Hydrodynamic properties of connective-tissue polysaccharides

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The major hydrodynamic properties of the connective-tissue polysaccharides are those that describe polysaccharide–water interaction as embodied in their osmotic-pressure and hydraulic-conductivity properties. This study shows that, for polysaccharides such as chondroitin sulphate, hyaluronate and the heparin-like polysaccharides, their hydrodynamic properties depend primarily on the presence of the uronic residue and the nature of the glycosidic linkage. Other parameters such as the degree of *N*-acetylation and sulphation were found not to influence these properties to any great extent. These studies particularly delineate structural–functional aspects of the connective-tissue polysaccharides in terms of their primary structure.

INTRODUCTION

Connective-tissue polysaccharides (or glycosaminoglycans), which include chondroitin 4- and 6-sulphate, dermatan sulphate and the heparin-like polysaccharides, have a wide distribution in animal tissues. They are found predominantly in extracellular matrices, on cell surfaces and in cytoplasmic granules. These polysaccharides are highly anionic, with sulphate substitution in the range of one to four sulphate groups per disaccharide unit and overall linear charge densities in the range of 1–5 charges per nm. These charges are fully ionized under physiological conditions, so that these molecules confer high concentrations of negative charge on regions that contain them.

The connective-tissue polysaccharides appear to be multifunctional molecules. Two different structure–function approaches have emerged. One view has been put forward to explain the functional properties of the glycosaminoglycans and their proteoglycan counterparts in terms of their physicochemical properties. The physicochemical properties of primary importance are those that regulate water content in tissues (Comper & Laurent, 1978), namely osmotic pressure, which can be represented as the effective concentration of the solute (which for polyanions is determined to a large extent by the activity of their constituent counterions), and specific hydraulic conductivity (which represents the ability of water/solvent to flow over the surface of polymer chains so that surface-to-volume ratios of the chains will be a major quantitative parameter in determining conductivity). The water content of matrices is important, as it defines tissue volume, creates spaces for molecular transport and for molecular organization and offers compressive resistance (as water itself is essentially incompressible). The soluble phase of the extracellular matrix is always associated with dissolved and retained polysaccharides (or proteoglycans); therefore any consideration of water properties in this system must take into account the role of the polysaccharide. The polysaccharide will have influence in resisting flow of water (hydraulic conductivity) and may generate imbibition of water in the tissue through relaxation of its concentration gradient (mutual diffusion). The major polysaccharides that have a role in terms of water regulation will be the chondroitin sulphate proteoglycans in tissues undergoing mechanical compression such as cartilage, hyaluronate in joint fluids and the heparin-like polysaccharides on cell surfaces and in basement membranes, particularly those

associated with tissue/capillary exchange. The physicochemical structure–function approach is pertinent to tissues that contain relatively concentrated solutions (> 5 mg/ml) of these polysaccharides.

The other structure–function concept is one that is focused at the primary structural level of these molecules and that equates biological activity to electrostatic interaction with macro-ions (Comper & Laurent, 1978; Lindahl & Höök, 1978). This approach is more amenable in discussing ‘dilute’ concentrations of the polysaccharides. The sulphated connective-tissue polysaccharides have been shown to participate in co-operative electrostatic binding of low-to-moderate affinity with many extracellular-matrix proteins and growth factors (Ruoslahti, 1988). Similar interactions have been demonstrated in model protein–glycosaminoglycan interacting systems (Blackwell *et al.*, 1977) and glycosaminoglycan–enzyme interactions (Avila & Convit, 1975, 1976; Elbein, 1974). In many of these studies it was demonstrated that unsulphated polysaccharides interacted to a lesser degree. High-affinity sequence-dependent binding has been shown to occur with some heparin-like polysaccharides in their interaction with antithrombin (for review see Lindahl *et al.*, 1986). The present study sets out to examine the role of various aspects of the primary structure of the connective-tissue polysaccharides on their physicochemical properties.

THEORY

The sedimentation-diffusion technique in the analytical ultracentrifuge provides a powerful tool for evaluation of the physicochemical parameters of water interaction, namely specific hydraulic conductivity and thermodynamic non-ideality.

