Non-electrostatic factors govern the hydrodynamic properties of articular cartilage proteoglycan

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The hydrodynamic frictional resistance to water flow exerted by articular cartilage proteoglycan is shown to be similar to that of proteoglycan isolated from Swarm rat chondrosarcoma, and independent of the state of aggregation of the proteoglycan. Frictional resistance is dependent, however, on the chain segments of the constituent chondroitin-sulphate and keratan-sulphate chains of the proteoglycan. Frictional resistance offered by chondroitin sulphate was independent of pH over the range 3.2–8.7. This confirms previous studies, associated with varying ionic strength and chemical modification of ionic groups of chondroitin sulphate, which showed that the frictional resistance offered by this molecule is independent of electrostatic factors. Water-structure-breaking and hydrogen-bond-breaking solvents were also without major effects on the flow resistance offered by chondroitin sulphate. An overall secondary structure of chondroitin sulphate was not evident, as it showed no significant difference to dextran in terms of its temperature dependence of relative viscosity. Local regions of rigid secondary structure, as manifested through inter-residue hydrogen bonding between sugar residues, is likely to control flow resistance as periodate-oxidized chondroitin sulphate and periodate-oxidized and reduced preparations showed a significant decrease in their frictional resistance to water.

INTRODUCTION

The biomechanical properties of articular cartilage, particularly its compressive resistance, are determined, in part, by factors controlling fluid movement within, and out of, the tissue. These factors can be considered in two groups; (1) the pressure balance on the tissue fluid associated with the net pressure provided by the external mechanical load and the internal osmotic pressure of the tissue, and (2) the frictional resistance offered by the tissue to flow induced by the net pressure gradients (Comper, 1991). Compressive resistance of cartilage will be a function of both these factors. It has been established previously that the osmotic pressure of proteoglycan is important for pressure-balance considerations (Maroudas, 1975b; Urban et al., 1979). It is now evident, too, that the frictional resistance to fluid flow (or hydrodynamic resistance) provided by the cartilage proteoglycan is equally important. It was originally shown by Maroudas (1975b) that cartilage permeability correlated with the proteoglycan content of the tissue. More recently, it has been shown that the flow resistance of the proteoglycan could account for cartilage flow permeability (Zamparo and Comper, 1989).

This study extends investigations on the factors that control this frictional resistance or viscous dissipation of fluid over the proteoglycan molecule. We have previously established that proteoglycans similar to the large, aggregating-articular-cartilage proteoglycans, namely those isolated from Swarm rat chondrosarcoma, provide the highest resistance per unit mass or anhydrous volume of a wide range of polymers which we have tested. The major structural difference between the two proteoglycans reside in the fact that the Swarm rat chondrosarcoma proteoglycan has exclusively chondroitin 4-sulphate as its glycosaminoglycan component, whereas the large aggregating-articular-cartilage proteoglycan may contain both the 4- and 6-isomers of chondroitin sulphate, as well as keratan sulphate (Hascall and Kimura, 1981). These structural differences would not be expected to alter the magnitude of the flow resistance offered by these proteoglycans (see below). In view of these facts, our previous experiments established a specific structure–function relationship for the large aggregating-cartilage proteoglycan molecule.

We demonstrated that in semi-dilute solutions (> 10 mg/ml) the flow of water in proteoglycan solutions is essentially independent of the state of aggregation of the proteoglycan, but is determined by segments of its constituent chondroitin-sulphate chains. Studies of the effect of ionic strength and chemical modifications of the polysaccharide chain, including desulphation and carboxyl-group reduction, demonstrated that there was no significant change in the flow-resistance properties of the chondroitin-sulphate chain. There was no difference, too, between the 4- and 6-isomers of chondroitin sulphate (Zamparo and Comper, 1989). Comparative studies on a variety of polysaccharides (Zamparo and Comper, 1991) demonstrated that flow resistance correlated with the nature of the polysaccharide linkage. Glycosaminoglycans with alternating β-(1→3) and β-(1→4) glycosidic linkages, as in the polysaccharides chondroitin sulphate, hyaluronan, dermatan sulphate and keratan sulphate, showed the same maximal flow resistance (Comper and Zamparo, 1990). In fact, the manner in which hydraulic conductivity of polysaccharides with different glycosidic linkages varied seemed to correlate with the degree of inter-residue hydrogen bonding and segmental-chain flexibility (Zamparo and Comper, 1991).

