Characterization of Proteoglycan and the Proteoglycan–Hyaluronic Acid Complex by Electric Birefringence

By MARTIN ISLES,* ALAN R. FOWERAKER,* BARRY R. JENNINGS,*
TIM HARDINGHAM† and HELEN MUIR†

**Department of Physics, Brunel University, Uxbridge, Middx. UB8 3PH, U.K., and*
†*Kennedy Institute of Rheumatology, Bute Gardens, Hammersmith, London W6 7DW, U.K.*

(Received 7 November 1977)

An electric field causes partial alignment of macromolecules in a dilute solution. The accompanying changes in the solution birefringence offer a sensitive and quick means of monitoring the rates of particle orientation and hence the size of the solute molecules. Such measurements are reported for dilute solutions of proteoglycans in the absence and presence of added hyaluronic acid. The proteoglycan molecules are shown to be some 580 nm long. In the presence of hyaluronic acid they form aggregates that appear to be consistent with the model previously proposed in which the proteoglycans attach radially to the extended hyaluronic acid chain. The electric-birefringence relaxation rates indicate aggregates of similar length to that of the extended hyaluronic acid chain, with the proteoglycans spaced on average at 29 nm intervals. A proteoglycan sample the cystine residues of which had been reduced and alkylated showed no evidence of aggregation with hyaluronic acid up to the concentrations of the acid corresponding to 1% of the total uronic acid content. The electric-birefringence method is shown to have a large potential in the study of associating polysaccharide solutions.

Cartilage proteoglycans form a class of complex macromolecules in which a large number of unbranched polysaccharide chains (chondroitin sulphate and keratan sulphate) are covalently attached by their reducing ends to a common polypeptide 'backbone' (reviewed by Muir & Hardingham, 1975).

A large proportion of the proteoglycan in hyaline cartilage has been shown to be capable of existing as large aggregates in which many proteoglycan molecules bind to a chain of hyaluronate. Each proteoglycan molecule has a binding site at one end of the protein core (Hardingham & Muir, 1973*a*) that has a high affinity for a decasaccharide unit of hyaluronate (Hardingham & Muir, 1973*b*; Hascall & Heinegård, 1974).

The general structure of both the proteoglycan unit and of the aggregate has been confirmed by electron-microscopic studies of samples when spread in monolayers (Rosenberg *et al.*, 1975; Lohmander & Hjerpe, 1975). This procedure has also permitted some direct measurement in the dry state of the length of the extended polypeptide backbone that forms the major axis of each proteoglycan molecule.

A number of novel electro-optical methods have been developed by which the size and shape of macromolecules can be determined in dilute solution, by using birefringence, scattered intensity, dichroism, fluorescence and optical rotation as the monitoring

optical property (Stoylov, 1971; Jennings, 1977). The physical principles underlying the methods are as follows. Many macromolecules are optically anisotropic. In dilute solution, however, molecules adopt random orientations with respect to neighbours and the medium as a whole is optically isotropic. In an electric field, individual molecules experience a torque as inherent or field-induced dipoles interact with the applied field. Some degree of orientational order is imposed on the solute molecules and the medium becomes anisotropic. By monitoring the magnitudes of the changes in the optical properties of the solution, one can infer the orientational behaviour of the solute macromolecules. It is advantageous to apply the field as a short-duration pulse and to record simultaneously the rates of change in the optical properties. These rates are then a measure of the orientational relaxation times (τ) of the rotating macromolecules, and τ is itself an indication of the molecular size and shape.

In the present paper we report an electric-birefringence study on solutions of proteoglycan and its interaction with hyaluronic acid. Electric birefringence, often called the Kerr (1875) electro-optic effect, is the longest established of the electro-optical methods for the study of solutions and suspensions (Peterlin & Stuart, 1943; Benoit, 1951; Yoshioka & Watanabe, 1969). The method is shown herein to

provide a rapid and sensitive means of evaluating the size of proteoglycan molecules and the size and structure of the complex formed with hyaluronate.

