Heparan sulfate, keratan sulfate, chondroitin, chondroitin 4/6-sulfate (80% 4-sulfate and 20% 6-sulfate), and UDP-N-acetylgalactosamine 4-sulfate were used as acceptors for the measurement of 3'-phosphoadenylyl sulfate:glycosaminoglycan sulfotransferase activities in human serum. Chromatographic fractionation of the serum followed by determination of the sulfotransferase activities demonstrated the existence of at least four different sulfotransferases capable of introducing sulfate to 1) position 6 of the internal N-acetylgalactosamine units of chondroitin, 2) position 6 of the nonreducing terminal N-acetylgalactosamine 4-sulfate unit of chondroitin 4/6-sulfate, 3) position 2 (amino group) of the glucosamine units in heparan sulfate, and 4) the sugar units in keratan sulfate, respectively. The fourth activity was separated into two subfractions with different specificities for the structure of neighboring sugars of the sulfate-accepting sugar units. No major variations in the sulfotransferase activities on added receptors were found to occur in sera from individuals 22–41 years old. In contrast, the activities in sera of various mammalian and avian species showed a species-specific variation. With mouse skin fibroblasts cultured in serum-free medium, preferential secretion of several sulfotransferases was observed. The endogenous sulfate acceptor for the measurement of 3'-phosphoadenylyl sulfate (PAPS) was synthesized from inorganic sulfate and ATP with ATP sulfurylase and adenyllyl sulfate kinases purified from Bakers' yeast (22). The specific activity was 3.0 × 10^6 cpm/μmol. A chondroitin sulfate-free keratan sulfate sample, prepared from fresh bovine cornea by proteolytic degradation with Pronase-P, ethanol fractionation, and digestion with chondroitinase ABC (29), was kindly donated by Dr. K. Nakazawa, Meijo University, Nagoya, Japan. Since the sample contained a significant amount of heparan sulfate, it was further treated with 0.24 M NaOH in 1.8 M acetic acid for 80 min (27) to degrade the contaminant. The resulting mixture was dialyzed against running tap water and then mixed with 2 volumes of 95% (v/v) ethanol containing 1.3% (w/v) potassium acetate. The precipitate was washed successively with ethanol and ether and dried over P₂O₅ in a vacuum. The final preparation had a molar ratio of sulfite to hexosamine = 1.13. Chondroitin 4/6-sulfate (average M₀ = 26,000; 80% 4-sulfate and 20% 6-sulfate) from whale cartilage, and heparan sulfate from bovine kidney (molar ratio of 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Mes, 2-(N-morpholino)ethanesulfonic acid.

Enzymes involved in the sulfation of growing glycosaminoglycan chains of proteoglycans are localized in the Golgi apparatus (1–4). Microsomal preparations and extracts derived therefrom have been used for investigation of the sulfation process (for reviews, see Refs. 5–7). When these preparations are incubated with [³⁵S]PAPS, rapid incorporation of [³⁵S]sulfate into endogenous proteoglycans or exogenous glycosaminoglycans is observed. The endogenous sulfate acceptors may represent incomplete proteoglycan molecules in all states of glycosaminoglycan chain extension which could serve as primers for both sulfation and polysaccharide polymerization (8). However, it is not possible to present methods in this paper. The abbreviations used are: PAPS, 3'-phosphoadenylyl sulfate; ATP, adenosine triphosphate; ADP, adenosine diphosphate; UDP, uridine diphosphate; GalNAc, N-acetylgalactosamine; Gal, galactose; Glc, glucose; GlcA, glucuronic acid; GAG, glycosaminoglycan. Several sulfotransferases have been demonstrated in human serum and the distribution of the sulfotransferase activities in various mammalian and avian sera. In the following paper (24), we will compare the properties of PAPS:GalNAc 4-sulfate 6-O-sulfotransferase preparations isolated from human serum and squid cartilage.

EXPERIMENTAL PROCEDURES

Materials—Adult human sera were prepared from fresh blood donated by volunteers, and other sera were from fresh blood specimens collected from ACI rats, Japanese rabbits, White Leghorn roosters, Japanese quails, and BALB/c mice. [³⁵S]PAPS was synthesized from inorganic [³⁵S]sulfate and ATP with ATP sulfurylase and adenyllyl sulfate kinases purified from Bakers' yeast (25). The specific activity was 3.0 × 10^6 cpm/μmol. A chondroitin sulfate-free keratan sulfate sample, prepared from fresh bovine cornea by proteolytic degradation with Pronase-P, ethanol fractionation, and digestion with chondroitinase ABC (25), was kindly donated by Dr. K. Nakazawa, Meijo University, Nagoya, Japan. Since the sample contained a significant amount of heparan sulfate, it was further treated with 0.24 M NaOH in 1.8 M acetic acid for 80 min (27) to degrade the contaminant. The resulting mixture was dialyzed against running tap water and then mixed with 2 volumes of 95% (v/v) ethanol containing 1.3% (w/v) potassium acetate. The precipitate was washed successively with ethanol and ether and dried over P₂O₅ in a vacuum. The final preparation had a molar ratio of sulfite to hexosamine = 1.13. Chondroitin 4/6-sulfate (average M₀ = 26,000; 80% 4-sulfate and 20% 6-sulfate) from whale cartilage, and heparan sulfate from bovine kidney (molar ratio of 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Mes, 2-(N-morpholino)ethanesulfonic acid.

