A soluble sulfotransferase that could 6-sulfate both chondroitin sulfate and corneal keratan sulfate was purified 27,500-fold using a sequence of affinity chromato-
graphy steps with heparin-Sepharose, wheat germ agglutinin-agarose, and 3',5'-ADP-agarose. The essentially pure enzyme had a specific activity 40 times greater than the most purified chondroitin 6-sulfotransferase previously reported and exhibited a single sharp Coomassie Blue-stained and a heavy silver-stained protein band of 75 kDa on SDS-polyacrylamide gel electrophoresis. Chromatography of the purified enzyme on Sephacryl demonstrated a size of 150 kDa, which indicated that the native enzyme exists as a dimer. In addition to 6-sulfation of nonsulfated GalNAc, the purified enzyme had the ability to sulfate GalNAc 4-sulfate residues to give GalNAc 4,6-disulfate residues. The purified enzyme exhibited a $K_m$ of 40 $\mu$M for adenosine 3'-phosphate 5'-phosphosulfate when either chondroitin sulfate or corneal keratan sulfate were used as the acceptors. Use of both chondroitin sulfate and keratan sulfate in the same experiment demonstrated mutual competition, establishing that the sulfation of these substrates is by the same enzyme. Photoaffinity labeling of the purified enzyme with 2-azidoadenosine 3',5'-di[5-32P]phosphate occurred only with the 75-kDa protein, confirming that this is the chondroitin 6-sulfotransferase/keratan sulfotransferase.

Proteoglycans contain sulfated glycosaminoglycans that are covalently attached to a wide range of core protein families. In addition to the considerable variation and heterogeneity in the size and number of glycosaminoglycan chains that are linked to a core protein, there is heterogeneity in the position and degree of sulfation. The multiple functions of these molecules appear to be contributed, at least in part, by defined sulfate substitution of the glycosaminoglycan chains, so that the process of sulfation may have a direct effect on function.

During biosynthesis of the polysaccharide portions of proteoglycans, sulfation of nascent polysaccharide chains is considered to be accomplished by specific sulfotransferases that are located in juxtaposition to the polymer-forming enzymes in the Golgi (1, 2). Thus, sulfation in chondroitin is a complicated process that can result in the presence of GalNAc 4-sulfate and GalNAc 6-sulfate in the same glycosaminoglycan chains (3) by the presence of occasional GalNAc 4,6-disulfate and GlcA 2-sulfate (4) or by the presence of iduronic 2-sulfate (5) when dermatan sulfate is formed. Different chondroitin sulfotransferases are involved for each type of sulfate substituent, and a number of these have been examined (6-10). It has been generally assumed in the case of keratan sulfate that the sulfotransferase involved in the sulfation of GlcNAc residues is different from the sulfotransferase involved in the sulfation of the Gal residues (11). However, this has not been clearly established. With heparin or heparan sulfate there are three distinct O-sulfotransferases and one N-sulfotransferase that are involved in the modification of these polymers (12).

Although there had been a number of reports on the utilization of soluble or solubilized sulfotransferases with soluble polysaccharide substrates, it became apparent many years ago that sulfation in intact cells only takes place efficiently with microsomal membrane-bound enzymes (13). Thus during biosynthesis, the nascent membrane-bound proteoglycan is presented to the membrane-bound sulfotransferases for sulfation to take place during the process of polymerization (14). During cell growth and/or turnover, there is a release of soluble sulfotransferases into the circulatory system of intact animals (8, 9, 15, 16) or into the media of cell cultures (9, 17), but this released enzyme plays no apparent role in biosynthesis. In order to understand the specificity and mechanism of these membrane-bound enzymes and their secreted forms, one needs to obtain purified enzymes.

