The Distribution of Sulphate Residues in the Chondroitin Sulphate Chain

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1. Electrophoresis of chondroitin sulphate, before and after partial degradation with testicular hyaluronidase, revealed charge heterogeneity of the degraded but not of the intact polymer. 2. Hyaluronidase-treated chondroitin sulphate was fractionated by gel chromatography. Two subfractions which were essentially monodisperse with regard to molecular weight (values of 8600 and 4800, respectively) were separated further by chromatography on Dowex 1. The resulting subfractions differed considerably with respect to their sulphate/disaccharide molar ratios. 3. Amino acid and neutral-sugar analyses of the Dowex 1 subfractions showed that the less sulphated fragments contained the carbohydrate-protein linkage region, whereas the high-sulphated fragments essentially lacked this constituent. It was concluded that chondroitin sulphate contains relatively less sulphate in the vicinity of the carbohydrate-protein linkage region than in the more peripheral portion of the polysaccharide chain.

The glycosaminoglycuronans of mammalian tissues are all polymers consisting of different numbers of uronosylhexosamine disaccharide repeating units (for detailed accounts on their structures see, e.g., Brimacombe & Webber, 1964; Muir, 1964; Fransson, 1970; Lindahl & Axelsson, 1971). Most of these polymers are sulphated and occur in the native state as proteoglycans composed of several polysaccharide chains linked to a polypeptide backbone. The carbohydrate-protein linkage region of these macromolecules involves a glucuronylgalactosylgalactosylxylosyl-O-serine sequence, which is apparently identical for all proteoglycans of this group (Rodén, 1968; Lindahl & Rodén, 1971).

The structures of the various glycosaminoglycuronans have most commonly been depicted by referring to the relevant repeating disaccharide unit, thus indicating a large measure of uniformity within each polymer. However, recent studies have shown that several of these compounds display considerable molecular heterogeneity, involving such structural parameters as chain length (Wasteson, 1971a), composition (Fransson, 1970; Lindahl & Axelsson, 1971) and degree of sulphation (Meyer, Davidson, Linker & Hoffman, 1956; Calatroni & Di Ferrante, 1968; Mathews, Duh & Person, 1962; Kawai, Seno & Anno, 1966).

With refinements in the techniques of isolation, the existence of galactosaminoglycuronans with sulphate/disaccharide ratios deviating from the usual equimolar one, has become increasingly apparent. Oversulphated as well as undersulphated

specimens have been described (see the references above); further, the variability in sulphate distribution has been demonstrated by the isolation of oligosaccharides with different sulphate/hexosamine ratios from enzymic digests of several preparations (Mathews & Decker, 1968; Suzuki *et al.* 1968; Harada, Murata, Fujiwara & Furuhashi, 1969). No information has been available about the distribution of sulphate in the galactosaminoglycuronan chain. Generally, sulphate residues may be more-or-less evenly distributed along the polymer or they may be accumulated at certain regions of the molecule.

The distribution of sulphate groups in chondroitin sulphate from bovine nasal cartilage is the subject of the present study. Evidence is presented indicating that the sulphate density is lower in the vicinity of the carbohydrate-protein linkage region than in the remainder of the molecule.

EXPERIMENTAL

Materials. The isolation, fractionation and partial degradation of chondroitin sulphate from bovine nasal septa were described in detail previously (Wasteson, 1971b); for clarity a brief recapitulation will be given.

After proteolytic digestion of the tissue the liberated polysaccharide was isolated by precipitation with cetylpyridinium chloride and was then converted into the sodium salt. The weight-average ($\bar{M}_w$) and number-average ($\bar{M}_n$) molecular weights of this preparation were 19800 and 15500 respectively (Wasteson, 1971a). The sulphate/uronic acid molar ratio was 1.0. One portion of this material was fractionated by gel chromatography

into a number of subfractions (fractions 1–6). Another portion of the same preparation was digested with testicular hyaluronidase until a 50% decrease in the viscosity number was attained. The digest was then fractionated by gel chromatography into fractions 7–11. The detailed characterization of fractions 1–11 was described previously (Wasteson, 1971a,b); some relevant analytical data are given in Table 1.

