Study of hyaluronic acid flexibility by electric birefringence

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Transient-electric-birefringence experiments were conducted on four samples of hyaluronic acid over the molecular-mass (M) range 5×10^4 – 4×10^6 in dilute aqueous solution. The geometrical, optical and electrical characteristics were monitored via the rotary relaxation times, optical-polarizability anisotropies and electrical polarizabilities respectively. Each indicates the molecular conformation to be consistent with some degree of rigidity at low M but that this does not persist at high M . The molecules do not become true random coils, but are best characterized in terms of a persistence length of 20 nm or 20 disaccharide units.

Hyaluronic acid is a naturally occurring linear polymer composed of repeating disaccharide sub-units. It is the only glycosaminoglycan that always appears to have the same chemical composition represented by:

Hyaluronic acid is found in the vitreous humour of the eye, Wharton's jelly of the umbilical cord, synovial fluid and articular-bone cartilage (Laurent, 1970). It is present in very small quantities (<1% of the total glycosaminoglycan composition) in connective tissue, where it plays an important role in the hierarchy of the tissue matrix. It is known to bind extensively to proteoglycan (Hardingham & Muir, 1974), thereby forming a hydrophilic aggregate that causes the inflation of the cartilage matrix. These extended aggregates are constrained within a network of collagen fibres. The breakdown of this hierarchy appears to be one of the biochemical changes directly associated with osteoarthritis (McDevitt & Muir, 1975). This conclusion has been reached from the observations that this disorder is often accompanied by both an increase in the quantity of unassociated proteoglycan and a disordering of the collagen network in the cartilage. In the light of these findings it has been suggested that the addition or replacement of cartilage hyaluronic acid may be beneficial in the treatment of arthrotic

disorders. Preliminary trials in which hyaluronic acid of high molecular mass (M) has been injected into horse knee cartilage as an alleviatory treatment for osteoarthritis have met with some success (Gingerich *et al.*, 1980). For this reason, and because of earlier uncertainty over the solution conformation of the molecule, there has been a current renewed quest for a better understanding of the conformational behaviour of hyaluronic acid in dilute solution.

Ogston & Stanier (1951) reported a hydrodynamic study on aqueous hyaluronic acid solutions in which they used viscosity and streaming-birefringence measurements to conclude that the polymer was a spherically symmetric random coil in solution. This was followed by extensive studies by light-scattering (Laurent & Gergely, 1955; Preston *et al.*, 1965; Cleland & Wang, 1970), which all concluded that sodium hyaluronate adopted a solution conformation that was far more rigid than the random coil, although the possibility of domains of freely coiling chains was not excluded. The chain rigidity was attributed to inter-residue hydrogen-bonding, and was further supported by evidence from periodate oxidation and n.m.r. experiments (Scott & Tigwell, 1978; Welti *et al.*, 1979). In addition, the electron-microscopic data of Rosenberg *et al.* (1975) on high- M material indicated extended molecular chains with lengths close to those of the fully extended polymer molecules.

More recently, transient-electric-birefringence measurements on dilute biopolymer solutions have been shown to be a means of rapidly evaluating the electrical, optical and geometrical characteristics of solute molecules (see Hawkins *et al.*, 1979). A pulsed electric field is applied to the solution and the

induced birefringence is recorded. The amplitude of the birefringence is used to evaluate the optical polarizability and the electric dipole moments of the molecules, and the rates of change of the birefringence relate directly to the size and shape of the molecules. In the present paper we report such measurements on aqueous solutions of hyaluronic acid of different M values to characterize the conformation of these molecules.

Theory of the method

For dilute solutions of macromolecules, the overall birefringence depends on the volume concentration of the molecules (c) as well as on their inherent optical anisotropy ($g_1 - g_2$), with g the optical polarizability per unit volume along the major (subscript 1) and minor (subscript 2) axes respectively. At an arbitrary field strength, Δn is given by (Peterlin & Stuart, 1943):

$$\Delta n = \frac{2\pi \cdot c}{n} (g_1 - g_2) \phi \quad (1)$$

Here n is the refractive index of the solvent and ϕ is the orientation function, which is a measure of the degree of orientation under a given field strength.