This paper describes the partial separation and characterization of four distinct sulfotransferases from human serum and the distribution of the sulfotransferase activities in various mammalian and avian sera. In the following paper (24), we will compare the properties of PAPS:GalNAc 4-sulfate 6-O-sulfotransferase preparations isolated from human serum and squid cartilage.

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To whom correspondence should be addressed. The abbreviations used are: PAPS, 3'-phosphoadenylyl sulfate; ADP–1–4, ADP–1–6, and ADP–4,6-bisulfate; O–β–d-glucuronosyl-(1–3)–N-acetylgalactosamine 4-sulfate, 6-sulfate, and 4,6-bisulfate, respectively; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Mes, 2-(N-morpholino)ethanesulfonic acid.

Glycosaminoglycan Sulfotransferases in Human and Animal Sera*

(Received for publication, November 6, 1985)
Table I

| Enzyme* | Exogenous acceptor |
|---------|---------------------|
| 1. GalNac 4-O-sulfotransferase | Chondroitin (9) |
| 2. GalNac 6-O-sulfotransferase | Chondroitin (9, 10) |
| 3. GalNac 4-sulfate 6-O-sulfotransferase | Chondroitin 4-sulfate; ΔΔi-4S; GalNac 4-sulfate (11) |
| 4. Terminal GalNAc 4-sulfate 6-O-sulfotransferase | UDP-GalNAc 4-sulfate; chondroitin 4-sulfate (10) |
| 5. GlcN 2-N-sulfotransferase | N-Desulfogaluronic acid; N-desulfogaluronic acid (12, 13) |
| 6. L-iduronic acid 2-O-sulfotransferase | Heparin (14) |
| 7. GlcNSO3 6-O-sulfotransferase | Heparin, heparan sulfate, and their partially desulfated derivatives (15, 16) |
| 8. Gal 6-O-sulfotransferase | Bovine cornea keratan sulfate and its partially desulfated derivatives (15-17); agarse (17) |
| 9. GlcNAc 6-O-sulfotransferase | Bovine cornea keratan sulfate and its partially desulfated derivatives (15-17) |

*The enzyme names are given to show the position of hydroxyl group (e.g. 6-O-) or amino group (e.g. 2-N-) of the monosaccharide unit (e.g. GalNAc) in acceptor molecule to which the sulfate group is transferred.

**The sulfate transfer onto position 6 of the nonreducing GalNac 4-sulfate end group.

The 2-O-sulfation of L-iduronic acid units appears to occur only in conjunction with the formation of such units by 5-epimerase reaction (14).

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ride monosulfate, GlcNAc(6-sulfate)-Gal, represents the sulfate groups incorporated into the glucosamine units of keratan sulfate. The radioactivities recovered as higher oligosaccharides, on the other hand, should represent the sulfate groups incorporated into either the galactose units or the glucosamine units adjacent to a galactose unit already bearing a sulfate (33).

**Culture of Mouse Dermal Fibroblasts**—Mouse dermal fibroblasts were obtained from newborn BALB/c mice skin by treatment with trypsin and collagenase as previously described (39). The cells were inoculated into 60-mm Falcon tissue culture dishes at a density of 6 x 10^6 cells/ml of medium (Dulbecco's modified Eagle's medium supplemented with glucose (2 g/liter), ascorbic acid (50 mg/liter), Hepes (10 mM), fetal calf serum (10%), penicillin (50,000 units/liter), and streptomycin (50 mg/liter)). The cells were cultured for 2.5 days in a 37°C incubator with a humidified atmosphere of 95% air/5% CO2 (culture medium was changed about 24 h after the inoculation). The cells were washed twice with Dulbecco's modified Eagle's medium and fed with either the serum-containing medium as above or a serum-free medium (Dulbecco's modified Eagle's medium supplemented with glucose (2 g/liter), ascorbic acid (50 mg/liter), Hepes (10 mM), epidermal growth factor (50 µg/liter), fibroblast growth factor (10 µg/liter), insulin (2 mg/liter), transferrin (5 mg/liter), penicillin (50,000 units/liter), and streptomycin (50 mg/liter)). The cells were grown at 37°C in a humidified atmosphere containing 5% CO2. About 1 day, culture medium was changed (the cultures at this stage are referred to as "time 0 cultures"). At the time indicated (12 and 24 h) one set of two dishes, each with the cells and either the serum-containing medium or the serum-free medium, was removed and the number of cells in each dish was counted. To examine the role of enzyme secretion, the spent medium was collected from the serum-free cultures, dialyzed against 0.02 M Tris-HCl, pH 7.2, containing 10% (w/v) glycerol and 0.5 mM diithiothreitol (solution A), concentrated to 0.1 volume by centrifugation on a Centricon 10 membrane, and assayed for glycosaminoglycan sulfotransferases (see above) and lactate dehydrogenase (for the assay method see Ref. 40). Other Methods—Protein in the eluates from chromatography columns was monitored by the Coomassie Blue method (42). In all other cases, protein was determined by the method of Lowry et al. (41) with bovine serum albumin (Fraction V) as a standard. Hexose was determined by the anthrone-H2SO4 method (43) with N-galactose as a standard.

