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Measurement of Activities of Human Serum Sulfotransferases Which Transfer Sulfate to the Galactose Residues of Keratan Sulfate and to the Nonreducing End *N*-Acetylglucosamine Residues of *N*-Acetyllactosamine Trisaccharide: Comparison between Normal Controls and Patients with Macular Corneal Dystrophy[1]

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Human serum sulfotransferase activities were measured in normal controls and patients with macular corneal dystrophy (MCD), an inherited disorder characterized by the decreased sulfation of keratan sulfate in the corneal stroma and serum, using two kinds of acceptor: partially desulfated keratan sulfate and a trisaccharide with a GlcNAc residue at the nonreducing terminal, GlcNAcβ1-3Galβ1-4GlcNAc. When partially desulfated keratan sulfate was used as the acceptor, only sulfotransferase activity which transfers sulfate to position 6 of the Gal residues was detected. In contrast, when GlcNAcβ1-3Galβ1-4GlcNAc was used as the acceptor, sulfotransferase activity which transfers sulfate to position 6 of the nonreducing terminal GlcNAc residue could be detected. Although keratan sulfate levels in the sera of MCD patients determined by ELISA were much lower than those in normal controls, there were no detectable differences in either the sulfotransferase activity responsible for the sulfation of position 6 of Gal residues or that responsible for the sulfation of position 6 of nonreducing end GlcNAc residues between normal controls and MCD patients. These results suggest that the sulfotransferase involved in the sulfation of keratan sulfate, which is assumed to be deficient in MCD patients, may not be secreted into the serum, and that direct measurement of the sulfotransferase activity present in affected tissues such as the cornea instead of serum may be necessary to confirm the postulated deficiency in the biosynthesis of keratan sulfate in MCD.

Key words: ELISA, keratan sulfate, macular corneal dystrophy, serum, sulfotransferase.

Keratan sulfate (KS) proteoglycans are the major proteoglycans in the corneal stroma, and they are thought to facilitate the acquisition of transparency by the developing cornea (*1-4*) and the maintenance of the transparency of the adult cornea (*5-7*). KS is known to be based on the repeating poly-*N*-acetyllactosamine sequence of -3Galβ1-4GlcNAcβ1-, which is generally sulfated on the C-6 position of GlcNAc with additional sulfate groups potentially present on C-6 of Gal residues. The sulfation of KS is thought to be catalyzed by two different sulfotransferases; one enzyme catalyzes sulfation at position 6 of a Gal residue and the other one catalyzes sulfation at position 6 of a GlcNAc residue. We previously cloned chondroitin 6-sulfotransferase (*8*) and keratan sulfate Gal-6-sulfotransferase (*9*). Both these cloned sulfotransferases transferred sulfate to position 6 of Gal residues present in KS or partially desulfated KS but were unable to sulfate internal GlcNAc residues (*9, 10*). Sulfotransferase activity acting on the nonreducing end GlcNAc residue in glycoproteins has been reported. Microsomal preparations from rat liver (*11*) and human bronchial mucosa (*12*) were found to catalyze the sulfation of nonreducing terminal GlcNAc residues and showed no activity when the GlcNAc residue was substituted with Gal. Recently, GlcNAc 6-*O*-sulfotransferase was cloned (*13, 14*). This enzyme transferred sulfate to position 6 of the nonreducing end of the GlcNAc residue of a trisaccharide, GlcNAcβ1-3Galβ1-4GlcNAc, but showed no activity toward a tetrasaccharide, Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAc. This enzyme was shown to be involved in the biosynthesis of the sulfated sialyl *N*-acetyllactosamine

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Abbreviations: $AMan_R$, 2,5-anhydro-D-mannitol; $AMan_R(6SO_4)$, 6-*O*-sulfo-2,5-anhydro-D-mannitol; $Gal(6SO_4)$, 6-*O*-sulfo-D-galactose; $Gal_R(6SO_4)$, 6-*O*-sulfo-D-galactitol; $GlcNAc(6SO_4)$, 6-*O*-sulfo-*N*-acetyl-D-glucosamine; Gal6ST, galactose 6-*O*-sulfotransferase; GlcNAc6ST, *N*-acetyl-D-glucosamine 6-*O*-sulfotransferase; KS, keratan sulfate; KSGal6ST, keratan sulfate galactose 6-sulfotransferase; MCD, macular corneal dystrophy; Oligo A, GlcNAcβ1-3Galβ1-4GlcNAc; PAPS, adenosine 3′-phosphate 5′-phosphosulfate.

and the sulfated sialyl Lewis x structures (*13, 14*). Although, at present, it is not clear whether or not the cloned GlcNAc 6-*O*-sulfotransferase is also involved in the biosynthesis of KS, it is likely that a similar sulfation mechanism might also work in the biosynthesis of KS.

Macular corneal dystrophy (MCD) is an autosomal recessive disorder characterized by corneal opacification, and is subdivided into two basic immunophenotypes (*15-17*). This disorder begins in the first decade of life, manifesting as a bilateral central corneal stromal haze. Eventually, the haze develops into grey-white nodules within the stroma (*18*). Keratan sulfate synthesized by corneal explants from type I MCD cases was reported to be undersulfated (*19, 20*). An immunoassay for KS, which is highly specific for sulfate residues of KS (*21, 22*), showed that the cornea and serum from patients with type I MCD lacked normally sulfated KS (*23-25*). These observations suggest that either or both sulfotransferase activities that participate in the biosynthesis of KS are affected in type I MCD. To determine the nature of the metabolic defect in MCD, accurate determination of the sulfotransferase level involved in the synthesis of keratan sulfate is important.

