The physiological role of sulfatases in mammalian tissues has long been a subject for speculation (for a review see Ref. 1). In recent years, investigation into the enzymatic defects of mucopolysaccharidoses has been accompanied by a number of important observations on the role of sulfatases in the metabolism of sulfated glycosaminoglycans. Thus, the enzymatic deficiencies that have been identified are glucosamine-N-SO_3 sulfatase relating to a novel mucopolysaccharidosis characterized by inadequate catabolism of keratan sulfate and heparan sulfate. It has also been demonstrated that 6-SO_3 hydrolysis by these enzymes occurs only on nonreducing residues (16). Much of the information regarding these sulfatases is obtained using cultured human skin fibroblasts as enzyme source, suggesting that many different types of sulfatase necessary for glycosaminoglycan metabolism reside in normal skin. However, as yet few of the sulfatases have been obtained in a purified state and much further work is required before the specificities of the sulfatases are understood and their separate identities made certain. We report here the results of our systematic study on the desulfation of chondroitin 4/6-sulfate, dermatan sulfate, heparan sulfate, heparin, and keratan sulfate by extracts of rat skin, and the separation and characterization of the enzymes responsible for these reactions.

**EXPERIMENTAL PROCEDURES**

**Materials**

Newborn rats (Moriyama strain), weighing 8 to 9 g, were purchased from the Moriyama-so, Nagoya. Chemicals and enzymes were obtained from the following sources: potassium p-nitrophenyl sulfate, Nakarai Kagaku Co., Kyoto; potassium 4-nitrocatechol sulfate, p-nitrophenyl β-D-glucuronide, p-nitrophenyl N-acetyl-β-D-glucosaminide, N-p-nitrophenyl β-D-galactoside, p-nitrophenyl N-acetyl-β-D-galactosaminide, and bovine liver β-glucuronidase, Sigma; chymotrypsinogen, hen's egg albumin, bovine serum albumin, and aldolase (molecular weight standards), Boehringer Mannheim, West Germany; testicular hyaluronidase, Worthington; H_3^32S0_3 (carrier-free), Radio-Isope Association, Tokyo; [N-[15S]SO_3]heparin (8.4 × 10^6 cpm/μmol of hexuronate), Calaton, Los Angeles; Sephadex G-200 and Sephadex G-50 superfine, Pharmacia; Bio-Gel P-2, Bio-Rad Lab; DEAE-cellulose, Brown Co., Diaflo PM-10 membrane, Amicon; chondroitinase-AC, chondroitinase-ABC, and the reference unsaturated disaccharides AGlcUA-GalNAc-(4-SO_4) and AGlcUA-GalNAc(G-SO_4) Sei-kagaku Kogyo Co., Tokyo; pronase from Kaken Kagaku Co., Tokyo. Generous gifts of the following materials are acknowledged: phenyl α-D-iduronide, Dr. H. Kuchida, Kyoto General Medicalochemical Laboratory; the tetrasaccharide disulfate and hexasaccharide trisulfate

**REFERENCES**

1. The abbreviations used are: GalNAc-4-SO_3, GalNAc-6-SO_3, and GalNAc-4,6-bis-SO_3, N-acetylgalactosamine 4-sulfate, 6-sulfate, and 4,6-bissulfate, respectively; GlcUA-GalNAc-(4-SO_4) and GlcUA-GalNAc(G-SO_4) Sei-kagaku Kogyo Co., Tokyo; pronase from Kaken Kagaku Co., Tokyo.

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5. The abbreviations used are: GalNAc-4-SO_3, GalNAc-6-SO_3, and GalNAc-4,6-bis-SO_3, N-acetylgalactosamine 4-sulfate, 6-sulfate, and 4,6-bissulfate, respectively; GlcUA-GalNAc-(4-SO_4) and GlcUA-GalNAc(G-SO_4) Sei-kagaku Kogyo Co., Tokyo; pronase from Kaken Kagaku Co., Tokyo.

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8. The abbreviations used are: GalNAc-4-SO_3, GalNAc-6-SO_3, and GalNAc-4,6-bis-SO_3, N-acetylgalactosamine 4-sulfate, 6-sulfate, and 4,6-bissulfate, respectively; GlcUA-GalNAc-(4-SO_4) and GlcUA-GalNAc(G-SO_4) Sei-kagaku Kogyo Co., Tokyo; pronase from Kaken Kagaku Co., Tokyo.

9. The abbreviations used are: GalNAc-4-SO_3, GalNAc-6-SO_3, and GalNAc-4,6-bis-SO_3, N-acetylgalactosamine 4-sulfate, 6-sulfate, and 4,6-bissulfate, respectively; GlcUA-GalNAc-(4-SO_4) and GlcUA-GalNAc(G-SO_4) Sei-kagaku Kogyo Co., Tokyo; pronase from Kaken Kagaku Co., Tokyo.

10. The abbreviations used are: GalNAc-4-SO_3, GalNAc-6-SO_3, and GalNAc-4,6-bis-SO_3, N-acetylgalactosamine 4-sulfate, 6-sulfate, and 4,6-bissulfate, respectively; GlcUA-GalNAc-(4-SO_4) and GlcUA-GalNAc(G-SO_4) Sei-kagaku Kogyo Co., Tokyo; pronase from Kaken Kagaku Co., Tokyo.
preparation from chondroitin 4/6-sulfate by digestion with testicular hyaluronidase, Dr. K. Kimata in this laboratory; and Pseudomonas endo-β-d-galactosidase and the tetrasaccharide trisulfate prepared from corneal keratan sulfate, Dr. K Nakazawa, Meijo University, Nagoya.

UDP-GalNAc-4-[35S]SO₄ (2 x 10⁷ cpm/μmol) and GalNAc-4-[35S]SO₄ (2 x 10⁷ cpm/μmol) were prepared as previously described (17).

