A distinct terminal structure in newly synthesized chondroitin sulphate chains

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A method was developed for the analysis of non-reducing terminal structure of radiolabelled chondroitin sulphate chains with the aid of *N*-acetylgalactosamine 4-sulphatase ('terminal 4-sulphatase'), *N*-acetylgalactosamine 6-sulphatase ('terminal 6-sulphatase'), β -glucuronidase and β -*N*-acetylhexosaminidase. Studies with this method on the non-reducing terminal structure of [35 S]sulphate- and [3 H]glucose-labelled chondroitin sulphate chains from rat and chick-embryo cartilages showed that the presence of a high proportion of 4-sulphated hexosamine residues is a common feature of the termini of newly synthesized chondroitin sulphate chains. Of the non-reducing terminal 4-sulphated hexosamine residues, about 14% (chick embryo) or 46% (rat) contained an additional sulphate group at position 6. The internal portion of the chondroitin sulphate chains, in contrast, contained little or no 4,6-bis-sulphated hexosamine residue, suggesting that 4,6-bis-sulphated structure may play a role in biosynthetic control at the level of chain termination.

Telser *et al.* (1966), Rodén *et al.* (1972) and Silbert (1978) have shown that chondroitin sulphate chains ending in GalNAc-6-SO₄ are able to accept a glucuronic acid, but that those ending in GalNAc-4-SO₄ are not capable of accepting a glucuronic acid. Sulphation of the non-reducing terminal would therefore have an important influence on the regulation of chondroitin sulphate chain elongation. The development of a technique for the structural analysis of very small quantities of non-reducing terminal sugars of newly synthesized chondroitin sulphate is clearly needed to test this possibility.

Chondroitinase ABC (chondroitin ABC lyase, EC 4.2.2.4) has been used for the determination of

Abbreviations used: terminal 4-sulphatase and terminal 6-sulphatase, sulphatases catalysing the hydrolysis of non-reducing *N*-acetylgalactosamine 4-sulphate and *N*-acetylgalactosamine 6-sulphate end groups respectively (for further information of their properties, see the text); GalNAc-4-SO₄, GalNAc-6-SO₄ and GalNAc-4,6-bisSO₄, *N*-acetylgalactosamine 4-sulphate, 6-sulphate and 4,6-bis-sulphate respectively; Δ Di-OS, 2-acetamido-2-deoxy-3-*O*-(β -D-glucopyranosyluronic acid)-D-galactose; Δ Di-4S, Δ Di-6S and Δ Di-4,6-bisS, derivatives of Δ Di-OS bearing a sulphate group at position 4, a sulphate group at position 6 and two sulphate groups at position 4 and position 6 respectively of the hexosamine moiety; GlcA, glucuronic acid.

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non-reducing terminal sugars of oligosaccharides from chondroitin sulphate on the basis of the fact that degradation by this enzyme gives rise to sulphated unsaturated disaccharides (Saito *et al.*, 1968) and releases the non-reducing terminal monosaccharides or disaccharides. When a uronic acid is terminal, saturated disaccharides are found, whereas if sulphated *N*-acetylgalactosamine is terminal, it is recovered as such (Sjöberg *et al.*, 1973). We previously applied this procedure to the analysis of a chondroitin sulphate from chick-embryo cartilage that had been metabolically labelled with [35 S]sulphate, and obtained GalNAc-4-[35 S]SO₄ and GalNAc-4,6-bis-[35 S]SO₄ as the predominant monosaccharide products (Tsuji *et al.*, 1980). This is consistent with the prediction that sulphation at position 4 of a terminal *N*-acetylgalactosamine would stop the polymerization of chondroitin sulphate.

During these studies, we noticed that there is a source of error in the analysis of chondroitin sulphate of relatively large size. Since the amounts of terminal sugars are extremely small relative to the disaccharide units of the interior portion, and since sulphated unsaturated disaccharides released from the interior portion are sensitive to weak alkali and high temperature, it will happen that, during the lengthy procedures for isolation and analysis of monosaccharides and unsaturated disaccharides, a small, but significant, portion of the latter com-

pounds is decomposed to give rise to monosaccharides, including the same compounds as those derived directly from the non-reducing termini. An attempt was therefore made to develop an alternative method for determining more precisely the non-reducing terminal structure of large-sized chondroitin sulphate chains by employing sulphatases and glycosidases that act specifically on the non-reducing terminal units. The present paper shows how the method was adapted to the structural analysis of radiolabelled chondroitin sulphates from different sources. The relevance to the chondroitinase method, and to the mechanism of chondroitin sulphate chain termination, is discussed.

Experimental

Materials

Na₂³⁵SO₄ (carrier-free) was purchased from Japan Radioisotope Association, Tokyo, Japan; D-[1-³H]glucose (15 mCi/μmol) was from New England Nuclear Corp., Boston, MA, U.S.A.; foetal-calf serum was from Grand Island Biological Co., Grand Island, NY, U.S.A.; bovine serum albumin (fraction V) was from Sigma Chemical Co., St. Louis, MO, U.S.A. ΔDi-0S, ΔDi-4S, ΔDi-6S and ΔDi-4,6-bisS were prepared by the method of Suzuki *et al.* (1968). UDP-*N*-acetylgalactosamine 6-[³⁵S]sulphate (6 × 10⁷ d.p.m./μmol) (Otsu *et al.*, 1984), heparan [³⁵S]sulphate (2 × 10⁷ d.p.m./μmol) (Hassell *et al.*, 1980) and keratan [³⁵S]sulphate (1 × 10⁷ d.p.m./μmol) (Oike *et al.*, 1980) were prepared as described previously. Quails (*Coturnix coturnix japonica*) and quail eggs (not over 2 days old) were obtained locally. β-Glucuronidase from rat preputial gland (Himeno *et al.*, 1975) was kindly provided by Dr. K. Kato, Kyushu University, Fukuoka, Japan. The preparation had a specific activity of 35 units/mg of protein as measured by the method with *p*-nitrophenyl β-D-glucuronide (Himeno *et al.*, 1975). When the enzyme preparation was examined for activities of terminal 4-sulphatase, terminal 6-sulphatase and β-*N*-acetylhexosaminidase under the conditions described below, none of those activities was detected. β-*N*-Acetylhexosaminidase was purified from a commercial β-*N*-acetylhexosaminidase preparation (6 units/mg; Boehringer Mannheim Yamanouchi, Tokyo, Japan) as follows. The crude enzyme preparation (19 units/0.6 ml) was dialysed for 24 h against three changes of 500 ml each of 20 mM-sodium acetate/acetic acid buffer, pH 5.6, and applied on to a Blue Sepharose CL-6B column (0.4 cm × 1 cm) equilibrated with the acetate buffer. The column was washed with 2 ml of the same buffer and then eluted with 2 ml of 0.05 M-KCl in the same buffer. The eluate was concentrated to