**RESULTS**

**Sulfate Transfer Catalyzed by Human Serum**—Fresh human serum was assayed at pH 7.2 for transfer of ^35S from [35S]PAPS to endogenous acceptors. Paper chromatography (in solvent A) of the resultant reaction mixture indicated that a small amount of ^35S was incorporated into material remaining at the origin on the paper chromatogram; the rate of incorporation was about 220 cpm/h/10 µl of serum, in the presence of 2 x 10^6 cpm of [35S]PAPS (Table II). The labeled products could be recovered directly from the reaction mixture on a preparative scale by digestion with Pronase followed by ethanol precipitation. Analysis with chondroitinase ABC, chondroitinase AC II, nitrous acid, and keratanase indicated that about 65 and 5% of the incorporated radioactivity were accounted for by chondroitinase ABC (or AC II)-sensitive materials (mainly chondroitin 6-sulfate) and nitrous acid-sensitive materials (such as heparan sulfate and heparin), respectively. The remainder (about 30%) of the incorporated radioactivity was refractory to the action of chondroitinase ABC, keratanase, and HNO2 (as ascertained by gel-chromatographic criteria), and presumably represents nonglycosaminoglycan-type sulfated polysaccharides. This resistant material was not studied further.

As Table II shows, the formation of radioactive material remaining at the origin was greatly stimulated by the addition of chondroitin, chondroitin 4/6-sulfate (80% 4-sulfate and 20% 6-sulfate), heparan sulfate, and keratan sulfate. Maximal stimulation was obtained at pH 6.5 with chondroitin (6-fold), at pH 6.9 with chondroitin sulfate (5-fold), at pH 8.0 with heparan sulfate (220-fold), and at pH 7.2 with keratan sulfate (2.5-fold).

The radioactive products obtained by incubation with chondroitin and chondroitin 4/6-sulfate were recovered from the reaction mixtures by digestion with Pronase followed by ethanol precipitation. Analysis with chondroitinase ABC (Table II) indicated that the serum catalyzed the formation of various types of sulfate ester bonds on the exogenous acceptors, e.g. 4-sulfate, 6-sulfate, and 4,6-bissulfate on the interior N-acetylgalactosamine units and 4,6-bissulfate on the nonreducing terminal N-acetylgalactosamine units. It is noteworthy that the type of the sulfate incorporated is largely dependent on the chemical nature of added acceptor. Thus, when chondroitin was the acceptor, about 98.5% of the sulfate groups incorporated were onto the interior N-acetylgalactosamine units (14% 4-sulfate, 82% 6-sulfate, and 2.5% 4,6-bissulfate). In contrast, when chondroitin 4/6-sulfate was used as the acceptor, about 30% of the sulfate groups were onto the nonreducing terminal N-acetylgalactosamine units (28% 4,6-bissulfate and 2% 4- or 6-sulfate).
of simplicity in product assay interference from other sulfotransferases which might be as acceptors. Although chondroitin 4-sulfate may also be used, the use of UDP-GalNAc 4-sulfate has the advantage of simplicity in product assay (10) and is less subject to interference from other sulfotransferases which might be present; e.g. UDP-GalNAc 4-sulfate does not serve as an acceptor for GalNAc 6-0-sulfotransferase, the enzyme introducing 6-sulfate to occasional N-acetylgalactosamine units in the interior portion of chondroitin sulfate (10). Table III shows the summary of enzyme purification.

Fresh serum was subjected to affinity chromatography on heparin-Sepharose CL-6B (Fig. 1). Automatically dialyzed fractions from the column were assayed for sulfotransferase activities toward chondroitin, UDP-GalNAc 4-sulfate, heparan sulfate, and keratan sulfate. As Fig. 1 shows, the affinity chromatography served as an effective method in separating

| Fraction     | Total protein (mg) | UDP-GalNAc 4-sulfate | Chondroitin sulfate | Heparan sulfate | Keratan sulfate |
|--------------|--------------------|----------------------|--------------------|----------------|----------------|
| Serum        | 21,100             | 2.5                  | 0.2                | 5.1            | 0.2            |
| Heparin-Sepharose CL-6B<sup>a</sup> |                     |                      |                    |                |                |
| Fraction I   | 409                | 5.3                  | 1.9                | 222            | 4.4            |
| Fraction II  | 126                | 352                  | 118                | 4.0            | 7.4            |
| Fraction III | 29                 | 82.8                 | 27.7               | 1.2            | 15.7           |
| Matrex Blue B<sup>b</sup> |                 |                      |                    |                |                |
| Fraction II-a| 35                 | 136                  | 35.1               | ND<sup>c</sup> | ND             |
| Fraction II-b| 28                 | 839                  | 17.3               | 3.9            | 0.4            |