Chondroitin 4-[35S]sulfate (1.9 x 10⁷ cpm/μmol of hexuronic acid) was prepared from newborn rat epiphyseal cartilages. Cartilage slices (300 mg) were suspended in 3 ml of Krebs-Ringer medium to which 300 units of penicillin and 120 μCi of H₂¹⁴SO₄ (carrier-free) was added. After 6 h of incubation at 37°C, the tissue slices were triced with 5 mg of pronase for 24 h at 50°C. A 5% concentration of trichloroacetic acid was made and the solution was centrifuged. The supernatant fluid after dialysis against water was mixed with potassium acetate (1%), and ethanol (2 volumes). The precipitate was added. After 6 h of incubation at 37°C, the tissue slices were triced with 0.24 mg of /3-glucuronidase for 16 h. The reaction was stopped by ethanol precipitation, and was treated further with 0.15 M potassium acetate (1%) and ethanol (2 volumes). The total radioactivity of the sample was 1.6 x 10⁷ cpm. When examined by digestion with chondroitinase-AC (18), the sample gave 93.3% converted into ΔGalUA-GalNAc-4-[35S]SO₄, 1.2% into ΔGalUA-GalNAc-6-[35S]SO₄, 1.0% into ΔGalUA-GalNAc-4,6-bis-[35S]SO₄, and 1.5% into unidentified compounds. Three per cent of the radioactive material remained undigestible. Chondroitin 4/6-[35S]sulfate (1.9 x 10⁷ cpm/μmol) was labeled with 35S-SO₄⁻ by paper chromatography in Solvent A.

The preparation was digested with chondroitinase-AC, and it gave 47.8% converted into ΔGalUA-GalNAc-4-[35S]SO₄, 45.9% into ΔGalUA-GalNAc-6-[35S]SO₄, 11.0% into ΔGalUA-GalNAc-4,6-bis-[35S]SO₄, and 2.2% into unidentified compounds. Three per cent of the radioactive material remained undigestible.

Heparan [35S]sulfate (9.0 x 10⁶ cpm/μmol of hexuronic acid) and dermanan [35S]sulfate (1.9 x 10⁷ cpm/μmol of hexuronic acid) were prepared from the crude glycosaminoglycan sample thus obtained, as described above. The crude glycosaminoglycan sample thus obtained was treated with 0.4 mg of /3-glucuronidase for 16 h. The reaction was stopped by ethanol precipitation, and was treated further with 0.24 mg of β-glucuronidase for 16 h. The reaction was stopped by ethanol precipitation, and the mixture was centrifuged at 10,000 x g for 15 min. The mixture was centrifuged at 800 x g for 15 min. The pellet (P₁) was retreated with the homogenizer and the mixture was centrifuged at 10,000 x g for 15 min. The supernatant fluid was centrifuged at 120,000 x g for 15 min to yield supernatant "S," and pellet "P₁." The pellet, (equivalent to about 36 mg of Lowry protein), was washed with 20 ml of buffer A containing 20 mM NaCl in 10% ethanol as eluant. Fractions corresponding in position to tetra-, hexa-, octa-, and deca saccharide (Kₑₑ = 0.77, 0.67, 0.58, and 0.50, respectively) were separately collected, concentrated, and desalted by gel filtration on Bio-Gel P-2. A portion (1.5 x 10⁶ cpm) of each oligosaccharide fraction was further treated with 0.4 mg of β-glucuronidase for 16 h. The reaction was stopped by ethanol precipitation, and the mixture was centrifuged at 10,000 x g, and the supernatant fluid was mixed with potassium acetate (1%) and ethanol (2 volumes). The total radioactivity of the sample was 1.6 x 10⁷ cpm. When examined by digestion with chondroitinase-AC, the sample gave 93.3% converted into ΔGalUA-GalNAc-4-[35S]SO₄, 1.2% into ΔGalUA-GalNAc-6-[35S]SO₄, 1.0% into ΔGalUA-GalNAc-4,6-bis-[35S]SO₄, and 1.5% into unidentified compounds. Three per cent of the radioactive material remained undigestible.

Heparan [35S]sulfate and heparan [35S]sulfate were separated from each other by the method of Cifonelli et al. (20) using CuSO₄; yield of dermanan [35S]sulfate, 1.66 x 10⁶ cpm. Heparan [35S]sulfate was further purified by treatment with chondroitinase-ABC (to remove dermanan sulfates) followed by ethanol precipitation (19); yield, 1.74 x 10⁶ cpm. When the dermanan [35S]sulfate preparation was digested with chondroitinase-ABC, it gave 82.7% converted into ΔGalUA-GalNAc-4-[35S]SO₄, 3.9% into ΔGalUA-GalNAc-6-[35S]SO₄, 3.3% into ΔGalUA-GalNAc-4,6-bis-[35S]SO₄, 1.4% into ΔGalUA-GalNAc-4,6,8-tris-[35S]SO₄, and 0.9% into unidentified products; 4.6% of the radioactive material remained undigestible. When the heparan [35S]sulfate preparation was treated with 0.24 μM of NaNO₂ in 1.5 M acetic acid for 80 min (21), all the radioactivity was released as small fragments, as judged by gel filtration on Sephadex G-50.

A portion (2.6 x 10⁶ cpm) of the dermanan [35S]sulfate sample was treated successively with testicular hyaluronidase (120 IU) and β-glucuronidase (2 mg), according to the method of Fransson and Roden (22), and the degraded polysaccharide was obtained by gel filtration on Sephadex G-50. This material will be referred to as "hyaluronic acid/β-glucuronidase digests." The hyaluronic acid/β-glucuronidase digests (for the characterization of the reference compounds) were separated by gel filtration on Sephadex G-50.

IdUA(2-[35S]SO₄)-Anhydromann(6-[35S]SO₄) was prepared from rat lung [35S]heparin (containing heparan [35S]sulfate) essentially as described by Jansson et al. (28). Rat lung slices (2.1 g) were suspended in 20 ml of Krebs-Ringer medium containing 2,000 units of penicillin and 0.1 mg of β-glucuronidase (2 mg). The tissue slices were treated with 20 mg of pronase for 16 h at 50°C, and crude glycosaminoglycans were collected by ethanol precipitation, as described above. A mixture of [35S]heparin and heparan [35S]sulfate was obtained from the crude glycosaminoglycan preparation by degrading chondroitin sulfate and heparan sulfate (26). GaNAc-6-[35S]SO₄ was prepared from GI-2C-GalNAc-6-[35S]SO₄ by digestion with Flavobacterium glucuronidase (26, 27).

