The utilization of $^{35}$S-sulfate in the synthesis of mucopolysaccharides by the retina

Michael O. Hall, David E. Ocumpaugh, and Richard W. Young

Utilization of $^{35}$S-sulfate by the retina of the rat was studied by both autoradiographic and biochemical techniques. One day after the intraperitoneal injection of the labeled sulfate in 7-week-old animals, radioactivity was present in significant amounts in the plexiform layers of the retina, and was particularly concentrated in the inner segments of the photoreceptors. Biochemical analysis showed that, although mucopolysaccharides constituted less than 5 percent of the retinal dry weight, essentially all of the bound $^{35}$S could be recovered in this fraction.

It is now generally recognized that acid mucopolysaccharides (MPS) are present in the photoreceptor region of the retina. This conclusion is based on histochemical staining procedures of various types applied in many species of animals. The MPS are believed to constitute a form of extracellular ground substance in which the photoreceptor outer segments are embedded. Early autoradiographic work failed to detect the incorporation of $^{35}$S-sulfate in this region. This led to the subsequent conclusion that the stainable MPS were nonsulfated. Autoradiographic evidence has recently been presented, however, which demonstrates that there is a significant incorporation of $^{35}$S-sulfate within the retina of the rat. In animals one month of age or older, labeling intensity was greatest in the photoreceptor layer, within which a progressive displacement of radioactivity from inner to outer segment was observed. One day after injection, the interval used for further analysis in this report, the concentration of autoradiographic silver grains over the inner segments considerably exceeded that over the outer segments. One week later, this relationship was reversed.

Biochemical investigation of retinal MPS seems to be limited to one report, describing the isolation from beef retinas of two metachromatic substances, which appeared to be neither keratosulfate nor chondroitin sulfate A or C, and an abstract stating that the bovine retina contains one nonsulfated and two sulfated acid MPS, as well as one nonsulfated neutral MPS.

The following report describes the results of a study in which retinal MPS were isolated from rats given an intraperitoneal injection of $^{35}$S-sulfate 24 hours prior to sacrifice. It will be shown that the vast majority of retinal radioactivity can be recovered in the sulfated acid MPS fraction.

**Methods**

**Experimental design.**

A. Two groups of twenty-five 7-week-old Long-Evans rats were injected intraperitoneally with 5
per gram body weight of carrier-free $^{35}$S-sulfate* which had been diluted with isotonic saline to contain 2 mc. per milliliter. The animals in group I ranged in weight from 150 to 175 grams (mean, 162 grams). Those in group II weighed 150 to 190 grams (mean, 167 grams). The rats were sacrificed by an overdose of chloroform 24 hours after injection. The eyes were enucleated, and the retinas removed. Under the dissecting microscope, each retina was carefully freed of vitreous, ciliary body, choroid, and pigment epithelium. The dissected retinas were then immediately frozen in an aluminum foil boat maintained on dry ice.

B. Ten additional 7-week-old rats were similarly injected with $^{35}$S-sulfate, sacrificed at 24 hours, and the retinas prepared as described. This material was used in the analysis of the free amino acid fraction.

C. The efficacy of the dissection procedure was investigated by histologic and autoradiographic technique in another small series of animals. The preparative procedure was identical except that the retinas, instead of being frozen, were fixed in Bouin-Holland solution or in buffered neutral formalin and subsequently treated for staining and autoradiography by methods described elsewhere.14

**Analytical procedure.** The initial extraction sequence was designed to (1) isolate the crude mucopolysaccharide fraction, and (2) account for all the radioactivity retained in the retinas.

