A study of factors influencing hydration of sodium hyaluronate from compressibility and high-precision densimetric measurements

Anthony DAVIES,* John GORMALLY,* Evan WYN-JONES,* David J. WEDLOCK†‡ and Glyn O. PHILLIPS†

*Department of Chemistry and Applied Chemistry, University of Salford, Salford M5 4WT, U.K., and

†Research Division, School of Natural Sciences, North East Wales Institute of Higher Education, Deeside, Clwyd CH5 4BR, Wales, U.K.

(Received 1 March 1983/Accepted 5 April 1983)

A study of the factors influencing the hydration of the biopolymer hyaluronic acid was made by compressibility and density measurements. The factors investigated were the hydration changes on glycosidic bond formation, and also the influence of counterion type, solution ionic strength and temperature. The results indicate that, with this biopolymer, the hydration of the glucuronate residue is significantly more than that of the *N*-acetylglucosamine residue, and further that the biopolymer is less hydrated than the sum of its component monosaccharide residues. Change of the counterion salt form of this polyelectrolyte from univalent to bivalent counterion type (Na^+ to Ca^{2+}) leads to a small though significant increase in the total hydration sheath surrounding the polymer. An increase in the background ionic strength of the solvent leads to a quantifiable lowering of the hydration of the polymer at physiological ionic strength compared with its value in salt-free aqueous solution. A decrease in hydration with increase in temperature in the range 20–50°C is the opposite of previous reports, and was observed when the polymer was dissolved both in pure water and in 0.15 M-NaCl.

Hyaluronic acid, a co-polymer of *N*-acetyl-D-glucosamine and D-glucuronic acid, is a glycosaminoglycan found in the ground substance of connective tissue. It is a hydrophilic polysaccharide with a number of roles and biological characteristics. These include contributing to the lubricating and viscoelastic capacity of synovial fluid and homeostatic influences in connective tissue in general (Comper & Laurent, 1978). Certain special preparations have found pharmaceutical applications since the successful use of hyaluronic acid as a vitreous replacement agent (Balazs & Sweeney, 1966). We have shown previously (Davies *et al.*, 1982) that the view proposed by some workers (Jacobson & Laurent, 1954; Jacobson, 1955) that the hyaluronic acid molecule had a long-range influence over solvent water is not manifest in water-binding studies involving the use of compressibility measurements. We now report further studies of hyaluronic acid hydration with the use of this technique.

The hyaluronic acid molecule in the physiological environment exists in a moderately-high-ionic-

strength solvent in an almost exclusively sodium salt form. Certain hydrodynamic functions have been studied as a function of ionic strength, and shown to undergo critical discontinuities at or near to the physiological ionic strength, equivalent to 0.15 M-NaCl solvent (Blumberg *et al.*, 1955). We hence thought it of importance to study the hydration characteristics of the hyaluronate molecule in the physiological ionic milieu and also at and around the physiological temperature of 37°C.

The hydration of hyaluronic acid (sodium salt form) has been investigated by using differential scanning calorimetry, by determining non-freezing water (Ikada *et al.*, 1980), but the limitations of this technique are that polymer–water interactions in high-ionic-strength environments cannot be studied because of the uncertain effect of the large associated freezing-point depression on the enthalpy of fusion of water, as well as the extra uncertainty due to supercooling effects (Ling, 1980). Further, hydration of the polymer, although usually temperature-dependent, can only be studied in the temperature range of the fusion event.

In the present work a systematic investigation of the influence of ionic environment on hydration of

‡ To whom correspondence should be addressed.

hyaluronic acid, in terms both of added simple electrolyte and of influence of counterion salt form, were investigated and quantified. It has been shown, in the region of the physiological temperature, that increase in temperature is accompanied by loss of oriented or bound water, as distinct from the findings obtained by Suzuki & Uedaira (1970), who showed the opposite. We observed this effect in both pure water and physiological-ionic-strength saline solvents. We have further quantified the number of water molecules associated with the individual constituent sugar units, oligosaccharides and the high-polymer form of sodium hyaluronate, and from this propose a hydration model for the polymer in aqueous solution.