In the present study, we attempted to measure human serum sulfotransferase activity using two acceptors; partially desulfated KS and a trisaccharide having the backbone of KS, GlcNAcβ1-3Galβ1-4GlcNAc. Sulfotransferase activity toward position 6 of Gal residues should be detected using partially desulfated KS, and sulfotransferase activity toward position 6 of nonreducing terminal GlcNAc residues seems to be detected using the trisaccharide. As a result we successfully detected both sulfotransferase activities in serum, and found that both activities in the serum of MCD patients were almost equal to those in normal serum.

MATERIALS AND METHODS

Materials—The following commercial materials were used: $H_2{}^{35}SO_4$ from Dupon/NEN; [^{3}H]$NaBH_4$ (16.3 GBq/mmol) from Amersham Japan, Tokyo. Unlabeled PAPS, Gal(6SO$_4$), and 3-*O*-sulfo glucosamine from Sigma, St. Louis, MO; Fast Desalting Column HR 10/10 and Hiload Superdex 30 16/60 from Pharmacia Biotech, Tokyo; chondroitinase ABC, *Streptococcus* β-galactosidase, biotin conjugated anti-keratan sulfate monoclonal antibody 5-D-4, and shark cartilage keratan sulfate from Seikagaku Corporation, Tokyo; Partisil 10-SAX from Whatman, Clifton, NJ; and 96 well microtiter plates (Immulon II) from Dynatech Laboratories, Alexandria, VA.

Adult human sera were prepared from fresh blood donated by 24 healthy volunteers (age 24-65, 11 males and 13 females) and 5 MCD patients (age 38-54, 2 males and 3 females). All patients had the clinical features of MCD, and the diagnosis of two of these patients was confirmed histopathologically.

KS from bovine cornea and Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAc (L1L1) were generously donated by Seikagaku Corporation. [^{35}S]PAPS was prepared as described (*26*). Partially desulfated KS (sulfate/glucosamine=0.62) was prepared from bovine corneal KS according to Nagasawa *et al.* (*27*). The oligosaccharide, GlcNAcβ1-3Galβ1-4GlcNAc (referred to as Oligo A), was prepared from oligosaccharide Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAc (L1L1) by digestion with *Streptococcus* β-galactosidase for 60 min at 37°C in 2.5 μmol of sodium acetate buffer, pH 5.5, 50 nmol of L1L1 and 10 mU enzyme, in a final volume of 50 μl (*28*). After digestion, Oligo A was purified by Superdex 30 chromatography. [^{3}H]Gal(6SO$_4$)β1-4AMan$_R$(6SO$_4$), a mixture of [^{3}H]Gal(6SO$_4$)β1-4AMan$_R$ and [^{3}H]Galβ1-4AMan$_R$-(6SO$_4$), and [^{3}H]AMan$_R$(6SO$_4$) were prepared from KS as described previously (*10, 29*). [^{3}H]Gal$_R$(6SO$_4$) was prepared from Gal(6SO$_4$) as described (*10*). [^{3}H]AMan$_R$-(3SO$_4$) was prepared from 3-*O*-sulfo glucosamine by deamination and reduction with NaB^3H_4.

Assay of Sulfotransferase Activity—The reaction mixture contained 2.5 μmol of imidazole-HCl, pH 6.8, 0.5 μmol of $MnCl_2$, 0.1 μmol 5′-AMP, 1 μmol of NaF, 25 nmol (as glucosamine for KS) of acceptor, 50 pmol of [^{35}S]PAPS (about 1×10^6 cpm), and serum, in a final volume of 50 μl. After incubation at 30°C for 5 h, the reaction was stopped by immersing the reaction tubes in a boiling water bath for 1 min. The denatured proteins formed on heating were solubilized by digestion with Pronase-P for 2 h at 37°C. The ^{35}S-labeled glycosaminoglycans, which were formed when KS or partially desulfated KS was used as the acceptor, were separated from $^{35}SO_4$ and [^{35}S]PAPS with the fast desalting column as described previously (*30*). The ^{35}S-labeled glycosaminoglycans were digested with chondroitinase ABC as described previously (*31*) to remove possible contaminating radioactive chondroitin sulfate formed from endogenous acceptors, and then the incorporation of $^{35}SO_4$ into KS or partially desulfated KS was determined. The ^{35}S-labeled oligosaccharides, which were formed when Oligo A was used as the acceptor, were separated from [^{35}S]PAPS and $^{35}SO_4$ by Superdex 30 gel chromatography, and then the radioactivity of the oligosaccharide fractions was determined.

Analysis of ^{35}S-Labeled Products—^{35}S-labeled KS isolated as above was *N*-deacetylated with 70% hydrazine containing 1% hydrazine sulfate at 96°C for 24 h (*29*). The *N*-deacetylated materials were subsequently subjected to deaminative cleavage and $NaBH_4$ reduction as described previously (*10, 29*). The degraded materials were separated by paper chromatography, together with [^{3}H]Gal(6SO$_4$)-β1-4AMan$_R$(6SO$_4$), [^{3}H]Gal(6SO$_4$)β1-4AMan$_R$, [^{3}H]Gal-β1-4AMan$_R$(6SO$_4$), and [^{3}H]AMan$_R$(6SO$_4$) as internal standards. The fractions corresponding to [^{3}H]Gal(6SO$_4$)-β1-4AMan$_R$ and [^{3}H]Galβ1-4AMan$_R$(6SO$_4$) were analyzed by Partisil 10-SAX HPLC as described below after purification by paper electrophoresis. The fraction corresponding to [^{3}H]Gal(6SO$_4$)β1-4AMan$_R$(6SO$_4$) was purified by paper electrophoresis, and then hydrolyzed with 0.1 M HCl at 100°C for 40 min. The hydrolysate was separated by paper chromatography again. The fractions corresponding to [^{3}H]Gal(6SO$_4$)β1-4AMan$_R$ and [^{3}H]Galβ1-4AMan$_R$-(6SO$_4$) formed on the partial acid hydrolysis were analyzed by Partisil 10-SAX HPLC after removal of $^{35}SO_4$ by paper electrophoresis.