The frozen retinas were lyophilized, then homogenized in a 2:1 (v/v) solution of chloroform-methanol. The homogenate was stirred in this solution for 1.5 hours, then filtered. The filtrate (lipid extract) was retained for radioactivity measurements. The residue was next successively digested with papain and trypsin, according to the method of Moretti and Whitehouse,17 in order to hydrolyze the retinal protein. The digests were dialyzed extensively to remove free amino acids and free sulfate. All dialysis solutions were subsequently concentrated to small volumes, and aliquots measured for radioactivity. Residual protein, remaining after enzymatic digestion, was precipitated by adding cold trichloroacetic acid (TCA) to a final concentration of 10 per cent, separated by centrifugation, and its radioactivity content determined. The precipitates was dissolved in a minimum of 4M potassium acetate, with warming at 60° C, and the Rivanol removed by passage through a column of Sephadex G-25, previously equilibrated with 4M potassium acetate. The MPS are eluted in the void-volume, whereas the Rivanol moves only slowly down the column. The eluates were dialyzed, lyophilized, and the residues taken up in water. Aliquots of each were counted for radioactivity.

Further fractionation of the sulfated acid MPS was achieved on a column of Sephadex A-25 according to the procedure of Schmidt.18 An aliquot of the MPS was hydrolyzed with 4 N HCl for 2 hours at 100° C. HCl was removed in vacuo over KOH and P.O3, and the residue spotted on sheets of Whatman No. 1 filter paper. Ascending chromatography was carried out twice with pyridine:ethyl acetate:acetic acid:water (5: 5:1:3, by volume) as solvent.19 The separated sugars were detected with alkaline silver nitrate.20 Kodak liquid x-ray fixer was used to remove background color.21

Samples to be assayed for radioactivity content were plated on weighed copper planchets, dried, reweighed, and counted in a Nuclear-Chicago gas flow G-M counter, equipped with a thin end.

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1Worthington Biochemical Corp., Freehold, N. J.
2California Corporation for Biochemical Research, Los Angeles, Calif.

**Table I.** Initial extraction sequence for isolating the mucopolysaccharide fraction, and for determining total retinal radioactivity

<table>
<thead>
<tr>
<th>Extraction sequence</th>
<th>Radioactivity measurement</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homogenize in chloroform: methanol Filter</td>
<td>Lipid extract (filtrate)</td>
</tr>
<tr>
<td>Digest residue with papain Dialyze</td>
<td>Papain dialysis fluid</td>
</tr>
<tr>
<td>Digest residue with trypsin Dialyze</td>
<td>Trypsin dialysis fluid</td>
</tr>
<tr>
<td>Precipitate with TCA</td>
<td>Protein precipitate</td>
</tr>
<tr>
<td>Dialyze supernatant</td>
<td>MPS dialysis fluid</td>
</tr>
<tr>
<td>Lyophilize</td>
<td></td>
</tr>
<tr>
<td>Dissolve in water</td>
<td>MPS fraction</td>
</tr>
</tbody>
</table>

the partitioning of radioactivity among these several subfractions.

The mucopolysaccharides were fractionated with Rivanol* by the procedure of Moretti and Whitehouse,17 with the following modification: The MPS fractions from each of the two series were combined, and an aliquot containing 50 per cent of the total counts separated into sulfated and nonsulfated acid MPS, and into neutral MPS. Each of the precipitates was dissolved in a minimum of 4M potassium acetate, with warming at 60° C, and the Rivanol removed by passage through a column of Sephadex G-25, previously equilibrated with 4M potassium acetate. The MPS are eluted in the void-volume, whereas the Rivanol moves only slowly down the column. The eluates were dialyzed, lyophilized, and the residues taken up in water. Aliquots of each were counted for radioactivity.

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window. Counts were corrected for background, isotope decay, and self-absorption. Several aliquots from each fraction were analyzed. The weights, counts per minute (c.p.m.), and standard errors were then averaged.

The twenty retinas obtained in the second experiment (B) were homogenized in 5 ml. water, and the supernatant obtained by centrifugation. The nonprotein fraction of this aqueous extract was then obtained by ultrafiltration through dialysis tubing under vacuum in the cold. The ultrafiltrate was lyophilized, taken up in a small volume of water, and separated by two-dimensional thin-layer chromatography on silica gel G (Brinkmann Instruments, Westbury, N. Y.). The first dimension was developed in butanol:acetic acid:water (3:1:1, by volume). The second dimension was developed in phenol:water (4:1, w/v) or developed twice in ethanol:58 per cent ammonia: water (18:1:1, by volume). Spots were detected by spraying with 0.2 per cent ninhydrin in butanol. The separations were then analyzed autoradiographically with Kodak Blue Brand x-ray film, exposed for periods up to one month.

