Incorporation of sulfate-$^{35}$S, N-acetylglucosamine-$^{14}$C, glucose-$^{14}$C, and galactose-$^{14}$C into calf and beef corneal glycosaminoglycans

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Beef corneas were incubated in vitro with N-acetylglucosamine-$^{14}$C, glucose-$^{14}$C, and galactose-$^{14}$C. Calf and beef corneas were incubated in vitro with inorganic sulfate-$^{35}$S. Corneal glycosaminoglycans were isolated and the mixture resolved by use of ECTEOLA anion-exchanger and further fractionation with ethanol. After incubation with glucose-$^{14}$C, a higher specific activity was found in keratan sulfate than in chondroitin-chondroitin sulfate, whereas the opposite was found after incubation with galactose-$^{14}$C and sulfate-$^{35}$S. Incubation of corneas with N-acetylglucosamine-$^{14}$C resulted in approximately the same specific activity in keratan sulfate and chondroitin-chondroitin sulfate. The results are discussed in terms of the normal biosynthesis of corneal glycosaminoglycans.

Glycosaminoglycans in cornea have been isolated and identified as keratan sulfate, chondroitin, and chondroitin sulfate.1 The development of methods for the isolation of small amounts of corneal glycosamino-

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trophoretic mobility as authentic keratan sulfate. No sulfate-$^{35}$S-labeled chondroitin sulfate could be demonstrated readily by the methods used in that study.9

Glucose-$^{14}$C has been found to be incorporated into the hexosamines of corneal glycosaminoglycans; glucosamine had a higher specific activity than did galactosamine.10 Cornea contains UDPglcA and UDPglc as well as other nucleotides and has the capacity to synthesize glucosamine 6-phosphate from glucose 6-phosphate or fructose 6-phosphate and glutamine.10, 11

Keratan and chondroitin sulfates may be synthesized at different rates in cornea. The biosynthesis of chondroitin and keratan sulfates can be independently influenced by steroids in structures such as nucleus pulposus and cartilage.12-14 A greater comprehension of the biochemical events and mechanisms involved in corneal wound healing and pathology will be obtained as the biosynthesis and interactions of keratan and chondroitin sulfates are more fully elucidated. For this purpose, calf and beef corneas were studied. Corneas were incubated with inorganic sulfate-$^{35}$S in a concentration of 20 $\mu$Ci per milliliter. The corneas were incubated for 6 hours at 38° in a shaker-water-bath, rinsed twice in tap water, shaken in saturated Na$_2$SO$_4$ at 4° overnight, rinsed in tap water, and then washed in distilled water for an additional 5 hours.

The corneas were homogenized, digested with collagenase and trypsin, lyophilized, and stored according to published methods.2 The digest was redissolved, dialyzed against water, centrifuged, and the supernatant fluid applied to a 1 by 7 cm. Ecteola column. The initial elution with 0.02M HCl was followed by stepwise elution with 0.15, 0.25, and 1.95M NaCl in 0.05M HCl.18 The eluates were dialyzed against water and lyophilized. Portions of these lyophilized fractions were hydrolyzed in 6N HCl for approximately 18 hours at 100° and the hexosamine content determined by a Gardell16 modification of the Elson and Morgan17 method.

The corneal glycosaminoglycans isolated by the method described above were further fractionated by stepwise elution from 2 by 30 cm. cellulose columns with 80, 50, 35, 25, and 0 per cent ethanol in 0.3 per cent barium acetate.15 Hexuronic acid and hexose were measured in the eluates by the carbazole19 method and the anthrone20 reaction, respectively. The eluates were then dialyzed against water and lyophilized. Total hexosamine as well as glucosamine and galactosamine contents were determined after acid hydrolysis.18 The sulfate-$^{35}$S was precipitated and counted as barium sulfate.4, 7, 9

Incorporation of various precursors into keratan and chondroitin sulfates by beef cornea. Corneas were obtained from fresh beef eyes and incubated in TC 199 tissue culture medium (Difco Laboratories, Detroit, Mich.) with streptomycin (6 $\mu$g per milliliter) and penicillin G (54 units per milliliter).9 Inorganic sulfate-$^{35}$S (Oak Ridge National Laboratory, Oak Ridge, Tenn.), N-acetylglucosamine-$^{1-14}$C, glucose-$^{1-14}$C or galactose-$^{1-14}$C (New England Nuclear Corp., Boston, Mass.) were added in concentrations of 1 $\mu$Ci per milliliter. The corneas were incubated for approximately 18 hours at 38° in a shaker-water-bath. Glycosaminoglycans were isolated and further resolved into fractions which contained keratan sulfate and chondroitin-chondroitin sulfate.1, 3, 9

Results

Incorporation of sulfate-$^{35}$S into chondroitin and keratan sulfates in calf cornea. Ecteola fraction A contained small amounts of hexosamine and low radioactivity (see Fig. 1). Most of the radioactivity was eluted in Ecteola fraction B. A partial separation of keratan sulfate from chondroitin sulfate can be achieved with further fractionation based on ethanol solubility. The glycosaminoglycan which was eluted from the cellulose column in the absence of ethanol contained approximately 93 per cent chondroitin sulfate. The glycosaminoglycan which was eluted from the cellulose column with 35 per cent ethanol contained approximately 90 per cent keratan sulfate.