The ^{35}S-labeled Oligo A was degraded through the same reaction sequence of hydrazinolysis, deaminative cleavage and $NaBH_4$ reduction as for KS, except that the reaction products obtained on hydrazinolysis were isolated with Superdex 30, followed by paper electrophoresis. The *N*-deacetylated materials were subjected to deamination and $NaBH_4$ reduction, and then separated by paper chromatography together with [^{3}H]AMan$_R$(6SO$_4$). The ^{35}S-radioac-

tivity superimposed on the ^{3}H-radioactivity of [^{3}H]AMan$_R$-(6SO$_4$) was determined as the incorporation into AMan$_R$-(6SO$_4$).

Superdex 30 Chromatography, Paper Electrophoresis, Paper Chromatography, and HPLC—The Hiload Superdex 30 16/60 column was equilibrated at 1 ml/min with 0.2 M NH_4HCO_3. Fractions of 1 ml were collected and the radioactivity was determined by liquid scintillation counting in 4 ml of Clearsol (Nacalai Tesque, Kyoto). Paper electrophoresis was carried out in pyridine/acetic acid/water (1:10:400, by volume, pH 4) at 30 V/cm for 40 min or 60 min using Whatman No. 3 strips (2.5×57 cm). For paper chromatography, samples were spotted on strips (2.5×57 cm) of Whatman No. 3 paper and developed with 1-butanol/acetic acid/1 M NH_3 (3:2:1, by volume). The dried paper strips after paper electrophoresis or paper chromatography were cut into 1.25 cm segments and then the radioactivity was determined by liquid scintillation counting. The recovery of the radioactivity after paper chromatography and paper electrophoresis was more than 80%. The Whatman Partisil 10-SAX column was equilibrated with 5 mM KH_2PO_4 at 40°C. The column was developed with 5 mM KH_2PO_4 isocratically at the flow rate of 1 ml/min.

Immunoassay for Serum Keratan Sulfate—Inhibition ELISA was performed as previously described (*32*) with minor modifications. Washing of the plates between the incubation steps was carried out with PBS containing 0.05% Tween 20 at room temperature. The plates were coated with 50 μl of 5 μg/ml purified bovine nasal cartilage proteoglycan monomer. After washing, the plates were incubated for 1 h with the diluted (1:25,000) biotin-conjugated anti-KS monoclonal antibody, 5-D-4 (*33*), which was preincubated for 1 h with an equivalent volume of serum or purified shark cartilage KS in the concentration range of 20-0.0002 μg/ml. After washing, the plates were further incubated for 30 min with avidin-labeled horseradish peroxidase. Color development was achieved using a Bio-Rad ELISA development kit and the absorbance was measured at 450 nm. The concentrations of the KS antigen present in sera were calculated by comparison with a calibration curve constructed for known concentrations of shark cartilage KS.

RESULTS

Keratan Sulfate Antigen in Serum—The levels of KS in sera from normal controls and patients with MCD were measured by the inhibition ELISA technique. In the normal controls, the level of KS was 152±48 ng/ml, whereas it was less than 3 ng/ml, which was the lower detectable limit for this assay method, in 5 patients with MCD (Table I), indicating that these patients had type I MCD. The sera of two normal controls (24-years-old male, control 1, and 30-years-old female, control 2) and two MCD patients (38-year-old male, MCD4, and 31-year-old male, MCD5) were used for the following experiments.

Gal 6-O-Sulfotransferase Activity in Serum—We determined serum sulfotransferase activity, which catalyzes the transfer of sulfate to the KS polysaccharide, using bovine corneal KS or partially desulfated KS as the sulfate acceptor.

The incorporation of $^{35}SO_4$ into KS proceeded linearly up to 5 h (Fig. 1A). The rate of incorporation was 0.55 pmol/h/mg serum protein for control 1, 0.50 pmol/h/mg serum protein for MCD5, and 0.49 pmol/h/mg serum protein for MCD4. The observation that the rate of sulfation of KS by MCD serum was nearly the same as that of sulfation by normal serum seems to agree with the previous observation of Hassell and Klintworth (*34*).

To determine the position to which sulfate was transferred, we degraded the ^{35}S-labeled product, which was formed by incubating the partially desulfated KS with [^{35}S]PAPS and serum, through the reaction sequence of *N*-deacetylation, deaminative cleavage, and $NaBH_4$ reduction. When the degraded materials were separated by paper chromatography, three peaks were obtained (Fig. 2). Peak b migrated to the same position as a mixture of [^{3}H]-Galβ1-4AMan$_R$(6SO$_4$) and [^{3}H]Gal(6SO$_4$)β1-4AMan$_R$ (monosulfated disaccharide fraction), and peak a migrated to the same position as [^{3}H]Gal(6SO$_4$)β1-4AMan$_R$(6SO$_4$) (disulfated disaccharide fraction). The radioactive materials remaining at the paper origin seemed to represent undegraded materials and were not examined further. Since standard AMan$_R$(6SO$_4$) migrated faster than peak b, AMan$_R$(6SO$_4$), if present, could be removed from both peak a and peak b.

