sulfated mucopolysaccharides in the retina of the rat

David E. Ocumpaugh and Richard W. Young

The distribution and synthesis of retinal mucopolysaccharides (MPS) in the rat have been studied by autoradiography, following injection of $^{35}$S-sulfate, supplemented by histochemical procedures. The results indicate that sulfated MPS occur in the plexiform and optic nerve fiber layers, but are most heavily concentrated in the photoreceptor zone. In this zone, production of MPS becomes significant when the rod outer segments start to develop. Sulfated MPS are synthesized in the region of the rod inner segments, and are subsequently displaced into the extracellular spaces between the outer segments, where they undergo rapid breakdown. A considerable proportion of the retinal sulfated mucopolysaccharide in the rat is hyaluronidase-labile.

Preliminary examination of autoradiograms of retinas from adult rats injected with $^{35}$S-sulfate revealed an appreciable concentration of the radioactive material in the photoreceptor layer, a finding which was in contrast with the results of earlier studies. Curran and Kennedy reported that, in newborn mice killed 4 to 18 hours after injection of labeled inorganic sulfate, there was an indication of low uptake in the inner plexiform layer. Other layers were free of radioactivity. In older mice, no significant retinal incorporation was found. Smelser and Ozanics found little detectable reaction in any part of the retina in either the embryonic or early postnatal rabbit 24 hours after labeled sulfate administration, although there was a concentration of silver grains at the interface between the retina and vitreous. A similar absence of significant $^{35}$S-sulfate uptake was observed by Dohlman in autoradiograms of retinas of adult rabbits killed 3 days after injection. Larsen's report on the guinea pig was in agreement with these findings. In mature animals killed 24 hours after administration of $^{35}$S-sulfate, "very little or no radioactivity" was evident in autoradiograms of the retina.

Since our observations were in conflict with those of previous workers, further investigation of the retinal utilization of inorganic sulfate appeared warranted. Histochemical, autoradiographic, and radiobiological techniques have been employed. The biochemical study reported previously demonstrated that $^{35}$S-sulfate is incorporated almost entirely into the acid mucopolysaccharide fraction of retinas from 8 week old rats killed 24 hours after injection. The histochemical and auto-
radiographic analyses, which deal with rats of various ages, are presented below.

Methods

Experimental design. Eyes from 71 Long-Evans rats in five groups, 1, 2, 4, 8, and 18 weeks of age, were used. The younger animals were unselected as to sex. Females were used in the two oldest groups. Rats were injected intraperitoneally with 4 to 8 μc per gram of body weight of carrier-free 35S-sulfate (Oak Ridge National Laboratory, Oak Ridge, Tenn.), diluted with isotonic saline solution so that the required dose was contained in a volume of 0.1 to 0.5 ml. The animals were then killed at intervals of from half an hour to 3 weeks after injection.

Autoradiography. The eyes were fixed in 10 per cent buffered neutral formalin for 2 days, or in Bouin-Hollande solution for 3 days, and were then double-embedded in nitrocellulose-paraffin. Anteroposterior sections were cut at 5 μ, mounted on glass slides, deparaffinized, and coated with Kodak NTB2 liquid emulsion by the dipping technique. The preparations were exposed in a dry atmosphere at 4° C. for 1 to 12 weeks, then developed in Dektol (Eastman Kodak, Rochester, N. Y.) for 2 minutes at 17° C. Sections were stained with hematoxylin after development. Some were also stained with periodic acid-Schiff (PAS) before coating with emulsion.

The thickness of each of the several retinal layers was measured near the posterior pole in the sectioned material by means of an ocular micrometer. The concentration of developed silver grains over these layers (Table I) and over the corneal stroma was determined with an ocular micrometer. Counts due to background were then subtracted from the counts obtained over the sections. The net silver grain concentration (grains per square) over the various retinal layers was then converted to a percentage of the concentration over the corneal stroma in the same section, to eliminate differences due to emulsion thickness, exposure time, dosage, and development. The corneal stroma was chosen as the basis for comparison because of the relatively long biologic half-life of incorporated 35S-sulfate in this tissue.4-7

Histochemistry. Mowry's modification of the colloidal iron technique8 for the identification of acid mucopolysaccharides was used. A PAS counterstain was sometimes employed.

Following formalin fixation, some sections from 8- and 18-week-old rat eyes were incubated for 3 hours at 37° C. in bovine testicular hyaluronidase (Wyeth Laboratories, Philadelphia, Pa.). Enzyme concentration was 150 U.S.P. units per milliliter in 0.1M phosphate buffer, pH 6.0, containing 0.3 per cent sodium chloride. Control sections were treated by the same method, except that the enzyme was omitted from the incubating medium. The sections were then prepared for autoradiography, or stained by the colloidal iron or PAS procedures.