Analytical methods. Uronic acid was determined by an automated version (Balazs, Berntsen, Karossa & Swann, 1965) of the procedure described by Bitter & Muir (1962). Sulphate was determined by the method of Antonopoulos (1962); each determination was carried out in quadruplicate. Amino acid and amino sugar analyses were performed with a Bio-Cal model BC-200 amino acid analyser after hydrolysis of samples *in vacuo* with 6M-HCl at 110°C for 24 h.

Neutral sugars were determined as alditol acetates by g.l.c. (Lindahl, 1970). Before analysis samples were hydrolysed with 2M-trifluoroacetic acid at 100°C for 2 h; these conditions are optimum for the determination of xylose, but the liberation of galactose from the carbohydrate–protein linkage region is incomplete (Lindahl, 1970).

Electrophoresis of polysaccharides was carried out on strips of cellulose acetate in 0.1M-HCl (1.9 V/cm for 2 h) by the method of Wessler (1971). Since the carboxyl groups of glycosaminoglycuronans are undissociated in this medium, the migration rate of any individual polysaccharide depends exclusively on its degree of sulphation.

Analytical gel chromatography of chondroitin sulphate on Sephadex G-200 was performed by the method of Wasteson (1971a).

Chromatography on Dowex 1 of hyaluronidase-treated chondroitin sulphate. Fractions 9 and 10 (100 and 93 mg respectively) of the hyaluronidase-treated chondroitin sulphate were each dissolved in 10 ml of water and the solutions were applied to columns (2 cm × 15 cm) of Dowex 1 (X2; Cl[−] form; 200–400 mesh). The columns were washed with 150 ml of water and were then eluted in a stepwise manner with 150 ml portions of the salt solutions

indicated in Table 2. The corresponding effluent fractions were concentrated in a rotatory evaporator and finally desalted by passage through a column of Sephadex G-25.

RESULTS

Electrophoresis of chondroitin sulphate. The electrophoresis patterns of the various chondroitin sulphate fractions are shown in Fig. 1. Each of the fractions 1–6 (representing the entire elution profile obtained on gel chromatography of the undegraded polysaccharide) (Wasteson, 1971b) showed one distinct spot. With the exception of fraction 6 (the low-molecular-weight extreme of this profile) all fractions had migrated like the unfractionated polysaccharide preparation. In contrast, fractions 7–11, which had been obtained by gel chromatography of hyaluronidase-treated chondroitin sulphate, were markedly heterogeneous, showing both high- and low-sulphated components not detected in the undegraded fractions. The heterogeneity

Table 1. *Analytical data of chondroitin sulphate fractions*

Fractions 5 and 6 are the two most low-molecular-weight subfractions of the undegraded polysaccharide preparation, whereas fractions 7–11 account for the total hyaluronidase-treated material (Wasteson, 1971b). See Wasteson (1971a,b) for further details of molecular weights. Each fraction may be regarded as close to monodisperse with respect to molecular weight.

Fraction	Sulphate/uronic acid molar ratio	Molecular weight
5	1.07	13900
6	—	11100
7	0.98	18300
8	0.96	12400
9	0.97	8600
10	0.95	4800
11	—	2400

Fig. 1. Electrophoresis in 0.1M-HCl of chondroitin sulphate fractions. CS, unfractionated, undegraded material; fractions 5 and 6, subfractions of undegraded material; fractions 7–11, subfractions of hyaluronidase-treated material; fractions 10–0.5M to 10–1.7M, subfractions of fraction 10, obtained by chromatography on Dowex 1. The electrophoresis patterns of fractions 1–4 were identical with that of fraction 5. The subfractions of fraction 9 migrated analogously to those of fraction 10. Since the suspended-strip method was used the relationship between migration distance and degree of sulphation is non-linear (Wessler, 1971).

was more pronounced in the lower-molecular-weight fractions 9–11 than in the higher-molecular-weight fractions 7 and 8.

Chromatography on Dowex 1 of hyaluronidase-treated fractionated chondroitin sulphate. Two fractions (nos. 9 and 10) of the hyaluronidase-treated chondroitin sulphate were further separated by chromatography on Dowex 1. By stepwise elution with sodium chloride solutions of increasing concentration, subfractions were obtained, the yields of which are given in Table 2. The total amounts of uronic acid in the subfractions of fractions 9 and 10 corresponded to 95% and 98%, respectively, of the uronic acid contents of the parent fractions.