Irreversible thermodynamics provides us with an expression for solute flux (J_1) in a two-component system associated with sedimentation velocity in the centrifuge with respect to a volume (assume to be the same as the cell)-fixed frame of reference (Fujita, 1962):

$$(J_1) = (s_1)_v C_1 \omega^2 r - (D_1)_v (\partial C_1 / \partial r) \quad (1)$$

where ω is the angular speed of the rotor (radians/s), r is the distance from the centre of the rotor, $(s_1)_v$ is the sedimentation coefficient and $(D_1)_v$ is the mutual diffusion coefficient of solute (defined as component 1) in a volume-fixed frame of reference,

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and C_1 is the concentration of component 1 in mass/volume units. Expressions for $(s_1)_v$ and $(D_1)_v$ have been derived previously (Comper *et al.*, 1986) such that:

$$(s_1)_v = (1 - \rho \bar{v}_1)(1 - \phi_1)M_1/f_{12} \quad (2)$$

and

$$(D_1)_v = (1 - \phi_1)^2(M_1/f_{12})(\partial \Pi^*/\partial C_1)_{T, \mu_2} \quad (3)$$

Substituting eqns. (2) and (3) into eqn. (1) gives:

$$J_1 = (1 - \phi_1)(M_1/f_{12}) \left[C_1 \omega^2 (1 - \rho \bar{v}_1) \left(\frac{\partial r^2/2}{\partial r} \right) - (1 - \phi_1)(\partial \Pi^*/\partial r)_{T, \mu_2} \right] \quad (4)$$

where ρ is the solution density, $\bar{v}_1$ is the partial specific volume component of 1, ϕ is the volume fraction of 1, M_1 is the relative molecular mass of component 1, f_{12} is the hydrodynamic frictional interaction between the solute and solvent (component 2), Π^* is the osmotic pressure, T is the temperature and μ_2 is the chemical potential of solvent. This equation then represents a balance of a mechanical force $[C_1 \omega^2 (1 - \rho \bar{v}_1) \partial r^2/2]$ and an osmotic force $[(1 - \phi_1) \partial \Pi^*]$ on the solute. The coefficient of eqn. (4) is directly related to the effective diffusion coefficient of component 1 ($= RT/f_{12}$, where R is the universal gas constant), which governs the kinetics of both pressure-driven processes, namely hydraulic flow and osmosis.

At high rotor speeds the flux J_1 is determined primarily by the sedimentation coefficient. A direct relationship between specific hydraulic conductivity, k , and the sedimentation coefficient has been derived by Mijnlief & Jaspers (1971):

$$k = \eta_2 (s_1)_v / C_1 \left[1 - \left(\frac{\bar{v}_1}{\bar{v}_2} \right) \right] \quad (5)$$

where η_2 is the viscosity of the solvent. At low rotor speeds, where sedimentation is negligible, the mutual diffusion coefficient of the solute may be evaluated. The combination of both the D_1 and s_1 data may then be used to calculate the thermodynamic non-ideality term:

$$(\partial \Pi^*/\partial C_1)_{T, \mu_2} = (1 - \rho \bar{v}_1) D_1 / (1 - \phi_1) s_1 \quad (6)$$

Integration of eqn. (6) with respect to C_1 may then yield the osmotic pressure. This equation is used with the knowledge that it is only applicable for monodisperse solutes or molecular-mass-independent solutes. For polydisperse dextran fractions we have previously demonstrated that in semi-dilute solutions the M_1/f_{12} parameter in eqns. (2) and (3) as derived from sedimentation and diffusion respectively are essentially the same, whereas in dilute solution they exhibit differences (Comper *et al.*, 1986). Therefore osmotic pressures evaluated by eqn. (6) for polydisperse solutes would only be valid in semi-dilute solution, where molecular-mass-independent behaviour is apparent. This has been established for the chondroitin sulphate polysaccharides (Comper & Williams, 1987; Zamparo & Comper, 1989).

Many experiments are performed with macromolecular solutes in thermodynamic equilibrium with physiological saline, pH 7.4. Under these conditions for polyelectrolytes the equation for the mutual diffusion coefficient takes on the same form as eqn. (3) (Varoqui & Schmitt, 1972). The equation for the sedimentation coefficient of polyelectrolytes is also the same as that described by eqn. (2), as simple salt effects on $(s_1)_v$ (or electrolyte dissipation) have been shown to be negligible for connective-tissue polysaccharides (Zamparo & Comper, 1989).