This study specifically sets out to measure flow resistance of articular cartilage proteoglycan to enable a comparison with other proteoglycan studies. We extend the examination of solvent conditions by measurement of flow resistance of chondroitin-sulphate chains as a function of pH, and also in the presence of hydrogen-bond-breaking solvents. We also examine the influence of chain flexibility on flow resistance through periodate oxidation which may break the $C_{(2)}$–$C_{(3)}$ bond of the uronic acid residue of chondroitin sulphate (Scott and Tigwell, 1973; Scott et al., 1976).

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THEORY

For the system of a negatively charged polyelectrolyte (P) with valence Z in the presence of simple electrolyte XY we define the electroneutral component PX_z as component 1 and the combination of XY + water as component 2. The equation associated with the sedimentation of the negatively charged polyion, for solutions of relatively low osmotic pressure, has been derived (Eisenberg, 1976) such that the polyion velocity v_p is given as

$$v_p = (\overline{M}_{p^*}\omega^2 r/f_p) + (iZe/f_p)(d\psi/dr) \quad (1)$$

where ω is the angular velocity, $d\psi/dr$ is the internally created field generated by the charge separation of the polyanion and its constituent counterions, i is an effective-charge parameter, f_p is the frictional coefficient per mole of the effective ionic species (p^*), and the molecular-mass term $\overline{M}_{p^*} = \overline{M}_p + (1-i)Z\overline{M}_X$. The influence of charge on sedimentation velocity, either through the concentration of XY or through variation of the pH, will be through its corresponding effect on the potential ψ.

EXPERIMENTAL

Materials

Chondroitin sulphate from whale and shark cartilage (mixed-sulphate isomers) (81F-3887) was from Sigma Chemical Co., St. Louis, MO, U.S.A. Sephacryl S-200 super fine (PD08266), dextran ($\overline{M}_w$ approx. 12000) fraction 11640-2 (FrIV) and dextran T-70 ($\overline{M}_w$ approx. 70000) were from Pharmacia Fine Chemicals, Uppsala, Sweden. Bovine-articular-cartilage aggrecan and proteoglycan aggregate were from the metacarpophalangial joint of bovine hocks and were prepared as D1D1 (protein content 7.5%) and A1 fractions respectively by methods described previously (Comper and Williams, 1987). All other chemicals used were of analytical grade.

Methods

Chemical methods

Tritiation of chondroitin sulphate was carried out as described by Van Damme et al. (1982). The oxidation of chondroitin sulphate by sodium periodate and subsequent borohydride reduction was carried out following the method of the Smith degradation used by Sandy (1979). The periodate-treated chondroitin sulphate was purified by dialysis (using Visking dialysis tubing) against 100 vol. of water (repeated five times), 50 vol. of 1 M NaCl, then 100 vol. of water (repeated three times) and freeze-dried. [^{3}H]Chondroitin sulphate, included in the initial periodate-oxidation reaction mixture and co-purified with unlabelled chondroitin sulphate, gave an elution profile on Sephacryl S-200 as shown in Figure 1. While there appeared to be some breakdown associated with the periodate-treated chondroitin sulphate it was certainly eluting at a lower K_{av} than dextran of $\overline{M}_w$ 12000. Estimation of the ratio of ^{3}H label to uronic-acid content of the untreated and treated samples demonstrated that periodate treatment resulted in the disappearance of 58.2% of the uronic acid chromogen. The concentration of the periodate-treated chondroitin sulphate samples was determined by analysis of dry weight (see below).

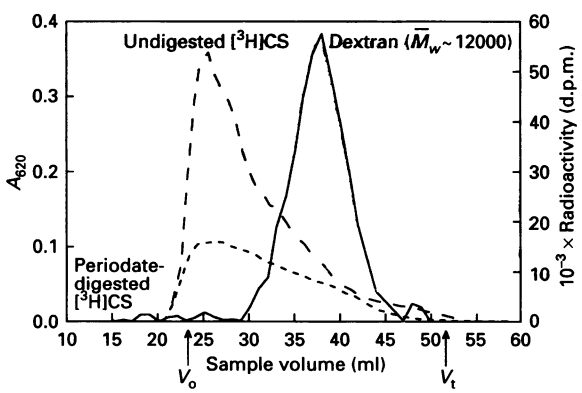

Figure 1 Chromatographic elution profile of periodate-treated chondroitin sulphate

Elution profile obtained for [^{3}H]chondroitin sulphate, periodate-treated [^{3}H]chondroitin sulphate and dextran ($\overline{M}_w$ approx. 12000) (hexose assay at 620 nm) on Sephacryl S-200 column (1.5 cm × 27 cm) in phosphate-buffered saline (140 mM NaCl/2.68 mM KCl/1.5 mM KH_2PO_4/8.1 mM Na_2HPO_4, pH 7.5).