To determine the amount of possible proteolytic activity present in the hyaluronidase preparations, the method of Kunitz9 was employed. Hemoglobin served as the substrate. Both enzyme-substrate and control groups were incubated in the same buffer-saline solution used in the enzyme extraction procedures.

Results

Retinal growth. The outer segments of the photoreceptor cells, which consist entirely of rods in the rat, began to develop at about 11 days of age, then rapidly increased in length over the next few weeks. By 8 weeks of age, growth had ceased.

Table I. Labeling concentration* over different layers of the retina at 24 hours and 1 week after injection of 35S-sulfate

| Age group (wk.) | Time after injection | Optic nerve | Ganglion cells | Inner plexiform | Inner nuclear | Outer plexiform | Outer nuclear | Photoreceptor
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<tbody>
<tr>
<td>1</td>
<td>24 hr.</td>
<td>14</td>
<td>8</td>
<td>19</td>
<td>1</td>
<td>16</td>
<td>3</td>
<td>13  1</td>
</tr>
<tr>
<td>1</td>
<td>1 wk.</td>
<td>3</td>
<td>1</td>
<td>6</td>
<td>1</td>
<td>4</td>
<td>1</td>
<td>3  1</td>
</tr>
<tr>
<td>4</td>
<td>24 hr.</td>
<td>9</td>
<td>4</td>
<td>13</td>
<td>7</td>
<td>19</td>
<td>3</td>
<td>45  16</td>
</tr>
<tr>
<td>1</td>
<td>1 wk.</td>
<td>7</td>
<td>1</td>
<td>6</td>
<td>1</td>
<td>4</td>
<td>2</td>
<td>21  37</td>
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<tr>
<td>8</td>
<td>24 hr.</td>
<td>13</td>
<td>14</td>
<td>28</td>
<td>8</td>
<td>32</td>
<td>16</td>
<td>105 33</td>
</tr>
<tr>
<td>1</td>
<td>1 wk.</td>
<td>16</td>
<td>12</td>
<td>15</td>
<td>9</td>
<td>9</td>
<td>9</td>
<td>30 106</td>
</tr>
<tr>
<td>18</td>
<td>24 hr.</td>
<td>14</td>
<td>19</td>
<td>27</td>
<td>13</td>
<td>22</td>
<td>16</td>
<td>103 36</td>
</tr>
<tr>
<td>1</td>
<td>1 wk.</td>
<td>15</td>
<td>13</td>
<td>20</td>
<td>14</td>
<td>16</td>
<td>16</td>
<td>32 105</td>
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*Concentration is expressed as a percentage of the labeling concentration over the corneal stroma.
†Photoreceptor outer segments are undeveloped at 1 week of age.
During this same period, the inner segments increased in length, the plexiform layers increased in thickness, and the nuclear layers became narrower. These age changes were essentially completed by 8 weeks. No variation in thickness of the ganglion cell or optic nerve layers occurred during the period studied.

Bouin-Hollande fixation caused shrinkage and distortion of the outer segments. Consequently, autoradiographic analyses were confined to formalin-fixed material.

**Autoradiography.** In 1-week-old animals, the heaviest silver grain concentration occurred over the inner and outer plexiform and optic nerve layers (Table I). The weakest labeling was observed over the nuclear strata. There was a slight reaction over the photoreceptor inner segments. One week after administration of $^{35}$S-sulfate, a significant decrease in grain concentration was observed over all layers of the retina.

In the group injected at 4 weeks of age, the most intense initial labeling was seen over the rod inner segments. The plexiform layers were again more heavily labeled than the optic nerve fiber and nu-

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**Fig. 1.** A, retina of an 8-week-old rat killed 24 hours after injection of $^{35}$S-sulfate. The greatest autoradiographic reaction occurs over the rod inner segments (arrow). There is also a moderate labeling of the inner and outer plexiform layers. The reaction is weaker over other retinal strata. (Autoradiogram, PAS and hematoxylin. $\times 400$.) B, a comparable section stained with the colloidal iron technique for mucopolysaccharides. The most intense staining reaction occurs in the photoreceptor layer below the pigmented choroid. There is also a strong reaction in the inner and outer plexiform layers. ($\times 400$.)
clear layers. One week after injection, the heaviest labeling occurred over the rod outer segments, where there was a rise in concentration relative to earlier intervals. A drop in autoradiographic reaction was seen, however, over the other regions of the retina (Table I).

