CROATICA CHEMICA ACTA CCACAA 56 (4) 803—808 (1983)

CCA-1416

YU ISSN 0011-1643
UDC 541.123
Conference Paper

The Study of Cartilage Proteoglycan Interactions by Electric Birefringence*

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Received November 5, 1982

Electric birefringence has been used to study the solution properties of the three main components of articular bone cartilage, proteoglycan, hyaluronic acid, and collagen. The method has shown particular applicability in following the interactions and associations of these cartilage components. The size and shape of the molecules and aggregates, in the light of their possible relevance to arthrotic disorders, has been investigated.

INTRODUCTION

There are three main classifications of diseases that affect bone joints.[1] The first, rheumatoid arthritis, is defined as inflamation of the joint and is treated by anti-inflamatory and immuno-suppressive drugs. The second, and most common, is osteoarthrosis which involves the progressive degeneration of bone cartilage. There is no known chemothereputic agent for this disease. The third, metabolic deposition arthropathy, is caused by mineralization in the synovial fluid of metabolic by-products. The main focus of this study is understanding the molecular interactions that exist in normal cartilage in order to provide a fuller scientific basis for the formulation of new treatments for osteoarthrosis.[2]

Normal articular bone cartilage is made of three main constituents whose interactions provide the viscoelastic properties needed to support the mechanical stress associated with movement. The three components are collagen, proteoglycan and hyaluronic acid. The tensile strength is due to the extensive protein matrix of collagen fibres while the elastic properties are consequences of the hydrophilic nature of the proteoglycan-hyaluronate aggregate. When pressure is applied, water is lost slowly from the matrix due to the osmotic force of the highly charged proteoglycan-hyaluronate. Release of the pressure is accompanied by expansion of the network and the influx of water, as controlled by the collagen network.

Proteoglycans are complex molecules consisting of a core protein, about 300 nm long, with a globular head[3] (Figure 1a). To this core, linear polysac-

* Based on a contributed paper presented at the »Ruđer Bošković« Institute's International Symposium on *Precipitation and Interfacial Phenomena in Mineralization in Biological and Biopolymer Matrices,* Cavtat/Dubrovnik, Croatia, Yugoslavia, June 1982.

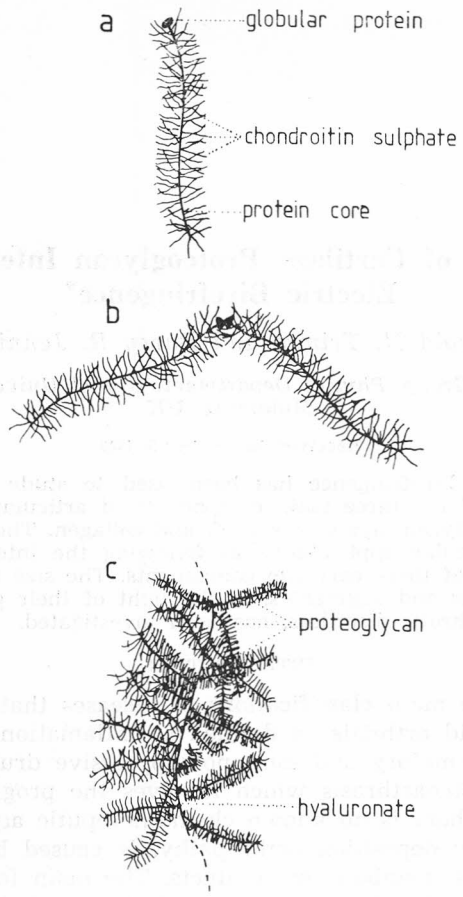

Figure 1. Schematic representations of (a) Proteoglycan monomer (b) Proteoglycan dimer and (c) Proteoglycan-hyaluronate complex.