Results

Purity of the tissue. Histologic examination of retinas dissected according to the procedures used in this study indicated that the tissue was free of contamination by vitreous, ciliary body, and choroid (Fig. 1). Scattered remnants of the distal processes of pigment epithelial cells were occasionally present. This was evidently due to a close adherence of these cellular extensions to the photoreceptor outer segments, since in no instance were intact pigment cells observed in the dissections.

Autoradiographic analysis of such retinas, dissected from animals injected with $^{35}$S-sulfate 24 hours before sacrifice, showed a significant incorporation of the labeled material into this tissue (Fig. 2). The autoradiographic reaction was weakest over the nuclear layers, stronger over the plexiform layers, and most intense overlying the layer of the photoreceptors. Within the photoreceptor layer, it was particularly concentrated over the inner segments. The remainder of the present report deals with the characterization of the material labeled in the sections.

Chemical fractionation of the retinas. The results of the initial extraction sequence for isolating retinal mucopolysaccharides and accounting for total retinal radioactivity (experiment A) are summarized in Table II. Although the total radioactivity recovered in the two duplicate series differs noticeably (possibly because of an error in dosage), the percentage of the total counts recovered in each of the six fractions is remarkably similar, indicating that the findings are reproducible.
Table II. Distribution of radioactivity in different fractions of retinas

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Group I</th>
<th></th>
<th>Group II</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Net counts per minute*</td>
<td>Counts per minute (per cent of total)</td>
<td>Net counts per minute*</td>
<td>Counts per minute (per cent of total)</td>
</tr>
<tr>
<td></td>
<td>Mean</td>
<td>SE</td>
<td></td>
<td>Mean</td>
</tr>
<tr>
<td>Lipid extract</td>
<td>1,203 ± 32</td>
<td>3.2</td>
<td>964 ± 28</td>
<td>6.2</td>
</tr>
<tr>
<td>Papain dialysis fluid</td>
<td>8,800 ± 94</td>
<td>22.8</td>
<td>3,414 ± 48</td>
<td>22.1</td>
</tr>
<tr>
<td>Trypsin dialysis fluid</td>
<td>497 ± 91</td>
<td>1.3</td>
<td>242 ± 90</td>
<td>1.6</td>
</tr>
<tr>
<td>Protein precipitate</td>
<td>133 ± 9</td>
<td>0.3</td>
<td>132 ± 10</td>
<td>0.9</td>
</tr>
<tr>
<td>MPS dialysis fluid</td>
<td>558 ± 93</td>
<td>1.4</td>
<td>72 ± 74</td>
<td>0.5</td>
</tr>
<tr>
<td>MPS fraction</td>
<td>27,385 ± 185</td>
<td>71.0</td>
<td>10,600 ± 79</td>
<td>68.7</td>
</tr>
</tbody>
</table>

*Total net counts per minute contained in each fraction. Corrected for background, decay, and for self-absorption.

Only 3 to 6 per cent of the total activity was obtained in the lipid extract. About 22 per cent was recovered in the dialysis fluid associated with the papain digestion. Less than 2 per cent of the total retinal activity was present in each of the next three fractions—trypsin dialysis fluid, TCA protein precipitate, and the dialysis fluid obtained from dialyzing the supernatant of the TCA precipitation. When this dialyzed supernatant was lyophilized and subsequently dissolved in water, it proved to contain more than two thirds (69 to 71 per cent) of the total retinal radioactivity. Thus, the vast majority of the retinal radioactivity was recovered in the crude MPS fraction.