EXPERIMENTAL

Materials

Heparin (pig mucosal) (M_{sd} approx. 12000) (lot 78F-0633)

(with 1.9–2.3 mol of sulphate/mol of glucosamine; Taylor *et al.*, 1973) and chondroitin sulphate (bovine trachea) (lot 58F0616) were from Sigma Chemical Co., St. Louis, MO, U.S.A. Keratan sulphate, from bovine intervertebral disc, was a gift from Dr. H. C. Robinson (Department of Biochemistry). The other polysaccharides used in this investigation have been previously described (Zamparo & Comper, 1989). All other reagents were of analytical grade.

Methods

Chemical methods. Desulphation and carboxy-group methylation of connective-tissue polysaccharides was performed by the method of Kantor & Schubert (1957). Desulphation of chondroitin sulphate was essentially complete (Zamparo & Comper, 1989) and that of heparin was about 90% complete as determined by a spectrophotometric assay (Farndale *et al.*, 1982). Carboxy-group reduction was by the method of Wolfrom & Juliano (1960).

Ultracentrifugal methods. Sedimentation velocity. Sedimentation coefficients were measured in the analytical ultracentrifuge at 20 °C by the method of Zamparo & Comper (1989). Normally the solutions are so concentrated that the concentration gradient that develops refracts light completely out of the cell. The result is that a band develops that moves according to the sedimentation of the material. The sedimentation was recorded by monitoring the movement of the band median by a series of ten photographs taken over a time period of up to 48 h. Measurements of the movement of the front or back of the band yielded very similar sedimentation coefficients to the movement of the median. The accuracy of the sedimentation-coefficient measurement was normally to within $\pm 3\%$. However, with low sedimentation coefficients ($< 0.45 \times 10^{-13}$ s) the error could be as high as $\pm 10\%$. Because of the very low sedimentation coefficients, boundary distances moved during the course of the measurement were small, namely 0.03–0.05 cm, which would indicate that radial dilution effects were insignificant (less than 1% dilution effect).

Mutual diffusion. Mutual diffusion coefficients were measured in the analytical ultracentrifuge, with a synthetic-boundary cell, by the method of Comper *et al.* (1986). Concentration gradients of less than 5 mg/ml were employed to generate net solute movement.

Osmotic pressure from polynomial regression analysis. Osmotic pressures of various polysaccharide preparations were evaluated from sedimentation and diffusion data by using eqn. (6). Linear-regression analysis was used to fit the sedimentation data (in terms of $\log k$ versus $\log C_1$), and the diffusion data were fitted to a polynomial regression by using a univariate curvilinear-regression model with orthogonal polynomials on a Vax model 11/780 computer (Digital Equipment Corp.). At least five measurements of each type of transport were obtained. Values of $\partial \Pi^*/\partial C_1$ so obtained were similarly fitted to a polynomial regression. The virial coefficients obtained were used to evaluate the variation of osmotic pressure with C_1 .

Solution densities. These were measured on a DMA 55 density meter (Anton Paar, Graz, Austria). Estimates of the partial specific volume for each polymer are shown in Table 1.

Solution preparation. To represent physiological ionic conditions, polymers used in this study were dialysed extensively against phosphate-buffered saline (140 mM-NaCl/2.68 mM-KCl/1.5 mM-KH₂PO₄/8.1 mM-Na₂HPO₄, pH 7.5) before use unless otherwise stated.

Table 1. Partial specific volumes of polysaccharides

The solvent was phosphate-buffered saline unless otherwise stated.

Material	Partial specific volume (ml/g)
Chondroitin	0.54
Chondroitin sulphate	0.47
Chondroitin sulphate in 1 M-NaCl	0.47
Carboxy-group-reduced chondroitin in water	0.56
Carboxy-group-methylated chondroitin in water	0.66
Swarm rat chondrosarcoma proteoglycan subunit	0.49
Heparin	0.46*
Heparin in 1 M-NaCl	0.44
Desulphated heparin	0.51
Hyaluronate	0.54
Keratan sulphate	0.49
Deacetylated chondroitin sulphate	0.45

* From data reviewed by Comper (1981).

Analytical procedures. Details of chemical assays and conversion factors for the connective-tissue polysaccharides have been described elsewhere (Comper & Williams, 1987; Zamparo & Comper, 1989).