Experimental

Materials

All measurements were made on samples of pig laryngeal proteoglycan prepared as described by Hardingham & Muir (1974). The sample was studied at a concentration of $209\mu\text{g}$ of uronic acid/ml (approx. 0.8 g of proteoglycan/litre) in water. It was prepared by dialysing proteoglycan in 0.5M-sodium acetate, pH6.8, against water adjusted to pH7.0 with 0.1M-NaOH. Preliminary experiments had established that this was in the middle of a concentration range in which there were no large changes in Δn or τ as a result of excessive intermolecular interactions.

The hyaluronic acid was from BDH Chemicals, Poole, Dorset, U.K., and was fractionated by gel chromatography on a column ($165\text{cm} \times 1.1\text{cm}$) of Sepharose 2B eluted at 7 ml/h in 0.5M-sodium acetate at pH6.8 and 4°C . The fraction of narrow size range (characterized by the position of elution with K_{av} , between 0.02 and 0.20) had a limiting specific viscosity of $[\eta]=1296\text{ml/g}$ measured with an Ostwald capillary viscometer. This corresponded to a viscosity-average molecular mass (M) of 6.7×10^5 by using the relationship:

$$[\eta]=KM^a \quad (1)$$

where $K=0.0228\text{ml/g}$ and $\alpha=0.816$ for hyaluronate in 0.2M-NaCl (Cleland & Wang, 1970). From this relative molecular mass the length of the extended chain was estimated by assuming 1 disaccharide unit $=1\text{nm}$.

Method

A convenient apparatus for electric-birefringence studies is shown schematically in Fig. 1. Light from a lower-power helium/neon laser passes through a polarizer-analyser pair that is set in the mutually crossed arrangement. Between these polarizing devices a cell is placed. This holds the solution under test between two stainless-steel electrodes. These electrodes are parallel and some 2.34mm apart. They extend the whole 5.10cm length of the cell. The system is adjusted so that the uniform electric field, obtained when an electric potential difference is applied between the electrodes, is at an azimuth of 45° with respect to that of the polarizer. The cell body is made of polytetrafluoroethylene and holds some 3ml of solution. It is fitted with inlet and outlet ports that enable connection to be made to a peristaltic pump and reservoir for remote control and modification of the solution under study. The light that penetrates the optical system is incident on a high-gain low-noise photomultiplier, whose output is displayed directly on to an oscilloscope and photographed for analysis at leisure. The photomultiplier response is proportional to $(\Delta n)^2$ when the polarizer and analyser are crossed or offset by a few degrees respectively (Badoz, 1956). The factor Δn represents

Fig. 1. Schematic diagram of the apparatus

Light from the laser penetrates a polarizing prism (pol), the sample cell, aperture stops (s) and an analysing prism (an) before falling on the cathode of a photomultiplier (PM). Pulsed electric potential differences originate at the pulse generator (PG) and are applied simultaneously to the cell electrodes and, via a high-voltage probe (PR), to the oscilloscope (CRO).

the birefringence in the cell and is defined as the difference between the refractive indices of the solution parallel and perpendicular to the direction of the applied field.

When the field is applied in the form of a pulse, the corresponding response in the photomultiplier output is transient in form. The initial build-up region of the birefringence, corresponding to the alignment of the solute molecules, is followed by the attainment of an equilibrium condition. The amplitude under this situation can, at least for rigid non-interacting molecules, be related directly to the electrical dipolar properties of the molecules. Of greater interest in the present study, however, is the birefringence decay following the termination of the electric pulse. The birefringence amplitude at any time (t) after the cessation of the field ($t=0$) obeys the equation (Benoit, 1951):

$$\Delta n = \Delta n_0 \exp(-t/\tau) \quad (2)$$

for a monodisperse solute. In this equation, Δn_0 is the value of the birefringence at zero time and τ is the molecular orientational relaxation time. It is a useful characteristic of the molecular geometry, which is obtained by analysing the decay rate of the birefringence in terms of the slope of the graph of $\ln(\Delta n/\Delta n_0)$ versus t .