The monosulfated disaccharide fraction (peak b in Fig. 2) was analyzed by SAX-HPLC (Fig. 3, A and B). The

TABLE I. **Serum keratan sulfate levels in normal controls and patients with MCD.** The level of KS was determined by ELISA using monoclonal antibody 5-D-4 as described under "MATERIALS AND METHODS." The values for normal controls are averages±SD. The number of samples is indicated in parentheses.

Subject group	Keratan sulfate level[a] (ng/ml)	Age
Normal controls	152±48 (24)	40.1±10.6
MCD patients		
MCD1	<3	40
MCD2	<3	49
MCD3	<3	54
MCD4	<3	38
MCD5	<3	31
Average	<3	46.2±12.4

[a]The lowest of KS level detectable with this method was 3 ng/ml.

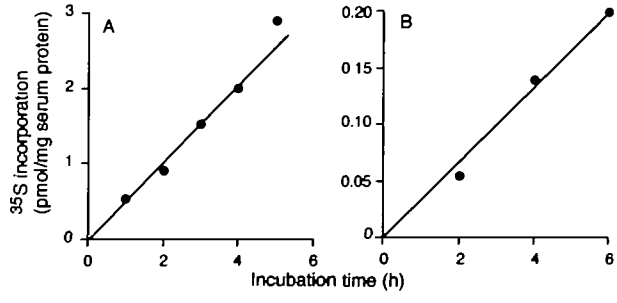

Fig. 1. **Time courses of incorporation of $^{35}SO_4$ into keratan sulfate (A) and Oligo A (B).** The incorporation of $^{35}SO_4$ into keratan sulfate and Oligo A was determined as described under "MATERIALS AND METHODS" using 400 μg protein of control 1 serum.

radioactive materials appeared as a single peak at the retention time of [^{3}H]Gal(6SO$_4$)β1-4AMan$_R$. On the other hand, no peak of ^{35}S-radioactivity was detected at the retention time of [^{3}H]Galβ1-4AMan$_R$(6SO$_4$). To determine which sulfate of Gal(6SO$_4$)β1-4AMan$_R$(6SO$_4$) carried the ^{35}S-radioactivity, [^{35}S]Gal(6SO$_4$)β1-4AMan$_R$(6SO$_4$) (peak a in Fig. 2) was subjected to partial acid hydrolysis, and then the hydrolysate was separated by paper chromatography and paper electrophoresis. The ^{35}S-labeled materials that migrated to the position of Galβ1-4AMan$_R$(6SO$_4$) on both paper chromatography and paper electrophoresis were analyzed by SAX-HPLC after reduction with NaBH$_4$ (Fig. 3, C and D). The major ^{35}S-radioactivity was observed at the position of Gal(6SO$_4$)β1-4AMan$_R$, a small amount of radioactivity being detected at the position of Gal$_R$(6SO$_4$). These observations indicated that the partially desulfated KS was only sulfated at position 6 of the Gal residue of the Galβ1-4GlcNAc or Galβ1-4GlcNAc(6SO$_4$) unit by both normal and MCD serum, and that the sulfotransferase activity responsible for the sulfation of GlcNAc residues could not be detected even if the partially desulfated KS was used as the acceptor.

GlcNAc 6-O-Sulfotransferase Activity in Serum—We previously detected GlcNAc 6-*O*-sulfotransferase activity which transfers sulfate to position 6 of the nonreducing end of GlcNAc using a trisaccharide, GlcNAcβ1-3Galβ1-4GlcNAc (referred to as Oligo A), as the acceptor (*13*). To investigate the possibility that the level of GlcNAc 6-*O*-sulfotransferase activity may be lower in MCD serum, we determined the sulfotransferase activity in serum using Oligo A as the acceptor. Figure 4 shows the elution profiles on Superdex 30 chromatography of the sulfated Oligo A formed on the incubation with control serum and MCD serum. The sulfated product was eluted at 87 min, which was about 2 min earlier than the elution time of Oligo A used as the acceptor. In a study on the sulfation of sialyl *N*-acetyllactosamine oligosaccharides, we previously observed that the retention time of monosulfated sialyl *N*-acetyllactosamine trisaccharide on Superdex 30 chromatography was 2 min earlier than that of sialyl *N*-acetyllactosamine trisaccharide (*35*). The observed retention time of the sulfated Oligo A thus seems to coincide with that of monosulfated Oligo A. This assumption was confirmed by the mobility of the sulfated product on paper electrophoresis; the sulfated products migrated near Galβ1-4GlcNAc(6SO$_4$) (data not shown).