This finding is even more striking when the weights of the several retinal fractions are taken into account. The dry weight of the retinas was slightly less than 90 mg. in each group (Table III). (Dry weight was obtained by adding the weights of the residue and supernatant of the chloroform : methanol extraction.) The dry weight of the crude MPS fraction was only 3 to 4 mg. Consequently, the crude MPS fraction represented less than 5 per cent of the total retinal dry weight, but contained some 70 per cent of retinal radioactivity. Expressed in terms of specific activity (counts per minute per milligram), the crude MPS fraction was over 50 times more radioactive than the remainder of the retina. As will be shown, this fraction was a mixture of components, some containing little or no 35S. Thus, the specific activity of the 35S-labeled mucopolysaccharide component(s) was even greater than indicated by these calculations.

Fractionation of the crude MPS. The crude MPS fraction was further purified by Rivanol precipitation, yielding subfractions representing (1) sulfated acid MPS, (2) nonsulfated acid MPS, and (3) neutral MPS. As anticipated, the vast majority (85 per cent) of the radioactivity was recovered in the sulfated acid MPS component, and the remainder (15 per cent) was obtained in the resulting supernatant (neutral MPS).

An attempt was made to purify further the crude MPS fraction by column chromatography on Sephadex A-25. Because of the small quantity of material available, the presence of radioactivity in the effluent was used to detect the MPS. Two peaks were eluted from the column, accounting for 94 per cent of the radioactivity applied (Fig. 3). The effluent was accordingly combined in two fractions, dialyzed, lyoph-

Table III. Weights and specific activities of the crude mucopolysaccharide and non-mucopolysaccharide fractions of retinas

<table>
<thead>
<tr>
<th>Measurement</th>
<th>Group I</th>
<th>Group II</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dry weight of retina (mg.)</td>
<td>88.5</td>
<td>83.3</td>
</tr>
<tr>
<td>Dry weight of crude MPS (mg)</td>
<td>4.1</td>
<td>3.0</td>
</tr>
<tr>
<td>Specific activity* of non-MPS fraction</td>
<td>135</td>
<td>61</td>
</tr>
<tr>
<td>Specific activity* of MPS fraction</td>
<td>6,673</td>
<td>3,533</td>
</tr>
</tbody>
</table>

*Counts per minute per milligram.
Constituents of the MPS fractions. Peak I, representing about one third of the radioactivity recovered from the column, contained all the components normally found in MPS: glucuronic acid, glucosamine, galactosamine, and galactose, as well as glucose, mannose, and an unidentified reducing sugar (Fig. 4). Peak II showed only traces of glucosamine, galactose, and the unidentified sugar, and a large amount of glucose. This peak contained about two thirds of the radioactivity applied to the column.

Presence of unbound \(^{35}S\)-sulfate. Thin-layer chromatographic separations of the free amino acid fraction of the twenty additional rat retinas showed as many as twenty-one ninhydrin-positive components. A single radioactive spot was detected by autoradiography, which component was ninhydrin-negative, and did not match any of the sulfur-containing control amino acids run under comparable conditions. The labeled substance was identified as free sulfate by comparison with autoradiograms of thin-layer chromatographic separations of the \(^{35}S\)-sulfate solution developed in the same manner.

Discussion

The histologic data indicate that the experimental material consisted strictly of retinas, essentially free of contamination from associated tissue structures. Autoradiographic analysis further demonstrated
that $^{35}$S-sulfate, injected into the peritoneal cavity, is ultimately incorporated into the retina in significant amounts. This finding, first reported by Ocumpaugh, is depicted in Fig. 2. Twenty-four hours after injection, the greatest activity is found in the photoreceptor inner segments. Lesser amounts of radioactivity are present in the plexiform layers. Very little is bound to the nuclear strata.

Work in progress has shown that when retinal sections are stained with Mowry's colloidal iron method for detection of acid MPS, the most intense reaction occurs over the photoreceptor layer. A strong staining of the plexiform layers is also observed. Extraction of sections with testicular hyaluronidase, under conditions which yield no proteolysis, removes most of the stainable material, and over half of the radioactivity as well.

It should be noted that the autoradiographic and biochemical analyses are in a sense not directly comparable. The rigors of autoradiographic tissue preparation (fixation, embedding, etc.) inevitably result in the loss of most small molecular-weight, water-soluble tissue constituents, including free $^{35}$S-sulfate, which is present in the retina 24 hours after injection, as shown above. Therefore, the autoradiograms demonstrate bound $^{35}$S, whereas the biochemical procedures were designed to recover all retinal radioactivity, bound and unbound.