For relatively low-amplitude applied fields, the orientation function depends on E^2 and has the form (Fredericq & Houssier, 1973):

$$\phi = \frac{E^2}{15} \left(\frac{\mu_1^2}{k^2 T^2} + \frac{(\alpha_1 - \alpha_2)}{kT} \right) \quad (2)$$

Here μ_1 is the permanent dipole moment, assumed to be along a major molecular axis, $(\alpha_1 - \alpha_2)$ is the electrical-polarizability anisotropy, k is the

parameter τ is the rotary relaxation time. For a polydisperse or heterogeneous sample, the sum of composite exponentials is encountered and the equation becomes:

$$\Delta n = \sum_i (\Delta n_0)_i \cdot \exp(-t/\tau_i) \quad (4)$$

An experimental average value of τ can be obtained, by analysing the initial slope of a semi-logarithmic plot of $\ln(\Delta n/\Delta n_0)$ versus t or by considering that time after which the birefringence amplitude has decayed to e^{-1} of its initial value. Various equations exist for the interpretation of the experimental τ in terms of the size of molecules of model shape. In the case of rods of length l , and diameter d (Broersma, 1960)

$$\tau = \frac{\pi \eta l^3}{18 kT} \left[\ln \left(\frac{2l_r}{d} \right) - 0.5 \right]^{-1} \quad (5)$$

Here η is the viscosity of the solvent at the absolute temperature T in question. For a randomly coiled molecule the relaxation time has been related to the relative molecular mass (M) by the equation (Stockmayer & Baur, 1964):

$$\tau = \frac{\chi \cdot [\eta] \cdot \eta_0 \cdot M}{RT} \quad (6)$$

Here χ is a constant equal to 1.21 or 0.85 for freely draining and non-freely draining coils respectively, R is the gas constant and $[\eta]$ is the intrinsic viscosity of the molecules in a solvent of viscosity η_0 . Evaluations of M via this equation are designated M_{rc} .

A model that allows for flexible molecules is the worm-like chain (WLC) for which the relaxation time is given by (Hearst, 1963):

$$\tau = \frac{\eta_0 \cdot q \cdot L^2}{12 kT} \left[1.26 \left(\frac{L}{q} \right)^{\frac{1}{2}} + 0.159 \ln \left(\frac{2q}{b} \right) - 0.387 + 0.16 \left(\frac{b}{a} \right)^{-1} \right] \quad (7)$$

Boltzmann constant and T is the absolute temperature. Under very-high-amplitude fields ($E \rightarrow \infty$) all of the molecules become fully oriented and the orientation function equates to unity. From eqn. (1) one can see that under such conditions the optical-polarizability parameter ($g_1 - g_2$) can be evaluated. By combining this result with experimentally obtained low-field data, the parameters in eqn. (2) can then be calculated.

Termination of the applied field is accompanied by a decay of the birefringence to its pre-field value, according to the equation (Benoit, 1951):

$$\Delta n = \Delta n_0 \cdot \exp(-t/\tau) \quad (3)$$

where Δn is the birefringence at any time t after the pulse cessation for which $t = 0$ and $\Delta n = \Delta n_0$. The

Here L is the contour length of the polymer, and q is the persistence length, which is defined as the length of projection of the molecule as if it were infinitely long, along the direction of the first bond (Kratky & Porod, 1949). Mathematically:

$$q = \lim_{L \rightarrow \infty} \left(\frac{h^2}{2L} \right) \quad (8)$$

The mean square end-to-end separation length of the molecules is given by h , and a and b are parameters used in modelling the frictional properties of larger molecules. For a rod ($a=b$) is the diameter of the rod, to a close approximation (Jennings & Brown, 1971). It has been shown elsewhere (Hearst & Stockmayer, 1962) that a and b can in the first

instance be approximated to the diameter of the polymer backbone. The persistence length is a useful indicator of polymer flexibility. For very rigid molecules approximating thin rods, such as polypeptides in helix-promoting solvents, q is as large as or larger than the contour length (L). Randomly coiled systems, such as polystyrene in benzene, may have a persistence length of less than 1 nm (Holzwarth, 1981). Typical of intermediate cases in high- M DNA, for which q is approx. 60 nm.