IdUA(2-[35S]SO₄)-Anhydromann(6-[35S]SO₄) was isolated from this mixture by gel filtration on Sephadex G-50, followed by paper electrophoresis for 60 min, as recommended by Jansson et al. (28). From 0.67 x 10⁶ cpm of [35S]heparin-heparan [35S]sulfate, 7.0 x 10⁶ cpm of [35S]heparin-heparan [35S]sulfate, 7.0 x 10⁶ cpm of IdUA(2-[35S]SO₄)-Anhydromann(6-[35S]SO₄) was obtained. IdUA(2-[35S]SO₄)-Anhydromann(6-[35S]SO₄) was prepared from a commercial heparin sample in a similar way.

Preparation of Crude Enzymes from Rat Skin—The fractionation scheme is shown in Scheme 1. Fresh skins, obtained from 10 newborn rats, were placed in ice-cold 0.5% NaCl freed from adhering tissues, and cut into small pieces (about 5 x 5 mm). All subsequent procedures were carried out at 0-4°C. Skin pieces, 10 g (wet weight), were homogenized in 50 ml of 0.02 M Tris-HCl pH 7.2, containing 0.25 M sucrose (Buffer A) and incubated with 0.25 M sucrose (Buffer A) and incubated with 20 mM of Buffer A + 1 mM of Polytron (Kinetamix CM 280; Luer-Schweiz) and the homogenate was centrifuged at 800 x g for 15 min. The pellet (P₂) was reconstituted with the homogenizer, and the mixture was centrifuged at 800 x g. The two supernatants obtained by Polytron homogenization were combined and centrifuged at 10,000 x g for 15 min to yield superenatant "S," and pellet "P." The pellet (equivalent to about 30 mg of Lowry protein), 1 mg of Buffer A + 1 mM of Polytron (Kinetamix CM 280; Luer-Schweiz) and the homogenate was centrifuged at 800 x g for 15 min. The supernatant fluid was centrifuged at 10,000 x g for 15 min and the super-
phenyl a-L-iduronide as substrate. The incubation mixture contained 0.2 μmol of substrate, 2.5 μmol of sodium acetate/acetic acid buffer, pH 5.0, 7.5 μg of bovine serum albumin, and enzyme in a final volume of 50 μl. After incubation for 1 h at 37°C, the reaction was stopped by addition of 0.5 ml of 0.1 M NaHCO3 (when the p-nitrophenyl glycosides were used) or Folin-Ciocalteu phenol reagent (30) (when the phenyl glycoside was used) and precipitated protein was removed by centrifugation. The liberated p-nitrophenol was measured spectrophotometrically at 425 nm. The liberated phenol was measured, after a blue color was developed by addition of 2 volumes of 12% Na2CO3, at 660 nm.

Analysis of 35S-labeled Glycosaminoglycans by Digestion with Chondroitinase Digestion of chondroitin sulfates (or their oligosaccharide derivatives) and dermatan sulfates with chondroitinase-AC and chondroitinase-ABC, respectively, gives rise to unsaturated disaccharides (27). When N-acetylgalactosamine sulfate is nonreducing terminal, this monosaccharide is liberated, whereas nonreducing terminal uronosyl moieties are recovered in the form of various saturated disaccharides (31). As shown in Scheme 2, these monosaccharides can be resolved by paper chromatography and paper electrophoresis. Thus, by counting the radioactivity of the split products on paper strips, sufficient data can be obtained to permit a formulation of the types and relative amounts of 35S sulfatide residues of a given 35S-labeled glycosaminoglycan sample.

**Scheme 1.** Flow diagram of the disruption and fractionation of rat skin. Buffer A, 0.02 M Tris-HCl, pH 7.2, containing 0.25 M sucrose.

Arylsulfatase activities were assayed with p-nitrophenyl sulfate or 4-nitrocatechol sulfate as substrate. The incubation mixture contained 0.5 μmol of substrate, 2.5 μmol of sodium acetate/acetic acid buffer, pH 5.0, 7.5 μg of bovine serum albumin, and enzyme in a final volume of 50 μl. After incubation for 3 h at 37°C, the reaction was stopped by addition of 0.5 ml of 0.1 M NaHCO3. When necessary, the mixtures were clarified by centrifugation before measuring the absorbance of the liberated p-nitrophenol or 4-nitrocatechol at 425 nm. The liberated phenol was measured, after a blue color was developed by addition of 2 volumes of 12% Na2CO3, at 660 nm.

**Scheme 2.** Method of determination of the proportions of different types of 35S sulfate linkages in chondroitin 35S-sulfates and dermatan 35S-sulfate. Aliquots of 35S-labeled glycosaminoglycans are digested with chondroitinase-AC (or ABC), and the degradation products are resolved by paper chromatography in Solvent A (48 h). Sulfated compounds are detected with a strip scanner or by radioautography. 1, undegraded polysaccharides (or higher oligosaccharides); 2, ΔGlcUA(SO3)-GalNAc-6-SO4; 3, ΔGlcUA(SO3)-GalNAc-4,6-bis-SO4; 4, ΔGlcUA-GalNAc-4,6-bis-SO4; 5, GlcUA-GalNAc-6-SO4; 6a, GlcUA-GalNAc-4,6-bis-SO4; 6b, GlcUA-GalNAc-6-SO4; 6c, GalNAc-4,6-bis-SO4; 7a, GlcUA-GalNAc-4,6-bis-SO4; 7b, SO3; 8, GalNAc-6-SO4; 9, GalNAc-4-SO4. To separate 6a, 6b, and 6c from one another, the corresponding zone is cut out and eluted with water. Aliquots of the eluate are examined by paper chromatography in Solvent B (24 h) and by paper electrophoresis (60 min), as indicated. Similarly, 7a and 7b are eluted from the strip of the initial chromatography and separated by paper electrophoresis. The zone corresponding to each degradation product is cut out and counted for radioactivity in a scintillation spectrometer.
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(in the digestion of dermatan sulfate) at 37°C for 2 h. The digest was chromatographed on paper in Solvent A. By this procedure, some compounds were not separated from one another, as indicated in Scheme 2. To separate these, 35S-labeled materials in the corresponding zones were eluted with water and subjected to paper chromatography in Solvent B or to paper electrophoresis, as indicated in Scheme 2. radioactive materials on paper strips were localized with a strip scanner. The radioactive zones were cut out, placed in scintillation vials, and counted.