Experimental

Materials

Hyaluronate polymer samples of various molecular weights were produced and characterized from a sample of high-molecular-weight sodium hyaluronate, a gift from Pharmacia, Uppsala, Sweden. The preparation and characterization have been described previously (Davies *et al.*, 1982). These same samples of various molecular weights are identified by their intrinsic-viscosity values (Cleland & Wang, 1970). Hyaluronate concentration was determined by using the modified carbazole reaction (Bitter & Muir, 1962), with D-glucuronolactone as standard.

Sodium hyaluronate oligosaccharides were prepared from the same high-molecular-weight sodium hyaluronate sample by enzymic degradation. Sodium hyaluronate was exhaustively dialysed against distilled water. Bovine testicular hyaluronidase (980 NF units/mg; Sigma Chemical Co., St. Louis, MO, U.S.A.) coupled to CNBr-activated Sepharose 6MB (Pharmacia), was added to the dialysed sodium hyaluronate (100 cm³, 10 mg/cm³) and transferred to an Amicon ultrafiltration cell (250 cm³) containing a PM10 filter. Filtration was carried out under pressure of 5 kg/cm². Additional water was added until 1 litre of filtrate had been collected. The solution was reduced by rotary evaporation and freeze-dried. This gave oligosaccharides of a range of molecular weights but mainly deca-saccharide (Cowman *et al.*, 1981).

Calcium hyaluronate was prepared by dialysing sodium hyaluronate against an excess of 0.1 M-HCl at 4°C, and then removing the excess of acid by dialysis against distilled water. The hyaluronic acid solution was then titrated to pH 7.7 with Ca(OH)₂ solution. The resulting solution was then exhaustively dialysed against distilled water to remove any excess Ca²⁺ ions and freeze-dried. Determination of the Ca²⁺ ion content by atomic absorption spectroscopy showed 90% conversion into the calcium salt form. The weight-average

molecular weight ($\bar{M}_w$) of this sample was 3.95×10^5 , determined from size-exclusion chromatography, after the freeze-drying procedure.

N-Acetyl-D-glucosamine and sodium D-glucuronate were obtained from Sigma Chemical Co.

All other reagents were of analytical grade and obtained from BDH Chemicals, Poole, Dorset, U.K.

Density measurements

These were made with a high-precision oscillatory densitometer (type DMA-602; Anton Paar, Graz, Austria) (precision ± 0.00001 g/cm³).

Ultrasonic-velocity measurements

These were measured interferometrically relative to the velocity of sound through water at 25°C, i.e. 1496.58 ± 0.04 m/s (Barlow & Yazgan, 1966). The equipment used was an ultrasonic resonance cavity, after the design of Eggers (1968); ultrasonic velocities were measured at 2, 3 and 4 MHz, then averaged. All samples were degassed in a glass syringe before measurement. A sound-velocity-temperature profile of water and 0.15 M-NaCl in the range 20–50°C was obtained.

Results and discussion

Hydration parameters

The volume fraction of water not interacting with the sodium or calcium hyaluronate, and simple electrolyte if present, can be reasonably assumed to have the same bulk physical properties as water in the absence of polyelectrolyte, and several studies (Laurent, 1957; Balazs *et al.*, 1959; Davies *et al.*, 1982) indicated the lack of any long-range interaction between water and sodium hyaluronate.

The speed of longitudinal sound waves through a medium is given by:

$$u^2 = \frac{1}{d\beta} \quad (1)$$

where u is the measured ultrasound velocity, β is the adiabatic compressibility of the medium and d is the density of the medium. Further, the adiabatic compressibility of the medium is given by the relationship:

$$\beta = -\frac{1}{V} \cdot \left(\frac{\partial V}{\partial P} \right)_s \quad (2)$$

where V is the medium volume, P the pressure acting on that system, and s denotes entropy. The product βV is additive with respect to the various components within the system:

$$\beta V = - \left(\frac{\partial V}{\partial P} \right)_s \quad (3)$$

This product can thus be regarded as derived from the sum of products of component compressibilities and volumes of hydrated polyelectrolyte, hydrated counterion, hydrated co-ion (if appropriate) and also the bulk non-interacting solvent, expressed as:

$$\beta_{\text{soln.}} V_{\text{soln.}} = \beta_{\text{CH}_{\text{aq.}}} V_{\text{CH}_{\text{aq.}}} + \beta_{\text{Na}_{\text{aq.}}} V_{\text{Na}_{\text{aq.}}} + \beta_{\text{Cl}_{\text{aq.}}} V_{\text{Cl}_{\text{aq.}}} + \beta_{\text{solv.}} V_{\text{solv.}}$$

where $\beta_{\text{soln.}}$, $\beta_{\text{CH}_{\text{aq.}}}$, $\beta_{\text{Na}_{\text{aq.}}}$, $\beta_{\text{Cl}_{\text{aq.}}}$ and $\beta_{\text{solv.}}$ represent the compressibilities of the solution, of hydrated hyaluronic acid (or saccharide unit), the hydrated Na^+ counterion, the hydrated Cl^- ion and solvent respectively, and subscripted values of V represent the respective volumes of each component. The solution is regarded either as just the carbohydrate solution or as carbohydrate plus simple electrolyte solution. The hydrated polymer and hydrated counterions and co-ions can be reasonably regarded as incompressible in comparison with the bulk solvent (Passinsky, 1938); then:

$$\beta_{\text{CH}_{\text{aq.}}} = \beta_{\text{Na}_{\text{aq.}}} = \beta_{\text{Cl}_{\text{aq.}}} \approx 0$$

and

$$\beta_{\text{soln.}} V_{\text{soln.}} = \beta_{\text{solv.}} V_{\text{solv.}}$$

Therefore:

$$\frac{\beta_{\text{soln.}}}{\beta_{\text{solv.}}} = \frac{V_{\text{solv.}}}{V_{\text{soln.}}} \quad (4)$$

Therefore:

$$V_{0, \text{H}_2\text{O}} = \frac{\beta_{\text{soln.}}}{\beta_{\text{solv.}}} \quad (5)$$

where $V_{0, \text{H}_2\text{O}}$ is the experimentally determined volume fraction of non-interacting solvent. The mass of water in unit volume of solution, M_1 , is given by:

$$M_1 = d - C_{\text{CH}} - C_{\text{NaCl}} \quad (6)$$

where d is the density of the solution and C_{CH} and C_{NaCl} are the concentrations of the carbohydrate and simple electrolyte components respectively in solution in g/cm^3 . Thus:

$$M_2 = M_1 - \frac{\beta_{\text{soln.}}}{\beta_{\text{solv.}}} \cdot d_0 \quad (7)$$

where M_2 is the mass of water interacting with carbohydrate and simple electrolyte solute and d_0 is the solvent density. The mass of water interacting with the simple electrolyte component, M_3 , is given by:

$$M_3 = d_{\text{NaCl}} - C_{\text{NaCl}} - \left(\frac{\beta_{\text{NaCl}}}{\beta_{\text{solv.}}} \right) \cdot d_0 \quad (8)$$

β_{NaCl} refers to the adiabatic compressibility of the NaCl solution, and d_{NaCl} and C_{NaCl} to the appropri-

ate densities and concentrations. This allows determination of the water interacting with the carbohydrate components by the difference between eqn. (7) and eqn. (8). Eqn. (5) is applicable to the calcium counterion salt form of hyaluronic acid.

Effect of electrolyte

Fig. 1 shows the adiabatic compressibility of sodium hyaluronate solutions in water and in various saline concentrations as a function of concentration of sodium hyaluronate. The salient features are that the compressibilities at each saline concentration are molecular-weight-independent and that the slopes or compressibility increment, $d\beta/dc$, are substantially independent of simple electrolyte concentration (Table 1).