1 ml by ultrafiltration on a Amicon PM-10 membrane. About 80% of the applied activity was recovered. On incubation with *p*-nitrocatechol sulphate (see below for the conditions), the starting enzyme preparation showed a sulphatase activity of about 2.7 munits/mg of protein, whereas the purified sample showed little sulphatase activity (<0.18 munit/mg of protein). Terminal 6-sulphatase (0.7 munit/mg of protein) was purified from quail oviduct as described previously (Nakanishi *et al.*, 1979), except that molecular-sieve chromatography was performed on Sephacryl S-200 instead of Sephadex G-200. Chondroitinase ABC from *Proteus vulgaris* N.C.T.C. 4636 (Yamagata *et al.*, 1968) was a product of Seikagaku Kogyo Co., Tokyo, Japan, and was kindly given by Mr. M. Mizutani of the company.

Determination of enzyme activities

Terminal 4-sulphatase activity was determined with UDP-*N*-acetylgalactosamine [6-³⁵S]4,6-bis-sulphate as substrate (Otsu *et al.*, 1984). Since previous studies (Fluharty *et al.*, 1975; Tsuji *et al.*, 1980) had shown the common identity of sulphatases acting on chondroitin 4-sulphate, UDP-*N*-acetylgalactosamine 4,6-bis-sulphate and *p*-nitrocatechol sulphate, advantage was taken in the experiments for enzyme purification of the fact that the efficiency of the purification method can be readily monitored by using *p*-nitrocatechol sulphate as a substrate (for the assay conditions, see Tsuji *et al.*, 1980). One unit of enzyme was defined as the quantity that catalysed the formation of 1 μmol of *p*-nitrocatechol/min.

Terminal 6-sulphatase activity was determined with UDP-GalNAc-6-[³⁵S]SO₄ as a substrate (Nakanishi *et al.*, 1979). One unit of enzyme was defined as the quantity that catalysed the formation of 1 μmol of SO₄²⁻/min.

Activities of β-glucuronidase (Himeno *et al.*, 1975) and β-*N*-acetylhexosaminidase (Kresse *et al.*, 1981) were determined with *p*-nitrophenyl β-D-glucuronide and *p*-nitrophenyl *N*-acetyl-β-D-glucosaminide respectively as substrate. In each case 1 unit of enzyme was defined as the quantity that catalysed the formation of 1 μmol of *p*-nitrophenol/min.

Purification of terminal 4-sulphatase

Unless otherwise indicated, all operations were conducted at 4°C.

Crude enzyme preparation. About 375 ml of quail egg-white was homogenized in 1500 ml of 20 mM-sodium acetate/acetic acid buffer, pH 5.6 (buffer A), for 2 min in a Polytron homogenizer. After centrifugation, the supernatant solution was dialysed for 48 h against two changes of 12 litres each of buffer A.

Step I (chromatography on CM-cellulose CM-52). The non-diffusible material (1830ml) was loaded on a CM-cellulose CM-52 column (4.8cm × 56cm) equilibrated with buffer A. The column was then washed with 3 litres of buffer A containing 0.1M-NaCl. Adsorbed material was developed at a flow rate of 180ml/h with a linear NaCl concentration gradient consisting of 2 litres of buffer A containing 0.1M-NaCl at the initial concentration and 2 litres of the same buffer containing 0.3M-NaCl at the final concentration. Enzyme-containing fractions (1360ml; 0.23M-NaCl to 0.3M-NaCl fractions) were pooled and dialysed for 4h against 12 litres of buffer A.

Step II (second chromatography on CM-cellulose CM-52). The non-diffusible material (1360ml) was loaded on a CM-cellulose CM-52 column (2.2cm × 15cm) equilibrated with buffer A. The column was then washed with 200ml of buffer A containing 0.1M-NaCl. Adsorbed material was developed at a flow rate of 40ml/h with a linear NaCl concentration gradient consisting of 100ml of buffer A containing 0.1M-NaCl at the initial concentration and 100ml of the same buffer containing 0.35M-NaCl at the final concentration. Enzyme-containing fractions (92ml; 0.23M-NaCl to 0.32M-NaCl fractions) were pooled and concentrated to 12ml by ultrafiltration on a PM-10 membrane.

Step III (chromatography on Sephacryl S-300). The concentrated solution was loaded on a Sephacryl S-300 column (2.6cm × 110cm) equilibrated with buffer A containing 0.1M-NaCl. The column was then eluted at a flow rate of 40ml/h with buffer A containing 0.1M-NaCl. Enzyme-containing fractions (100ml; K_d 0.45 to K_d 0.70 fractions) were pooled, concentrated to 10ml by ultrafiltration on a PM-10 membrane and stored at -80°C.

Incubation of cartilages in radioisotope-containing media

The medium was a modified Eagle's minimal essential medium, in which the amounts of inorganic sulphate and glucose were decreased to give 0.3mM-SO₄²⁻ and 1.6mM-glucose respectively, and which contained 10% (v/v) dialysed foetal-calf serum, 0.005% (w/v) ascorbic acid and either 1.5mCi of carrier-free Na₂³⁵SO₄/ml or 15mCi of [³H]glucose/ml. For each incubation, about 100mg of fresh tissues were placed in 1 ml of warm (37°C) medium. The incubation was performed at 37°C for 5h in a humidified atmosphere containing 5% CO₂.