Chromatographic and Electrophoretic Techniques—Ion exchange chromatography of enzymes was done at 4°C on a small column (1 x 5.5 cm) of DEAE-cellulose, equilibrated with 0.02 M Tris-HCl, pH 7.2. Samples (4 ml) to 20 mg as protein) were applied to the column. After washed with 16 ml of the same buffer, the column was developed by linear gradient elution with 30 ml of 0.02 M Tris-HCl, pH 7.2, in the mixing flask and 30 ml of 0.4 M NaCl in the same buffer in the reservoir. Effluent fractions of 1 ml were collected at a rate of 10 ml/h and analyzed for protein and enzyme activity.

Gel chromatography of enzymes was done at 4°C on a column (1.2 x 85 cm) of Sephadex G-200, equilibrated with 0.2 M NaCl in 0.02 M Tris-HCl, pH 7.2. Samples (1.0 ml, 0.3 to 1.6 mg as protein) were applied to the column, and were then eluted with the same salt solution. Effluent fractions of 1 ml were collected at a rate of 7 ml/h and analyzed for protein and enzyme activity.

Paper electrophoresis was carried out on 60-cm strips of Toyo No. 51A paper in the apparatus described by Markham and Smith (32) at a potential gradient of 30 V/cm for the periods indicated in individual experiments. The buffer used was 0.05 M ammonium acetate/acetic acid, pH 5.0.

Paper chromatography was carried on 60-cm strips of Toyo No. 51A paper at room temperature by the descending method. The solvent systems used were: Solvent A, butyric acid/O.5 M ammonia (5:3, by volume) and Solvent B, l-butanol/acetic acid/water (10:3:5, by volume).

Other Methods—Hexuronate was determined by the orcinol method (33), and protein by the method of Lowry et al. (34).

RESULTS

In initial experiments, the 10,000 x g supernatant (S1) and pellet (P1) fractions from rat skin, prepared as shown in Scheme 1, were assayed at pH 5.0 for liberation of [35S]SO42- from dermatan [35S]sulfate and IdUA([35S]SO4)-Anhydroman([35S]SO4). The rates of [35S]SO42- liberation (expressed as cpm x 10^-7/h/mg of protein) by P1 were 4.27 from dermatan [35S]sulfate, and 73.0 from IdUA([35S]SO4)-Anhydroman([35S]SO4) compared to 3.96 from dermatan [35S]sulfate and 12.0 from IdUA([35S]SO4)-Anhydroman([35S]SO4) by S1. Since the data indicated that P1 is a main locus of both of the sulfatase activities, this fraction has been used as a first source of enzyme.

Solubilization and Fractionation of Sulfatases from 10,000 x g Pellet (P1)—When the 10,000 x g pellet (P1) was treated in a sonic oscillator and the supernatant of the sonicate (P2) was assayed using chondroitin 4/6-[35S]sulfate as substrate, it was shown that essentially all of the sulfatase activity could be solubilized by sonication for 3 min.

In separate experiments, P1 was exposed to 2% Tween 20 or 0.1% Triton X-100 for 30 min at 0°C, after which it was sedimented at 10,000 x g for 10 min. When the resulting supernatant solutions were assayed as above, more than 95% of the sulfatase activity in P1 could be found in the Triton X-100 extract whereas little activity was detected in the Triton X-100 100 extract.

DEAE-cellulose chromatography of P1, yielded several fractions with sulfatase activity as measured by hydrolysis of chondroitin 4/6-[35S]sulfate, dermatan [35S]sulfate, heparan [35S]sulfate, [N-[35S]SO4]-heparin, and keratan [35S]sulfate (Fig. 1, a to e).

Peak I (Fractions 5 to 10) acted on heparan [35S]sulfate and keratan [35S]sulfate. Since the substrates share common 6-[35SO4] linkages to N acetylgalactosamine not present in the other substrates, the activity may be due to the occurrence of an N-acetylgalactosamine 6-SO4 sulfatase.

With keratan [35S]sulfate, a second peak appeared just after and overlapping the first peak (Fig. 1e). This peak (Peak II) differed from Peak I in that it had activities towards chondroitin 4/6-[35S]sulfate (Fig. 1a) and dermatan [35S]sulfate (Fig. 1b), but not toward heparan [35S]sulfate (Fig. 1e). The specificity can be accounted for by the occurrence of a sulfatase specific for the 6-SO4 linked to sugars with the galactose configuration.

Peak III (Fractions 29 to 37) differed from Peak II in that it had little activity toward keratan [35S]sulfate. Thus, the enzyme in this peak must be an N-acetylgalactosamine 4-SO4 sulfatase.

Peak IV (Fractions 35 to 44) acted on heparan [35S]sulfate and [N-[35S]SO4]-heparin, consistent with the presence of an N-SO4 sulfamidase.

As far as these glycosaminoglycan substrates were used, no apparent peak was observed corresponding to an idurionate-SO4 sulfatase (which should act on dermatan [35S]sulfate and heparan [35S]sulfate). That the enzyme did indeed occur in the descending limb of Peak IV was indicated when the fractionation by DEAE-cellulose was monitored with a more specific substrate, IdUA([35S]SO4)-Anhydroman([35S]SO4) (Fig. 1f; designated Peak V).

Also shown in Fig. 1, g and h, are the patterns obtained...
with GalNAc 4[^35S]SO₄, (GlcUA-GalNAc 4[^35S]SO₄), and GalNAc-4/6[^35S]SO₄-GalUA-GalNAc-4/6[^35S]SO₄ as substrates. With the 4-sulfated heptasaccharide only one peak was observed corresponding to Peak III (Fig. 1g), whereas with the 4/6-sulfated heptasaccharide an additional peak was obtained corresponding to Peak II (Fig. 1h). The results are consistent with the suggestions that Peak II and III represent a sulfatase specific for the 6-SO₄ linked to sugars with the galactose configuration and an N-acetylgalactosamine-4-SO₄ sulfatase, respectively.

When the fractionation by DEAE-cellulose was monitored with a keratan sulfate tetrasaccharide trisulfate, GlcNAc-6[^35S]SO₄-Gal-6[^35S]SO₄-GalUA[^35S]SO₄-Gal, only one peak was observed corresponding to Peak I (Fig. 1i). In this assay system, incubation was carried out for 16 h, since the velocity obtained with the tetrasaccharide was much lower than that obtained with keratan[^35S]sulfate. Regardless of this difference in assay condition, it is apparent from the results in Fig. 1, e and i, that the Peak II enzyme cannot release[^35S]SO₄ at all when the corneal keratan[^35S]sulfate is replaced by its tetrasaccharide derivative. Since the[^35S]SO₄ residue that is present in the keratan[^35S]sulfate but not in the tetrasaccharide is linked to nonreducing terminal galactose, the results not only support the view that the Peak II enzyme is a 6-SO₄ sulfatase for sugars with the galactose configuration, but also indicate that desulfation by this enzyme occurs at the nonreducing terminal.