Ecteola fraction C contained glycosaminoglycans which were further resolved into keratan and chondroitin sulfates with ethanol. The total amount of glycosaminoglycans was approximately 93 per cent chondroitin sulfate. The glycosaminoglycan which was eluted from the cellulose column with 35 per cent ethanol contained approximately 90 per cent keratan sulfate.
Activities of corneal glycosaminoglycans

Fig. 1. Distribution of corneal glycosaminoglycans after elution from Ecteola column with chloride followed by further fractionation with ethanol on a cellulose column. The ethanol-eluted fractions were analyzed for glucosamine-galactosamine ratio. The specific activity is given under each ethanol fraction and expressed as sulfate-35S counts per minute per microgram of hexosamine. Open columns represent ethanol-eluted fractions with insufficient amounts of hexosamine for ratio determination.

Table I. Beef corneal glycosaminoglycans

<table>
<thead>
<tr>
<th>Glycosaminoglycan fraction</th>
<th>60% ethanol insoluble (chondroitin-chondroitin sulfate)</th>
<th>70% ethanol insoluble (keratan sulfate)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sulfate-35S</td>
<td>1,784</td>
<td>1,090</td>
</tr>
<tr>
<td>N-acetylglucosamine-1-14C</td>
<td>5,070</td>
<td>5,020</td>
</tr>
<tr>
<td>Glucose-1-14C</td>
<td>2,635</td>
<td>4,200</td>
</tr>
<tr>
<td>Galactose-1-14C</td>
<td>9,750</td>
<td>6,800</td>
</tr>
</tbody>
</table>

Composition of fraction verified by passage through Ecteola; specific activities include contribution of hexosamine.

Discussion

It is evident that calf and beef cornea will incorporate sulfate-35S not only into keratan sulfate but also into chondroitin sulfate, in vitro. Calf and beef cornea incorporate sulfate-35S into glycosaminoglycans to the extent that chondroitin sulfate demonstrated a higher activity than did keratan sulfate (Fig. 1, Table I). Corneal chondroitin sulfate is known to have a variable sulfate content and, more recently, the variability of the sulfate content of glycosaminoglycan which was eluted from the cellulose column in the absence of ethanol was found to be chondroitin sulfate.

Incorporation of various precursors into chondroitin and keratan sulfates in beef corneas. Chondroitin sulfate incorporated more sulfate-35S than did keratan sulfate (see Table I). N-acetylglucosamine-1-14C was incorporated into the two glycosaminoglycans with a resulting equal specific activity in chondroitin and keratan sulfates. Incubation of cornea with glucose-1-14C led to a higher specific activity in keratan sulfate than in the chondroitins, whereas the reverse situation was found after incubation of corneas with galactose-1-14C.
keratan sulfate has been demonstrated. However, the average sulfate:hexosamine ratio of chondroitin sulfate has been found to be much lower than that of keratan sulfate. These data suggest that the turnover of sulfate groups in chondroitin sulfate may be higher than that of keratan sulfate of cornea. Keratan sulfate in cartilage has a much longer half-life than does chondroitin sulfate. When earlier results are reviewed in terms of new information, it is suggested that the longer half-life of sulfate-35S in cornea compared with that in sclera may be related to the absence of keratan sulfate in sclera rather than the absence of a blood supply in cornea.

It is not known at which stage of polymerization of corneal glycosaminoglycans sulfation occurs. While the present study does not offer conclusive data, it was suggested in a previous study that the shorter chain-length keratan sulfate may be a better sulfate acceptor. Published data suggest that in other connective tissues sulfation occurs after synthesis of the polymer. Resolution of glycosaminoglycans on Ecteola is in part related to the number of anionic groups and to the molecular size. The specific activity of keratan sulfate was higher in Ecteola fraction D than in fraction B (see Fig. 1). Resolution of keratan sulfate on Ecteola results in fractionation on the basis of molecular weight and keratan sulfate in fraction D would be expected to be the higher molecular weight glycosaminoglycan. The keratan sulfate which binds more strongly to Ecteola may represent the glycosaminoglycan in a more complete or in a more active stage of biosynthesis. Furthermore, the larger weight glycosaminoglycans are replaced by smaller molecular weight glycosaminoglycans in regenerating corneal wounds and much greater concentration of radioactivity can be observed by radioautography at a corneal wound after sodium sulfate-35S administration. In addition, the smaller chain-length keratan sulfate shows greater sulfate-acceptor properties than does the larger chain-length keratan sulfate. Glucose and galactose are not incorporated so as to yield the same specific activity in both corneal glycosaminoglycans. It is speculated that both corneal glycosaminoglycans may not turn over at the same rate, and the glycosaminoglycans may be sulfated, as acceptor sites are made available during biosynthesis.

Although cell-free extracts from beef cornea epithelium are capable of incorporating inorganic sulfate-35S into glycosaminoglycans, it seems unlikely that the stromal glycosaminoglycans are synthesized in the epithelium. Corneal stroma can incorporate inorganic sulfate-35S at a normal rate in some species in vitro and in vivo when denuded of epithelium and endothelium. It is more logical to conclude that epithelial cells have the capacity to synthesize 3'-phosphoadenosine 5'-phosphosulfate (active sulfate) and to transfer sulfate groups to suitable acceptor compounds.

REFERENCES