Previous work with partially purified chondroitin 6-sulfotransferases from a variety of sources (8, 15, 18, 19) has suggested that the sulfation of Gal residues in keratan sulfate may be catalyzed by the same enzyme, since the activities were not separable. In addition, it has been previously shown that the two enzyme activities show similar properties (18, 20) and similar developmentally associated changes (16). However, other work with cornea has suggested that the chondroitin 6-sulfotransferase and keratan sulfotransferase may be separable (11). Furthermore, partially purified chondroitin 6-sulfotransferase from mouse liver was reported to have no activity toward corneal keratan sulfate (21). The most highly purified chondroitin 6-sulfotransferase obtained to date was from the cartilage medium of chick embryo chondrocytes (19) and was cultured in the presence of [32P]phosphate occurred only with the 75-kDa protein, confirming that this is the chondroitin 6-sulfotransferase/keratan sulfotransferase.

This research was supported by the Veterans Affairs Medical Research Service and by National Institutes of Health Grants AR-41649 (to G. S.) from the National Institutes of Health. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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kDa band on SDS-PAGE was quite broad, raising questions regarding its purity. Thus it is still not clear whether two separate sulfotransferases are involved in the 6-sulfation of GalNAc residues in chondroitin sulfate and 6-sulfation of Gal residues in keratan sulfate or whether both glycosaminoglycans are substrates for the same enzyme.

In this report, we present our results on the purification of chondroitin 6-sulfotransferase/keratan Gal 6-sulfotransferase from chicken serum. Our 27,500-fold purified enzyme preparation had a specific activity 40 times greater than that previously reported (19). The two activities exhibited identical chromatographic properties throughout the purification steps. In addition, we have found that the two substrates are mutually competitive in accepting [35S]sulfate, indicating that the two activities are associated with the same enzyme molecule.

Chondroitin 6-sulfotransferase activity was determined by measuring the incorporation of [35S]sulfate from PAPS into desulfated chondroitin and or bovine corneal keratan sulfate. Reaction mixtures contained 0.05 M MES buffer, pH 6.5, 0.015 mM MgCl2, 2 mM CaCl2, 1 mM dithiobisretol, 0.02% Triton X-100 (buffer A) plus 0.15 M NaCl. The column was washed with 15–20 column volumes of the same buffer, and the bound proteins were then eluted by changing the concentration of NaCl to 0.75 M. Fractions of 5 ml were collected and assayed for enzyme activity.

Fractions containing the sulfotransferase activity were pooled and diluted with buffer A to adjust the concentration of NaCl back to 0.15 M and used for the next step.

Step 2: Chromatography on WGA-Agarose—The enzyme from the above step was added to 10 ml of WGA-agarose that had been previously equilibrated with buffer A, 0.15 M NaCl, and mixed for 3 h at 4 °C in a sealed column. After this period, the column was washed with 20 column volumes of buffer A, 0.15 M NaCl. Bound proteins were eluted by the addition of 0.5 M GlcNAc to the buffer. Fractions containing the sulfotransferase activity were pooled and dialyzed first against buffer A without NaCl and then against buffer A, 0.05 M NaCl.

Step 3: Chromatography on 3',5'-ADP-Agarose (NaCl elution)—Dialyzed fractions from step 2 were mixed for 5 h with 5 ml of 3',5'-ADP-agarose in a sealed column (1.5 × 10 cm) that had been previously equilibrated with buffer A, 0.05 M NaCl. The column was washed with 10 column volumes of buffer A, 0.15 M NaCl, and bound proteins were then eluted with buffer A, 1.0 M NaCl. Fractions containing the sulfotransferase activity were pooled and dialyzed first against buffer A and then against buffer A, 0.05 M NaCl.

Step 4: Rechromatography on 3',5'-ADP-Agarose (3',5'-ADP elution)—The eluate from step 3 was loaded on 5 ml of 3',5'-ADP-agarose column previously equilibrated with 200 ml of buffer A, 0.05 M NaCl. After washing the column, the bound proteins were eluted with buffer A, 0.05 M NaCl containing 0.5 mM 3',5'-ADP. The fractions containing the sulfotransferase activity were pooled and dialyzed first against buffer A, 1.0 M NaCl and then against buffer A, 0.05 M NaCl. The purified enzyme was stored at −20 °C until use.