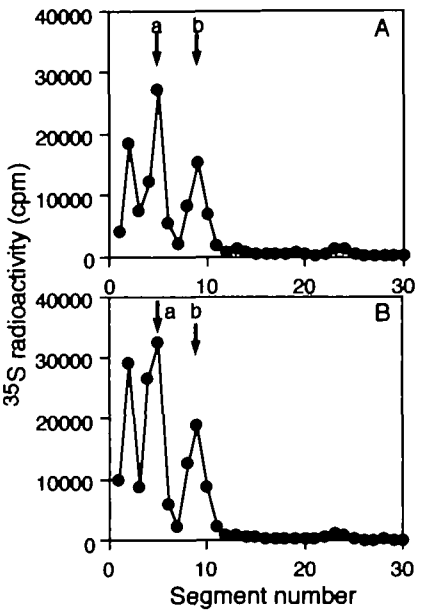

Fig. 2. **Paper chromatographic separation of the degradation products formed through the reaction sequence of hydrazinolysis, deaminative cleavage, and NaBH$_4$ reduction from ^{35}S-labeled partially desulfated keratan sulfate.** Partially desulfated KS was labeled by incubation with [^{35}S]PAPS and control serum (A) or MCD serum (B). Arrow a indicates the migration position of [^{3}H]Gal(6SO$_4$)-AMan$_R$(6SO$_4$), and arrow b that of the mixture of [^{3}H]Gal-AMan$_R$(6SO$_4$) and [^{3}H]Gal(6SO$_4$)-AMan$_R$, respectively. The peak fractions of disulfated disaccharides (indicated by arrow a) and monosulfated disaccharides (indicated by arrow b) were pooled and purified by paper electrophoresis for further analysis.

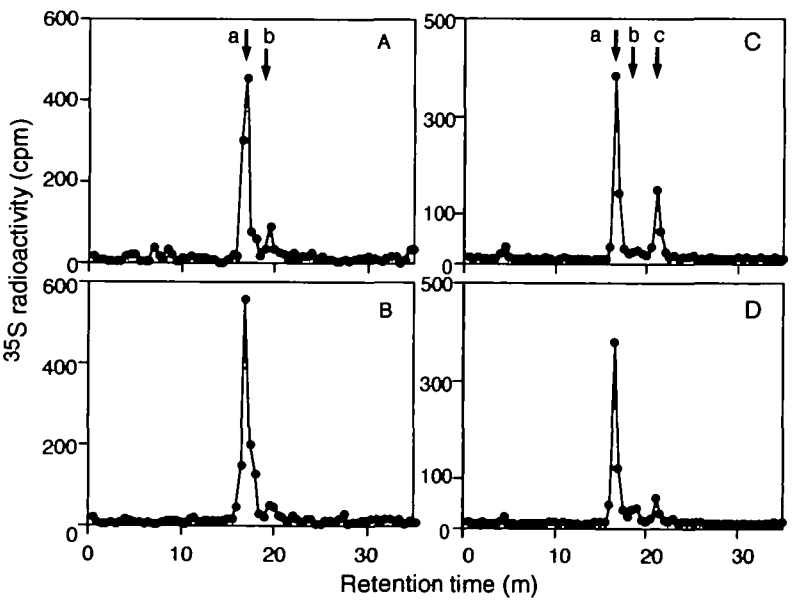

Fig. 3. **HPLC separation of the monosulfated disaccharide fraction derived from ^{35}S-labeled partially desulfated keratan sulfate.** ^{35}S-labeled partially desulfated KS was formed by incubation of control serum (A and C), or MCD serum (B and D). A, monosulfated disaccharide fraction from peak b in Fig. 2A; B, monosulfated disaccharide fraction from peak b in Fig. 2B; C, monosulfated disaccharide fraction obtained on partial acid hydrolysis of peak a in Fig. 2A; and D, monosulfated disaccharide fraction obtained on partial acid hydrolysis from peak a in Fig. 2B. The partial acid hydrolysis and separation of the hydrolysates by paper chromatography and paper electrophoresis were carried out as described under "MATERIALS AND METHODS." Arrows a, b, and c indicate the retention times of [^{3}H]Gal(6SO$_4$)-AMan$_R$, [^{3}H]-Gal-AMan$_R$(6SO$_4$), and [^{3}H]Gal$_R$(6SO$_4$), respectively.

The incorporation of sulfate into Oligo A proceeded linearly up to 6 h under the assay conditions used (Fig. 1B). The rate of incorporation when sera from normal controls were used was nearly the same as that when sera from MCD patients were used (Fig. 5).

We investigated whether or not sulfate was transferred to position 6 of the non-reducing terminal GlcNAc residue. The strategy for the structural analysis of the sulfated product is shown in Fig. 6. When sulfate is transferred to the nonreducing terminal GlcNAc residue, radioactive $AMan_R(6SO_4)$ should be recovered after the reaction sequence of hydrazinolysis, deaminative cleavage, and $NaBH_4$ reduction. However, we previously observed that the deacetylation of the GlcNAc residue at the reducing end of sulfated *N*-acetyllactosamine with hydrazine/hydrazine sulfate did not proceed quantitatively; about half of the reducing end GlcNAc residue was transformed to an unidentified byproduct without a positive charge derived from a free amino group (*35*). It is, therefore, expected that the hydrazinolysis of sulfated Oligo A should result in the formation of at least two deacetylated products with one or two free amino groups. When the sulfated Oligo A was subjected to paper electrophoresis after hydrazinolysis, two major peaks and one minor peak were actually obtained (Fig. 7). From its mobility, peak 1 appears to be a product with two free amino groups at both the reducing and nonreducing ends, and peak 2 a product with a free amino group at only the non-reducing end. Peak 3 seems to be unreacted materials, judging from its mobility on paper electrophoresis, and was not examined further. Peaks 1 and 2 were recovered and subjected to paper chromatography after deaminative cleavage and $NaBH_4$ reduction (Fig. 8). For both peaks 1 and 2 in Fig. 7, two peaks were obtained; one peak migrated to around the position of a mixture of $[^3H]Gal(6SO_4)\beta1\text{-}4AMan_R$ and $[^3H]Gal\beta1\text{-}4AMan_R(6SO_4)$, and the other peak migrated to the position of $[^3H]AMan_R\text{-}(6SO_4)$. In our previous study on GlcNAc 6-*O*-sulfotransferase, we observed that, under the hydrazinolysis conditions used here, almost all the ^{35}S-radioactivity transferred to Oligo A was cleaved from the sulfated Oligo A and