Preparation of radiolabelled chondroitin sulphate and trisaccharide

From the labelled cartilages (100mg), chon-

droitin sulphate chains were prepared as previously described (Oike *et al.*, 1980). The resulting chondroitin sulphate chains were chromatographed on a column (1cm × 64cm) of Sepharose CL-6B equilibrated and eluted with 0.2M-ammonium acetate, pH6.0. The peak fractions (11 ml) of labelled chondroitin sulphate were pooled and freeze-dried; the yield from the labelled samples of chick-embryo epiphyseal cartilage was 1.0×10^8 d.p.m. of chondroitin [³⁵S]sulphate (sp. radioactivity 5.3×10^7 d.p.m./μmol of glucuronic acid) or 9.7×10^7 d.p.m. of [³H]chondroitin sulphate (sp. radioactivity 5.5×10^7 d.p.m./μmol of glucuronic acid), and the yield from the labelled samples of rat xiphisternal cartilage was 3.7×10^7 d.p.m. of chondroitin [³⁵S]sulphate (sp. radioactivity 6.1×10^7 d.p.m./μmol of glucuronic acid) or 3.4×10^7 d.p.m. of [³H]chondroitin sulphate (sp. radioactivity 6.8×10^7 d.p.m./μmol of glucuronic acid).

[³⁵S]Sulphated trisaccharide was prepared from the chondroitin [³⁵S]sulphate from chick-embryo epiphyseal cartilage by digestion with a mixture of testicular hyaluronidase and β-glucuronidase followed by preparative paper chromatography (Suzuki & Strominger, 1960); the yield was 1.0×10^6 d.p.m. (sp. radioactivity 1.1×10^7 d.p.m./μmol of glucuronic acid).

Paper electrophoresis and paper chromatography

Paper electrophoresis was done on 60cm strips of Toyo no. 51A paper at room temperature in the apparatus described by Markham & Smith (1952) at a potential gradient of 30V/cm for 60min. The buffer used was 0.05M-ammonium acetate/acetic acid, pH5.0.

Paper chromatography was done on 45cm strips of Toyo no. 51A paper at room temperature by the descending method with the following solvent systems: A, butanol/ethanol/water (13:8:4, by vol.); B, butanol/acetic acid/water (10:3:5, by vol.); C, butyric acid/0.5M-ammonia (5:3, v/v).

Procedures for structural analysis of radiolabelled chondroitin sulphate

Method I. This assay measures the amount of radiolabelled GalNAc-4-SO₄, GalNAc-6-SO₄, GalNAc-4,6-bisSO₄, ΔDi-4S, ΔDi-6S and ΔDi-4,6-bisS derived from chondroitin [³⁵S]sulphate or its trisaccharide fragment by chondroitinase ABC digestion. The reaction mixture contained 1.5μmol of Tris/HCl buffer, pH8.0, 1.5μmol of sodium acetate, test sample (1.3×10^3 – 10^5 d.p.m. as ³⁵S), chondroitin 4-sulphate (0.2μmol as hexuronate), chondroitin 6-sulphate (0.2μmol as hexuronate) and 0.5 unit of chondroitinase ABC, in the total volume of 50μl. The mixture was incubated at 37°C for 1h and then subjected to the separation

procedures consisting of paper electrophoresis and paper chromatography in solvent C (Nakanishi *et al.*, 1981). Radioactive materials on paper strips were located by β -ray camera photography. The zones containing labelled materials were cut out, placed in scintillation solution and counted for radioactivity.

Method II. This assay is a modification of Method I and is used for analysis of [^3H]glucose-labelled chondroitin sulphate. Incubation mixtures were the same as those used for Method I, except for the presence of [^3H]chondroitin sulphate (10^5 d.p.m. as ^3H) as substrate. After incubation at 37°C for 1 h, the mixture was subjected to paper electrophoresis. After drying, monosaccharides and disaccharides were located by staining appropriate reference compounds with aniline hydrogen phthalate. Five radioactive bands, corresponding to *N*-acetylgalactosamine (Band I), non-sulphated disaccharides (Band II), GalNAc-4- SO_4 plus GalNAc-6- SO_4 (Band III), monosulphated disaccharides (Band IV) and GalNAc-4,6-bis SO_4 plus bis-sulphated disaccharides (Band V) respectively were obtained. Radioactive *N*-acetylgalactosamine (Band I) was eluted from the corresponding paper strips with water and further purified by paper chromatography in solvent A. Radioactive components in the other bands were isolated by successive paper chromatography in solvents B and C as previously described (Nakanishi *et al.*, 1981; Habuchi *et al.*, 1979) and were counted for radioactivity as above.

Method III. The inorganic [^{35}S]sulphate released from chondroitin [^{35}S]sulphate or its trisaccharide fragment by treatment with terminal 4-sulphatase, terminal 6-sulphatase or a mixture of the two enzymes is a measure of the sum of 4-sulphate residues in both non-reducing GalNAc-4- SO_4 and GalNAc-4,6-bis SO_4 end groups, of 6-sulphate residues in non-reducing GalNAc-6- SO_4 end group or of the sum of 4-sulphate and 6-sulphate residues in non-reducing GalNAc-4- SO_4 , GalNAc-6- SO_4 and GalNAc-4,6-bis SO_4 end groups respectively. Therefore the amount of individual end groups can be calculated from the yields of inorganic [^{35}S]sulphate in the three systems. The standard reaction mixtures contained $2\mu\text{mol}$ of sodium acetate/acetic acid buffer, pH 4.4 (or pH 3.6 when the substrate was trisaccharide), $0.15\mu\text{mol}$ of 1,4-saccharolactone (inhibitor of β -glucuronidase; see Levvy & Conchie, 1966), test sample (about $0.12\text{--}2\text{ nmol}$ as hexuronate or $1.3 \times 10^3\text{--}10^5$ d.p.m. as ^{35}S) and 10 munits of terminal 4-sulphatase (tube 1), 0.05 munit of terminal 6-sulphatase (tube 2) or 10 munits of terminal 4-sulphatase plus 0.05 munit of terminal 6-sulphatase (tube 3), in the total volume of $30\mu\text{l}$. After incubation at 37°C for 5 h, the mixtures in tubes 2 and 3 were adjusted to