Fig. 1j indicates that the skin extract contains arylsulfatases which can be resolved into two fractions. It is noteworthy that one of the activities is closely associated with the Peak III activity for N-acetylgalactosamine-4-SO₄ linkages.

For further purification, several runs of DEAE-cellulose chromatography of P, samples were carried out, and peak fractions were separately pooled, concentrated, and subjected to gel filtration on a Sephadex G-200 column that had been chromatographed on a Sephadex G-200 column, as described under "Methods." The pH and substrates used for enzyme assay were: (a) GlcNAc-6[^35S]SO₄-GalUA[^35S]SO₄, pH 4.6; (b) GalUA[^35S]SO₄-GalNAc-4[^35S]SO₄, pH 4.6; (c) GalNAc-4[^35S]SO₄-IdUA[^35S]SO₄, pH 4.6; (d) [^35S]SO₄-(GlcUA-GalNAc-4[^35S]SO₄), pH 4.6; (e) [^35S]SO₄-(GlcUA-GalNAc-4[^35S]SO₄), pH 4.6 (for scale see left ordinate); (f) GalNAc-4[^35S]SO₄-(GlcUA-GalNAc-4[^35S]SO₄), pH 4.6 (for scale see right ordinate); (g) GalUA[^35S]SO₄-heparin, pH 5.0. Note the differences in the scale of the ordinate.

Table I shows the sensitivity of each enzyme to various inorganic salts which added to the standard incubation medium at optimal pH for each enzyme. The five sulfatases can be distinguished from one another by their sensitivities toward the inorganic ions: e.g. N-acetylgalactosamine-6-SO₄ sulfatase, N-SO₃ sulfamidase, and iduronate-SO₄ sulfatase were not inhibited to 50 to 60% by this concentration of NaCl or KCl whereas the other sulfatases were inhibited to 90 to 100% by this concentration of NaCl or KCl; a stimulation of N-SO₃ sulfamidase was seen with 10 mM MnCl₂ or MgCl₂, although moderate inhibition was noted on the activities of N-acetylgalactosamine-6-SO₄ sulfatase and N-acetylgalactosamine-4-SO₄ sulfatase when chondroitin-4-SO₄ was used in place of GalNAc-4[^35S]SO₄-(GlcUA-GalNAc-4[^35S]SO₄). Table II shows the sensitivity of each enzyme to various inorganic salts which added to the standard incubation medium at optimal pH for each enzyme. The five sulfatases can be distinguished from one another by their sensitivities toward the inorganic ions: e.g. N-acetylgalactosamine-6-SO₄ sulfatase, N-SO₃ sulfamidase, and iduronate-SO₄ sulfatase were not affected by 10 mM NaCl or KCl whereas the other sulfatases were inhibited to 90 to 100% by this concentration of NaCl or KCl; a stimulation of N-SO₃ sulfamidase was seen with 10 mM MnCl₂ or MgCl₂, although moderate inhibition was noted on the activities of N-acetylgalactosamine-6-SO₄ sulfatase and N-acetylgalactosamine-4-SO₄ sulfatase when chondroitin-4-SO₄ was used in place of GalNAc-4[^35S]SO₄-(GlcUA-GalNAc-4[^35S]SO₄).

When each enzyme solution equivalent to 1 mg of protein/
For the preparation of S1, P1, P2, and Peaks I to V from DEAE-cellulose and Sephadex G-200 columns see the text. The pH and substrate used for each enzyme assay were: pH 3.7, GlcNAc-6-[^35S]-SO4-6-SO4-Gal 6[^35S]-SO4-GlcNAc-6[^35S]-SO4-Gal for N-acetylglucosamine-6-SO4 sulfatase (I); pH 4.6, GalNAc-4/6[^35S]-SO4-4-[^35S]-SO4-(GlcUA-GalNAc-4/6[^35S]-SO4) for N-acetylgalactosamine-6-SO4 sulfatase (II); pH 4.6, GalNAc-4/6[^35S]-SO4-4-[^35S]-SO4-(GlcUA-GalNAc-4/6[^35S]-SO4) for N-acetylgalactosamine-6-SO4 sulfatase (III); pH 5.6, [N-[^35S]-SO4]-heparin for N-SO3 sulfamidase (IV); and pH 4.6, IdUA-[^35S]-SO4-Anhydromannitol[^35S]-SO4 for iduronate-SO4 sulfatase (V).

| Fraction          | I       | II      | III     | IV      | V       |
|-------------------|---------|---------|---------|---------|---------|
| S1                | 15 (0.07) | 1480 (7.1) | 576 (2.8) | 203 (1.0) | 262 (1.2) |
| P1                | 1.3 (0.05) | 600 (20.0) | 255 (8.5) | 87 (2.9) | 218 (7.3) |
| P2                | 1.3 (0.07) | 600 (30.0) | 200 (10.0) | 78 (3.7) | 116 (5.9) |
| DEAE-cellulose    | 1.2 (0.89) | 288 (63.0) | 100 (298) | 31 (61.5) | 30 (30.4) |
| Sephadex G-200    | 0.5 (3.1) | 19 (456) | 15 (332) | 15 (133) | 30 (9.8) |

| Enzyme activity* | (specific activity, units/mg protein) |
|------------------|------------------------------------|
| I                | 26 in S1 and 2.2 in P1; (II) 750 in S1 and 304 in P4; (III) 301 in S1 and 133 in P1; (IV) 24 in S1 and 10 in P1; (V) 390 in S1 and 311 in P1. With heparan [^35S]-SO4 as substrate, the values for Peak I enzyme are about 3 times greater than those indicated above. |

* Note that the values obtained with different sulfatases can not be compared directly, since no correction is made for the differences in specific radioactivities of labeled substrates. Total activities expressed as nanomoles of SO4^-2 released per h are: (I) 26 in S1 and 2.2 in P1; (II) 750 in S1 and 304 in P1; (III) 301 in S1 and 133 in P1; (IV) 24 in S1 and 10 in P1; (V) 390 in S1 and 311 in P1. With heparan [^35S]-SO4 as substrate, the values for Peak I enzyme are about 3 times greater than those indicated above.