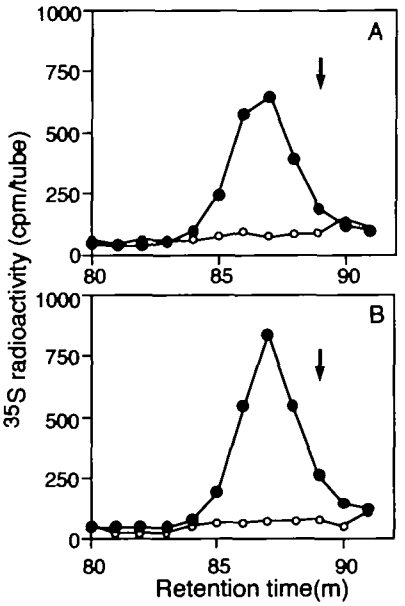

Fig. 4. **Superdex 30 gel chromatography of the ^{35}S-labeled products formed from Oligo A on incubation with [^{35}S]PAPS and human serum.** The sulfotransferase reactions were carried out in the presence (●) or absence (○) of Oligo A using control serum (A) or MCD serum (B). Arrows indicate the retention time of Oligo A. The peak fractions (85 to 90 min) were collected and purified by paper electrophoresis for further analysis.

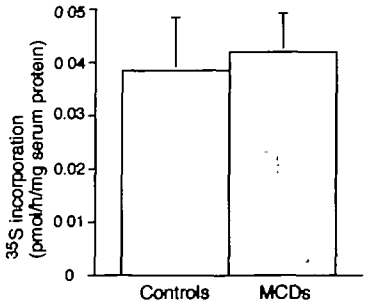

Fig. 5. **^{35}S incorporation into Oligo A on incubation with control serum and MCD serum.** The incorporation into Oligo A was calculated from the radioactivity of the fractions (85 to 90 min) obtained on Superdex 30 chromatography in Fig. 4. The radioactivity observed in the absence of Oligo A was subtracted. The open bar represents the average ± range for sera obtained from two normal persons, and the stippled bar the average ± range for sera obtained from two MCD patients.

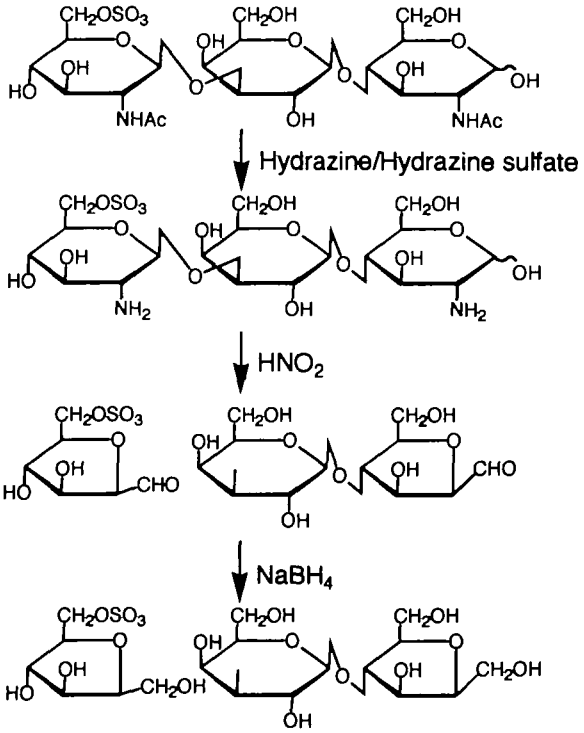

Fig. 6. **The strategy for structural analysis of sulfated Oligo A.** When $^{35}SO_4$ is transferred to position 6 of the nonreducing end GlcNAc residue from [^{35}S]PAPS, [^{35}S]6-*O*-sulfo-2,5-anhydromannitol [$AMan_R(6SO_4)$] should be recovered after the reaction sequence of hydrazinolysis, deaminative cleavage, and $NaBH_4$ reduction. *N*-Deacetylation of the GlcNAc residue at the reducing end, however, did not proceed quantitatively, and two reaction products were observed after separation by paper electrophoresis, as shown in Fig. 7.