Table 1 also shows the average value of hydration of sodium hyaluronate samples in the molecular-weight (M_w) range 5.06×10^4 – 3.22×10^6 in molecules of bound water per disaccharide residue for different concentrations of simple electrolyte. The

Fig. 1. Adiabatic compressibility of sodium hyaluronate solutions as a function of sodium hyaluronate concentration (mol of disaccharide/ dm^3), in solvents of different ionic strengths, for hyaluronic preparations of different intrinsic viscosity

Experimental details are indicated in the text. $\circ$, $[\eta] = 160 \text{ cm}^3/\text{g}$; $\bullet$, $[\eta] = 380 \text{ cm}^3/\text{g}$; Δ , $[\eta] = 1960 \text{ cm}^3/\text{g}$; $\square$, $[\eta] = 3160 \text{ cm}^3/\text{g}$. Top three graphs refer to left-hand ordinate. Bottom graph refers to right-hand ordinate.

Table 1. *Compressibility increment and hydration parameters of sodium hyaluronate as a function of ionic strength*

Experimental details are indicated in the text.

Concn. of NaCl (M)	$d\beta/dc$ ($N^{-1} \cdot m^3 \cdot mol^{-1}$)	Averaged hydration value (molecules of hydration water per disaccharide residue)
0	-1.65×10^{-13}	9.0
0.005	-1.59×10^{-13}	8.3
0.050	-1.54×10^{-13}	6.4
0.150	-1.45×10^{-13}	5.2

number of molecules of water of hydration decreases with increasing salt concentration. In saline solutions, as previously found (Davies *et al.*, 1982) for aqueous solutions, change of molecular weight in the range 5.06×10^4 – 3.22×10^6 did not give rise to significant change in hydration value, but increase in concentration of sodium hyaluronate in the range 0.94 – 14.72 mg/cm^3 led to a slight decrease in the hydration parameter. That the number of molecules of water of hydration decreases with increasing simple electrolyte concentration is to be expected, since the hyaluronate chain, having polyelectrolyte properties (Blumberg *et al.*, 1955; Cleland, 1977), will contract on addition of simple electrolyte, thus sterically decreasing the number of potential hydrogen-bonding sites. Further, the addition of simple electrolyte will decrease the electrostrictional field of the carboxy groups of the hyaluronate chain, giving rise also to a decrease in degree of hydration, and will also encourage intramolecular hydrogen-bonding (Atkins *et al.*, 1980), which will have a similar effect.

Effect of temperature

Sound velocities and solution densities were measured as a function of temperature in the range 25 – 50°C , in water and 0.15 M-NaCl at sodium hyaluronate concentrations in the range of 2 – 7 mg/cm^3 , the samples having $\bar{M}_w$ approx. 1×10^6 . The results, expressed as hydration values, are shown in Fig. 2. In both solvent systems, the sodium hyaluronate has a negative temperature coefficient of hydration. This accords with temperature-dependence of hydration obtained by using partial-specific-volume measurements for the polymers poly(ethylene oxide) and poly(vinylpyrrolidone), although dextran showed no temperature-dependence (I. D. Robb, personal communication). The hydration studies on these three polymers were performed in the range 15 – 45°C . All of these results are distinct from the findings reported by Suzuki & Uedaira (1970), who examined the temperature-dependence of hydration of potassium hyaluronate by compressibility measurement and found it had a

Fig. 2. *Plot of molecules of hydration water per disaccharide residue as a function of temperature*

Experimental details are indicated in the text. $\circ$, Sodium hyaluronate in water; $\square$, sodium hyaluronate in 0.15 M-NaCl .

positive temperature coefficient. We can offer no explanation for their findings, which do not follow the normal patterns. Counterion change will not account for this.