Ultracentrifugal methods

Sedimentation coefficients were measured in the analytical ultracentrifuge at 20 °C by the method described previously (Zamparo and Comper, 1989; Comper and Zamparo, 1990). Specific hydraulic conductivity, k, could be calculated from the sedimentation coefficient $(S_1)_v$ (in a volume-fixed frame) (see Comper and Zamparo, 1990)

$$k = \eta_2(S_1)_v/C_1[1-(\bar{v}_1/\bar{v}_2)] \quad (2)$$

where η_2 is the viscosity of solvent (component 2), C_1 is the concentration of component 1 in mass/volume units and $\bar{v}_i$ is the partial specific volume of i. This equation has been derived specifically with concentrated polymer solutions in mind. The concentration dependence of the sedimentation coefficient (due to molecular interactions in concentrated solutions) will be manifested directly in the concentration dependence of k.

Viscosity measurement

Viscosities were measured in an M4 capillary U-tube with a flow time for water of 30 s at 20 °C.

Analytical procedures

Details of chemical assays and liquid-scintillation counting have been described elsewhere (Comper and Williams, 1987; Zamparo and Comper, 1989). Dry-weight analysis for moisture content and concentration determination was performed with the sample being dried to constant weight under vacuum (133 Pa) at 60 °C in preweighed vials.

RESULTS

It was important to establish that our previous results on the hydraulic conductivity of Swarm rat chondrosarcoma proteoglycan were similar to that obtained for articular cartilage proteoglycan. The results in Figure 2 demonstrate the close similarity of the k values for aggrecan and proteoglycan aggregate isolated from these two tissue sources. These values are also similar to the values obtained for chondroitin-sulphate chains. This confirms previous findings that in semi-dilute solution k is essentially determined by critical segments of the chondroitin-sulphate chain. It is to be noted that direct comparisons of the proteoglycan with chondroitin sulphate on a mass concentration scale, will be influenced by the low protein content of the former.

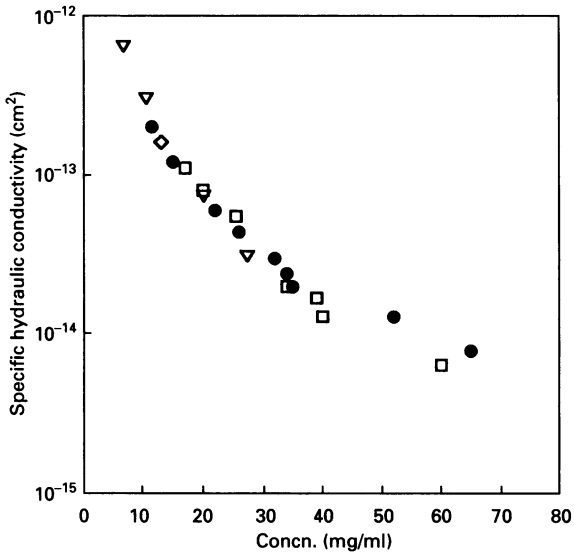

Figure 2 Variation of the specific hydraulic conductivity *k* as a function of concentration for chondroitin-sulphate proteoglycans

The proteoglycans were articular cartilage aggrecan (▽), articular cartilage proteoglycan aggregate (◇), Swarm rat chondrosarcoma aggrecan (□) (from Zamparo and Comper, 1989), and chondroitin sulphate (●) (from Comper and Zamparo, 1990). All polysaccharides were in phosphate-buffered saline.