recovered as $AMan_R(6SO_4)$, and that a tetrasaccharide, Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAc, could not serve as an acceptor for GlcNAc 6-*O*-sulfotransferase (*13*). These observations suggest that nearly quantitative removal of the nonreducing end GlcNAc residue bearing 6-*O*-sulfate could be achieved under the hydrazinolysis conditions, and that the reducing end GlcNAc residue in Oligo A is unlikely to be sulfated by GlcNAc 6-*O*-sulfotransferase. Taken together, the slower migrating materials appeared to be disaccharide derivatives bearing sulfate on Gal residues, which was possibly introduced by the Gal 6-*O*-sulfotransferase described above. Although a sulfotransferase capable of sulfating reducing end GlcNAc residues has not been found, the possibility that the slower migrating materials might be disaccharide derivatives bearing sulfate on reducing end GlcNAc residues could not be excluded. We did not examine the slower migrating materials further. In a separate series of experiments involving chick embryo cornea, we confirmed that the material that migrated to the position of $[^3H]AMan_R(6SO_4)$ on paper chromatography behaved identically with $[^3H]AMan_R(6SO_4)$ on both paper electrophoresis and SAX-HPLC, and that the material was clearly separated from $[^3H]AMan_R(3SO_4)$ on SAX-HPLC, as described previously (*13*) (data not shown). These observations indicated that a significant part of Oligo A was sulfated at position 6 of the nonreducing terminal GlcNAc residue. As shown in Fig. 8, the proportion of $AMan_R(6SO_4)$ formed from peak 1 in Fig. 7 was almost the same as that from peak 2. The proportion of 6-*O*-sulfation at nonreducing GlcNAc residues as to the total sulfation of Oligo A was thus calculated from the mean values for peaks 1 and 2, and was on average 14.3% for controls and 13.8% for MCDs. From the incorporation into Oligo A shown in Fig. 5 and the proportion of 6-*O*-sulfation at nonreducing GlcNAc residues, the incorporation of $^{35}SO_4$ at position 6 of nonreducing GlcNAc was calculated to be 5.5 ± 0.6 fmol/mg serum protein for the controls and 5.9 ± 1.3 fmol/mg serum protein for MCDs. These results indicate again that no significant difference was observed between normals and MCDs.

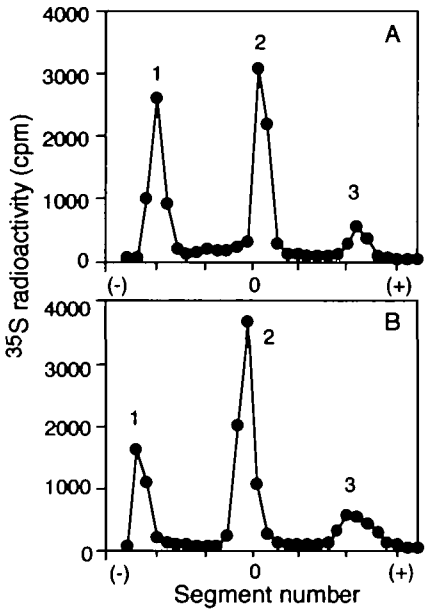

Fig. 7. **Paper electrophoresis of the products formed on hydrazinolysis from ^{35}S-labeled Oligo A.** ^{35}S-labeled Oligo A formed on incubation with control (A) or MCD (B) serum was subjected to hydrazinolysis as described under "MATERIALS AND METHODS," and the products were separated by paper chromatography after purification by Superdex 30 chromatography. Peak 1 appears to be a product with two free amino groups at both the reducing and nonreducing ends, and peak 2 to be a product with a free amino group only at the non-reducing end. Peak 3 seems to be unreacted materials. Peaks 1 and 2 were recovered and subjected to the reaction sequence of deamination and $NaBH_4$ reduction.

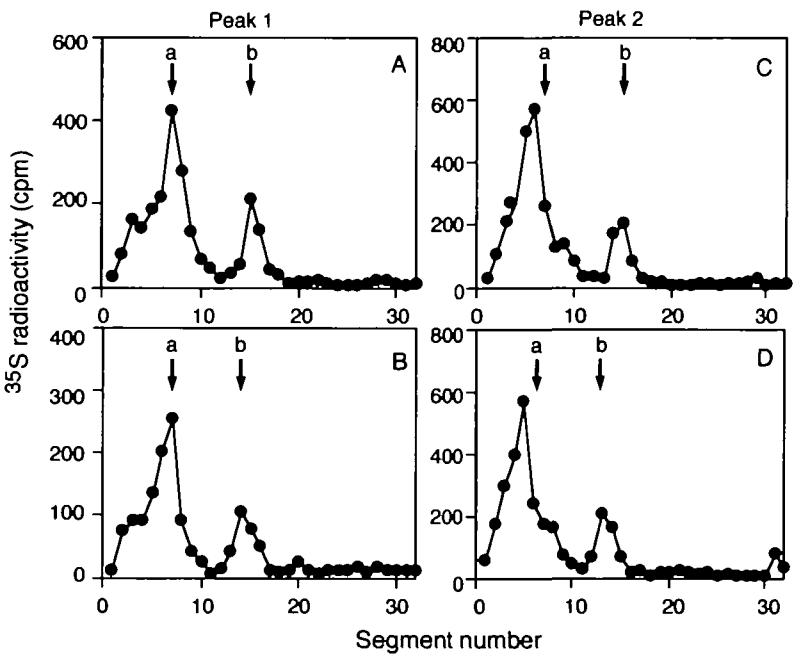

Fig. 8. **Paper chromatography of ^{35}S-labeled materials formed from ^{35}S-labeled Oligo A through hydrazinolysis, deaminative cleavage, and $NaBH_4$ reduction.** ^{35}S-labeled Oligo A was prepared by incubation with control serum (A and C) or MCD serum (B and D), and then subjected to hydrazinolysis. After separation of the products of hydrazinolysis by paper electrophoresis, as shown in Fig. 7, peaks 1 and 2 in Fig. 7 were treated with HNO_2 and then reduced with $NaBH_4$. A, peak 1 in Fig. 7A; B, peak 1 in Fig. 7B; C, peak 2 in Fig. 7A; and D, peak 2 in Fig. 7B. Arrow a indicates the migration position of a mixture of $[^3H]Gal\text{-}AMan_R(6SO_4)$ and $[^3H]Gal\text{-}(6SO_4)\text{-}AMan_R$, and arrow b that of $[^3H]AMan_R\text{-}(6SO_4)$..