Hydration of individual sugars and oligosaccharides

Fig. 3 shows the adiabatic compressibility of sodium glucuronate and *N*-acetylglucosamine solutions in water as a function of molar concentration. It can be seen that the solution adiabatic compressibility of *N*-acetylglucosamine is greater than that of sodium glucuronate at equimolar concentrations. When the results are expressed in terms of hydration parameters, we find an average value of 1.0 molecule of water of hydration per *N*-acetylglucosamine residue, and 11.3 molecules of water of hydration per sodium glucuronate residue. This gives an average value of 12.3 molecules of water of hydration per saccharide pair. Fig. 4 shows the total variation in hydration as a function of saccharide concentration: the hydration value decreases with increase in concentration, as previously noted for hyaluronate polymer. As expected, the charged sugar unit sodium glucuronate had the much higher hydration value. If we compare the hydration parameters of high-molecular-weight sodium hyaluronate ($\bar{M}_w > 5 \times 10^4$) with those of *N*-acetylglucosamine and sodium glucuronate at

Fig. 3. Plot of adiabatic compressibility of *N*-acetylglucosamine ($\square$) and sodium glucuronate ($\circ$) as a function of molar concentration

Experimental details are indicated in the text.

comparable concentrations (approx. 10 mM in each saccharide residue), we get a value of 8.9 molecules of water per disaccharide for high-molecular-weight sodium hyaluronate and 14.1 molecules of water of hydration per saccharide pair. This means some 5 molecules of water of hydration are lost on forming the two glycosidic linkages associated with the polymerization process, and from this one can infer that on average approximately one molecule of water of hydration is associated with each of the two anomeric hydroxy groups and one each with the C-3 in *N*-acetylglucosamine and C-4 in sodium glucuronate. End effects can be seen to be manifest in the value of hydration obtained for sodium hyaluronate oligosaccharides, mainly of decasaccharide chain length (Fig. 5), where the average hydration value is 11.5 water molecules per disaccharide residue, intermediate between the value for the hypothetical disaccharide of individual sugar units and that for the highly polymeric sodium hyaluronate shown for comparison. Similar end effects have been observed for hydration values of highly polymerized dextran and oligomeric dextran with M_n below 2000 (Gekko & Noguchi, 1971). The molecular weight of sodium hyaluronate decasaccharide is 2005.

Fig. 4. Plot of molecules of hydration water per saccharide residue as a function of concentration in mg/cm^3

Experimental details are indicated in the text. $\square$, *N*-Acetylglucosamine; $\triangle$, sodium glucuronate.

Fig. 5. Plot of molecules of hydration water per disaccharide residue as a function of concentration in mg/cm^3

Experimental details are indicated in the text. $\circ$, Sodium hyaluronate ($\bar{M}_w = 5 \times 10^4$); $\square$, sodium hyaluronate oligomers.

Effect of counterion

The adiabatic compressibilities of both sodium and calcium salts of hyaluronic acid are shown in Fig. 6, as a function of uronic acid concentration. When the results are expressed in terms of hydration values (Fig. 7), it is seen that the average hydration value of calcium hyaluronate is slightly greater than that of sodium hyaluronate, by 1.5 molecules of water per disaccharide residue (Davies *et al.*, 1982). Ikada *et al.* (1980), using differential scanning calorimetry to measure non-freezing water, found a

Fig. 6. Plot of adiabatic compressibility of sodium hyaluronate (□) and calcium hyaluronate (○) as a function of molar concentration. Experimental details are indicated in the text.

Fig. 7. Plot of molecules of hydration water per disaccharide residue as a function of concentration in mg/cm^3 for sodium hyaluronate (□) and calcium hyaluronate (○). Experimental details are indicated in the text.

qualitatively similar trend for sodium and calcium salts of chondroitin, a similar glycosaminoglycan composed of *N*-acetylgalactosamine and glucuronic acid. We have shown (A. Davies, J. Gormally, E.

Wyn-Jones, D. J. Wedlock & G. O. Phillips, unpublished work) that Ca^{2+} ion interaction with the hyaluronate chain is less than predicted by current polyelectrolyte theories, and almost certainly does not occur with the expected loss of water from the hydration shell of the Ca^{2+} . This accords with the slightly higher hydration value observed for the calcium salt form of hyaluronate.