pH 5.6 with $3\mu\text{l}$ of 1M-sodium acetate. Fresh terminal 6-sulphatase (0.05 munit) was added to tubes 2 and 3, and the mixtures were incubated at 37°C for 22 h (if necessary, 0.025 munit of fresh enzyme was added at 12 h). To the mixtures in tubes 1, 2 and 3 was added $0.1\mu\text{mol}$ of Na_2SO_4 (carrier) and the mixtures were subjected to paper electrophoresis. After drying, the zone corresponding to inorganic sulphate was located by staining with the BaCl_2 /Rhodizonate reagent (Schneider & Lewbart, 1956), cut out, placed in scintillation vials and counted for radioactivity.

It should be noted that the optimum desulphation rate by terminal 6-sulphatase against trisaccharide was at pH 3.6, against chondroitin sulphate from chick-embryo cartilage at pH 4.4 and against chondroitin sulphate from rat xiphisternal cartilage at pH 5.6. Therefore re-treatment of the sample with terminal 6-sulphatase at pH 5.6 (see above) was essential to ensure complete hydrolysis of 6-sulphate residues, particularly when the chondroitin sulphate from rat xiphisternal cartilage was analysed.

Method IV. The *N*-acetyl[^3H]galactosamine released from [^3H]glucose-labelled chondroitin sulphate under the influence of (1) β -*N*-acetylhexosaminidase, (2) terminal 4-sulphatase and β -*N*-acetylhexosaminidase, (3) terminal 6-sulphatase and β -*N*-acetylhexosaminidase and (4) terminal 4-sulphatase, terminal 6-sulphatase and β -*N*-acetylhexosaminidase is a measure of non-reducing GalNAc end group, of the sum of non-reducing GalNAc and GalNAc-4- SO_4 end groups, of the sum of non-reducing GalNAc and GalNAc-6- SO_4 end groups and of the sum of non-reducing GalNAc, GalNAc-4- SO_4 , GalNAc-6- SO_4 and GalNAc-4,6-bis SO_4 end groups respectively. On the other hand, the [^3H]glucuronic acid released by β -glucuronidase digestion is a measure of non-reducing GlcA end group. Therefore the amounts of five different types of end groups can be calculated from the amounts of released acetyl[^3H]galactosamine and [^3H]glucuronic acid.

The reaction mixtures contained $2\mu\text{mol}$ of sodium acetate/acetic acid buffer, pH 4.4, test sample (about 2 nmol as hexuronate or 10^5 d.p.m. as ^3H) and 30 munits of β -glucuronidase (tube 1), 12 munits of β -*N*-acetylhexosaminidase (tube 2), 10 munits of terminal 4-sulphatase and 12 munits of β -*N*-acetylhexosaminidase (tube 3), 0.05 munit of terminal 6-sulphatase and 12 munits of β -*N*-acetylhexosaminidase (tube 4) or 10 munits of terminal 4-sulphatase, 0.05 munit of terminal 6-sulphatase and 12 munits of β -*N*-hexosaminidase (tube 5), in the total volume of $30\mu\text{l}$. [Since the β -*N*-acetylhexosaminidase preparation used in the present study contained a small, but significant, amount of arylsulphatase, the enzyme was added to the reac-

tion mixture after the digestion with terminal 4-sulphatase or 6-sulphatase (or both) had been completed (5h), and incubation was continued for another 5h in the presence of $1.5\mu\text{mol}$ of PO_4^{3-} (arylsulphatase inhibitor).] 1,4-Saccharolactone (β -glucuronidase inhibitor), $0.15\mu\text{mol}$, was also added to tubes 2–5. After incubation at 37°C for 5h, $0.05\mu\text{mol}$ each of glucuronic acid and *N*-acetylgalactosamine (carriers) were added to the mixtures, and the sugars were isolated by paper chromatography in solvent A. The sugars were located by staining guide strips with the AgNO_3 reagent. The zones corresponding to glucuronic acid and *N*-acetylgalactosamine were cut out and eluted with water. Radioactive materials in the eluates were counted in a liquid-scintillation spectrometer.

When the [^3H]chondroitin sulphate from rat xiphisternal cartilage was a substrate, the buffer in tube 4 was replaced by $2\mu\text{mol}$ of sodium acetate/acetic acid buffer, pH 5.6, and enzyme treatment in tube 5 was performed sequentially as follows: (1) 5h digestion with 10 munits of terminal 4-sulphatase at pH 4.4, (2) 12h digestion with 0.025 munit of terminal 6-sulphatase at pH 5.6, (3) 1h digestion with 0.1mg of Pronase P at pH 8 (for deproteinization), (4) precipitation of the resulting chondroitin sulphate with 3 vol. of 95% (v/v) ethanol in the presence of 1.3% (w/v) of potassium acetate and (5) 4h digestion of the precipitate (dissolved in $30\mu\text{l}$ of 0.1 M-acetate buffer, pH 4.4) with 12 munits of β -*N*-acetylhexosaminidase.

Other analytical methods

Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin (fraction V) as a standard, and glucuronic acid by the method of Bitter & Muir (1962), with glucuronolactone as a standard.