DISCUSSION

In this paper we showed that human serum contains two sulfotransferase activities; one is the activity responsible for the sulfation of position 6 of internal Gal residues (referred to as Gal6ST activity), and the other is that responsible for the sulfation of position 6 of nonreducing end GlcNAc residues (referred to as GlcNAc6ST activity). Sulfotransferase activity which is capable of transferring sulfate to KS *in vitro* has been evaluated in various animal sera with KS or partially desulfated KS as the sulfate acceptor (*36-38*); nevertheless, it is not clear which activity was evaluated. Our present data revealed that only Gal6ST activity could be detected when KS or partially desulfated KS was used as the acceptor. Consequently, polysaccharide acceptors appeared not to be suitable for the detection of GlcNAc6ST activity.

We used a trisaccharide, GlcNAcβ1-3Galβ1-4GlcNAc (Oligo A), instead of KS as the acceptor to detect the sulfotransferase activity acting on the nonreducing end GlcNAc residue. Sulfated products were obtained by gel filtration after incubating Oligo A with [^{35}S]PAPS and serum, and then cleaved through the reaction sequence of hydrazinolysis/deaminative cleavage/$NaBH_4$ reduction to yield $AMan_R(6SO_4)$ from the nonreducing terminal GlcNAc($6SO_4$) residue. Thus, the ^{35}S-radioactivity of $AMan_R(6SO_4)$ should represent the amount of $^{35}SO_4$ incorporated into the nonreducing terminal GlcNAc. Actually we could detect sulfotransferase activity toward nonreducing terminal GlcNAc residues. However, at present, it is not evident whether the sulfotransferase activity toward the nonreducing end GlcNAc is involved in the sulfation of KS or in the biosynthesis of sulfated glycoproteins.

The GlcNAc residues in KS from bovine cornea were reported to be fully 6-*O* sulfated (*29, 39*), suggesting that the 6-*O* sulfation of GlcNAc residues may be tightly coupled with the elongation of sugar chains. On the assumption that GlcNAc6ST transfers sulfate exclusively to the nonreducing terminal GlcNAc residues, 6-*O* sulfation of GlcNAc residues should precede the transfer of Gal to GlcNAc. Funderburgh *et al.* reported that partially sulfated KS was synthesized by cultured keratocytes (*40*). GlcNAc6ST activity might be decreased in cultured keratocytes, resulting in the formation of partially sulfated KS through the transfer of Gal residues before the sulfation of nonreducing terminal GlcNAc residues.

On the basis of the results of histochemical and biochemical analysis of the cornea, serum, and cartilage, MCD has been believed to include a synthetic disorder of KS, especially in the sulfation process. The serum KS levels are extremely low, as judged on immunological quantification of KS using 5-D-4 monoclonal antibody. These observations also indicate an apparent defect in the sulfation of KS in MCD, because 5-D-4 monoclonal antibody was reported to recognize sulfated large oligo- or polysaccharides rather than nonsulfated lactosaminoglycans or short sulfated oligosaccharides (*21, 22*). It has thus been expected that the reduced synthesis of KS observed in MCD patients may be caused by a deficiency of sulfotransferases responsible for the synthesis of KS.

Hassell and Kintworth reported that sera from MCD patients contained the same levels of KS sulfotransferase activity as found in controls (*34*); however, they did not identify the actual sulfation site on KS. In the present study, we demonstrated that KS is sulfated exclusively at position 6 of Gal residues. Taking these results into account, the ability of 6-*O* sulfation of Gal residues was assumed to be equal in normal controls and MCD patients. In addition, our experiments showed that GlcNAc6ST activity in the sera of normal controls and MCD patients was also nearly equal. Accordingly, the sera of the MCD patients contained normal levels of both Gal6ST and GlcNAc6ST activity. There are several possible explanations for these observations contrary to expectation. (a) We have reported that the sulfation of Gal residues in KS is catalyzed by two sulfotransferases; chondroitin 6-sulfotransferase (C6ST) and keratan sulfate Gal-6-sulfotransferase (KSGal6ST) (*8-10*). Inoue *et al.* (*36*) and Sugahara *et al.* (*38*) reported that C6ST activity was present in human serum. It is therefore possible that the serum sulfotransferase activity which transfers sulfate to keratan sulfate *in vitro* may be due mainly to C6ST, and that KSGal6ST activity may be masked by the excess C6ST activity. It also seems possible that, unlike C6ST, KSGal6ST might not be secreted into the serum. (b) There may be multiple isozymes of GlcNAc6ST responsible for the synthesis of KS, and most of the activity we detected in the serum may reflect the activity derived from tissues other than cornea such as cartilage. (c) The GlcNAc6ST responsible for the sulfation of KS may be different from the enzyme responsible for the sulfation of glycoprotein oligosaccharides, and the activity we detected in the serum may be mostly for the synthesis of glycoprotein. It is possible that the GlcNAc6ST activity for the glycoprotein synthesis may mask the GlcNAc6ST activity responsible for the synthesis of KS, that might be decreased or completely missing in MCD serum. (d) The GlcNAc6ST responsible for the synthesis of KS might not be secreted into the serum from the tissues in which KS is actively produced. Considering these points, direct measurement of sulfotransferase activity in extracts of the cornea as well as determination of the expression of sulfotransferase genes in the cornea are required for characterization of the disorder of KS biosynthesis in MCD patients. The analytical methods developed for the determination of Gal6ST and GlcNAc6ST activities in serum in this study will provide a useful approach for determination of these activities in the cornea.

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