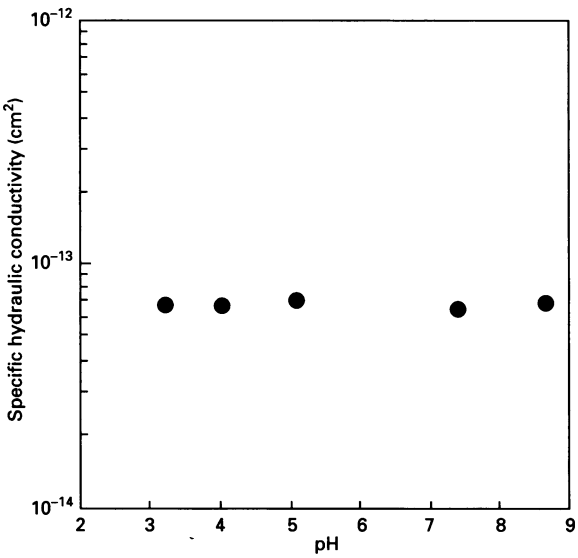

Figure 3 pH dependence of the specific hydraulic conductivity of chondroitin sulphate

Chondroitin sulphate was present at a concentration of 22.2 mg/ml in Michael's sodium barbital/sodium acetate buffers at various pHs (3.2–8.7). The buffers were always at an Na^+ concentration of 0.15 M.

With the knowledge that local micro-environmental factors most probably govern the properties of the critical chain segments in chondroitin sulphate we set about examining the different solvent conditions that may affect the chain. Variation of pH from 3.2 to 8.7, with Michael's sodium barbital/sodium acetate buffer, with the sodium concentration being maintained at 0.15 M, demonstrated that the hydraulic conductivity was essentially unaffected over this range of pH (Figure 3). The lack of electrostatic effects, particularly as we move through the pK_a

Table 1 Hydraulic conductivities of chondroitin sulphate in various solvents

Abbreviations: PBS, phosphate-buffered saline; GuHCl, guanidine hydrochloride.

Chondroitin sulphate concn. (mg/ml)	Solvent	$10^{13} \times k(cm^2)$
12.6	PBS	1.60
12.6	4 M GuHCl	1.57
12.0	8 M Urea	1.15
12.6	0.5 M NaF	1.79

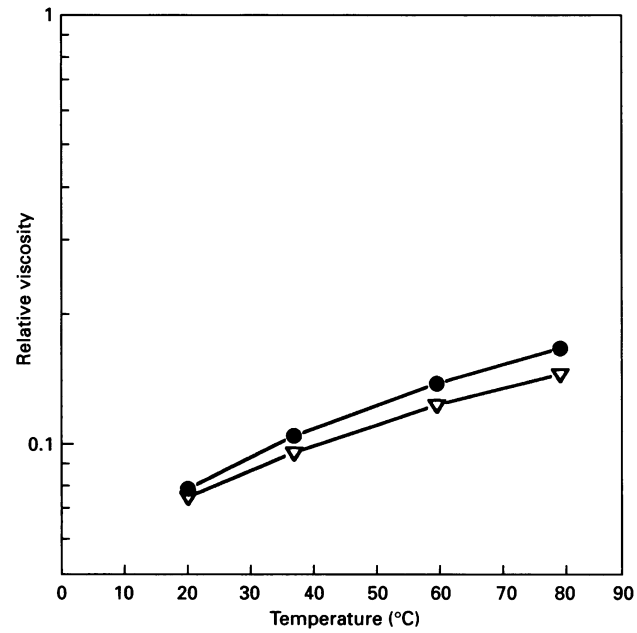

Figure 4 Temperature dependence of the relative viscosity of various polysaccharides

The temperature dependence was corrected for the change in solvent viscosity. Studies were made on chondroitin sulphate at 40 mg/ml (●) and dextran T-70 at 100 mg/ml (▽).

(≈ 4) of the carboxyl group of the uronic-acid residue, confirms previous results where we demonstrated that *k* remained constant as NaCl concentration was varied from 0.03 M to 1.5 M, or when the charge groups of chondroitin sulphate were chemically modified or removed (Comper and Zamparo, 1990).

The influence of various water-structure-breaking and hydrogen-bond-breaking solvents on the hydraulic conductivity of chondroitin sulphate is shown in Table 1. The concentration of 12 mg/ml was chosen as a compromise between the concentration required to manifest critical segment-length control of conductivity and that needed to get measurable sedimentation, particularly with the relatively high-viscosity solvents used, such as 8 M urea. Guanidine hydrochloride (4 M) was without effect on the conductivity of chondroitin sulphate, whereas an 11% increase in *k* was registered with 0.5 M NaF. An unexplainable effect was seen with 8 M urea where we actually recorded a decrease in conductivity [it is to be noted that this conductivity is already corrected for solvent viscosity as embodied in eqn. (2)].