The procedure for the birefringence measurements was as follows. A known quantity (10ml) of the proteoglycan solution was introduced into the reservoir and pumped into the birefringence cell. Care was taken to avoid air-bubble formation. A pulsed electric field was then applied to the solution and the photomultiplier transient output was recorded on the oscilloscope. Some 0.1 ml of a stock solution containing $20\mu\text{g}$ of uronic acid/ml, equivalent to $50\mu\text{g}$ of hyaluronic acid/ml, was then added to the reservoir and the total solution was circulated for complete mixing. The mixture was then left for some 15 min to allow the proteoglycan to interact with the hyaluronic acid. After this interval, a second measurement was recorded. Similar additions of hyaluronic acid were made successively, and the above procedure was repeated until the birefringence amplitudes remained constant with further addition of hyaluronic acid. Throughout the course of the experiment, the applied pulse width was adjusted to accommodate the apparent changes in the particle relaxation time τ . For data reported herein, a field strength of 360V/cm was used. This was within the range of fields (E) for which the birefringence was proportional to E^2 . All measurements were made at an ambient temperature of 22°C .

Results and Discussion

The study is best considered in three parts: proteoglycan solutions, the proteoglycan-hyaluronic acid complex and reduced and alkylated proteoglycans.

Vol. 173

(a) Proteoglycan solutions

A typical transient response of the photomultiplier is shown in Fig. 2(a) for a proteoglycan solution of concentration 0.8mg/ml . The birefringence is positive, indicating that the optical polarizability of the molecules is greatest along their major geometric axis. Treating the molecules in the first instance as prolate ellipsoids or cylinders, the major axis corresponds to the extended protein core of the molecules. Fig. 3 is an analysis of the decay of the transient of Fig. 2(a) according to eqn. (1). The curvature of the semi-logarithmic plot indicates the polydisperse nature of the sample as each species present contributes to the exponential decay process. The most reliable data are those of the initial slope (Schweitzer & Jennings, 1972), for which a value of $3.6 \pm 0.3\text{ms}$ was obtained for τ . It is instructive to relate this to the dimensions of an equivalent ellipsoid by using the following equation (Perrin, 1934) with D the rotary diffusion coefficient, k the Boltzmann constant and T the absolute temperature:

$$D = \frac{1}{6\tau} = \frac{3kT}{16\pi\eta a^3} \left(\frac{p}{p^2-1} \right) \left\{ \frac{(2p^2-1)}{p\sqrt{(p^2-1)}} \ln[p + \sqrt{(p^2-1)}] - 1 \right\} \quad (3)$$

Here η is the viscosity of the solution, with a and b the semi-major and minor axial lengths and p the axial ratio (a/b). Eqn. (3) is insensitive to the value chosen for the semi-minor axis owing to the strong dependence of τ on a^3 . For the molecular dimensions calculated below, a variation of some 10% in the assumed minor axis results in only a 2% change in the calculated molecular length. Assuming a value of 40nm for the semi-minor axis, corresponding to the extended length of chondroitin sulphate chains of 20000mol.wt. (Hardingham & Muir, 1973a), a molecular length of 580nm is obtained for the proteoglycan molecules. This is comparable with the estimate by Rosenberg *et al.* (1975), where a length of approx. 400nm was suggested. The value of 580nm found in the present study would correspond to the proteoglycan being highly extended at these low ionic strengths. This would not be surprising when one considers the mutually repulsive charges of the neighbouring polysaccharide side chains as evidence by an increase in radius of gyration of proteoglycan as low ionic strength in a light-scattering study (Pasternak *et al.*, 1974).

(b) Proteoglycan-hyaluronate complex

As the hyaluronic acid was progressively added to the proteoglycan solution, the birefringence transients changed. The following factors were observed when pulsed fields of 360V/cm and 10ms duration were used.

Fig. 2. Transient birefringence for proteoglycan with added hyaluronic acid

In each part, the applied field amplitude is 360 V/cm, and the proteoglycan concentration corresponds to 209 μg of uronic acid/ml. (a) Response with no added hyaluronic acid. The amplitude of the transient corresponds to $\Delta n = 6.4 \times 10^{-8}$ and the decay to $\tau = 3.6$ ms. (b) The 'fast' positive birefringence contribution in the presence of hyaluronic acid at a concentration of 140 μg of uronic acid/ml. The transient amplitude corresponds to $\Delta n = 9.8 \times 10^{-9}$ and $\tau = 3$ ms. Note that in this situation the tail of the transient goes negative with respect to its pre-field value owing to the presence of the 'slow' negative contribution. (c) The 'slow' negative contribution in the presence of hyaluronic acid at a concentration of 160 μg of uronic acid/ml. For this component, $\tau = 650$ ms. The amplitude of the negative optical response is 16% of the amplitude of the trace of frame (a).