Results

Purification and properties of terminal sulphatases

Sulphatases capable of hydrolysing the 4-sulphate and 6-sulphate residues respectively in non-reducing hexosamine end groups of chondroitin

sulphate would be extremely useful tools for structural studies on chondroitin sulphate chains and for investigating the biosynthesis and function of proteoglycans. We previously purified a GalNAc-4- SO_4 4-sulphatase from hen oviduct (Tsuji *et al.*, 1980) and a GalNAc-6- SO_4 6-sulphatase from quail oviduct (Nakanishi *et al.*, 1979) and showed that these enzymes catalyse the hydrolysis of sulphate of the non-reducing GalNAc-4- SO_4 and GalNAc-6- SO_4 end groups respectively of chondroitin sulphates. It was noticed in these previous studies that crude extracts of the tissues contain high β -glucuronidase and β -*N*-acetylhexosaminidase activities, which bring about further breakdown of the chondroitin sulphate chain after the initial desulphation, thereby leading to loss of specificity. In the case of the 6-sulphatase from quail oviduct, these interfering activities could readily be removed by a modification of the procedures previously described (Nakanishi *et al.*, 1979). In the case of the 4-sulphatase from hen oviduct, however, at least six steps of purification procedures were necessary to remove the interfering activities (Tsuji *et al.*, 1980). We have since examined several other sources and found that crude extracts of the quail egg-white contain a high activity of a GalNAc-4- SO_4 sulphatase with little or no β -glucuronidase activity (see also Lush & Conchie, 1966).

The three steps of purification (see the Experimental section) were enough to obtain a 4-sulphatase preparation suitable for the present purpose (Table 1).

Since previous studies had shown that the sulphatase activities towards chondroitin 4-sulphate and *p*-nitrocatechol sulphate are due to one common enzyme (Fluharty *et al.*, 1975; Tsuji *et al.*, 1980), advantage was taken in the present study of the fact that the efficiency of the purification method can be readily monitored by using *p*-nitrocatechol sulphate as a substrate. The final preparation had a specific activity of 6 units/mg of protein, compared with 2 units/mg of protein for the sulphatase from hen oviduct. On incubation for 5h with appropriate substrates, the enzyme prepar-

Table 1. Summary of purification of terminal 4-sulphatase from quail egg-white
For details see the Experimental section.

Step	Total protein (mg)	Total activity (units)	Specific activity (units/mg of protein)	Purification (-fold)	Yield (%)	β -Hexosaminidase (unit/mg of protein)
Crude enzyme solution*	36600	77	0.002	1	100	0.002
1st CM-cellulose CM-52	63.4	37	0.58	276	48	0.005
2nd CM-cellulose CM-52	13.2	35	2.8	1330	45	0.004
Sephacryl S-300*	5.2	31	6.0	2860	40	<0.001

* No β -glucuronidase activity was detected.

ation was free of β -glucuronidase and sulphatases for UDP-*N*-acetylgalactosamine 6- ^{35}S sulphate, keratan ^{35}S sulphate and heparan ^{35}S sulphate.

As shown below, the GalNAc-4-SO₄ sulphatase from quail egg-white and the GalNAc-6-SO₄ sulphatase from quail oviduct are able to distinguish non-reducing GalNAc-4-SO₄ and GalNAc-6-SO₄ end groups from each other and from the sulphated groups in the interior position. Therefore we refer to these enzymes as terminal 4-sulphatase and terminal 6-sulphatase respectively.

Samples (1300 d.p.m.) of the trisaccharide derived from chondroitin ^{35}S sulphate (source: chick-embryo epiphyses) were incubated with excess of terminal 4-sulphatase and terminal 6-sulphatase (for details of the procedures, see Method III in the Experimental section). The results in Fig. 1 show that 26% (340 d.p.m.) and 22% (285 d.p.m.) of the added radioactivity were released as inorganic ^{35}S sulphate by terminal 4-sulphatase and terminal 6-sulphatase respectively within 6 h and that thereafter no release was observed. Addition of fresh enzymes at 6 h had little effect. The results are consistent with the view that only one of the two sulphate residues in a trisaccharide molecule is released by these enzymes.

A 1300 d.p.m. portion of the trisaccharide substrate was digested with the mixture of terminal 4-sulphatase and terminal 6-sulphatase. Untreated and sulphatase-treated samples were then digested with chondroitinase ABC and the resulting products analysed by paper electrophoresis followed by paper chromatography (see Method I in the Experimental section). As shown in Table 2, chondroitinase digestion of the untreated ^{35}S sulphated trisaccharide sample demonstrated radioactive GalNAc-4-SO₄ and GalNAc-6-SO₄ derived from the non-reducing terminal and radioactive $\Delta\text{Di-4S}$ and $\Delta\text{Di-6S}$ derived from the reducing terminal. After incubation with a mixture of

terminal 4-sulphatase and terminal 6-sulphatase, there was a complete disappearance of radioactive GalNAc-4-SO₄ and GalNAc-6-SO₄, with no detectable change in the amount of radioactive unsaturated disaccharides. It is clear therefore that

Fig. 1. Time course of ^{35}S sulphate release from trisaccharide ^{35}S sulphate by terminal 4- and 6-sulphatases. Each incubation mixture containing 1300 d.p.m. of trisaccharide ^{35}S sulphate and either 10 munits of terminal 4-sulphatase (●) or 0.05 munit of terminal 6-sulphatase (○) was incubated for the indicated periods (see Method III in the Experimental section). Radioactive sulphate released was isolated by paper electrophoresis. At the time indicated by the arrows, 5 munits of terminal 4-sulphatase or 0.03 munit of 6-sulphatase was added to the corresponding tube, and the reaction allowed to continue for an additional 4 h.

Table 2. Chondroitinase ABC digestion of ^{35}S sulphated trisaccharide before or after treatment with a mixture of terminal 4-sulphatase and terminal 6-sulphatase for 6 h

For details see the text. Triplicate assays showed a coefficient of variation of 1–3%.

Product from chondroitinase digestion	Substrate ...	Radioactivity			
		Untreated trisaccharide		Sulphatase-treated trisaccharide	
		(d.p.m.)	(% of that added)	(d.p.m.)	(% of that added)
$\Delta\text{Di-4S}$		395	30	390	30
$\Delta\text{Di-6S}$		255	20	255	20
$\Delta\text{Di-4,6-bisS}$		Trace*	<0.8	Trace*	<0.8
GalNAc-4-SO ₄		335	26	0	0
GalNAc-6-SO ₄		285	22	0	0
GalNAc-4,6-bisSO ₄		15	1.2	0	0

* Less than 10 d.p.m.

sulphate release was exclusively from the non-reducing terminal.