charides of chondroitin 4 sulphate (~ 45 nm in length) and less frequently keratin sulphate (~ 20 nm in length) are attached radially via a covalent trisaccharide-serine residue interaction. The molecular weight of an average molecule is 2.5×10^6 and contains 100 chondroitin sulphate and 50 keratan sulphate chains. In solution, proteoglycans have been shown (a) to self associate[4] (Figure 1b), and (b) to bind to hyaluronate.[5]

Hyaluronic acid is a linear polysaccharide whose negatively charged side groups cause it to be fully extended in water. Proteoglycan forms aggregates with hyaluronic acid through non-covalent interactions of the globular heads with disaccharide units of the hyaluronic acid (Figure 1c). Hyaluronic acid of molecular weight 1×10^6 (2.4 μm long) can bind up to 100 proteoglycan molecules. The proteoglycan/hyaluronate complex is trapped within the collagen framework owing to both the size of the complex and the attraction between proteoglycan and collagen. In arthrotic disorders, the amount of proteoglycan which is easily extractable from the articular cartilage is more than that from healthy cartilage, indicating a decrease in the aggregatability

of the proteoglycan. Physical studies of the size, interaction and association of proteoglycan with hyaluronic acid and collagen are thought to provide a molecular basis for the understanding of, and hence treatment for, arthrotic disorders.

When subjected to a pulsed electric field, solutions of proteoglycan, hyaluronic acid and collagen all become birefringent. By measuring the rate of loss of birefringence upon termination of the field, the rotary diffusion coefficient (D) of the solute molecules can be obtained.[6] This parameter is related to the reciprocal of the cube of the major molecular dimension and is thus strongly dependent on molecular size and morphology. Furthermore, multiple components of D can be determined and used, in favourable cases, to determine the behaviour of one molecular species in the presence of another which indicates its superiority over other methods, such as light scattering[7] and ultrafiltration,[8] used in the study of proteoglycan systems.

EXPERIMENTAL

The samples of pig laryngeal proteoglycan and hyaluronic acid were obtained from Drs. T. Hardingham and H. Muir of the Kennedy Institute. Their preparation has been described elsewhere.[9] The proteoglycan was studied at a concentration of 200 μg/ml uronic acid. The collagen sample was obtained from Dr. A. Bailey of the Meat Research Institute, Bristol. It was acid soluble and kept at pH 3.2. All samples were of high purity and narrow polydispersity. The solutions were dialysed against deionised water, at pH 7, to produce samples of low ionic strength. This was done to minimise experimental difficulties due to conductance.

The electric birefringence apparatus used has been described elsewhere.[5] The recent replacement of the photomultiplier with a photodiode has increased both the sensitivity and dynamic range of the instrument. The present apparatus is capable of measuring birefringence values as small as 10^{-9} and has a response time of 200 ns. The sample cell has been modified for biochemical studies in that it can handle samples of 0.5 ml while retaining a 5 cm path length. The high voltage for the electric fields was generated using a Cober model 605 pulse generator. Up to 2.2 kV was applied across electrodes 2 mm apart to orientate the solute molecules. The pulse width was varied from 1 μs to 10 ms. The electric birefringence transients obtained were digitised and stored in a Datalab DL 922 transient recorder. The data was then transferred into a computer and the parameters of the exponential decays were determined by use of a multiparametric curve fitting routine.[10] Besides speed and convenience, this allowed both the analysis of multiple exponential decays and the removal of noise form weak signals via digital filtering techniques.

RESULTS

The decay of the birefringence for a single relaxing species follows the equation:

$$\Delta n = \Delta n_o \, e^{-t/\tau} \tag{1}$$

Multiple relaxing species of i components, each of birefringence Δn_{oi} and relaxation time τ_i give rise to the equation

$$\Delta n = \sum_i \Delta n_{oi} \, e^{-t/\tau_i} \tag{2}$$

When subjected to pulsed electric fields, solutions of proteoglycans exhibited birefringence transients which consisted of three components (Figure 2). By varying the concentrations, temperature of the solution and the field strength and pulse width, the relative proportions of each component could be seen to change. Using equation 2 and a curve fitting procedure, the relative contributions to any experimental transient could be isolated.