Only small amounts (3 to 6 per cent) of the radioactivity were removed from the retinal homogenate by chloroform-methanol extraction. Although a minor incorporation of $^{35}$S into sulfated lipids is not excluded, it seems more likely that this activity is due to free sulfate, solubilized in the methanol.

It would be anticipated that any free sulfate remaining in the retinal homogenate would be dissolved in water and appear in the dialysis fluid of the first (papain) enzyme extraction. Indeed, 22 per cent of the total retinal activity was recovered in that fraction. If any of this radioactivity had been due to the release of amino acid-$^{35}$S by protein breakdown, significant amounts would also be expected to be present in the dialysis fluid of the subsequent (trypsin) enzyme digestion. This was not observed (Table II), nor was there any significant radioactivity in the TCA-precipitated residual protein. Furthermore, autoradiographic analysis of thin-layer chromatograms of the free amino acid fraction of comparable retinas was negative, yielding a radioactive spot for $^{35}$S-sulfate only. Consequently, most if not all of the activity in these several fractions may be attributed to unbound $^{35}$S-sulfate.

Dialysis of the TCA supernatant yielded no significant radioactivity, but the nondialyzable, water-soluble residue proved to be highly radioactive. This crude mucopolysaccharide fraction represented less than 5 per cent of the total retinal dry weight, but contained about 70 per cent of the total retinal radioactivity. Practically all (85 per cent) of this activity was precipitated by procedures designed to isolate sulfated acid MPS. (Trials with standard MPS mixtures indicated that the maximum efficiency of the precipitation is about 90 per cent.) Chromatography on Sephadex A-25 suggested that two types of sulfated acid MPS were present. The first of these (one third of the total radioactivity) contained glucuronic acid, glucosamine, galactosamine, and galactose, as well as glucose, mannose, and an unidentified sugar. The second (two thirds of the total radioactivity) yielded only small amounts of glucosamine, galactose, and an unidentified sugar, but considerable glucose. Although these findings are compatible with, and indeed strongly support, the conclusion that the $^{35}$S-sulfate had been incorporated into MPS, they do not allow the assignment of the MPS to currently available categories.

The conclusion that $^{35}$S-sulfate was in-
corporated into sulfated MPS is based on the following findings:

1. Histochemical MPS stains reacted most intensely with the retinal layers which contained the most bound radioactivity.

2. Extraction of retinal tissue sections with testicular hyaluronidase removed much of the bound radioactivity. This enzyme is known to act specifically on certain classes of acid MPS.

3. The material of the fraction considered to be MPS was obtained by an extraction sequence demonstrated to isolate MPS in other tissues.

4. The material of this fraction was macromolecular, since it was not dialyzable.

5. This material was nonlipid, since it was insoluble in chloroform:methanol.

6. It was nonprotein, since it was not destroyed by digestion with papain or trypsin, nor was it precipitated with 10 per cent TCA. Furthermore, no free $^{35}$S-labeled amino acids were detected.

7. This fraction contained most of the retinal radioactivity (and possibly all of the bound $^{35}$S). It has repeatedly been demonstrated that mammalian utilization of $^{35}$S-sulfate is almost entirely restricted to the synthesis of sulfated MPS.

8. The material was precipitated by Rivanol, which is known to precipitate acid MPS.

9. Hydrolysis of the material yielded all of the sugars normally found in acid MPS.

In summary, the foregoing report indicates that inorganic sulfate is a significant precursor in the metabolism of the retina in the rat. Essentially all of the sulfate bound by the retina is utilized in the synthesis of sulfated acid mucopolysaccharides. The sulfated MPS, which constitute less than 5 per cent of the retinal dry weight, are mainly located in the plexiform and photoreceptor layers. In the latter, they appear to be synthesized in the inner segments.$^5$,$^13$

We gratefully acknowledge the technical assistance of Mrs. Mirdza Berzins.

REFERENCES