Analysis of chondroitin [35 S]sulphate from chick-embryo epiphyses

A 10^5 d.p.m. portion of the chondroitin [35 S]sulphate isolated from chick-embryo epiphyses was incubated with terminal 4-sulphatase or terminal 6-sulphatase or both (Method III). The results in Fig. 2 show that maximal release corresponded to 2.05% (by terminal 4-sulphatase) and 0.28% (by terminal 6-sulphatase) of the added radioactivity. When, however, the same substrate was incubated with a mixture of terminal 4-sulphatase and terminal 6-sulphatase, 2.64% of the added radioactivity was released as inorganic [35 S]sulphate. Apparently, this amount of sulphate release is in excess of that ($2.05 + 0.28\%$) expected if the non-reducing end groups contain only GalNAc-4-SO₄ and GalNAc-6-SO₄ and if the two sulphatases act independently on these groups. The difference, 0.31%, can be taken as indicating the occurrence in the non-reducing termini of a third group, GalNAc-4,6-bisSO₄, because the 6-sulphate residue in this group is susceptible to terminal 6-sulphatase only after removal of the adjacent 4-sulphate residue by terminal 4-sulphatase (Nakanishi *et al.*, 1979). Thus, although the sulphate released by terminal 6-sulphatase represents only the GalNAc-6-SO₄ end group, the sulphate released by terminal 4-sulphatase represents both GalNAc-4-SO₄ and the 4-sulphate group of GalNAc-4,6-bisSO₄. Since the sulphate released by the mixture of the two sulphatases should represent all the sulphated end groups (GalNAc-4-SO₄, GalNAc-6-SO₄ and GalNAc-4,6-bisSO₄), and since specific radioactivities of the two sulphate residues of non-reducing terminal GalNAc-4,6-bisSO₄ were almost the same (as ascertained by the method of Nakanishi *et al.*, 1981), calculations can be made from the following equations:

$S_3 - S_2 - S_1$ = amount of non-reducing GalNAc-4,6-bisSO₄ end group, expressed as radioactivity of one of the two sulphate residues

S_2 = amount of non-reducing GalNAc-6-SO₄ end group, expressed as radioactivity of the sulphate residue

$S_1 - (S_3 - S_2 - S_1)$ = amount of non-reducing GalNAc-4-SO₄ end group, expressed as radioactivity of the sulphate residue

where S_1 , S_2 and S_3 represent radioactivity (d.p.m.) of [35 S]sulphate released by terminal 4-sulphatase, by terminal 6-sulphatase and by the mixture of the two sulphatases respectively.

A second portion (10^5 d.p.m.) of the chondroitin [35 S]sulphate from chick-embryo epiphyses was digested with chondroitinase ABC, and the resulting products were isolated by paper electrophoresis followed by paper chromatography.

Fig. 2. Time course of [35 S]sulphate release from chondroitin [35 S]sulphate (source: chick-embryo cartilage) by terminal 4-sulphatase or terminal 6-sulphatase, or both. Each incubation mixture containing 10^5 d.p.m. of chondroitin [35 S]sulphate and 10 munits of terminal 4-sulphatase (●), 0.05 munit of terminal 6-sulphatase (○), or both (△), was incubated for the indicated periods (see Method III in the Experimental section). Radioactive sulphate released was isolated by paper electrophoresis. At the time indicated by the arrows, 5 munits of terminal 4-sulphatase or 0.03 munit of 6-sulphatase, or both, was added to corresponding tube, and the reaction allowed to continue for an additional 2 h. The horizontal broken line indicates the sum of the maximum amounts of [35 S]sulphate released by terminal 4-sulphatase and terminal 6-sulphatase respectively.

About 98% of the total radioactivity was recovered as the products shown in Table 3. The amounts of the monosaccharide products representing non-reducing ends are slightly higher than those estimated by the sulphatase method, perhaps owing to partial degradation of 35 S-labelled unsaturated disaccharides (from the interior portion) to the corresponding monosaccharides during the analytical procedures. At any rate, it is clear that

Table 3. *Chondroitinase ABC digestion of chondroitin [³⁵S]sulphate from chick-embryo epiphyses*

For details see the text. About 2% of the added radioactivity (10⁵d.p.m.) was found in fractions other than the six fractions listed here. Triplicate assays showed a coefficient of variation of 2–4%.

Product from chondroitinase digestion	Radioactivity	
	(d.p.m.)	(% of that added)
From interior portion		
ΔDi-4S	50 150	52.7
ΔDi-6S	44 620	46.9
ΔDi-4,6-bisS	460	0.48
Sum	95 230	100
From non-reducing end		
GalNAc-4-SO ₄	1970	70.8
GalNAc-6-SO ₄	310	11.2
GalNAc-4,6-bisSO ₄	500	18.0
Sum	2780	100

about 89% of the [³⁵S]sulphate residues of non-reducing ends are in the 4- and 4,6-bis-sulphated GalNAc forms, whereas the proportion of such forms in the interior portion is only 53%.

Analysis of [³H]glucose-labelled chondroitin sulphate from chick-embryo epiphyses

An attempt was made to modify the sulphatase method to allow the end-group assay of [³H]-glucose-labelled chondroitin sulphate. The procedure is based on the measurement of the [³H]-glucuronic acid and acetyl[³H]galactosamine formed by the following reactions:

where R_{1–5} indicate the interior portions of chondroitin sulphate chains. Calculations can be made from the following equations:

$$\begin{aligned}
 U &= \text{amount of non-reducing GlcA end group, expressed as radioactivity} \\
 A_1 &= \text{amount of non-reducing GalNAc end group, expressed as radioactivity} \\
 A_2 - A_1 &= \text{amount of non-reducing GalNAc-4-SO}_4 \text{ end group, expressed as radioactivity} \\
 A_3 - A_1 &= \text{amount of non-reducing GalNAc-6-SO}_4 \text{ end group, expressed as radioactivity} \\
 A_4 - A_3 - A_2 + A_1 &= \text{amount of non-reducing GalNAc-4,6-bisSO}_4 \text{ end group, expressed as radioactivity}
 \end{aligned}$$

where *U* represents radioactivity of glucuronic acid released by β-glucuronidase, and A_{1–4} represent radioactivities of N-acetylgalactosamine released by β-N-acetylhexosaminidase, by terminal 4-sulphatase and β-hexosaminidase, by terminal 6-sulphatase and β-N-acetylhexosaminidase and by terminal 4-sulphatase, terminal 6-sulphatase and β-N-acetylhexosaminidase respectively (for details of the procedures, see Method IV in the Experimental section).