Previous analyses of fractions 1–11 indicated chondroitin sulphate as the only polysaccharide present in significant amounts (Wasteson, 1971b). Analysis of the subfractions of fractions 9 and 10 gave essentially similar results (Table 2). Amino acid analysis showed serine to be the predominant amino acid, with smaller amounts of glycine, glutamic acid, aspartic acid, proline, alanine and threonine. This pattern, typical of proteolytically degraded chondroitin sulphate (Muir, 1958; Gregory Laurent & Rodén, 1964), applied to all subfractions except the minor fraction 9–3.0M, which contained proline (molar ratio to glucuronic acid, 1/4), glutamic acid and threonine in amounts exceeding those of serine. Although the glucuronic acid/galactosamine molar ratio of this fraction was 0.9, considerable amounts of glucosamine were also detected (Table 2), suggesting the presence of keratan sulphate. Judging from the relative amounts of xylose and galactose, keratan sulphate appeared to be present, in small amounts, also in the more sulphated subfractions of fraction 10 (Table 2). However, this contaminant would seem to be insignificant in terms of its contribution to the total sulphate contents of the fractions; further, it should not affect the xylose/uronic acid ratios.

The subfractions of fractions 9 and 10 showed appreciable variation with respect to sulphate contents, as shown by electrophoresis (Fig. 1) as well as by direct sulphate determination (Table 2). As expected, fractions eluted at higher ionic strength from the ion-exchange resin contained more sulphate per repeating disaccharide unit than did those eluted at lower ionic strength.

Distribution of carbohydrate–protein linkage region. The distribution between the subfractions of fractions 9 and 10 of the carbohydrate–protein linkage region may be estimated from the serine/uronic acid ratios (Table 2). These ratios were considerably higher in the low-sulphated than in the high-sulphated subfractions. Since each of the various subfractions showed the same molecular weight on gel chromatography as did the corre-

sponding parent fractions, the low-sulphated subfractions apparently consisted of relatively more chains containing the carbohydrate–protein linkage region than did the high-sulphated ones. In the following passages, chondroitin sulphate chains containing the carbohydrate–protein linkage region will be denoted 'linkage fragments', as opposed to 'peripheral fragments' lacking this region.

The presence of xylose is a characteristic feature of the glycosaminoglycuronan–protein linkage region, and therefore a specific quantitative measurement of linkage fragments could be obtained by determining the amounts of this pentose in the chondroitin sulphate subfractions. Only fraction 10 was subjected to analysis, as its constituent subfractions covered a wider range of sulphate/disaccharide ratios than did those of fraction 9. The results revealed an inverse relation between the xylose and the sulphate contents of the subfractions (Table 2). As the molecular weights of the subfractions were all identical with that of the parent fraction 10 (Table 1) the number of xylose residues per polysaccharide chain, and hence, the proportion of linkage fragments within each subfraction could be calculated (see Table 2 for further details). The two high-sulphated subfractions, 10–1.3M and 10–1.5M, thus consisted of more than 95% of peripheral fragments, whereas the low-sulphated subfractions, 10–0.5M and 10–0.7M, contained linkage fragments exclusively.

By relating the total yield of polysaccharide in each subfraction to the corresponding ratio of linkage fragments to peripheral fragments the absolute amounts of the two types of fragments were estimated. From these values the distribution of linkage fragments and peripheral fragments between the subfractions of fraction 10 were calculated (Fig. 2). As shown, 42% of the linkage fragments but only 5% of the peripheral fragments had an average sulphate/disaccharide ratio of 0.82 or less, whereas 13% and 87% of the respective fragment types showed ratios of 0.95 or more.

DISCUSSION

The electrophoresis patterns shown in Fig. 1 demonstrate that treatment of chondroitin sulphate with an endoglycosidase (hyaluronidase) yielded fragments of varying charge density. Since the non-degraded, parent polysaccharide appeared homogeneous with respect to charge density (Fig. 1) this observation suggested a non-randomness in the distribution of sulphate residues along the polysaccharide chain.