Results quoted for hydration of sodium hyaluronate itself obtained by using differential scanning calorimetry (Ikada *et al.*, 1980) give hydration numbers between 2 and 3 times greater than we find, although the calorimetric technique is prone to giving elevated hydration values because of supercooling of polymer solutions. However, in view of the temperature-dependence of hydration parameter observed by us, a higher hydration value at such depressed temperatures is to be expected.

In conclusion, it may be said that the hyaluronic acid molecule, existing in an almost exclusively sodium salt form under normal physiological ionic conditions and temperature, is not an excessively hydrated molecule, but rather its homeostatic properties are probably due to its non-ideal osmotic characteristics and not to water-binding. In aqueous solution at 25°C it is closely associated with not less than 9 molecules of water of hydration, whereas at physiological temperature and ionic strength this is decreased to approx. 4. These parameters are expressed as lower limiting values, since the finite intrinsic compressibility of sodium hyaluronate will result in higher hydration parameters. Suzuki & Uedaira (1970) showed that taking a value of the intrinsic compressibility of dextrin as equal to the value of intrinsic compressibility for potassium hyaluronate increased the value of the hydration parameter by approx. 100%. Sample availability and practical problems prevented determination of the intrinsic compressibility of sodium hyaluronate, and use of data for a neutral polysaccharide would be inappropriate, since we have shown (Fig. 3) that the solution adiabatic compressibility of the hydrated sodium glucuronate residue is less than that of the hydrated *N*-acetylglucosamine residue. A component of this difference could be due to a lower relative intrinsic adiabatic compressibility for the sodium glucuronate residue, and therefore the spread in hydration values obtained by using values for a neutral sugar polysaccharide will be less than inferred by Suzuki & Uedaira (1970).

References

- Atkins, E. D. T., Meader, D. & Scott, J. E. (1980) *Int. J. Biol. Macromol.* 2, 318–319
- Balazs, E. A. & Sweeney, D. B. (1966) in *Modern Problems in Ophthalmology* (Streiff, E. B., ed.), vol. 4, pp. 230–232, S. Karger, Basel and New York

- Balazs, E. A., Bothner-By, A. A. & Gergely, J. (1959) *J. Mol. Biol.* **1**, 147–159
- Barlow, A. J. & Yazgan, E. (1966) *Br. J. Appl. Phys.* **17**, 807–811
- Bitter, T. & Muir, H. M. (1962) *Anal. Biochem.* **4**, 330–334
- Blumberg, B. S., Osler, G. & Meyer, K. (1955) *J. Clin. Invest.* **34**, 1454–1461
- Cleland, R. L. (1977) *Arch. Biochem. Biophys.* **180**, 57–68
- Cleland, R. L. & Wang, J. L. (1970) *Biopolymers* **9**, 799–810
- Comper, W. D. & Laurent, T. C. (1978) *Physiol. Rev.* **58**, 255–315
- Cowman, M. K., Balazs, E. A., Bergman, C. & Meyer, K. (1981) *Biochemistry* **20**, 1379–1385
- Davies, A., Gormally, J., Wyn-Jones, E., Wedlock, D. J. & Phillips, G. O. (1982) *Int. J. Biol. Macromol.* **4**, 436–438
- Eggers, F. (1968) *Acoustica* **19**, 323–329
- Gekko, K. & Noguchi, H. (1971) *Biopolymers* **10**, 1513–1524
- Ikada, Y., Suzuki, M. & Iwata, H. (1980) *ACS Symp. Ser.* **127**, 287
- Jacobson, B. (1955) *Sven. Kem. Tidskr.* **67**, 1–17
- Jacobson, B. & Laurent, T. C. (1954) *J. Colloid. Sci.* **9**, 36–40
- Laurent, T. C. (1957) *Ark. Kemi* **11**, 503–512
- Ling, G. (1980) in *Water and Aqueous Solutions* (Horne, R. A., ed.), p. 664, Wiley-Interscience, New York
- Passinsky, A. (1938) *Acta Physiochim. URSS* **8**, 835–839
- Suzuki, Y. & Uedaira, H. (1970) *Bull. Chem. Soc. Jpn.* **43**, 1892–1894