An alternative model (Hearst, 1963) that uses the parameter q is the weakly bending rod (WBR), for which:

$$\tau = \frac{\pi \cdot \eta_0 \cdot L^3}{6kT} \left\{ 3 \ln \left(\frac{L}{2b} \right) - 4.92 + 4 \left(\frac{b}{a} \right) + \frac{L}{4q} \left[4.5 \ln \left(\frac{L}{2b} \right) - 10.2 + 4 \left(\frac{b}{a} \right) \right] \right\}^{-1} \quad (9)$$

Experimental

Four hyaluronic acid samples (sodium salt, from rooster comb cartilage) covering the M range $5 \times 10^4 < M_w < 4 \times 10^6$ were studied. These were a gift from E. Wyn-Jones and A. Davies of the University of Salford, who also provided M_w and M_n data from their size-exclusion chromatography measurements. The samples were identical with those reported in Davies *et al.* (1982). All samples were highly birefringent and were studied at concentrations of

approx. $10 \mu\text{g/ml}$ at neutral pH in freshly distilled and deionized water.

A schematic of the electric-birefringence apparatus is given in Fig. 1. A helium/neon laser generated at a light-beam that passed through a high-quality polarizer, a sample cell, an optical quarter-wave plate and a second analysing polarizer. This was followed by a low-noise photodiode detector whose output was amplified and displayed directly on an oscilloscope, whose trace was recorded photographically. In all cases the response was transient (Fig. 2). All applied electric pulses were generated by a commercial d.c. pulse generator.

Pulses of up to $1.8 \times 10^6 \text{ V} \cdot \text{m}^{-1}$ and of durations up to 8 ms were used for each sample.

Results

Regular birefringence transients were always obtained (Fig. 2a). From the steady-state amplitude of these transients, birefringence values were determined at various field strengths for each sample. In each case a Kerr-Law region [i.e. $\Delta n \propto E^2$; as eqn.

Fig. 1. Schematic diagram of an electric-birefringence apparatus

Light from an He/Ne laser passes through a polarizer, sample cell, quarter-wave plate and analyser to fall on to a photodiode detector. A high-voltage pulse generator creates a square-wave d.c. electric field across the electrodes, and the induced birefringence signal is stored digitally on a transient recorder and processed by a micro-computer.

Fig. 2. Electric-birefringence transients of aqueous solutions of sodium hyaluronate

(a) Typical birefringence response from a solution of sodium hyaluronate ($\bar{M}_w = 3.2 \times 10^6$) at a concentration of $32 \mu\text{g/ml}$. The electric field was $2.5 \times 10^5 \text{ V/m}$ and $\Delta n = 6.0 \times 10^{-8}$. The time scale is 0.2 ms/division . The two-component relaxation (one very fast, one very slow) is quite noticeable. (b) Birefringence response of sodium hyaluronate ($\bar{M}_w = 1.1 \times 10^6$) at a concentration of $57 \mu\text{g/ml}$ to a reversing-field ($1.0 \times 10^5 \text{ V/m}$) d.c. pulse. The rate of decay of the birefringence ($\Delta n = 2.1 \times 10^{-8}$) on field reversal indicates absence of permanent dipole moment. The time scale is 0.1 ms/division .

(2)] and a saturation region [Δn independent of E ; as eqn. (1) with $\phi = 1$] was manifest. From the high-field data, with the use of eqn. (1) the optical anisotropy ($g_1 - g_2$) was evaluated. Furthermore, by using these data in conjunction with the initial slopes of Fig. 3, one could evaluate the factor $[(\Delta\alpha/kT) + (\mu^2/k^2T^2)]$. This is essentially a summation factor of the permanent and induced dipolar contributions to the electrical characteristics of the molecules. An additional experiment was performed to determine the absolute values of the induced and permanent dipole moments. This consisted of applying pulses in juxtaposition of alternate polarity, i.e. the applied field was switched from a positive to a negative-going voltage instantaneously. Should the

Fig. 3. Kerr plot of birefringence versus the square of the electric field for sodium hyaluronate

The sodium hyaluronate had $\bar{M}_w$ 1.3×10^6 and was at a concentration of $5.7 \mu\text{g/ml}$. Low-field data are shown in the inset. Error bars indicate experimental uncertainty.

molecules have a permanent dipole moment, then such field reversal would be accompanied by a 180° rotation of the molecules to follow the polarity change, thereby causing an instantaneous fall and rise in the observed birefringence. Induced dipolar contributions involve instantaneous charge-displacement reversal in the molecular structure with no observable change in Δn on field reversal. Fig. 2(b) shows the absence of any birefringence change with the field reversal. It is thus concluded that permanent dipoles are absent and that the aforementioned factor has its origin solely in induced dipole mechanism. Values of the polarizability anisotropy parameter ($\alpha_1 - \alpha_2$) and the optical-polarizability anisotropies have been evaluated for each sample from equivalent data to those shown in Fig. 3. The values are displayed in Fig. 4.