In 8- and 18-week-old rats, initial labeling was also most intense over the rod inner segments (Figs. 1 and 2). Again, there was a strong reaction over the inner and outer plexiform layers, and a lesser labeling of the optic nerve fiber and nuclear layers. Subsequently, a gradual shift or displacement of radioactivity from the inner to the outer segment region was observed (Fig. 2). One day after injection, the greatest silver grain concentration occurred over the inner segments. At 3 days, labeling was more prominent over the junction between the inner and outer segments. By 1 week, the labeled material had reached the level of the outer segments, where it was uniformly distributed. Grain concentration in this zone now greatly exceeded that over the other retinal strata (Table I). In fact, the autoradiographic reaction over the rod outer segments, initially very low, rose to levels comparable to that over the corneal stroma at 1 week after injection. Thereafter, labeling over the outer segments dropped rapidly. Two weeks after injection it was only 15 per cent of the corneal stroma (Fig. 3).

The biologic half-life of the 35S-labeled material of the outer segment zone was obtained by analyzing the rate at which the concentration of radioactivity decreased in this region, using methods summarized by Comar. When corrected for loss of labeling from the corneal standard, the biologic half-life was calculated to be about 2.5 days.

**Histochetry.** Bouin-Hollande fixation yielded more discriminate staining with the colloidal iron technique than did formalin fixation. Heaviest staining occurred in the region of the photoreceptor outer segments (Fig. 1). The rod outer segments apparently did not bind the dye, except possibly on their outer surface. Rather, the material between the rods appeared to be stained. The outer segments themselves were heavily stained with PAS.

There was also heavy colloidal iron

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**Fig. 2.** A, retinal photoreceptor layer 8 hours after injection of 35S-sulfate. The concentration of labeling is most intense in the inner segments at this interval. B, three days after injection of labeled sulfate, labeling is heaviest near the junction of the rod inner and outer segments. C, at 1 week, the reaction is most intense over the outer segment region. (Autoradiograms, PAS and hematoxylin. ×800.)
staining of a material forming a fibrous meshwork through the outer and inner plexiform layers (Fig. 1). There was a moderate staining reaction over the fibrous processes of the optic nerve layer. The nuclear and ganglion cell layers were generally unstained. However, in some sections, positively-staining fibrous structures were seen extending discontinuously through these strata parallel to the axis of the rods.

At enzyme concentrations of 150 units per milliliter, the testicular hyaluronidase solution exhibited no proteolytic activity when incubated with hemoglobin for 3 hours at 37° C.

Hyaluronidase extraction prior to autoradiography resulted in a loss of some labeling from all layers of the retina in 8- to 18-week-old animals killed 1 hour to 2 weeks after injection (Table II). Labeling over the photoreceptor layer was reduced by 70 per cent compared to the concentration over the same region in control sections (Fig. 4 and Table II). From 23 to
Table II. Loss of labeling in different layers of the retina and the corneal stroma after hyaluronidase extraction

<table>
<thead>
<tr>
<th>Ocular tissue</th>
<th>Percentage loss compared to controls</th>
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<tbody>
<tr>
<td>Retina</td>
<td></td>
</tr>
<tr>
<td>Outer segment</td>
<td>70</td>
</tr>
<tr>
<td>Inner segment</td>
<td>67</td>
</tr>
<tr>
<td>Outer nuclear</td>
<td>23</td>
</tr>
<tr>
<td>Outer plexiform</td>
<td>48</td>
</tr>
<tr>
<td>Inner nuclear</td>
<td>31</td>
</tr>
<tr>
<td>Inner plexiform</td>
<td>34</td>
</tr>
<tr>
<td>Ganglion cells</td>
<td>43</td>
</tr>
<tr>
<td>Optic nerve</td>
<td>47</td>
</tr>
<tr>
<td>Corneal stroma</td>
<td>27</td>
</tr>
</tbody>
</table>

48 per cent was lost from other retinal strata. In the same sections, only about 27 per cent was lost over the corneal stroma. The capacity of the retina to stain with the colloidal iron technique was almost completely abolished by prior enzyme extraction. There was, however, some persistence of the staining reaction over the outer segment region. Staining with PAS was unimpaired.

Discussion

A significant incorporation of inorganic sulfate has been shown to occur in the rat retina. The discrepancy between this finding and those previously derived from retinas of the mouse, rabbit, and guinea pig may be a result of species differences in the chemical nature (e.g., degree of sulfation) of retinal mucopolysaccharides. Conceivably, it could also be due in part to recent improvements in autoradiographic technique.

Radiobiophysical studies have shown that the vast majority of bound retinal radioactivity in these rats exists in mucopolysaccharides (MPS). Fractionation of retinal extracts from 8-week-old rats killed 1 day after 35S-sulfate injection demonstrated that, although MPS constituted less than 5 per cent of the retinal dry weight, essentially all of the bound 35S could be recovered in this fraction.