Table 4 shows the results of such analyses of the [³H]chondroitin sulphate isolated from chick-embryo epiphyses that had been incubated with [³H]glucose: the percentages of chains ending in GlcA, GalNAc, GalNAc-4-SO₄, GalNAc-6-SO₄ and GalNAc-4,6-bisSO₄ are 24, 0, 57, 10 and 9 respectively. Analysis of the same sample by the chondroitinase method (Method II) gave a consistent result, except that the amounts of the monosaccharide products from chondroitinase digestion were slightly higher than those expected from the results of the sulphatase assay (Method IV) (for the explanation see above). The results, taken together, indicate that the proportion of 4- and 4,6-bis-sulphated GalNAc units is far lower in the interior portion than in the non-reducing end.

Analysis of newly synthesized chondroitin sulphate from rat xiphisternal cartilages

The same procedures were applied to the chondroitin sulphate isolated from rat xiphisternal cartilages that had been incubated with either [³⁵S]sulphate or [³H]glucose. The results (Table 5) reveal the end groups to be composed principally

of GalNAc-4-SO₄ and GalNAc-4,6-bisSO₄, and further suggest that the presence of a high proportion of 4-sulphated and 4,6-bis-sulphated GalNAc

Table 4. Analysis of [3H]glucose-labelled chondroitin sulphate from chick-embryo epiphyses

For details see the text. Triplicate assays showed a coefficient of variation 1–4%. Abbreviation: N.D., not determined.

Sugar unit	Radioactivity			
	Chondroitinase method		Sulphatase method	
	(d.p.m.)	(% of that added)	(d.p.m.)	(% of that added)
Interior portion				
GlcA-GalNAc	16900	17.4	N.D.	N.D.
GlcA-GalNAc-4-SO ₄	47170	48.6	N.D.	N.D.
GlcA-GalNAc-6-SO ₄	32750	33.8	N.D.	N.D.
GlcA-GalNAc-4,6-bisSO ₄	200	0.2	N.D.	N.D.
Sum	97020	100	N.D.	N.D.
Non-reducing end				
GlcA	N.D.	N.D.	350	24.3
GalNAc	130	8.3	0	0
GalNAc-4-SO ₄	920	59.0	820	57.0
GalNAc-6-SO ₄	280	18.0	140	9.7
GalNAc-4,6-bisSO ₄	230	14.7	130	9.0
Sum	1560	100	1440	100

residues is a common characteristic of the termini of newly synthesized chondroitin sulphate chains.

Discussion

The sulphation pattern and molar ratio of major repeating disaccharide units in chondroitin sulphate can most easily be determined by treatment of the chondroitin sulphate with chondroitinase ABC or AC followed by separation, identification and measurement of the released unsaturated disaccharides (Yamagata *et al.*, 1968). In principle, this method is also applicable to the analysis of a terminal structure. Hascall *et al.* (1972) and Oike *et al.* (1980) found that, when a proteoglycan was digested with chondroitinase ABC or AC, the disaccharide unit closest to the linkage oligosaccharides resisted digestion with the former, but was removed by the latter. Taking advantage of this difference in specificity, Oike *et al.* (1980) have shown that the *N*-acetylgalactosamine in this particular region is sulphated predominantly at position 4. On the other hand, Sjöberg *et al.* (1973) demonstrated that, when an oligosaccharide from chondroitin sulphate was digested with chondroitinase ABC or AC, the non-reducing terminal disaccharide (if a glucuronic acid is terminal) or monosaccharide (if a sulphated *N*-acetylgalactosamine is terminal) was released as such, whereas the disaccharide units in the remaining portion were released as unsaturated disaccharides. Determination of these products by means of paper or column chromatography has been shown to be useful for proving the proportion and structure of the terminal sugar (for example, see Table 2). This

technique could also be applied to the analysis of the non-reducing terminal structure of chondroitin sulphate of higher M_r . In this case, however, the amount of terminal sugars is extremely small relative to that of the disaccharide units in internal sequence, so that separation of the released monosaccharide and saturated disaccharide from unsaturated disaccharides by chromatography is much more laborious than in the analysis of oligosaccharide. In addition, artificial formation of sulphated *N*-acetylhexosamine, owing to degradation of sulphated unsaturated disaccharides, has often been observed (H. Inoue, R. Nakayama, K. Otsu & Y. Nakanishi, unpublished work).

The results described here demonstrate the validity of the sulphatase-utilizing method as a means of analysing the terminal structure of chondroitin sulphate. This method has the advantage of simplicity with respect to the process of product determination and is less subject to interference from the predominant sugar components in the interior portion. One drawback of the method is that the conditions (e.g. pH) required for completion of the reaction with terminal 6-sulphatase are rigorous and may vary depending on the chemical nature of substrate. It is necessary therefore that the optimal pH for terminal 6-sulphatase is determined beforehand on the sample to be analysed. If the optimal pH for terminal 6-sulphatase differs from that for terminal 4-sulphatase on the same substrate, treatments with these enzymes must be done stepwise under different conditions, as described in the Experimental section.