RESULTS AND DISCUSSION

The data for the concentration-dependence of specific hydraulic conductivity, k , for various polysaccharides including heparin, desulphated heparin, Swarm rat chondrosarcoma proteoglycan subunit, chondroitin sulphate, desulphated chondroitin sulphate, methylated chondroitin, deacetylated chondroitin sulphate, decarboxylated chondroitin, keratan sulphate and hyaluronate are shown in Fig. 1. These data do demonstrate that two groups of results are apparent. The polysaccharides with glycosidic linkages of alternating β 1,4- and β 1,3-linkages (chondroitin sulphate, keratan sulphate and hyaluronate) have lower k values than the heparin-like polysaccharides with either alternating β 1,4- and α 1,4-linkages or all linkages being in the α 1,4 form. The nature of the glycosidic linkage appears to be the only differentiating factor among the two groups of polysaccharides, where a range of parameters were varied including degree of sulphation and *N*-acetylation, the degree of carboxylation, ionic strength and relative molecular mass. None of these parameters, particularly sulphate content and ionic strength, appears to influence the k data to any significant extent. The hydraulic conductivity of these polysaccharides, then, represents viscous dissipation of water over a segment of the polysaccharide chain that offer its major resistance through its conformation as governed by its glycosidic linkage.

The mutual diffusion for Swarm rat chondrosarcoma proteoglycan subunit, chondroitin sulphate, chondroitin, heparin and desulphated heparin demonstrate that the diffusion coefficients of all these materials converge to similar values at high concentration (Fig. 2). Again, relative molecular mass and degree of sulphation do not appear to be important in governing the magnitude of the diffusion coefficient at high concentrations. These studies, together with the k data, indicate that the osmotic pressure would not be enhanced by increasing sulphation. Rather, as shown in Fig. 3, the more highly sulphated heparin has a lower

Fig. 1. Variation of the specific hydraulic conductivity k as a function of concentration for connective-tissue polysaccharides

The connective-tissue polysaccharides were heparin in phosphate-buffered saline (Δ) (from Zamparo & Comper, 1989) and in 1 M-NaCl ($\blacktriangle$), desulphated heparin in phosphate-buffered saline (∇), Swarm rat chondrosarcoma proteoglycan subunit in phosphate-buffered saline ($\blacksquare$) (from Comper & Williams, 1987), chondroitin sulphate in phosphate-buffered-saline ($\blacklozenge$) and in 1 M-NaCl ($\diamond$), desulphated chondroitin sulphate (chondroitin) in phosphate-buffered saline ($\square$) (from Zamparo & Comper, 1989), deacetylated chondroitin sulphate ($\blacktriangledown$), carboxy-group-methylated chondroitin in water ($\square$), carboxy-group reduced chondroitin in water ($\blacksquare$), keratan sulphate (bovine intervertebral disc) in phosphate-buffered saline ($\circ$) and hyaluronate (bovine synovial fluid, acid-hydrolysed) (from Zamparo & Comper, 1989) ($\times$).

Fig. 2. Variation of the mutual diffusion coefficient as a function of concentration for connective-tissue polysaccharides

Symbols are as defined in the legend to Fig. 1. Data for Swarm rat chondrosarcoma proteoglycan and chondroitin sulphate are from Comper & Williams (1987).

osmotic pressure than chondroitin and chondroitin sulphate proteoglycan, which will be due to, in part, counterion condensation on to the more highly charged sulphated molecules (Manning, 1979; Comper, 1981). It appears that only the uronic acid residue with its associated counterion is then required to contribute to the high osmotic activity of these materials, as the osmotic pressure of these polysaccharides at high salt

Fig. 3. Osmotic pressures of various polysaccharide preparations as a function of concentration

Curve A represents the Swarm rat chondrosarcoma proteoglycan subunit, curve B represents chondroitin and curve C represents heparin.

concentrations is significantly lowered (Urban *et al.*, 1979). It follows, too, that the hydrodynamic volume occupied by the sulphated polysaccharides, as determined by water-interaction parameters discussed above, would not be significantly different to or enhanced as compared with their unsulphated counterparts. Rather, differences would be registered by the nature of the glycosidic linkage and chain conformation.

This work demonstrates that the physicochemical properties associated with both equilibrium and dynamic water-polysaccharide interaction in concentrated polysaccharide solution is governed by the primary structure of the polysaccharide chain (as distinct from the proteoglycan structure, which may be required, by virtue of its size, to retain the polysaccharide chains

within a tissue compartment) (see also Urban *et al.*, 1979; Comper & Williams, 1987) in terms of the nature of its glycosidic linkage and the uronic acid content.

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