**Table I: Purification of rat skin sulfatases**

**Table II: Effect of inorganic salts on five sulfatase activities**

In each assay the conditions were those optimal for the enzymatic hydrolysis, as described in the text. The activity is expressed relative to control determination with no added inorganic salt, the activity being taken as 100%.

| Salt                      | I       | II      | III     | IV      | V       |
|---------------------------|---------|---------|---------|---------|---------|
| NaCl (10 mM)              | 58      | 100     | 53      | 113     | 105     |
| KC1 (10 mM)               | 56      | 102     | 53      | 110     | 100     |
| MgCl2 (10 mM)             | 45      | 72      | 32      | 132     | 97      |
| MnCl2 (10 mM)             | 30      | 70      | 35      | 200     | 90      |
| Na2SO4 (0.5 mM)           | <2      | 35      | 25      | 87      | 50      |
| Na2SO4 (2 mM)             | <2      | 20      | <2      | 62      | 22      |

* I, N-acetylglucosamine-6-SO4 sulfatase; II, N-acetylglucosamine (or galactose)-6-SO4 sulfatase; III, N-acetylgalactosamine-6-SO4 sulfatase; IV, N-SO3 sulfamidase; V, iduronate-SO4 sulfatase. The substrates and pH used for the assay were as in Table I.

ml, in 0.05 mM acetate buffer at optimal pH for each enzyme, was preincubated at 50°C for 15 min, it was observed that N-acetylglucosamine-6-SO4 sulfatase, N-acetylglucosamine-6-SO4 sulfatase, and N-acetylgalactosamine-6-SO4 sulfatase had lost approximately 90% of their initial activity while the other two enzymes had lost only 5 to 10% of the activity. When stored frozen (with occasional thawing and refreezing), N-acetylgalactosamine-6-SO4 sulfatase was found to be relatively more unstable than the other enzymes; i.e. about 80% of its initial activity was lost in 2 months while the other four enzymes retained more than 70% of the activities.

Under the standard conditions for enzyme assay (3 h incubation), there was a linear relationship between the amount of each sulfatase added and the amount of [^35S]-SO4^-2 released up to an enzyme amount equivalent to 0.5 µg (Peak II-, III-, and V-enzyme) or 1.0 µg (Peak I- and IV-enzyme) of protein. Maximum release of [^35S]-SO4^-2 (by digestion overnight with an excess of enzyme) corresponded to about 0.5% (from heparan [^35S]-SO4 sulfatase by Peak I enzyme), 4.1% (from chondroitin 4[^35S]-SO4 sulfatase by Peak III enzyme), 4.1% (from [N-[^35S]-SO4]-heparin by Peak IV enzyme), 1.1% (from heparan [^35S]-SO4 sulfatase by Peak IV enzyme), and less than 0.1% (from heparan [^35S]-SO4 sulfatase and dermatan [^35S]-SO4 sulfatase by Peak V enzyme) of the added radioactivity (1 × 10^-6 cpm). When the reaction mixtures were examined by gel filtration on Sephadex G-200, each sulfatase-treated sample showed essentially the same macromolecular pattern as the starting material, plus a very
small peak corresponding to inorganic sulfate. The results suggest that neither extensive removal of internal sulfate linkages nor breakdown of the polysaccharide chains occurs by the action of these sulfatase preparations.

When IdUA(\(^{35}\text{S}\text{SO}_4\))-Anhydroman(\(^{35}\text{S}\text{SO}_4\)) was digested with the most purified Peak V preparation, the compound was quantitatively converted to two radioactive products which behaved as inorganic sulfate and IdUA-Anhydro-\(\text{SO}_4\) was quantitatively converted to two radioactive products with the most purified Peak V preparation, the compound

The following compounds were tested at two to four concentrations in the ranges indicated in parentheses and found to be inactive: p-nitrophenyl sulfate (0.2 to 0.5 \(\mu\)mol/50 \(\mu\)l); UDP-N-acetylgalactosamine-4-\(^{35}\text{S}\text{SO}_4\); N-acetylgalactosamine-4-\(^{35}\text{S}\text{SO}_4\); N-acetylgalactosamine-6-\(^{35}\text{S}\text{SO}_4\); GalNAc-4-\(^{35}\text{S}\text{SO}_4\); GlcUA-GalNAc-4-\(^{35}\text{S}\text{SO}_4\); GalNAc-4,6-bis-(\(^{35}\text{S}\text{SO}_4\)); GlcUA-GalNAc-4-\(^{35}\text{S}\text{SO}_4\); GalNAc-4,6-bis-(\(^{35}\text{S}\text{SO}_4\)); GlcUA-GalNAc-4-\(^{35}\text{S}\text{SO}_4\); GalNAc-4,6-bis-(\(^{35}\text{S}\text{SO}_4\)).

Further studies with various oligosaccharide substrates demonstrated that only a nonreducing N-acetylgalactosamine-6-\(^{35}\text{S}\text{SO}_4\) end group is desulfated by the enzyme. Thus, even-numbered oligosaccharides bearing a glucuronic acid at the nonreducing terminal, (GlcUA-GalNAc-4-6-(\(^{35}\text{S}\text{SO}_4\)), were completely refractory to the action of this enzyme. In contrast, odd-numbered oligosaccharides, GalNAc-4,6-(\(^{35}\text{S}\text{SO}_4\); GlcUA-GalNAc-4,6-(\(^{35}\text{S}\text{SO}_4\)), gave positive results; i.e., upon incubation with 0.023 unit (determined with the heptasaccharide as substrate) of the enzyme for 3 h, 2.9, 6.9, 7.2, and 9.0% of the added \(^{35}\text{S}\) (1 \(\times\) 10^4 cpm) were released as \(^{35}\text{S}\text{SO}_4^2^-\) from the tri-, penta-, hepta-, and nonsaccharide, respectively.

When GalNAc-4,6-(\(^{35}\text{S}\text{SO}_4\)); GlcUA-GalNAc-4,6-(\(^{35}\text{S}\text{SO}_4\)); was treated with 0.037 unit of the enzyme for 3 h and the untreated and treated mixtures were analyzed by digestion with chondroitinase-AC (see "Methods"), it was shown that the percentage of N-acetylgalactosamine-6-(\(^{35}\text{S}\text{SO}_4\)) (derived from the nonreducing terminal) had reduced from 14 to 3% by the sulfatase action, with a corresponding increase of \(^{35}\text{S}\text{SO}_4^2^-\) from 0 to 11%. No detectable change in the amount of N-acetylgalactosamine-4-(\(^{35}\text{S}\text{SO}_4\)) (19%) and unsaturated disaccharide products (67%) was found.