<sup>a</sup> See Fig. 1
<sup>b</sup> See Fig. 2
<sup>c</sup> ND, not determined

6-sulfate. This pattern of acceptor-dependent variation in the type of sulfate transfer is similar to that observed with extracts from quail oviduct (10). In view of the known specificities of chondroitin/chondroitin 4-sulfate sulfotransferases (Table I), the most likely possible serum enzymes responsible for the observed activities are GalNAc 4-O-sulfotransferase, GalNAc 6-0-sulfotransferase, and terminal GalNAc 4-sulfate 6-O-sulfotransferase (for the enzyme nomenclature, see Table I). Since a small, but significant, amount of [35S]sulfate was found as N-acetylgalactosamine 4,6-bissulfate in the interior portion of chondroitin 4/6-sulfate, a 6-O-sulfotransferase for interior GalNAc 4-sulfate units might also be present in the serum.

The radioactive products obtained by incubation with heparan sulfate and keratan sulfate were similarly recovered from the reaction mixtures and treated with nitrous acid and keratanase, respectively. Sephadex G-50 chromatography of the treated samples showed that over 98% of the labeled products from heparan sulfate and about 76% of those from keratan sulfate could be degraded with nitrous acid and keratanase, respectively, to yield fragments appearing in retarded fractions from the gel. The results suggest that human serum contains some heparan sulfate (heparin) and keratan sulfate sulfotransferase similar to, if not identical with, those listed in Table I.

When fresh serum was kept frozen at -20°C with occasional thawing and refreezing, no significant loss of the sulfotransferase activities was observed over a period of 1 year, except for the activity of GalNAc 4-O-sulfotransferase for chondroitin, which decreased gradually on repeated freezing and thawing. Comparison of the sulfotransferase activities of a fresh serum sample with those of a fresh plasma sample obtained from the same source showed no significant difference, indicating that platelets could not be a source of the sulfotransferases.

Fractionation of Serum Sulfotransferases—On the basis of the observations described above, advantage was taken in the following fractionation experiments of the fact that most, if not all, of the individual sulfotransferase activities can be monitored with UDP-GalNAc 4-sulfate, chondroitin, heparan sulfate (molar ratio of sulfate to hexosamine = 0.99), and keratan sulfate (molar ratio of sulfate to hexosamine = 1.13) as acceptors. Although chondroitin 4-sulfate may also be used as an acceptor for terminal GalNAc 4-sulfate 6-O-sulfotransferase, the use of UDP-GalNAc 4-sulfate has the advantage of simplicity in product assay (10) and is less subject to interference from other sulfotransferases which might be present.

### Table III

Purification of sulfotransferases from human serum.

| Fraction     | Total protein (mg) | UDP-GalNAc 4-sulfate | Chondroitin sulfate | Heparan sulfate | Keratan sulfate |
|--------------|--------------------|----------------------|--------------------|----------------|----------------|
| Serum        | 21,100             | 2.5                  | 0.2                | 5.1            | 0.2            |
| Heparin-Sepharose CL-6B<sup>a</sup> |                     |                      |                    |                |                |
| Fraction I   | 409                | 5.3                  | 1.9                | 222            | 4.4            |
| Fraction II  | 126                | 352                  | 118                | 4.0            | 7.4            |
| Fraction III | 29                 | 82.8                 | 27.7               | 1.2            | 15.7           |
| Matrex Blue B<sup>b</sup> |                 |                      |                    |                |                |
| Fraction II-a| 35                 | 136                  | 35.1               | ND<sup>c</sup> | ND             |
| Fraction II-b| 28                 | 839                  | 17.3               | 3.9            | 0.4            |

<sup>a</sup> See Fig. 1
<sup>b</sup> See Fig. 2
<sup>c</sup> ND, not determined

![Fig. 1. Affinity chromatography of human serum on heparin-Sepharose CL-6B.](image-url)
sulfotransferase (as a whole) from other serum proteins; over 95% of the applied proteins were recovered in the washings (panel a), whereas essentially all of the sulfotransferase activities were found in the retentates.

The heparan sulfate-dependent activity was eluted in a single large peak with a maximum at tube 46 (panel c), whereas all the other activities eluted in peaks between tubes 50 and 70 (panels a, b, and d). The fractions containing most of the heparan sulfate-dependent activity (tubes 43–51) were pooled, concentrated on a PM-10 membrane by pressure ultrafiltration, and used for further studies as “Fraction I.”