The various active fractions were determined by 10% SDS-PAGE. The protein was performed with Coomassie Blue R-250 followed by destaining with isopropl alcohol, acetic acid, water (1:1:8 (v/v/v)) and by silver staining.

RESULTS AND DISCUSSION

Enzyme Purification—Various chicken tissues were screened to obtain a source that might be preferable to cartilage or chondrocyte growth medium as had been previously utilized by others (6, 19). Centrifugation of serum at 12,000 × g enabled the precipitation of 20–25% of the total protein, which had no chondroitin sulfotransferase or keratan sulfotransferase activity. Less than 2% of the supernatant chondroitin sulfotrans-
was bound and could be specifically eluted with 0.5M GlcNAc mixing for 3 h with WGA-agarose, all of the activity bound. However, after washing the column, elution was performed with 1.0 M NaCl as described under "Experimental Procedures." Fractions of 2 ml were collected and assayed for chondroitin 6-sulfotransferase (●) and keratan sulfotransferase (○) activities. Protein was monitored by measuring the absorbance at 280 nm (○). Elution with 1.0 M NaCl was started at fraction 31.

When the eluted protein was added to a WGA-agarose column, less than 50% of the activity was bound. However, after mixing the enzyme for 3 h with WGA-agarose, all of the activity was bound and could be specifically eluted with 0.5 M GlcNAc with no loss of activity. Both chondroitin 6-sulfotransferase and keratan sulfotransferase bound with strong affinity to a heparin-Sepharose column. The two activities were inseparable and eluted as a single peak from this column. This was in contrast to a previous report with human serum, which described two peaks of keratan sulfate sulfotransferase activity upon elution from heparin-Sepharose (8). The chromatography on heparin-Sepharose yielded a 43-fold purification with a 30% recovery of total activity (Table I). The pooled fractions from this step contained less than 0.05% fold purification with a 30% recovery of total activity (Table I).

(8). The chromatography on heparin-Sepharose yielded a 43-fold purification with a 30% recovery of total activity. Both chondroitin 6-sulfotransferase and keratan sulfotransferase activities had identical elution profiles from the WGA-agarose column. However, unlike a previously reported 40% loss in activity in purification of chondroitin 6-sulfotransferase with an overall recovery of 6.8%. The final specific activity with chondroitin as acceptor was 18 µmol of sulfate incorporated per min/mg of protein (40 times higher than that previously reported (19)) and with keratan sulfate as acceptor was 12 µmol of sulfate incorporated per min/mg of protein. Chondroitin 6-sulfotransferase and keratan sulfotransferase were inseparable throughout the purification, maintaining a constant activity ratio of approximately 1.5:1.

Coomassie Blue R-250 staining of the SDS gel after eluting directly with 3',5'-ADP without eluting first with NaCl resulted in only a 12,000-fold purification. However, as shown in Table I, by using the NaCl elution step prior to the 3',5'-ADP elution, we obtained a total 27,500-fold purification of chondroitin 6-sulfotransferase with an overall recovery of 6.8%. The final specific activity with chondroitin as acceptor was 18 µmol of sulfate incorporated per min/mg of protein (40 times higher than that previously reported (19)) and with keratan sulfate as acceptor was 12 µmol of sulfate incorporated per min/mg of protein. Chondroitin 6-sulfotransferase and keratan sulfotransferase were inseparable throughout the purification, maintaining a constant activity ratio of approximately 1.5:1.