The following compounds were tested at two to four concentrations in the ranges indicated in parentheses and found to be inactive: p-nitrophenyl sulfate (0.2 to 0.5 \(\mu\)mol/50 \(\mu\)l); UDP-N-acetylgalactosamine-4-(\(^{35}\text{S}\text{SO}_4\)) N-acetylgalactosamine-4-(\(^{35}\text{S}\text{SO}_4\)); N-acetylgalactosamine-6-(\(^{35}\text{S}\text{SO}_4\)) GalNAc-4-(\(^{35}\text{S}\text{SO}_4\)); GlcUA-GalNAc-4-(\(^{35}\text{S}\text{SO}_4\)); GalNAc-4,6-bis-(\(^{35}\text{S}\text{SO}_4\)); GlcUA-GalNAc-4-(\(^{35}\text{S}\text{SO}_4\)); GalNAc-4,6-bis-(\(^{35}\text{S}\text{SO}_4\)); GlcUA-GalNAc-4-(\(^{35}\text{S}\text{SO}_4\)); GalNAc-4,6-bis-(\(^{35}\text{S}\text{SO}_4\)); GlcUA-GalNAc-4-(\(^{35}\text{S}\text{SO}_4\)); GalNAc-4,6-bis-(\(^{35}\text{S}\text{SO}_4\)); GlcUA-GalNAc-4-(\(^{35}\text{S}\text{SO}_4\)); GalNAc-4,6-bis-(\(^{35}\text{S}\text{SO}_4\)); GlcUA-GalNAc-4-(\(^{35}\text{S}\text{SO}_4\)); GalNAc-4,6-bis-(\(^{35}\text{S}\text{SO}_4\)); GlcUA-GalNAc-4-(\(^{35}\text{S}\text{SO}_4\)); GalNAc-4,6-bis-(\(^{35}\text{S}\text{SO}_4\)).

Further evidence for the specificity of sulfate hydrolysis was obtained by examination of GalNAc-4,6-(\(^{35}\text{S}\text{SO}_4\)); GlcUA-GalNAc-4,6-(\(^{35}\text{S}\text{SO}_4\)); before and after treatment with the sulfatase; following incubation of 1 \(\times\) 10^4 cpm of the substrate with 0.013 unit of the enzyme for 3 h, there was a reduction of N-acetylgalactosamine-6-(\(^{35}\text{S}\text{SO}_4\)) (19 to 15%) and a corresponding appearance of \(^{35}\text{S}\text{SO}_4^2^-\) (from 0 to 4%) with no detectable change in the amount of N-acetylgalactosamine-6-(\(^{35}\text{S}\text{SO}_4\)) and unsaturated disaccharides.

The V_max and K_m calculated from the rates of sulfate release with varying concentrations of tri-, penta-, hepta-, and nonsaccharide 4-SO_4^- (Table III) showed that, if the calculation is based on the content of nonreducing N-acetylgalactosamine-4-SO_4^- end group, the V_max values increase with increasing chain length while the K_m values do not differ greatly.

The most purified enzyme preparation was tested for its ability to release \(^{35}\text{S}\text{SO}_4^2^-\) from various sulfate esters (concentration range, 1 - 4.2 \(\times\) 10^4 cpm/50 \(\mu\)l), i.e., UDP-GalNAc-4-(\(^{35}\text{S}\text{SO}_4\)); GalNAc-4,6-bis-(\(^{35}\text{S}\text{SO}_4\)); GlcUA-GalNAc-4-(\(^{35}\text{S}\text{SO}_4\)); hyaluronidase/β-glucuronidase-treated dermantan (\(^{35}\text{S}\)sulfate, GalNAc-4-(\(^{35}\text{S}\text{SO}_4\)) GalNAc-6-(\(^{35}\text{S}\text{SO}_4\)); GlcUA-GalNAc-4-(\(^{35}\text{S}\text{SO}_4\)); GalNAc-6-(\(^{35}\text{S}\text{SO}_4\)); GlcUA-GalNAc-4-(\(^{35}\text{S}\text{SO}_4\)); GlcUA-GalNAc-6-(\(^{35}\text{S}\text{SO}_4\)); GalNAc-6-(\(^{35}\text{S}\text{SO}_4\)); GlcUA-GalNAc-4,6-(\(^{35}\text{S}\text{SO}_4\))) among the sulfate esters, only the first three compounds gave positive results.

As shown in Figs. 1 and 2, there was a close parallelism between the activity for chondroitin-4-SO_4^- nonsaccharide...
and that for p-nitrophenyl sulfate. Moreover, the purified enzyme was shown to hydrolyze 4-nitrocatechol sulfate at a rate (150 μmol/h/mg of protein) higher than that for p-nitrophenyl sulfate (6 μmol/h/mg of protein). The results are consistent with the notion that arylsulfatase B and the N-acetylglactosamine-4-SO₄ sulfatase are one and the same (8, 10, 11).

Comparison of N-Acetylglactosamine-4-SO₄ and -6-SO₄ Sulfatases Localized in Soluble and Particulate Fractions— As described above, considerable proportions of the sulfatases in rat skin were released into the soluble fraction (S₁) by the first 3-min treatment in a Polytron homogenizer. The results suggest that some of the readily solubilized sulfatases might differ from their particulate counterparts.

To test this possibility, the soluble fraction (S₁) obtained by the initial Polytron treatment was chromatographed on a DEAE-cellulose column under the same conditions used for Pₚ (cf. Fig. 1). Fig. 5 illustrates the elution patterns of sulfatase activities towards GalNAc-4/6-[35S]SO₄-(GlcUA-GalNAc-4/6-[35S]SO₄)₃ and GalNAc-4-[35S]SO₄-(GlcUA-GalNAc-4-[35S]-SO₄)₃ together with those of arylsulfatase activities and proteins. As can be seen, the sulfatase patterns of S₁ and Pₚ were essentially identical, although their protein patterns were quite different from each other.