The field-free decay of the transient birefringence was expressed in terms of the semi-logarithmic plots mentioned above. In principle, single relaxation times might be expected for monodisperse samples. All experimental decays were analysed by using a Marquart-type curve-fitting procedure for the variation in the semi-logarithmic plot slope. Two predominant relaxation times were obtained for each sample. These were designated as τ_f and τ_s for the fast and the slow values, respectively. They are listed in Table 1. It is seen that, whereas τ_s varies from sample to sample, τ_f is approximately constant for each sample. It is concluded that τ_f is due to the rotational diffusion of the small oligosaccharide impurities in the samples. The slow relaxation

Table 1. Experimental data for hyaluronic acid samples

Weight-average molecular mass $10^{-6} \times M_w$	Number-average molecular mass $10^{-6} \times M_n$	Fast relaxation process τ_f (ms)	Slow relaxation process τ_s (ms)	Molecular mass of equivalent random coil $10^{-6} \times M_{rc}$
0.051	0.017	0.01	0.02	0.3
0.45	0.18	0.01	0.60	3.2
1.3	0.37	0.03	6.8	6.9
3.2	0.64	0.03	9.8	6.2

Fig. 4. Effect of molecular mass on the optical polarizability anisotropy ($g_1 - g_2$) and the electric polarizability ($\alpha_1 - \alpha_2$) of sodium hyaluronate

Fig. 5. Length of rod-like particle as a function of molecular mass
Curve A, theoretical length of hyaluronate chain if a rigid rod model is assumed. Curve B, experimentally determined length obtained from rotational diffusion coefficient.

process, τ_s , is that due to the tumbling motion of the hyaluronic acid molecules.
Values of τ_s have been analysed for each sample in terms of each of the four molecular models implied in eqns. (5)–(9). In each case, values of the equivalent rod length (l_r), the persistence length (q)

Fig. 6. Persistence length of hyaluronate as a function of molecular mass for two models which allow chain flexibility

Curve A, weakly bending rod (WBR) model. Curve B, worm-like chain (WLC) model.

from the WBR and WLC models, and the equivalent molecular mass (M_{rc}) from the Stockmayer–Baur random-coil model have all been calculated and are given either in the Table or in Figs. 5 and 6.

Discussion

Geometric parameters

Departure of the curve B of Fig. 5 from linearity shows that a rigid-rod model is inappropriate for hyaluronic acid over the M range studied. The initial slope of that Figure (A) represents the equivalent behaviour of a rigid rod having the extended-chain contour length of the polymer. As might be expected, the observed values do, however, approach numerical agreement with rod-like behaviour for limiting small M .

From Table 1 one also sees that the M values derived from the experimental τ in terms of eqn. (6) for an assumed freely flexible random coil are also unsatisfactory. It should be noted, however, that, whereas for the lowest M studied the discrepancy between M_w and M_{rc} is some 6-fold, this has decreased to only 2-fold for M of a few million. It is concluded that the molecular conformation becomes increasingly coiled with hyaluronic acid of the highest M values studied.

Analysis of the major relaxation time in terms of semi-flexible molecular models is thus preferred. The persistence length is a parameter that describes constant curvature in a molecular chain and that reaches a constant value at higher M values (see eqn. 8). The attainment of such a condition is seen in Fig. 6 for both the WBR and the WLC models. It is the WBR model that most clearly illustrates the departure of this polymer from a rigid to a flexible molecular conformation. For either model, the limiting persistence length at high M is approx. 20 nm. This corresponds to a high degree of molecular flexibility. As each disaccharide unit is approx. 1 nm in length (Bettelheim, 1959), the persistence length corresponds to 20 individual disaccharide subunits. A molecule of such low M might be expected to act as a rigid system. Increasing M would be accompanied by the increasing curvature and hence flexibility implicit in q . However, the system never reaches truly random-coiled behaviour.

Electrical and optical parameters

Similar conclusions are derived from an analysis of both the optical anisotropy per unit volume ($g_1 - g_2$) and the anisotropy of the electrical polarizability ($\alpha_1 - \alpha_2$). The dependence of these parameters on M is not expected to be the same, however. Optical anisotropy is a volume-dependent parameter and thus depends directly on the length of a rigid molecule, and hence on M . For rigid molecules the electrical anisotropy ($\alpha_1 - \alpha_2$) has been predicted theoretically to depend on the square (or even the cube) of the length of a rigid rod (Mandel, 1961; McTague & Gibbs, 1966; Oosawa, 1971; Schurr, 1971) and hence on M^x , with $x > 1$.