Coomassie Blue R-250 staining of the SDS gel after eluting directly with 3',5'-ADP resulted in a broad doublet around 75 kDa (not shown), while the use of the NaCl elution step prior to the 3',5'-ADP elution as described under "Experimental Procedures." The approximate molecular weight of the purified sulfotransferase, as determined by gel chromatography on a Sephacryl S-300 column (not shown), was found to be 150 kDa, confirming the previous report that the native enzyme exists as a dimer (19). It is of note that rat liver heparan sulfate N-sulfotransferase (27) and mouse mast cell heparin N-sulfotransferase (28) as well as heparin O-sulfotransferases appear to be monomers (29, 30).

**Photoaffinity Radiolabeling—** Although PAP<sup>35</sup>S has been shown to be a good photoaffinity label for phenol sulfotransferases (31), our attempts to use this ligand for photoaffinity labeling of the chondroitin 6-sulfotransferase/keratan sulfotransferase were unsuccessful. However, we found that an analog of 3',5'-ADP, worked well. Although PA<sup>35</sup>P had been used successfully for photoaffinity labeling of the PAPS transporter (32), we found that it was much less efficient than 2-N<sub>3</sub>-[<sup>32</sup>P]ADP for labeling the chondroitin 6-sulfotransferase/keratan sulfotransferase. There was no labeling when control incubations containing 2-N<sub>3</sub>-[<sup>32</sup>P]ADP were not subjected to UV irradiation or when heat-inactivated protein samples were UV-irradiated in presence of this ligand (not shown). When proteins from the early steps of purification were exposed to UV
light in the presence of 2-N$_3$-[32P]cADP, photoincorporation of the gel with Coomassie Blue R-250. (45 kDa), bovine carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21.5 kDa), and lysozyme (14.4 kDa). A, proteins visualized by staining the gel with a WGA-agarose column. Lane 1, 65 µg of protein eluted from a heparin-Sepharose column; lane 2, 30 µg of protein eluted from a WGA-agarose column; lane 3, 1 µg of protein eluted with 1 M NaCl from a 3'5'-ADP-agarose column; lane 4, 0.2 µg of protein eluted with 0.5 M 3',5'-ADP from a 3',5'-ADP-agarose column. B, radioactivity of the same bands visualized by autoradiography of the gel.

**Fig. 3.** SDS-PAGE analysis of photoaffinity-labeled preparations of chondroitin 6-sulfotransferase. Protein fractions from the different steps in the purification were subjected to photolabeling with 2-N$_3$-[32P]cADP, and the photolabeled samples were electrophoretically separated on a 10% SDS-PAGE as described under "Experimental Procedures." The following standards were used: rabbit muscle phospho- lase B (97.4 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45 kDa), bovine carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21.5 kDa), and lysozyme (14.4 kDa). A, proteins visualized by staining the gel with Coomassie Blue R-250. Lane 1, 65 µg of protein eluted from a heparin-Sepharose column; lane 2, 30 µg of protein eluted from a WGA-agarose column; lane 3, 1 µg of protein eluted with 1 M NaCl from a 3'5'-ADP-agarose column; lane 4, 0.2 µg of protein eluted with 0.5 M 3',5'-ADP from a 3',5'-ADP-agarose column. B, radioactivity of the same bands visualized by autoradiography of the gel.

**Fig. 4.** SDS-PAGE analysis of chondroitin/keratan 6-sulfotransferase preparations by silver staining. Proteins visualized by silver staining as follows: lane 1, 400 µg of protein eluted from a heparin-Sepharose column; lane 2, 320 µg of protein eluted from a WGA-agarose column; lane 3, 30 ng of protein eluted with 1 M NaCl from a 3'5'-ADP-agarose column; lane 4, 18 ng of protein eluted with 0.5 M 3',5'-ADP from a 3',5'-ADP-agarose column. Standards were as in Fig. 3.