The major activities on UDP-GalNAc 4-sulfate (panel a) and chondroitin (panel b) were eluted in tubes 54–64 (indicated as Fraction II). At this stage, their elution profiles overlapped each other. However, the recovered activity for chondroitin, but not the activity for UDP-GalNAc 4-sulfate, exceeded the applied one by a factor of 4.8 (Table III), suggesting that the two activities reside in different enzyme species and that the serum contains an inhibitor of the chondroitin-dependent sulfotransferase which can be removed by the affinity chromatography.

The keratan sulfate-dependent activity (panel d) was eluted in two peaks, the first one appearing in the zone for UDP-GalNAc 4-sulfate/chondroitin-dependent activities (Fraction II) and the second one in a slower eluting zone with some overlap with the first peak. As shown below, attempts to obtain a keratan sulfate sulfotransferase preparation devoid of chondroitin/chondroitin 4-sulfate sulfotransferase activities have so far been unsuccessful. In the present work, therefore, aliquots of the two peaks (tubes 54–64 and tubes 65–70) were separately pooled, dialyzed against solution A, concentrated on a PM-10 membrane by pressure ultrafiltration, and used as Fraction II and Fraction III, respectively.

In an attempt to separate the activities of chondroitin, UDP-GalNAc 4-sulfate, and keratan sulfate from one another, Fraction II from the heparin-Sepharose CL-6B column was pooled, concentrated by pressure ultrafiltration, and loaded on a Måtrex Blue B column equilibrated with solution A. The column was washed with solution A and then eluted with an increasing salt gradient from 0 to 0.6 M NaCl in solution A (Fig. 2). The chondroitin-dependent activity was recovered almost exclusively in the washings (panel b). The UDP-GalNAc 4-sulfate-dependent activity, in contrast, was eluted in a large peak (containing 90% of the total activity) with a maximum at tube 40 plus a small peak in the washings (panel a). Very little keratan sulfate-dependent activity was recovered from the column, perhaps owing to denaturation of the enzymes during the manipulation for column chromatography. The fractions (tubes 3–12) containing most of the chondroitin-dependent activity and those (tubes 34–49) containing the main activity for UDP-GalNAc 4-sulfate were separately pooled, dialyzed against solution A, concentrated by pressure ultrafiltration, and used for further studies in “Fraction II-a” and “Fraction II-b”, respectively.

Identification of the Products of Sulfotransferase Reactions—The following facts established that the 35S-labeled product formed by incubation of UDP-GalNAc 4-sulfate with [35S]PAPS in the presence of Fraction II-b (see Fig. 2) was UDP-N-acetylgalactosamine 4,6-bis-35S-sulfate. The 35S-labeled product had electrophoretic and chromatographic mobilities identical with authentic UDP-GalNAc 4,6-bissulfate (see Ref. 36 for the mobilities of the authentic compound). After treatment with 0.01 M HCl at 100 °C for 10 min, all the radioactivity was found in a compound with the electrophoretic and chromatographic mobilities of authentic N-acetylgalactosamine 4,6-bisulfate (see Ref. 36). Further treatment of the resulting radioactive compound with N-acetylgalactosamine 4-sulfate sulfatase did not release the label as inorganic [35S]sulfate but as a radioactive compound with the electrophoretic and chromatographic mobilities of authentic N-acetylgalactosamine 6-sulfate (see Ref. 36). The results indicate that the enzyme in Fraction II-b catalyzes the transfer of [35S]sulfate from [35S]PAPS to position 6 of the N-acetylgalactosamine 4-sulfate moiety of the nucleotide acceptor. As is the case of terminal GalNAc 4-sulfate 6-O-sulfotransferase from quail oviduct (10), the Fraction II-b enzyme also catalyzes the transfer of sulfate from PAPS to position 6 of the nonreducing N-acetylgalactosamine 4-sulfate end group of chondroitin 4/6-sulfate (see the accompanying paper for further details of the properties of Fraction II-b enzyme).

To determine the position of the [35S]sulfate groups incorporated into added chondroitin by Fraction II-a (see Fig. 2), the labeled product was recovered from the incubation mixture by Pronase digestion followed by ethanol precipitation. Digestion of this material with chondroitinase ABC resulted in the release of about 94% of the radiolabel as a compound with the paper chromatographic and paper electrophoretic mobilities of ΔDi-6S. Thus, the activity in Fraction II-a may actually represent GalNAc 6-O-sulfotransferase, the known enzyme capable of introducing 6-sulfate into the interior N-acetylgalactosamine units of chondroitin. The activity of introducing 4-sulfate to the interior N-acetylgalactosamine...
units, which is detectable in fresh serum (Table I), was not found in Fraction II-a. In view of its low stability (see above), it is possible that the enzyme has been denatured during the manipulation for purification.