Fig. 3. Analysis of the transient decay of the birefringence for proteoglycan solutions

The decay of the birefringence following the termination of the electric field, for the transient of Fig. 2(a). The initial slope (---) of the semi-logarithmic plot of the reduced birefringence with time corresponds to $\tau = 3.6$ ms. Curvature of the graph indicates sample polydispersity.

Fig. 4. Effect of hyaluronic acid on the birefringence of a proteoglycan solution

The proteoglycan concentration was 209 μg of uronic acid/ml. The field strength was 360 V/cm. Error bars indicate the maximum uncertainties in the measurements of the birefringence.

(i) The birefringence positive amplitude progressively decreased. The decline in the amplitudes of Δn with hyaluronic acid concentration is shown in Fig. 4: it is continuous.

(ii) Analysis of the decay curves showed the same initial slope and hence the same value of τ throughout the range of hyaluronic acid concentration.

(iii) For concentrations of hyaluronic acid greater than 0.57% the transients showed an increasing tendency to overshoot the base line at the end of the decay process. This is shown in Fig. 2(b).

This third factor appears to be due to the presence of a second rotatory process with apparent birefringence of opposite sign and of a much longer relaxation time. In an attempt to study this additional contribution, the field pulse length was extended to 160ms. A typical response is shown in Fig. 2(c). The characteristic relaxation time of this slow negative contribution was 650ms. It was noted that, as the hyaluronic acid concentration was increased, the positive fast contribution diminished and the negative slow process became increasingly dominant. We shall refer to these as the 'fast' and 'slow' contributions respectively. Auxiliary experiments were made on solutions of hyaluronic acid alone. For these molecules in isolation the relaxation time was as fast as a few microseconds. This excludes the possibility that these molecules could be responsible for the complex two-component behaviour observed in the present study.

In birefringence measurements, it is convenient to record the transmitted-light-intensity in the absence of the orienting field, as this is used to quantify the amplitude of Δn . It was noted that, as the hyaluronic acid was increasingly added to the proteoglycan solution, so this residual background intensity, which is the reciprocal of the apparent absorbance, progressively decreased (Fig. 5). Such an increase in the absorbance suggests that, as the hyaluronic acid was added, the scattering power of the solution increased, thereby depleting the light-flux that penetrated the

Fig. 5. Effect of hyaluronic acid on the absorbance of a proteoglycan solution

The relative change in absorbance $\Delta A = A_c - A_{c=0}$ of the solution is plotted as a function of added hyaluronic acid concentration c . The proteoglycan concentration was 209 μg of uronic acid/ml. Data were recorded at a wavelength of 633 nm.

medium. The growth in scattered intensity immediately indicated an increase in solute particle size.

A consistent interpretation of all the data is as follows. The constancy of the fast-component relaxation time and its identity with the value obtained for the proteoglycan molecules indicates the persistence of free proteoglycan molecules in solution on addition of small quantities of hyaluronic acid. Fig. 4 thus provides an indication of the declining quantity of free proteoglycan in the mixture. At higher concentrations of hyaluronic acid, the slow component became increasingly important as the fast component declined. The much greater but constant value of τ for the slow component, which was some 180 times that of the fast component, the corresponding increase of the absorbance and the accompanying change in the sign of the birefringence all suggest that a highly specific molecular interaction was present in ever-increasing quantity.

Furthermore, the data support the model (Hardingham & Muir, 1973a) for the complex formed between proteoglycans and hyaluronic acid chains (Fig. 6). The proteoglycans are visualized as being regularly spaced along the hyaluronic acid molecule and being radially attached via a specific interaction with a globular protein head that is located at the end of each proteoglycan molecule.