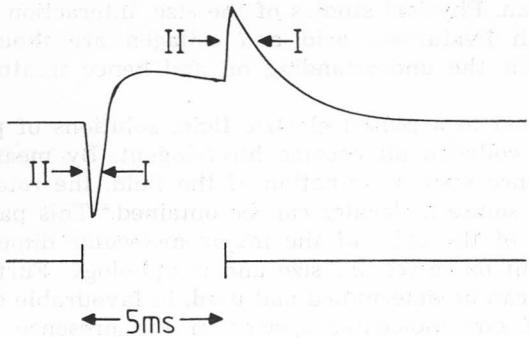

Figure 2. Electric birefringence transient (upper trace) of a proteoglycan solution at a concentration of 200 μg/ml in response to a field of 800 V cm^{-1} for 5 ms duration (lower traçe). I and II indicate the components I and II in the response.

From data obtained for proteoglycan solutions, the following characteristics were observed:

i) The existence of a slow relaxing species of positive birefringence with a relaxation time of 8 ms. This was component **I.**

ii) Also the presence of a faster relaxing species of negative birefringence with a relaxation time of 300 μs. It was component **II.**

iii) An adidtional positive birefringence signal, apparent only at high fields, with a relaxation time less than 2 μs. It was component **III.**

iv) The addition of dithiothreitol caused the loss of component **I.**

v) The addition of hyaluronic acid caused a loss of component **II** and the emergence of a new signal of positive birefringence and slow relaxation time.

vi) The addition of collagen[11] caused a decrease in component **II** and the energence of a new signal of positive birefringence and slow relaxation time.

DISCUSSION

The three components of the electric birefringence transients for proteoglycans were identified as follows. Component **I** was due to the presence of proteoglycan dimers. These dimers are thought to be joined head-to-head through the globular regions at one end of each protein core, (Figure 1b). Such a hypothesis was verified by the addition of dithiothreitol, an agent which disrupts the disulphide bridging of cysteine residues which are found only in the head region. This caused the loss of component **I** which was therefore deemed to be due to the presence of dimers. The second component (**II**) was due to the proteoglycan monomers. When the relaxation time obtained from this component was inserted in the equation for a prolate ellipsoid[6] the major dimension obtained (300 nm) corresponded well with that measured by other methods.[3] It is interesting to note that the value of the length of the dimer was less than twice that of the monomer. By assuming that the dimer adopted a distinct, regular geometry, the equation of Wegner et al.[12] was used to interpret the rotary diffusion coefficient and thus evaluate the structure. For proteoglycan dimers this corresponded to a hinge of two monomer units[13] subtending an angle of 120°. The third component (**III**) has been identified as the electrically induced and independent orientation of the polysaccharide side chains.[14]

When hyaluronic acid was added to the proteoglycan solutions, the aggregation of proteoglycan monomers to the extended hyaluronate chain was followed by the loss of component **II** and the formation of a new transient component corresponding to a large species with a major dimension equal to the length of the hyaluronate chain and a minor dimension of approximately the length of two proteoglycan molecules. The opposite sign of the birefringence of this component to that of isolated proteoglycan molecules indicated that the proteoglycans were now at right angles to their original orientation, as depicted in Figure 3. All this supports the 'bottle brush' model of the complex proposed by other investigators.[2]

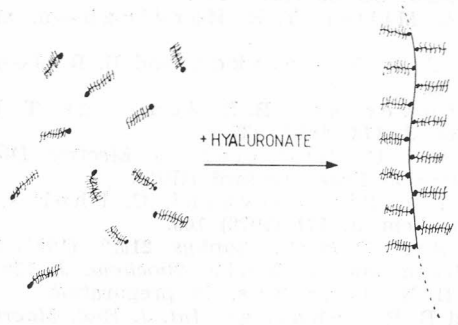

Figure 3. Association of proteoglycan monomers (left side) with the extended linear molecule of hyaluronic acid to form the open molecular complex (right side).