The position of the [\(^{35}\)S]sulfate groups incorporated into heparan sulfate by Fraction I (see Fig. 1) was determined by deaminative degradation of the [\(^{35}\)S]labeled product that was recovered from the incubation mixture by Pronase digestion followed by ethanol precipitation. Before nitrous acid treatment, the labeled product was almost entirely excluded from the position expected for inorganic sulfate (indicated by the arrow in Fig. 3). When the product of HNO\(_3\) degradation was further analyzed by paper electrophoresis, a single radioactive spot was detected by autoradiography (not shown). This radioactive spot coincided with inorganic sulfate (added as internal marker) located with the BaCl\(_2\)-rhodizonate reagent (45). The results indicate that the sulfate residues transferred to added heparan sulfate were specifically incorporated into 2-N-sulfate groups. Thus, the activity of Fraction I on added heparan sulfate may actually represent GlcN 2-N-sulfotransferase. This conclusion was further supported by the fact that chemically N-acetylated heparan sulfate was essentially inactive as an exogenous acceptor for Fraction I; the amount of [\(^{35}\)S]sulfate incorporated was less than 9% of that incorporated into the parent heparan sulfate sample.

To investigate whether Fractions II and III (see Fig. 1) catalyze the transfer of sulfate into either galactose or N-acetylgalactosamine unit, or both, of added keratan sulfate, the labeled products isolated from the reaction mixtures by Pronase digestion followed by ethanol precipitation were mixed with unlabeled keratan sulfate (carrier) and the mixtures were exhaustively digested with keratanase. The resulting digests were analyzed by Sephadex G-50 chromatography (Fig. 4). While all the undigested glycosaminoglycan samples were eluted in the void volume fraction, their elution profiles were altered in a pronounced manner after keratanase digestion. Thus, when measurements were made of hexose-positive material (which represents the bulk of the oligosaccharides derived from unlabeled keratan sulfate added as a carrier), about 40% of the total hexose was recovered after keratanase digestion in a peak with the \(K_d\) value of disaccharide monosulfate, GlcNAc(6-sulfate)-Gal (panel d; see Ref. 33 for the identification of the disaccharide). When measurements were made of \(^{35}\)S radioactivity (which represents the oligosaccharides derived from newly sulfated keratan sulfate in vitro), it was revealed that the introduction of [\(^{35}\)S]sulfate by Fraction II

![Fig. 3. Effect of nitrous acid treatment on the [\(^{35}\)S]sulfate-labeled product obtained by incubation of Fraction I (see Fig. 1) with [\(^{35}\)S]PAPS and heparan sulfate. The [\(^{35}\)S]-labeled glycosaminoglycan (8.4 x 10\(^5\) cpm) isolated from the reaction mixture by Pronase digestion followed by ethanol precipitation was applied to a Sephadex G-50 column (0.8 x 90 cm) before (C) and after (D) nitrous acid degradation. The column was equilibrated with 0.2 M ammonium acetate/acetic acid, pH 6, and elution was carried out with this buffer at a flow rate of 10 ml/h. Fractions of 0.46 ml were collected and analyzed for radioactivity. The void volume \((V_v)\) and the total volume \((V_t)\) of the column as well as the position of elution of SO\(_2\)\(^-\) are indicated by the arrows.](attachment:image.png)

![Fig. 4. Chromatography on Sephadex G-50 of keratanase digests of bovine cornea keratan sulfate (a) and [\(^{35}\)S]sulfate-labeled products derived from the keratan sulfate by incubation with [\(^{35}\)S]PAPS in the presence of Fraction II (b) or Fraction III (c). The [\(^{35}\)S]-labeled glycosaminoglycans (5.7 x 10\(^5\) cpm) were isolated from the reaction mixtures by Pronase digestion followed by ethanol precipitation. Each of the labeled samples was mixed with 1 mg of unlabeled keratan sulfate (carrier) and applied to a Sephadex G-50 column (0.8 x 40 cm) before (×) and after (○) keratanase digestion. The column was equilibrated with 0.2 M ammonium acetate/acetic acid, pH 6, and elution was carried out with this buffer at a flow rate of 10 ml/h. Fractions of 0.3 ml were collected and analyzed for hexose and radioactivity. The elution profiles obtained with the sulfate samples added as a carrier (a), and with the [\(^{35}\)S]-labeled products derived by incubation with either Fraction II (b) or Fraction III (c) are shown. The void volume \((V_v)\) and the total volume \((V_t)\) of the column as well as the positions of elution of keratan sulfate disaccharide, tetrasaccharide, and hexasaccharide (see Ref. 33) are indicated by the arrows.](attachment:image.png)
(panel b) and Fraction III (panel c) caused marked alterations in the molecular size distribution of the keratanase-derived oligosaccharide fragments. In both cases, the peaks of labeled oligosaccharide fragments were earlier than those of hexose-positive oligosaccharides. It is especially noteworthy that in no case was the label found in the zone for sialic acid. Since keratanase has been shown to hydrolyze keratan sulfate at the endogalactoside bonds of nonsulfated galactose units but not at a galactoside bond in which galactose 6-sulfate participates (33), the failure of the labeled product to yield 35S-labeled GlcNAc(6-sulfate)-Gal can be interpreted as indicating 1) that the newly introduced [35S]sulfate residues were on galactose units which rendered their galactoside bonds resistant to keratanase and/or 2) that the [35S]sulfate residues were on glucosamine residues adjacent to a galactose unit already bearing a sulfate residue. As Fig. 4, b and c indicate, there is a difference between the products of Fraction II and III in the size distribution of keratanase-derived [35S] oligosaccharides. This difference may be interpreted as reflecting the fact that Fraction II differs from Fraction III in transferring sulfate more preferentially into a region of keratan sulfate where keratanase-resistant galactoside bonds (i.e. galactose 6-sulfate-participating bonds) are in close proximity to the sulfate-accepting (nonsulfated) sugar units.