The electric-birefringence data enable us to discuss this model in a quantitative manner. Firstly, in such a model, the major axes of the proteoglycan molecules form the transverse or minor axes of the complex. Hence the birefringence of the complex should be of opposite sign to that of the individual proteoglycan molecules, as observed. Secondly, the complex may be approximated by a prolate ellipsoid in the first analysis, with its major axis defined by the extended hyaluronic acid chain. Under '(a) Proteoglycan solutions' the length of the proteoglycan molecules was estimated to be 580 nm. According to the model, this should provide the semi-minor axis of the complex. Using it as such, together with the value of 650 ms for τ in eqn. (3), leads to a semi-major

Fig. 6. Schematic representation of the proteoglycan-hyaluronate complex

axis of $1.5\mu\text{m}$, or $3\mu\text{m}$ for the overall length of the complex. This is to be compared with the average length of $1.6\mu\text{m}$ estimated for the individual hyaluronic acid molecules from the viscosity data. In addition, it supports the suggestion that the complex exists in a highly extended state at the low ionic strengths used in this study. Thirdly, from Fig. 4, the birefringence has fallen to zero when the hyaluronic acid concentration has risen to 0.86% of the total uronic acid content. If the positive birefringence be taken as indicative of the non-aggregating fraction of proteoglycan in the solution, then this particular hyaluronic acid concentration corresponds to the condition where all the proteoglycan is utilized in the formation of the complex. From this condition and assuming mol.wt. of 2×10^6 and 6.7×10^5 for the proteoglycan and hyaluronic acid respectively, the experimental data indicate a packing of one proteoglycan molecule approximately every 29 nm along the hyaluronic acid chain. This agrees well with previous estimates of 24 nm (Hardingham & Muir, 1973b), 24–48 nm (Hascall & Heinegård, 1974) and 20–30 nm (Rosenberg *et al.*, 1975).

(c) Reduced and alkylated proteoglycan

Proteoglycans were reduced in the presence of dithiothreitol and alkylated with iodoacetamide. Viscosity measurements, gel chromatography and sedimentation-velocity analysis showed that there was no significant decrease in the average molecular size, but they were no longer able to bind to hyaluronate (Hardingham *et al.*, 1976).

Electric-birefringence measurements were made on this reduced and alkylated sample by using fields of up to 240 V/cm and of 6 ms duration. The solution concentrations were identical with those used for the studies on the 'normal' proteoglycans. Data were recorded in the absence and presence of various amounts of hyaluronic acid. Typical transients are shown in Fig. 7. From these measurements we note the following.

(i) In the absence of added hyaluronic acid, for a solution of concentration $209\mu\text{g}$ of uronic acid/ml, the magnitude of the birefringence was 8×10^{-8} and the relaxation time was 2.6 ms. These should be compared with 6.8×10^{-8} and 3.6 ms respectively for the non-alkylated sample. The small differences in these two sets of parameters simply reflect a minor difference in molecular size of some 1.15 times only between the two preparations. The preparation of the reduced and alkylated form therefore has not involved gross changes in the molecular geometry.

(ii) The addition of hyaluronic acid did not affect the transient birefringence response of the solutions of reduced and alkylated proteoglycans. This was true even up to an hyaluronic acid content corresponding to $200\mu\text{g}$ of uronic acid/ml. We recall that

Fig. 7. Transient birefringence for reduced and alkylated proteoglycan

(a) With no added hyaluronic acid; $\Delta n = 8 \times 10^{-8}$ and $\tau = 2.6$ ms. (b) With hyaluronic acid added to a concentration corresponding to $200\mu\text{g}$ of uronic acid/ml. Both transients were obtained with electric fields of 240 V/cm amplitude and 6 ms duration on a proteoglycan solution of $209\mu\text{g}/\text{ml}$ of uronic acid concentration.

this was the maximum hyaluronic acid content used in experiments with the non-alkylated proteoglycans. In Fig. 7, the traces for both zero and this maximal hyaluronic acid content are shown when the reduced and alkylated material was used. It is instructive to compare Fig. 7 with Fig. 2.