The addition of collagen to proteoglycan solutions again resulted in the decrease of component **II** accompanied by the formation of a new, slow transient species, compatible with the formation of a large molecular complex, which was found to be irreversible. Discrete relaxation data were obtained which, via current continuing studies, are still being interpreted in attempts to elucidate the conformation of the complex. In this respect, the electric birefringence data show considerably greater promise than the other optical methods used to date (turbidity, scattered intensity) for such conformational analysis.

CONCLUSIONS

Measurements of proteoglycan interactions in solution using the electric birefringence method have led to the following conclusions.

i) Proteoglycans self associate to form dimers in aqueous solution of low ionic strength.

ii) The dimer is formed by the head-to-head association of two monomer segments so as to subtend an angle of 120^0.

iii) The interactions of both monomer and dimer can be studied simultaneously when co-existent together by analysing each component of the multicomponent birefringence transients in turn.

iv) The interaction of proteoglycans with hyaluronate can be followed and the site and geometry of the resulting complex can be estimated.

v) The association of proteoglycan with collagen can be followed and the size of the resulting aggregate determined.

vi) Electric birefringence offers a fast and sensitive method for the study of the interactions of biological macromolecules involved in connective tissue matrix formation.

Acknowledgement. — The authors wish to thank Drs. T. Hardingham and H. Muir of the Kennedy Institute of Rheumatology, Hammersmith, London and Dr. A. Bailey of the Meat Research Institute, Bristol for the samples supplied.

The work was funded by a grant from the Arthritis and Rheumatism Council.

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SAŽETAK

Proučavanje interakcije hrskavičnih proteoglikana s pomoću električkog dvoloma

H. H. Trimm i B. R. Jennings

Proučavana su svojstva otopina triju glavnih sastojaka hrskavice zglobne kosti: proteoglikana, hialuronske kiseline i kolagena. Upotrijebljena je metoda električkog dvoloma koja se pokazala posebno prikladnom za praćenje interakcija i asocijacije navedenih sastojaka hrskavice. Proučavana je moguća povezanost veličine i oblika molekula i agregata s poremećajima izazvanim artritisom.

CROATICA CHEMICA ACTA CCACAA **56** (4) 809—815 (1983)

CCA-1417

YU ISSN 0011-1643
UDC 541.18
Conference Paper

An Electric Birefringence Study of Aqueous Tetraethyleneglycol Dodecyl Ether Micelles*

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Received August 26, 1982

Pulsed electric birefringence measurements have been made in the isotropic micellar phase of the tetraethyleneglycol dodecyl ether/water system. Variations have been recorded in both the Kerr constant and the rotary relaxation times for this system (a) as the temperature was increased at constant surfactant/water composition and (b) as the surfactant content was increased at constant temperature. Each of these parameters varied markedly with both temperature and composition. Explanations for the observed phenomena are given in terms of either variable micelle geometry or changing micelle interactions. At present neither is able to fully satisfy the data.

INTRODUCTION

There is considerable current interest both in the structure of aqueous surfactant micelles and in experimental methods for the study of the same. Apart from the obvious commercial interest in these systems, academic studies are increasing owing to the revelation of numerous variable phases that can exist with dispersions under different conditions of temperature and surfactant/water composition[1]. In this report, we are concerned with one such surfactant, namely tetraethyleneglycol dodecyl ether (TGE). Its aqueous micellar phase has been studied using the electric birefringence technique.