The studies reported herein indicate that sulfated MPS are present at negligible levels in the nuclear and ganglion cell layers of the retina. However, representatives of this class of compounds are found in significant amounts in the fibrous (plexiform and optic nerve fiber) layers of the retina, as shown by both the autoradiographic and histochemical procedures. The precise, cytologic location of the MPS within these layers is not known.

There is an even higher concentration of sulfated MPS in the photoreceptor layer. The MPS in this region appear to occur, for the most part, extracellularly, as a component of the ground substance in which the photoreceptor outer segments are embedded. The blue, colloidal iron staining reaction was confined to the material surrounding the PAS-positive receptor elements, as shown previously in the human retina. Electron microscopic observations also indicate the extracellular location of the photoreceptor MPS.

The sulfated MPS of this zone appear to be synthesized in the rod inner segments, and are subsequently displaced into the extracellular spaces between the outer segments. This process is reflected by the shift of labeling from the inner to outer segment region in the autoradiograms. The displacement of radioactivity during the first week after injection was seen with particular clarity in the 8- and 18-week-old animals, in which the retina had attained its mature dimensions. Displacement of 35S-labeled material was also observed in 4-week-old animals, at which age the photoreceptor outer segments were still growing.

At 1 week of age, when the outer segments in the rat have not yet begun to develop, no concentration or displacement of radioactivity was detected in the photoreceptor layer, although a weak labeling was observed. Thus, the production of sulfated MPS by retinal rods becomes significant with the development of the photosensitive outer segments. Continued production of sulfated MPS takes place long after the mature dimensions of the photo-
receptor cells have been attained, however, and is accompanied by a balanced loss of MPS in the adult retinas. Such a balance between synthesis and breakdown is revealed by the similar rate of uptake and loss of $^{35}$S-labeled material in 8- and 18-week-old rats. The rate of renewal is very rapid. About half of the photoreceptor sulfated MPS is renewed every 2.5 days in both 8- and 18-week-age groups, a turnover rate more than ten times that of sulfated MPS in the corneal stroma.7

The nature of the ultrastructure of the myoid portion of the photoreceptor inner segments1112 suggests that this part of the cell is the site of an active synthesis of large molecular weight compounds, such as MPS. In fact, Fine and Zimmerman13 concluded from a study of the fine structure of the rod and cone retinae of the human retina that the major portion of the extracellular MPS is synthesized within the inner segments of the photoreceptors. Although a possible contribution by the microvillous processes of the Muller's cells which extend into this zone cannot yet be excluded, the present autoradiographic findings in the rat are consistent with the conclusion that the photoreceptor inner segments represent the major site of MPS synthesis. Droz13 and Young14 noted that labeled amino acids were incorporated in this zone in the mature retinae of rats and mice. As also occurs with $^{35}$S-sulfate, the labeled material was then displaced into the outer segment region, and subsequently disappeared. The observation14 that labeled protein was displaced as a discrete band, which ultimately disappeared at the interface with the pigment epithelium, led to the conclusion that the photoreceptor outer segments undergo continual renewal. Thus it appears that both the rod outer segments and the MPS-containing material in which they are embedded may be continually replaced in the adult retina.

The presence of MPS in the region of the photoreceptor outer segments has been recognized for many years.5111315 Initially, it was believed that these MPS were nonsulfated and hyaluronidase-resistant.61119 In the rat, however, at least part of the MPS in this and other retinal zones is sulfated, and includes fractions which are susceptible to digestion with bovine testicular hyaluronidase. In all layers of the retina, there was some loss of radioactivity after hyaluronidase extraction. About 70 per cent of the labeling was removed from the photoreceptor region, and about 20 to 50 per cent from the other retinal layers. None of the radioactivity was removed from the lens capsule in these preparations,22 and only 27 per cent was eliminated from the corneal stroma. The latter finding is in agreement with studies which indicate that about one third of the corneal MPS are hyaluronidase-labile.23

These results indicate the existence of sulfated MPS in the rat retina, some of which are hyaluronidase-labile, in association with others which are hyaluronidase-resistant. Similar findings have been reported in cattle.2425 Evidently, the different classes of sulfated MPS are not uniformly distributed in the retina, the hyaluronidase-labile fractions being predominant in the photoreceptor layer.

In summary, the sulfated MPS of the retinal photoreceptor zone in the rat are synthesized in the region of the rod inner segments, are displaced into the extracellular space between the outer segments, and undergo rapid renewal in the mature retina. Sulfated MPS are also present, in lower concentrations, in the remaining retinal strata, notably in the inner and outer plexiform layers. Some, but not all of the retinal MPS in the rat are hyaluronidase-labile.

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REFERENCES


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