Properties of Serum Sulfotransferases—The maximum rates of sulfate incorporation into heparan sulfate (with Fraction I), keratan sulfate (with Fraction III), chondroitin (with Fraction II-a), and UDP-GalNAc 4-sulfate (with Fraction II-b) occurred at pH 8.0 (in 0.05 M Tris-HCl buffer), at pH 7.2 (in 0.05 M Tris-HCl buffer), at pH 4.8 (in 0.05 M sodium acetate/acetic acid buffer), and at pH 5.2 (0.05 M sodium acetate/acetic acid buffer), respectively. It should be noted that the activity toward chondroitin (optimal pH = 4.8) exhibited about 50% of the maximal activity even at pH 7.0 (the pH of the serum) and that replacement of UDP-GalNAc 4-sulfate (acceptor) by chondroitin 4/6-sulfate resulted in the shift of optimal pH of the fourth activity from pH 5.2 to 6.9 (see above and Ref. 24).

As Fig. 5 shows, Ca2+ and Mg2+ stimulated the GlcN 2-N-sulfotransferase activity of Fraction I; at the optimal concentration of Ca2+ ion (10 mM), the activity increased about 16-fold. Keratan sulfate sulfotransferase (Fraction III) and GalNAc 6-O-sulfotransferase (Fraction II-a) were less sensitive to these cations. From the fact that MnCl2, NaCl, and KCl did not necessarily produce comparable effects on these reactions, it can be surmised that the chloride ion of itself does not account for the observed activations with calcium and magnesium salts (see Ref. 24 for the effect of cations on the terminal GalNAc 4-sulfate 6-O-sulfotransferase activity of Fraction II-b).

Sulfotransferase Activities in Various Serum Specimens—Ten serum specimens from healthy individuals (22-year-old men of blood type AB and blood type O, 22-year-old women of blood type A and blood type O, a 26-year-old man of blood type B, a 28-year-old man of blood type AB, and a 41-year-old man of blood type B) were analyzed for the sulfotransferase activities as described above. None of the enzyme activities could be demonstrated to correlate with age and blood type; variations of the specimens were within 20% of the mean values shown in Table IV. Serum specimens from seven patients of spondyloepiphyseal dysplasia were similarly analyzed. No significant deficiency of the sulfotransferase activities was detected in these sera.

As Table IV shows, the sulfotransferase activities in sera

![Figure 5: Effect of metal ions on the activities of GlcN 2-N-sulfotransferase of Fraction I (a), keratan sulfate sulfotransferase Fraction III (b), and GalNAc 6-O-sulfotransferase of Fraction II-a (c). The assay conditions were those described under "Experimental Procedures," except for the variation in the concentrations of CaCl2 (Δ), MgCl2 (●), MnCl2 (▲), KC1 (◆), and NaCl (○).](image)
that the apparent sulfotransferase activities may reflect, at least in part, the occurrence in sera of either some sulfotransferase inhibitors (see above) or glycosaminoglycan sulfatases which may interfere with the enzyme assays.

Secretion of Glycosaminoglycan Sulfotransferases by Skin Fibroblasts in Culture—Available evidence indicate that glycosaminoglycan sulfotransferases are localized in the Golgi apparatus where they are involved in sulfation of growing glycosaminoglycan chains (1-4). One may assume, therefore, that a source of the serum sulfotransferases is the enzymes in the Golgi apparatus. This would lead to the conclusion that the apparent sulfotransferase activities may reflect, at least in part, the occurrence in sera of either some sulfotransferase inhibitors (see above) or glycosaminoglycan sulfatases which may interfere with the enzyme assays.

Several experiments were separately assayed for glycosaminoglycan sulfotransferases, with UDP-GalNAc 4-sulfate, chondroitin, heparan sulfate, and keratan sulfate as acceptors. For comparison, the activity of lactate dehydrogenase, a marker enzyme for cytosol (51), was also measured.

Fig. 6. a and b shows that the sulfotransferase activities for UDP-GalNAc 4-sulfate and chondroitin in the medium increased linearly at about 100 and 5 units/plate/12 h, respectively. The corresponding activities in the cell layer also increased with incubation time but at far lower rates, so that after 24 h the activities for UDP-GalNAc 4-sulfate and chondroitin in the medium were about 12 and 3 times, respectively, the corresponding activities in the cell layer. The results suggest that terminal GalNAc 4-sulfate 6-O-sulfotransferase and GalNAc 6-O-sulfotransferase are actively synthesized and secreted by the fibroblast-like cells in culture.