The possibility that inter-residue hydrogen bonding occurs over the entire chain length of chondroitin sulphate was tested by the measurement of viscosity as a function of temperature. The viscosity of chondroitin sulphate at a concentration of 40 mg/ml

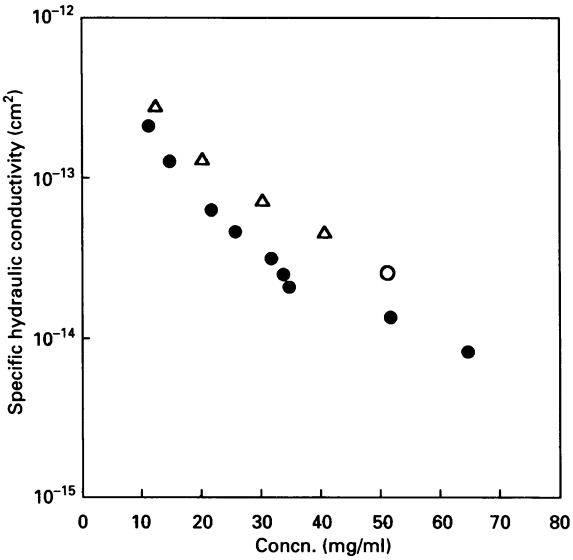

Figure 5 The variation of the specific hydraulic conductivity as a function of concentration for periodate-treated chondroitin sulphate

The periodate-treated chondroitin sulphates were the oxidized form (△) and the reduced form (○) compared with untreated chondroitin sulphate (●) (from Comper and Zamparo, 1990).

was compared with that of dextran (100 mg/ml) of similar viscosity. Dextran is known to be a flexible polymer with mainly α-(1 → 6) glycosidic linkages in which inter-residue hydrogen bonding is not prevalent. When the temperature dependence of solvent viscosity is accounted for, we find that the viscosity actually increases with temperature in a similar fashion for both chondroitin sulphate and dextran (Figure 4). These studies demonstrate that if inter-residue hydrogen bonding is present in chondroitin sulphate, then it does not extend over long distances along the chain.

Unlike solvent and temperature treatment, chemical modification of chondroitin sulphate with periodate (in which 58% of the uronic-acid residues were modified without extensive change in relative molecular mass) resulted in a marked increase in hydraulic conductivity when compared with that of the untreated material. In comparison to the values in Table 1, the oxidized periodate-treated chondroitin sulphate, at a concentration of 12.6 mg/ml, gave a k value of 2.63×10^{-13} cm², which is approx. 60% higher than the k value obtained for untreated chondroitin sulphate at the same concentration. The concentration dependence of k for oxidized periodate-treated chondroitin sulphate shows considerably higher values of k compared with the untreated material (Figure 5). Oxidized/reduced periodate-treated chondroitin sulphate also showed similar behaviour.

DISCUSSION

This study establishes that the hydraulic conductivity of proteoglycans isolated from articular cartilage is essentially governed by critical segments of its constituent polysaccharide chains, namely chondroitin sulphate and keratan sulphate [we have demonstrated previously that the conductivity of these two glycosaminoglycans is identical (Comper and Zamparo, 1990)]. There is now considerable evidence to suggest that charge effects in sedimentation, borne through finite values in the potential term, psi, in eqn. (1) are negligible in semi-dilute solution. This evidence comes from studies on the dependence of k on pH (this paper), ionic strength (Zamparo and Comper, 1989) and chemical modification, including desulphation and carboxyl-group reduction (Comper and Zamparo, 1990). These results should prove to be informative for developing improved models for the permeability of cartilage tissue. We have already shown that the Happel–Brenner approximation for flow resistance of glycosaminoglycan chains, particularly with alternate β-(1→3) and β-(1→4) glycosidic linkages, severely underestimated the degree of flow resistance offered by these chains (Zamparo and Comper, 1989). A number of treatments for modelling cartilage permeability have also included predicted charge effects exerted by the glycosaminoglycan chain. These interpretations have been of the form of open-circuit permeability (where current density is zero), where streaming potentials will be manifested (Eisenberg and Grodzinsky, 1988). (This process is equivalent to sedimentation flux of the polyanion in the centrifuge, where the potential, psi, would be the streaming potential, measured in tissues, due to proteoglycan–sodium counterion charge separation.) Alternatively, the charge effect has been expressed in terms of the proteoglycan-concentration dependence of frictional interactions between ions and water (Lai et al., 1991). Our experiments demonstrate that these charge effects on flow resistance, as measured directly by sedimentation analysis, are insignificant in semi-dilute solutions of chondroitin sulphate. These results are also consistent with the observations by Maroudas (1975a) that the hydraulic conductivity of cartilage is not influenced significantly by increasing the ambient NaCl concentration from 0.15 M to 1.0 M. It seems apparent that where ionic conditions have affected the mechanical properties of cartilage, this can be explained as being due to the ionic-strength dependence of either the osmotic component contributed by the glycosaminoglycan, or glycosaminoglycan electrostatic interaction with neighbouring extracellular-matrix molecules, rather than the viscous dissipation of water over the polysaccharide chain.