**TABLE I** Purification chart for sulfotransferase

| Purification Step | Total protein | Total activity | Specific activity | Purification | Recovery | Ratio of CS/KS$^a$ |
|-------------------|--------------|----------------|-------------------|--------------|----------|------------------|
| 12,000 × g supernatant | 20,300 | 13.6 | 0.00067 | 1 | 100 | 1.52 |
| Heparin-Sepharose | 145 | 4.2 | 0.029 | 43 | 31 | 1.48 |
| WGA-agarose | 23 | 4.45 | 0.19 | 200 | 33 | 1.44 |
| 3',5'-ADP-agarose (Elution by NaCl) | 1.25 | 1.6 | 1.3 | 1,900 | 12 | 1.47 |
| 3',5'-ADP-agarose (Elution by 3',5'-ADP) | 0.05 | 0.92 | 18 | 27,500 | 6.8 | 1.45 |

$^a$ Ratio of chondroitin 6-sulfotransferase activity to keratan sulfate sulfotransferase activity.

**FIG. 5.** Incorporation of [35S]sulfate from PAP$^{35}$S into exogenous glycosaminoglycan acceptors. Reaction mixtures containing varying amounts of glycosaminoglycan acceptors (0.4–40 µg) and 0.05 M MES buffer, pH 6.5, 0.015 M MnCl$_2$, 0.1% Triton X-100, 1 mM PAP$^{35}$S (1.5 Ci/mmol), 20 ng of purified enzyme protein in a total volume of 15 µl were incubated at 37°C for 30 min. The reaction mixtures were then spotted on Whatman No. 1 paper and chromatographed as described under "Experimental Procedures." The origins containing the sulfated glycosaminoglycans were eluted with water and analyzed for radioactivity. ●, chondroitin; ▲, chondroitin 4-sulfate; ■, chondroitin 6-sulfate; ●, dermatan sulfate; ○, bovine corneal keratan sulfate; and □, human costal cartilage keratan sulfate.

**Concentration-dependent fashion.** At a concentration of 16 µM there was 80% inhibition, and essentially 100% inhibition at 100 µM. Combined with the purification procedures, the results of 2-N$_3$-[32P]cADP confirmed that the protein migrating at 75 kDa was a single protein with both chondroitin 6-sulfotransferase and keratan sulfotransferase activities.

**Acceptor Substrate Specificity—** The sulfotransferase was found to be highly specific for desulfated chondroitin, chondroitin 6-sulfate, and bovine corneal keratan sulfate equally as sulfate acceptors, while chondroitin 4-sulfate appeared to be a slightly better acceptor (Fig. 5). Dermatan sulfate was only one-eighth as efficient as chondroitin 4-sulfate. Human costal cartilage keratan sulfate did not serve as a substrate, consistent with a previous report (20). Heparin or heparan sulfate did not serve as acceptors (not shown). Our finding that chondroitin sulfate served as a better substrate than the completely desulfated chondroitin (Fig. 5) was consistent with the observation that partially desulfated chondroitin sulfate was a better sulfate acceptor than completely desulfated chondroitin (33).

We have previously reported an apparent $K_m$ of 500 µM for PAPS when a chondroitin hexasacharide was used as the acceptor with soluble enzyme (34). However, in the present experiments using desulfated chondroitin or keratan sulfate with the purified enzyme, the $K_m$ for PAPS was found to be decreased significantly to 40 µM. These results would indicate that interaction of a full-length chondroitin with the enzyme had an effect upon the $K_m$ for PAPS.

All sulfation of chondroitin, as determined by analyzing the products of chondroitin ABC lyase degradation, was found to be
at the GalNAc 6-position with no detectable transfer to the 4-position. However, approximately 10–15% of the $^{35}$S-labeled disaccharides from chondroitin ABC lyase degradation of the labeled chondroitin 4-sulfate and dermatan sulfate chromatographed as ADI-4,6S, indicating that the chondroitin 6-sulfotransferase can add a 6-sulfate to an already 4-sulfated GalNAc residue.