In contrast to the above three enzymes, lactate dehydrogenase, a cytosol enzyme, was at a high level in the cell layer even on time 0 and the cell-associated activity decreased very slowly with a compensatory increase of the activity in the medium (Fig. 6e). As Fig. 6d shows, the rate of appearance of keratan sulfate sulfotransferase in the medium was almost the same as that of lactate dehydrogenase release. Furthermore, the activity of keratan sulfate sulfotransferase in the medium was only 10% or less of that in the cell layer even at the end of incubation. It appears, therefore, that keratan sulfate sulfotransferase is actively synthesized in the cell but, unlike the other three sulfotransferases, remains unreleased under the culture conditions.

DISCUSSION

Earlier studies showed that mammalian and avian sera were able to catalyze the transfer of sulfate from added PAPS to

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Footnote: Under the standard assay conditions, no sulfatase activity was detected in human serum with p-nitrocatechol sulfate and UDP-GalNAc-4,6-bis-[6-35S]sulfate as substrates (I. Inoue, K. Otsu, S. Suzuki, and Y. Nakanishi, unpublished observations).
exogenous chondroitin (19, 22), chondroitin sulfate (18), dermatan sulfate (18), and keratan sulfate (23), as well as endogenous chondroitin sulfate proteoglycans (22). We now show that human serum contains four distinct sulfotransferases, each having a high degree of specificity for the structure of glycosaminoglycan acceptor molecule. These include 1) a sulfotransferase sulfating position 6 of the N-acetylgalactosamine units in the interior portion of keratan (designated GalNAc 6-O-sulfotransferase), 2) a sulfotransferase introducing a second sulfate to position 6 of the nonreducing terminal N-acetylgalactosamine 4-sulfate unit of chondroitin sulfate (designated terminal GalNAc 4-sulfate 6-O-sulfotransferase), 3) a sulfotransferase sulfating position 2 (amino group) of the glucosamine units in heparan sulfate (designated GlcN 2-N-sulfotransferase), and 4) a sulfotransferase introducing sulfate to the sugar units in keratan sulfate (designated keratan sulfate sulfotransferase). The specificity of the serum GalNAc 6-O-sulfotransferase appears to be the same as that of the enzymes described in chick embryo cartilage (9) and quail oviduct (10). The pH and divalent cation optima and $K_a$ values are also similar for all three sources of GalNAc 6-O-sulfotransferase. Likewise, the specificities and properties of the other three serum sulfotransferases are similar to those found with the terminal GalNAc 4-sulfate 6-O-sulfotransferase from quail oviduct (10), the GlcN 2-N-sulfotransferase from calf arterial wall (13) and mouse mast cell tumor (12), and the Gal 6-O-sulfotransferase from bovine cornea (17), respectively. The similarity between the serum and tissue forms may reflect a relatively low species specificity of the enzymes and further suggests that the sulfotransferases found in serum are derived from denaturation during the manipulation. The results, taken together, indicate that the four enzymic activities represent different enzyme species.

The specificity of the serum GalNAc 6-O-sulfotransferase forms in human serum is not a fortuitous event due to nonspecific cell death and degeneration, but the result of an elaborate mechanism for enzyme secretion by a cell or tissue system. Studies in vivo through pulse-chase electron microscopy autoradiography with radioactive sulfate (3, 4) and studies in vitro through subcellular fractionation (1, 2) have shown that sulfation of nascent or fully elongated glycosaminoglycan chains is accomplished by membrane-bound sulfotransferases located in the Golgi apparatus. The secretory route from this area is considered to be discontinuous in that the proteoglycans must be packaged into vesicles to be conveyed to the next compartment, i.e., vesicles fusing with the plasma membrane (52). It may therefore be postulated that the membrane of the secretory granules bear on their inner surface some of the sulfotransferases. This suggests that when a vesicle bearing such enzymes fuses with the plasma membrane, the enzymes can be released, presumably in combination with sulfated proteoglycans, outside the cell. Our results obtained with mouse dermal cell cultures as a model system are consistent with this hypothesis and show that, whereas keratan sulfate transferase and lactate dehydrogenase (cytosolic marker) remain unreleased, the sulfotransferases toward UDP-GalNAc 4-sulfate, chondroitin, and heparan sulfate are released into medium at characteristic rates. The evidence presented here for the appearance of specific sulfotransferase species in sera and the results of other investigators indicating that there are glycosyltransferases appearing in sera (53) suggests the existence of a specific group of enzymes for glycoconjugate biosynthesis which are destined to be discharged into the blood stream. Further isolation and characterization of such enzymes and comparison between secretory and nonsecretory sulfotransferases should result in a better understanding of their topography and function. It is of note in this respect that all of the human serum sulfotransferases described here exhibit significant activities at the pH of the serum and that human serum contains chondroitin sulfate proteoglycans (54, 55). This suggests a role in serum-dependent sulfation, but what this is remains unknown.

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