TGE is represented by the chemical formula n-$C_{12}H_{25}(OCH_2CH_2)_4OH$. The composition vs. temperature phase diagram of the TGE/water system has been reported elsewhere[2] and is partially reproduced in Figure 1. The L_1 region is of particular interest for two reasons. Firstly, it is an optically transparent, isotropic micellar solution throughout. Secondly, it exhibits two interesting phase boundaries with different characteristics. If the temperature be increased under low surfactant composition condition, the medium eventually clouds as

* Based on a contributed paper presented at the »Ruđer Bošković« Institute's International Symposium on *Precipitation and Interfacial Phenomena in Mineralization in Biological and Biopolymer Matrices,* Cavtat/Dubrovnik, Croatia, Yugoslavia, June 1982.

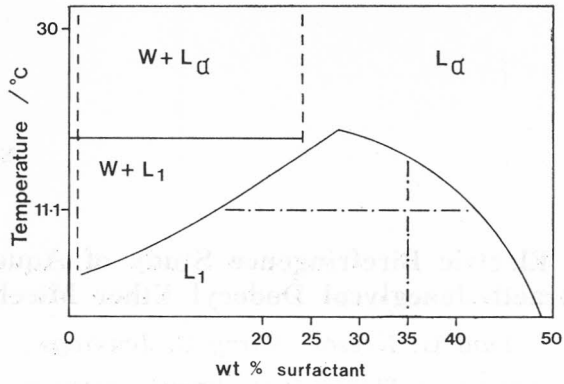

Figure 1. Partial phase diagram for the tetraethyleneglycol dodecyl ether/water system. Symbols represent L_1 — micellar phase; L_α — lamellar phase; $W + L_1$ — water plus micelle region; $W + L_\alpha$ — water plus lamellar region. —.—.—.— represents experimental scan lines.

the system passes into a water-plus-micellar dispersion region. For higher surfactant concentrations ($\gtrsim 25\%$ by weight), continuous temperature increase results in the formation of the optically birefringent lamellar phase (L_α). This L_1-to-L_α transition can also be induced at relatively low temperatures by increasing the surfactant concentration of the medium. It is of interest to know if the microscopic characteristics of the dispersion vary throughout the L_1 region, and if so, to highlight the properties precursive to the phase transitions.

A variety of physical methods have been used to study micelle geometry, including light scattering[3–6], NMR[7], sedimentation equilibrium[8] and electric birefringence[6,9]. The last mentioned has certain advantages in studying these media. The method consists of subjecting the solution to a short duration electric voltage pulse while recording the transient induction and decay of any birefringence in the system. The amplitude of the birefringence response indicates the inherent optical anisotropy of the dispersed components whilst the rates of change are sensitive indicators of the geometry of the dispersion. This combination of anisotropy and size parameters is beneficial in the analysis of associating systems. Hoffman[6] has used the method to study the TGE/water system at a single, very low surfactant composition below the cloud point. He showed that the micelles were non-spherical and, assuming them to be rod-like, derived a length of 110 nm. In the present work, we have surveyed the L_1 phase at much higher surfactant concentrations, under a variety of temperature and concentration conditions, in an attempt to understand the structure of the phase and its pretransitional properties.

EXPERIMENTAL

The TGE sample was obtained from the Nikkol Chemical Company of Kyoto, Japan, and was used without further purification. All experimental media were prepared by weighing surfactant directly into glass tubes and adding the appropriate amounts of doubly-distilled deionised water. The tubes were then sealed and their contents thoroughly mixed by heating, centrifugation and agitation.