**Table III**

| Substrate | Relative Vₘₐₓ | Kₘ (μM) |
|-----------|--------------|---------|
| GalNAc-4-SO₄-GlcUA-GalNAc-4-SO₄ | 12 | 0.027 |
| GalNAc-4-SO₄-(GlcUA-GalNAc-4-SO₄)₂ | 10 | 0.030 |
| GalNAc-4-SO₄-(GlcUA-GalNAc-4-SO₄)₃ | 18 | 0.049 |
| GalNAc-4-SO₄-(GlcUA-GalNAc-4-SO₄)₄ | 20 | 0.064 |

**FIG. 5**

DEAE-cellulose chromatography of the supernatant, S₁, of rat skin homogenate. Conditions for the chromatography are given under "Methods." Fractions of 1 ml were collected and assayed for sulfatase activities toward (a) GalNAc-4/6-[35S]SO₄-(GlcUA-GalNAc-4/6-[35S]SO₄)₃ at pH 4.6, (b) GalNAc-4-[35S]SO₄-(GlcUA-GalNAc-4-[35S]-SO₄)₃ at pH 4.6, and (c) p-nitrophenyl sulfate at pH 5.0. Bars denote the positions of Peaks II and III in Fig. 1.

**DISCUSSION**

The assay system used here is so designed as to permit the detection of most, if not all, of the sulfatase activities toward hitherto known glycosaminoglycans. Our survey of the skin extract with this assay system has led to the demonstration of five different sulfatases, specific, respectively, for certain suitably located (presumably nonreducing terminal) N-acetylglucosamine-6-SO₄, N-acetylglactosamine (or galactose)-6-SO₄, N-acetylglactosaminoglycan-4-SO₄, glucosamine-N-SO₄, and iduronate-SO₄ residue of a glycosaminoglycan chain. No other glycosaminoglycan sulfatase (e.g. isozymes, enzymes with broader substrate specificity, or enzymes capable of hydrolyzing sulfate linkages at many points within a glycosaminoglycan chain) were detected. Genetic disease studies, on the other hand, have provided evidence for the occurrence of five different glycosaminoglycan sulfatases in human fibroblasts (see the Introduction for the references). Our results with rat skin are in good agreement with these works on the human genetic diseases, and suggest that rat skin could prove useful not only as a source of sulfatases for enzymological studies but also as a model system to study more extensively the roles of the sulfatases in proteoglycan metabolism under various physiological and pathological conditions.

It is necessary to comment on the specificity of Peak II enzyme. That the enzyme releases SO₄²⁻ from the nonreducing N-acetylglactosamine-6-SO₄ end groups of chondroitin sulfates is established by the experiments using GalNAc-4/6-[35S]SO₄-(GlcUA-GalNAc-4/6-[35S]SO₄)₃ as substrate. The enzyme also shown to catalyze release of SO₄²⁻ from corneal keratan sulfate at about one fourth the rate of SO₄²⁻ release from chondroitin 4/6-sulfate; amount of SO₄²⁻ released from keratan sulfate in 1 h by 1 mg of enzyme protein is 2.4 nmol, compared to 9.6 nmol from chondroitin 4/6-sulfate. This release from keratan sulfate can not be attributed to hydrolysis on nonreducing N-acetylglucosamine-6-SO₄ residues, because an early portion of the peak fractions from Sephadex G-200 (Fractions 46 to 50 in Fig. 2b) has little activity toward keratan sulfate tetrasaccharide bearing N-acetylglucosamine-6-SO₄ at the nonreducing end (data not shown in Fig. 2). Thus, the release of SO₄²⁻ must be from nonreducing galactose 6-sulfate residues. The results, taken together, imply that the Peak II enzyme is a sulfatase specific for the 6-sulfate linked with the galactose configuration has previously been suggested by DiFerrante et al. (15) and Horwitz and Dorfman (16). These workers demonstrated that, as compared to extracts from normal skin fibroblasts, extracts from Morquio fibroblasts have greatly reduced sulfatase activities for galactosyl 6-sulfate, N-acetylglactosaminidol 6-sulfate (15), and 6-sulfated trisaccharide from chondroitin sulfate (16), but have
normal activity for N-acetylgalcosamine 6-sulfate (15) and its disaccharide derivative (16). Our results therefore support these earlier observations, although there is no direct evidence at this stage to indicate that the enzyme can indeed act on galactose 6-sulfate in the monosaccharide form as well as in polysaccharide forms.

From what is known of the substrate specificities of the sulfatases from rat skin, they should be able to act on sulfated glycoproteins and glycolipids which have the same nonreducing sugars as glycosaminoglycans have. Isolation of a sulfated glycoprotein from rat lung has enabled us to test this possibility. The sulfated glycoprotein was obtained, during the course of preparation of 1dUA-2[^35S]SO_4, Anhydromenan-6[^35S]SO_4 from [^35S]-labeled rat lung (see "Experimental Procedures"), as a fraction which was not depolymerized by exhausive treatment with chondroitinase-ABC, keratan sulfate-endo-β-galactosidase, and HN_2O. On paper electrophoresis, it gave a broad [^35S]-band with a mobility similar to hyaluronic acid. With this compound as substrate, Peak II enzyme (0.5 µg as protein) showed release of [[^35S]SO_4]^2- corresponding to 0.8% of the added radioactivity (3,000 cpm), in 1 h. Whether the [[^35S]SO_4]^2- release represents hydrolysis of nonreducing N-acetylgalactosamine 6-sulfate or galactose 6-sulfate end groups is not known, but the results strengthen the possibility that the enzymes will be used in structural studies of sulfated glycoproteins and glycolipids which, with few exceptions, have been very poorly characterized.

The rat skin extract appears to contain at least two different arylsulfatases as judged by chromatography on DEAE-cellulose (Fig. 1). One of these (the faster eluting component) was closely associated with the activity toward N-acetylgalactosamine-4-SO_4 in chondroitin sulfate and dermatan sulfate (Figs. 1 and 2). This enzyme was also shown to catalyze the desulfation of UDP-GalNAc-4-SO_4. These results taken together with those previously published (8-13, 36, 37) indicate that the activities toward the arylsulfate, sugar nucleotide, and glycosaminoglycans may be due to one, common enzyme. The other arylsulfatase component (Fig. 1; the slower eluting peak) was not studied in detail because it exhibited no signif-icant activity toward the [^35S]-labeled glycosaminoglycan sub-

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