As shown in Fig. 5, human costal cartilage did not serve as an acceptor for the sulfotransferase, while bovine corneal keratan sulfate served as an efficient acceptor. Furthermore, keratanase degradation of $^{35}$S-labeled bovine corneal keratan sulfate did not yield any disaccharide products, consistent with the previous report (19), indicating that the enzyme had no activity on any sulfated internal residue. It has previously been established that human costal cartilage keratan sulfate is essentially 100% Gal 6-sulfated (20), while 50% of the Gal residues of bovine corneal keratan sulfate are unsulfated (35), and it has also been established that Pseudomonas keratanase has no activity on internal residues where the Gal is 6-sulfated (36). Thus our results indicate that the sulfate was only incorporated onto the Gal of the keratan sulfate. In order to substantiate these results, we incubated PAP $^{35}$S with p-nitrophenyl galactoside, p-nitrophenyl N-acetylgalactosaminide, and p-nitrophenyl N-acetylglucosaminide. Incorporation of $^{35}$S-sulfate into these glycosides was found to be 17,000 and 22,000 cpm for the galactoside and N-acetylgalactosaminide, respectively, but less than 500 cpm for the N-acetylglucosaminide. These results confirm that the sulfated transfer was transferred to the 6-position of Gal residues in keratan sulfate.

Competition experiments with both chondroitin sulfate and keratan sulfate in the same incubation mixture were performed in order to determine whether the purified enzyme had a single catalytic site that is involved in the sulfation of both Cs and Gal residues or whether the enzyme was bifunctional with separate catalytic sites for the sulfation of chondroitin and keratan. We found (Table I) that the presence of increasing concentrations of chondroitin sulfate at a fixed concentration of 40 µg of keratan sulfate resulted in increasing inhibition of keratan sulfotransferase activity, and the presence of increasing keratan sulfate concentrations inhibited the chondroitin 6-sulfotransferase activity. These results, demonstrating that the two substrates were mutually competitive, indicate that the same catalytic site in the enzyme is involved in sulfation of both GaNAc and Gal residues.

Our purification, competition experiments, and the photoaffinity labeling have now provided definitive information regarding a single catalytic site for the 6-sulfation of GaNAc residues in chondroitin sulfate and 6-sulfation of Gal residues in keratan sulfate. Furthermore, our experiments have validated the use of 2-N$_2$-$^{32}$P-ADP as a specific photoaffinity probe for a sulfotransferase and would indicate that this analog might be useful as a specific photoprobe in characterizing other sulfotransferases.

Acknowledgment—We thank Dr. J. E. Silbert, Veteran’s Administration Medical Center, Bedford, and Harvard Medical School, Boston, for valuable comments and constant encouragement.

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**TABLE II**

| Variable substrate concentration | $[^{35}S]$Sulfate cpm into KS$^a$ | $[^{35}S]$Sulfate cpm into CS$^a$ | Inhibition of KS sulfotransferase | Inhibition of CS sulfotransferase |
|----------------------------------|----------------------------------|----------------------------------|----------------------------------|----------------------------------|
| KS (40 µg)                       |                                  |                                  |                                  |                                  |
| 0                                | 22,200                           | 0                                | 0                                |                                  |
| 0.4                               | 18,900                           | 1,300                            | 14.6                             |                                  |
| 4.0                               | 17,800                           | 6,970                            | 19.4                             |                                  |
| 40.0                              | 6,350                             | 18,800                           | 71.3                             |                                  |
| CS (40 µg)                       |                                  |                                  |                                  |                                  |
| 0                                | 34,400                           | 0                                | 0                                |                                  |
| 0.4                               | 650                              | 29,900                           | 13.0                             |                                  |
| 4.0                               | 1,900                            | 29,100                           | 15.4                             |                                  |
| 40.0                              | 6,350                             | 18,800                           | 45.3                             |                                  |

$^a$ KS, keratan sulfate.  
$^b$ CS, chondroitin sulfate.