Studies on the hydraulic conductivity over a wide range of different polysaccharides (Comper and Zamparo, 1990; Zamparo and Comper, 1991) have demonstrated that k is correlated strongly with the nature of the glycosidic linkage. Polysaccharides that showed the greatest resistance to flow were those in the group of glycosaminoglycans with alternating β-(1→3) and β-(1→4) linkages. On the other hand, those polysaccharides that showed least resistance to flow (when compared on a mass or volume concentration scale) were the flexible polysaccharides like β-(1→6) linked dextrans. The intermediate flow-resistant polysaccharides were the carboxymethyl celluloses with β-(1→4) linkages and the heparin-like polysaccharides with either alternating β-(1→4) and α-(1→4) linkages or all the linkages being in the α-(1→4) form. These experimental correlations seem to be remarkably similar to the degrees of intra-chain flexibility and inter-residue hydrogen bonding identified by Scott and coworkers (Scott and Heatley, 1982; Scott et al., 1984; Heatley and Scott, 1988; Scott, 1991) in mammalian glycosaminoglycans. A co-operative hydrogen-bonding pattern has been identified with hyaluronan (four bonds over a repeat trisaccharide), chondroitin sulphate (three bonds) and keratan sulphate (three bonds). For heparin-like polysaccharides, two possible inter-residue hydrogen bonds may form, yet the structure will have extra flexibility because of the presence of the iduronate residue (Casu et al., 1988). Therefore, the suggestion is made that a stiff-chain polysaccharide, generated through a sleeve of inter-residue hydrogen bonding, yields a structure of greater surface

area/volume ratio, and hence offers greater resistance to flow. For chondroitin sulphate rotation around both glycosidic linkages is restricted; restriction around the β-(1→3) linkage is caused by hydrogen bonding of the acetamido group C=O to hydroxyl group HO-2 of GlcA. Restriction around the β-(1→4) linkage would be greater as there are two hydrogen bonds generated from HO-3 of GlcA to O-5 of GlcNAc and from NH to COO^- of GlcA.

Efforts to disrupt the stiffness or hydrogen-bonded secondary structure of chondroitin sulphate through temperature and hydrogen-bond-breaking solvents were not particularly conclusive. This was evident from the conformational changes of the whole chain, as determined by the temperature dependence of viscosity, and the small influence that 0.5 M NaF had on critical segments in hydraulic conductivity measurements. The most probable conclusion from these experiments is that the persistent length associated with a continuously stiff chain was over a segment rather than the whole chain. This would be entirely consistent with the molecular-mass independence of the hydraulic conductivity.

A striking feature, however, of our efforts to affect the flexibility of the chain was the result obtained with periodate treatment. It was apparent that with approx. 58 % of the uronic-acid residues being altered in terms of the removal of the $C_{(2)}$–$C_{(3)}$ bond in the uronic-acid residue, then a marked increase in hydraulic conductivity was observed. We suggest that this was due to the increased flexibility of critical segments in the chondroitin sulphate chain.

In conclusion, this work substantiates earlier findings that the physicochemical properties of glycosaminoglycan chains associated with dynamic water–polysaccharide chain interaction in concentrated polysaccharide solutions are governed by the conformation and shape of the chain rather than its anionic nature. This work also confirms the lack of involvement of the sulphate groups of chondroitin sulphate in its hydrodynamic and physicochemical properties (Comper and Zamparo, 1990).

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