To localize the low- and high-sulphated sections of the molecule, two essentially monodisperse (with regard to molecular weight) fractions of

Table 2. *Analytical data of fractions of chondroitin sulphate obtained after partial degradation with hyaluronidase*

Fractions are denoted by the molarities of the NaCl solutions used for their elution from Dowex 1. 'Xylose/chondroitin sulphate molar ratio' is calculated from the xylose/uronic acid ratios and expressed as residues per polysaccharide chain (mol. wt. 4800). Calculations were based on the assumption that the chondroitin sulphate molecules in fraction 10 comprised on the average 9.6 repeating disaccharide units (mol. wt. 501, corresponding to the sodium salt of a disaccharide containing one sulphate residue). The presence in fractions 10-0.5M and 10-0.7M of xylose in excess of one residue per polysaccharide chain may partly reflect the contribution of the galactosylgalactosylxylosyl-peptide moiety to the molecular weight.

Fraction	Yield (mg of glucuronic acid)	Glucosamine/total hexosamine (%)	Sulphate/uronic acid molar ratio	$10^3 \times$ Serine/uronic acid molar ratio	$10^3 \times$ Xylose/uronic acid molar ratio	$10^3 \times$ Galactose/ uronic acid molar ratio	Xylose/chondroitin sulphate molar ratio
9-0.75M	0.3	9	0.84	100	—	—	—
9-1.0M	2.1	7	0.90	93	—	—	—
9-1.25M	10.4	2	0.90	44	—	—	—
9-1.5M	13.7	2	1.14	7.1	—	—	—
9-3.0M	0.9	62	2.80	—	—	—	—
10-0.5M	0.5	7	—	160	150	180	1.4
10-0.7M	0.7	4	0.65	170	120	170	1.1
10-0.9M	2.1	3	0.82	140	48	73	0.5
10-1.1M	6.0	1	0.92	56	38	76	0.4
10-1.3M	13.4	1	0.95	6.3	4.2	13	0.04
10-1.5M	2.7	1	1.10	11	1.9	18	0.02
10-1.7M	0.4	11	1.29	59	—	—	—

Fig. 2. Distribution of linkage fragments (●) and peripheral fragments (○) between the subfractions of fraction 10. Each subfraction is indicated by the molarity of the corresponding Dowex 1 eluent and by its sulphate/disaccharide molar ratio. The amounts of each fragment are expressed as a percentage of the total amounts, in fraction 10, of the respective fragment type.

hyaluronidase-treated chondroitin sulphate (fractions 9 and 10) were separated further by chromatography on Dowex 1. Both fractions, which displayed considerable charge heterogeneity on electrophoresis (Fig. 1), yielded subfractions of different sulphate contents (Fig. 1; Table 2). Analysis of these subfractions with respect to components of the carbohydrate-protein linkage region showed, as expected, a larger proportion of linkage fragments in the less extensively degraded fraction 9 than in fraction 10. From the results given in Table 2 it may be calculated that such fragments accounted for about 20% of fraction 10; assuming a serine/xylose ratio of 1.5 for the subfractions of fraction 9 the corresponding value for this fraction would be approx. 40%.

The distribution of the two types of polysaccharide fragments between the subfractions of fraction 10 (Fig. 2) shows that linkage fragments are generally less sulphated than peripheral ones. The serine contents of the subfractions of fraction 9 (Table 2) indicate a similar predominance of low-sulphated linkage fragments. It is concluded that the chondroitin sulphate molecule contains less sulphate in the vicinity of the carbohydrate-protein linkage region than in the more peripheral portions of the chain.

Previous investigations have demonstrated that the two glucosaminoglycans, heparin and heparan sulphate, both exhibit a relative lack of sulphate at the carbohydrate-protein linkage region (Lindahl, 1966; Cifonelli, 1968). The present study provides evidence for a similar phenomenon involving the galactosaminoglycan chondroitin sulphate.

Although the mechanism(s) responsible for these findings are unknown a hypothesis suggested by Lindahl (1966) may deserve further attention. The biosynthesis of glycosaminoproteoglycans is believed to occur by stepwise addition of monosaccharide units to a preformed protein acceptor, thus yielding a non-sulphated proteoglycan, which is subsequently sulphated [see, e.g., reviews by Stoolmiller & Dorfman (1969) and by Rodén (1971)]. It seems conceivable that the polysaccharide portion immediately adjacent to the protein core may be relatively inaccessible to the sulphotransferases concerned, and hence remains low-sulphated. This hypothesis appears attractive, as it may explain a structural feature shared by otherwise disparate polysaccharides; however, its validity remains to be established.

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