Several reports (e.g. Faltynek & Silbert, 1978; Kimata *et al.*, 1973) have shown that the chon-

Table 5. *Analysis of [³⁵S]sulphate-labelled chondroitin sulphate and [³H]glucose-labelled chondroitin sulphate from rat xiphisternal cartilage*
 For details of the procedures see Methods I-IV in the Experimental section. Triplicate assays showed a coefficient of variation of 1-4%. Abbreviation: N.D., not determined.

Sugar unit	Radioactivity					
	Chondroitin [³⁵ S]sulphate			[³ H]Chondroitin sulphate		
	Method I		Method III	Method II		Method IV
	(d.p.m.)	(% of that added)	(d.p.m.)	(% of that added)	(d.p.m.)	(% of that added)
Interior portion						
GlcA-GalNAc	N.D.	N.D.	N.D.	9010	9.4	N.D.
GlcA-GalNAc-4-SO ₄	82350	95.7	N.D.	84080	88.1	N.D.
GlcA-GalNAc-6-SO ₄	1650	1.9	N.D.	1540	1.6	N.D.
GlcA-GalNAc-4,6-bisSO ₄	2050	2.4	N.D.	860	0.9	N.D.
Sum	86050	100	N.D.	95490	100	N.D.
Non-reducing end						
GlcA	N.D.	N.D.	N.D.	510	15.3	230
GalNAc	N.D.	N.D.	N.D.	40	1.2	10
GalNAc-4-SO ₄	2920	31.6	38.3	1360	40.9	1110
GalNAc-6-SO ₄	210	2.3	0.5	90	2.7	10
GalNAc-4,6-bisSO ₄	6120	66.1	61.2	1330	39.9	940
Sum	9250	100	100	3330	100	2300
						100

droitin sulphate chains from chick-embryo cartilage are hybrid structures containing both 4-sulphated and 6-sulphated disaccharides in the same chain. The results described in the present paper have revealed further complexity of the structure of the chondroitin sulphate chains. The data in Table 4 show that the [^3H]chondroitin sulphate synthesized by chick-embryo cartilage has a 4-sulphate/6-sulphate ratio of 10:7 as a whole and consists of a mixture of chains, with some terminating in glucuronic acid (24% of the ^3H at non-reducing termini) and 3 times as many terminating in sulphated *N*-acetylgalactosamine. There was little or no chain terminating in non-sulphated *N*-acetylgalactosamine, suggesting that the introduction of an *N*-acetylgalactosamine into the non-reducing end must immediately be followed by the addition of a glucuronic acid (to position 3) or a sulphate (to position 4 or 6), so that chains ending in non-sulphated *N*-acetylgalactosamine could not accumulate to more than a trace amount. Of the chains terminating in sulphated *N*-acetylgalactosamine, more than 87% bear a sulphate at position 4. This type of modification at the terminal is even more prominent in the chondroitin sulphate synthesized by rat xiphisternal cartilage, where over 95% of the terminal *N*-acetylgalactosamine units are sulphated at position 4. The results are consistent with the findings by Rodén *et al.* (1972) that oligosaccharides ending in *N*-acetylgalactosamine 4-sulphate, when incubated with UDP-[^{14}C]glucuronic acid in the presence of a 100000g pellet from a homogenate of chick-embryo cartilage, were not capable of accepting a glucuronic acid, in contrast with oligosaccharides ending in non-sulphated or 6-sulphated *N*-acetylgalactosamine, which were active as acceptors for glucuronosyl transfer. Our data therefore give further support to the hypothesis that the chain termination is due, at least in part, to sulphation at position 4 of the terminal sugar so as to prevent the addition of a glucuronic acid for further elongation.

A unique feature of the chondroitin sulphate chains treated in this paper is the presence of 4,6-bis-sulphated *N*-acetylgalactosamine at the non-reducing terminal. The occurrence of this structure was previously predicted when we found in quail oviduct a sulphotransferase capable of catalysing the incorporation of sulphate into position 6 of a non-reducing *N*-acetylgalactosamine 4-sulphate end group of chondroitin sulphate (Nakanishi *et al.*, 1981). This enzyme (termed 'terminal 6-sulphotransferase') can be separated from a 6-sulphotransferase present in the same tissue that catalyses the incorporation of sulphate into the interior position of non-sulphated chondroitin. The acceptor specificities of terminal 6-sulphotransferase suggest that the enzyme is suited for the synthesis

of the 4,6-bis-sulphated terminal in cartilages. Our preliminary experiments have indeed shown that both chick-embryo and rat xiphisternal cartilage contain a terminal 6-sulphotransferase, the specificity of which is very similar to that isolated from quail oviduct (H. Inoue, K. Otsu, Y. Nakanishi & S. Suzuki, unpublished work). The biological role of bis-sulphated terminal structure in cells or tissues remains to be determined, but its appearance in various chondroitin sulphates of different origins is indicative of an important function.

In view of the results described here, one may assume that all the hexosamine and uronic acid units in a chondroitin sulphate chain can be removed sequentially by treatment with β -glucuronidase, terminal 4-sulphatase, β -*N*-acetylhexosaminidase, and terminal 6-sulphatase, provided that no 'unusual' unit (e.g. a sulphated hexuronate, see Suzuki *et al.*, 1968) exists in the chain. Preliminary experiments have been carried out to test the workability of this approach. The incubation mixture (30 μl) contained 2 μmol of acetate buffer, pH 4.4, 5×10^5 d.p.m. of chondroitin [^{35}S]sulphate, 10 munits of terminal 4-sulphatase plus 0.05 munit of terminal 6-sulphatase, 30 munits of β -glucuronidase, 10 munits of β -*N*-acetylhexosaminidase and a small amount of toluene. Incubation was carried out at 37°C for 48 h; during that time additional enzymes (the same amounts as above) were added at 24 h. Under these conditions, about 60% of the added radioactivity were released as inorganic sulphate. Whether the apparent insusceptibility of the remaining portion of the chain is due to the presence of an unusual sugar unit or to interference from the accumulation of reaction products is not known, but the above results suggest that further refinement of the terminal sulphatase/glycosidase method will provide a methodology necessary to characterize the fine structure of chondroitin sulphate chains.

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