Non-linear dependence of ($g_1 - g_2$) on M over the molecular-mass range studied is evidenced directly in Fig. 4. As in the analysis of τ , however, the data much more closely resemble a linear dependence at low M .

The variable power-dependence of ($\alpha_1 - \alpha_2$) on a particle length depends on the origins of the polarizability and the charged nature of the polymer. A commonly used equation for charged rods in conducting media is that due to Mandel (1961) and is given by:

$$\alpha_1 = \frac{Z^2 \cdot n \cdot e^2 \cdot l_r^2}{12kT} \quad (10)$$

where n is the total number of counterions each of valency Z and charge e associated with the surface of a rod molecule of length l_r . It is assumed that $\alpha_1 \gg \alpha_2$. The suitability of this equation and model are appraised in Fig. 7, where it is seen that ($\alpha_1 - \alpha_2$) does not maintain constant dependence on the experimentally observed rod length (Fig. 5) l_r^2 ,

Fig. 7. Electric polarizability ($\alpha_1 - \alpha_2$) of a rod-like particle as a function of the square of the rod length (l_r).

although an inclination to such dependence is again seen for low molecular length. Comparison of Fig. 7 with Fig. 4 shows the possibility of the dependence of ($\alpha_1 - \alpha_2$) on l^2 rather than on l . It is concluded that both ($\alpha_1 - \alpha_2$) and ($g_1 - g_2$) also support the interpretation that hyaluronic acid is stiff at low M values and exhibits increasing flexibility with increasing M .

The magnitudes of the optical anisotropy are typical of those for other long thin macromolecules. The electrical polarizability calculated from eqn. (10) is approx. $1 \times 10^{-30} \text{ F} \cdot \text{m}^2$, which is 10 times the experimental values. Such agreement is acceptable for two reasons. Firstly, the Mandel model assumes the completely uniform smearing of charge along the surface of the particles, with each charge having complete freedom to move in the electric field. Such a finite binding constant between Na^+ ions and carboxylic groups at low ionic strength is unrealistic. Secondly, actual polarization does not take place at the molecular surface, but rather at the plane of shear in the electric charge layer surrounding the molecules. The theory cannot account for this. In the light of these limitations, the order of magnitude agreement between eqn. (10) and experiment is encouraging.

General conclusions

Transient-electric-birefringence measurements give rise to geometrical, electrical and optical parameters. Each set of parameters for hyaluronic acid samples of various M values indicate the same molecular behaviour for this molecule. At very low M values, departure from any rigid conformation is evidence. Such an M value is well below 10^5 . With increasing M values, and up to 4×10^6 , increasing molecular flexibility is exhibited. Although the system never reaches a truly flexible randomly coiled conformation, it appears that the system is well described by a weakly bending rod with a persistence length of some 20 nm. Two further obser-

variations are made on this conclusion. Firstly, such a model is consistent with the high value of $\beta = 0.8$ in the Mark-Houwink equation:

$$[\eta] = KM^\beta \quad (11)$$

reported by Cleland & Wang (1970). Secondly, the data also explain the controversy in the literature as to the conformation of this molecule. Much of the early work was undertaken on material of a single molecular mass. Hence in cases where low M was used interpretations were given in terms of rigid-rod models (Cleland & Wang, 1970; Cleland, 1977). Related experiments on high- M material seemed more consistent with interpretation via truly random coils (Laurent & Gergely, 1955; Laurent, 1970). It would appear from the present work that hyaluronic acid is never described completely as a random coil or as a rigid rod, but that full descriptions can only be made in terms of models such as the weakly bending rod or the worm-like chain via the persistence-length concept.

Finally, transient electric birefringence presents itself as a method for studying such complicated systems. It has two major advantages. Firstly, the experiments and data acquisition are extremely fast. Secondly, data relating to the geometry and the optical and electrical parameters of the molecules are all simultaneously recorded. The method is well suited to the study of small sample volumes (500 μ l) and is not destructive. In the case of highly birefringent molecules, such as the hyaluronic acid studied in the present work, concentrations as low as 100 ng/ml can be analysed with ease, thereby minimizing the problem of solute-solute interactions. A disadvantage of the method, however, is that it cannot be used with highly conducting media, e.g. at extremes of pH or under high ionic strengths.

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