Electric birefringence measurements were made with a conventional apparatus[10]. Light of 633 nm wavelength (λ) from a 1 mW helium-neon laser entered the sample cell after being linearly polarised at 45° azimuth to the electric field direction. After leaving the cell, the light traversed a quarter-wave plate set in parallel

azimuth to the initial polarisation state and fell on an analysing polariser which was slightly offset from the 'crossed' azimuth relative to the initial polariser. This optical arrangement gave optimum detection sensitivity and enabled the sign of the induced birefringence to be evaluated[11]. Any light transmitted through the analyser fell on the photocathode of a photomultiplier whose output was displayed on an oscilloscope for visual inspection and photographed for permanent retention. In addition, the photomultiplier response was fed to a transient digitiser and microcomputer anssembly for direct analyses of birefringence amplitudes and decay rates. The cell consisted of a glass trough some 5 cm in length and held a pair of stainless steel electrodes spaced some 2 mm apart along its length. Some 1 ml of sample filled the interspace, across which pulsed electric potential differences of up to 2.2 kV were applied for durations between 30 and 500 μs. The duration was limited in each experiment to that just sufficient to allow the birefringence to attain a steady value. Heating and electrophoretic effects were minimised thereby. The cell fitted into a custom designed thermostated jacket through which water was pumped to vary the cell temperature and maintain the contents to a thermal stability of $\pm$ 0.1 °C. The sample temperature was monitored throughout all experiments using an iron/copper-nickel thermocouple linked to a digital display.

RESULTS

Birefringence responses were recorded for two specific situations. The first was with variable temperature for a sample with a constant composition of 34.8 weight % of TGE. The second was under isothermal conditions for media of variable composition. Thus, data were obtained corresponding to horizontal and vertical scan lines within the L_1 phase of Figure 1. In all cases, measurements were made for a variety of applied field strengths. From the measurements, the following observations were recorded:

(i) All birefringence responses were negative and of regular transient exponential form (Figure 2).

(ii) The steady birefringence amplitude (Δn) always had a quadratic dependence on the applied field amplitude (E). Thus Kerr constants (B) could be used to characterise the optical anisotropy of a sample, where[10]

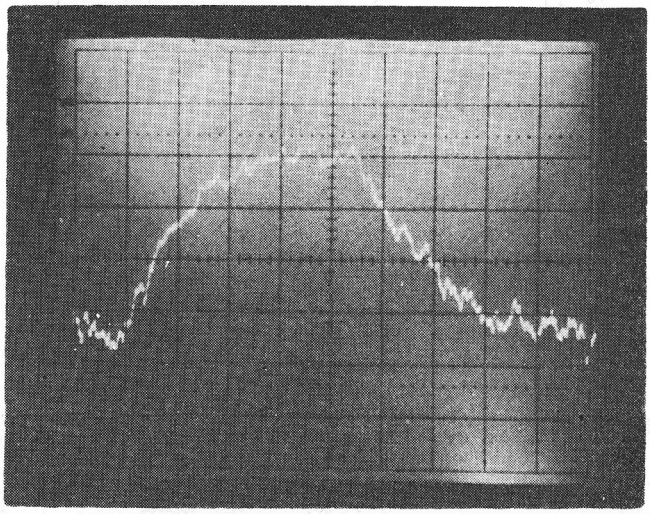

Figure 2. Transient electric birefringence response for a TGE/water micellar system. Surfactant concentration of 24.92 weight %, T = 11.1 °C, E = 6.94 kV cm^{-1}.

$$B = \mathrm{L}t_{E\to 0}\,(\Delta n/\lambda E^2) \quad (1)$$

(iii) At any time t during the post-field decay process, the birefringence satisfied the monoexponential function[12]

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

where Δn_o was the value of Δn at the cessation of the applied pulse for which $t = 0$. A single relaxation time τ thus characterised the decay process for all systems studied.

(iv) All transients were symmetrical with the build-up curve characterised by the same τ as the decay.

(v) For a given sample, τ was independent of E.

(vi) For a sample with a constant surfactant composition of 35 weight % TGE, increasing temperature was accompanied by the continuous increase in B but decrease in τ shown in Figure 3.