(iii) With the addition of hyaluronic acid, both the molecular relaxation time and the amplitude of the birefringence remained essentially constant. There was no gross decrease in Δn , no rapid growth in τ and no appearance of a 'slow' negative contribution to the birefringence. All of these factors indicate that, in the presence of hyaluronic acid, the reduced and alkylated proteoglycan molecules neither form the specific radial complex detected for natural proteoglycan nor indeed do they appear to form any other type of aggregate.

(iv) The 'free' hyaluronic acid in this case did not contribute noticeably to the observed birefringence. Neither did the absorbance of the solution change

significantly. This confirms the supposition that the 'slow' component encountered with the experiments on natural proteoglycan (as in Fig. 2) was not due to free hyaluronic acid.

(d) Conclusions

The results showed that the electric-birefringence technique is particularly suitable for investigating cartilage proteoglycans. At relatively low field strengths (360 V/cm) large changes in birefringence are encountered, reflecting a large natural and/or induced electrical dipole moment, which permitted measurements to be made at low concentration (below 1 mg/ml). The change in birefringence observed on binding to hyaluronate involved a loss of the free proteoglycan response and the appearance of a new, slower, component associated with the aggregate that was evident at the same field strength, but only with much longer field times. This was in complete agreement with the characteristics of aggregation established by other techniques (Muir & Hardingham, 1975).

At the present stage of development of the technique it was not possible to allow for polydispersity and non-ideality of the solution in the analysis of the results. However, assuming a simple ellipsoid model, the molecular dimensions calculated for the proteoglycan monomer and aggregate were reasonably close to those predicted from the known average molecular weight of the proteoglycans and hyaluronate used in this study.

The Science Research Council is gratefully acknowledged for the award of a research studentship to M. I. and the Arthritis and Rheumatism Council for general support for T. E. H., H. M. and a special grant to B. R. J.

References

- Badoz, J. (1956) *J. Phys. Radium* **17**, Suppl. **11**, 143A-149A
- Benoit, H. (1951) *Ann. Phys. (Paris)* **6**, 561-609
- Cleland, R. L. & Wang, J. L. (1970) *Biopolymers* **9**, 799-810
- Hardingham, T. E. & Muir, H. (1973a) in *Normal and Osteoarthrotic Articular Cartilage* (Ali, S. Y., Elves, M. W. & Leaback, D. H., ed.), pp. 51-63, Institute of Orthopaedics, London
- Hardingham, T. E. & Muir, H. (1973b) *Biochem. J.* **135**, 905-908
- Hardingham, T. E. & Muir, H. (1974) *Biochem. J.* **139**, 565-581
- Hardingham, T. E., Ewins, R. J. F. & Muir, H. (1976) *Biochem. J.* **157**, 127-143
- Hascall, V. C. & Heinegård, D. (1974) *J. Biol. Chem.* **249**, 4242-4249
- Jennings, B. R. (1977) *Adv. Polym. Sci.* **22**, 61-81
- Kerr, J. (1875) *Philos. Mag.* **50**, 337-348
- Lohmander, S. & Hjerpe, A. (1975) *Biochim. Biophys. Acta* **404**, 93-109
- Muir, H. & Hardingham, T. E. (1975) *MTP Int. Rev. Sci.* **5**, 153-222
- Pasternak, S. G., Veis, A. & Breen, M. (1974) *J. Biol. Chem.* **249**, 2206-2211
- Perrin, F. (1934) *J. Phys. Radium* **5**, 497-511
- Peterlin, A. & Stuart, H. A. (1943) *Handbuch und Jahrbuch der Chemischen Physik*, vol. 8, section 1B, Backer and Erler, Leipzig
- Rosenberg, L., Hellman, W. & Kleinschmidt, A. K. (1975) *J. Biol. Chem.* **250**, 1877-1883
- Schweitzer, J. F. & Jennings, B. R. (1972) *Biopolymers* **11**, 1077-1089
- Stoylov, S. P. (1971) *Adv. Colloid Interface Sci.* **3**, 45-110
- Yoshioka, K. & Watanabe, H. (1969) in *Physical Principles and Techniques of Protein Chemistry* (Leach, S., ed.), pp. 335-367, Academic Press, New York