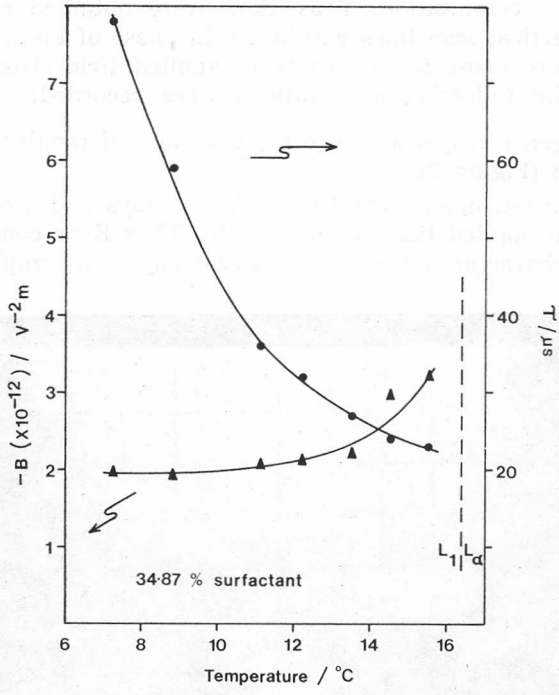

Figure 3. Temperature dependence of the birefringence data. Kerr constant (B) and relaxation time (τ) for a TGE composition of 34.9 weight %. $L_1 : L_\alpha$ denotes the position of the phase boundary under these conditions.

(vii) At a constant temperature of 11 °C, as the TGE content of the system was increased, so both B and τ increased as seen in Figure 4.

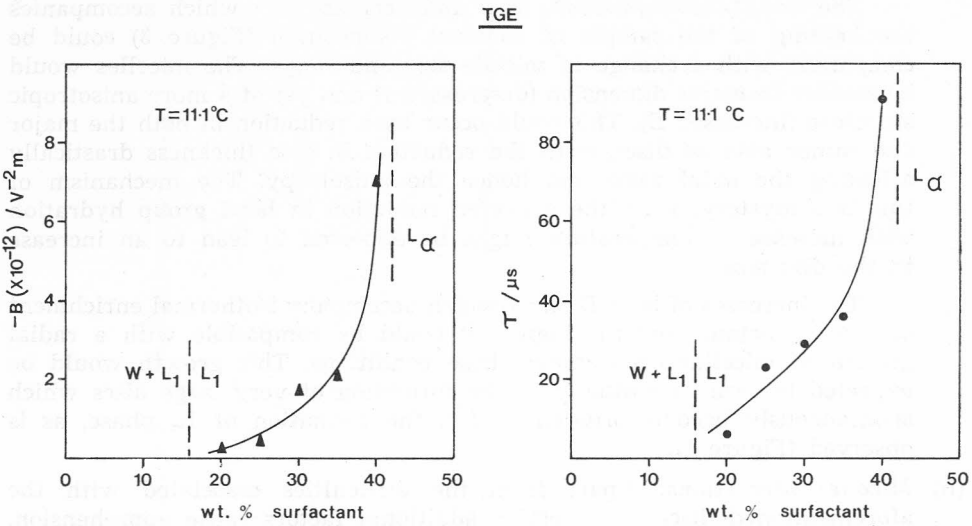

Figure 4. Variation of electric birefringence parameters with TGE composition in the micellar phase. Kerr constant (B) and relaxation time (τ) at a constant temperature of 11.1 °C. Indications (W + L_1) and (L_α) indicate neighbouring phases to the micellar phase L_1 (see Figure 1).

DISCUSSION

Despite the extensive literature on micelle structure and behaviour, there is a lack of firm evidence as to the geometry of micelles in situations equivalent to the L_1 phase of TGE. Whereas Hoffman et al.[6] interpreted their data at low surfactant composition in terms of an assumed rod-like structure, Tanford et al.[8] have indicated that micelles formed by such surfactants are discs. Consideration of the possible packing geometries of the individual molecules within the micelles[1], together with the fact that an increase of both surfactant concentration and temperature lead to transitions from the L_1 phase into the highly extended lamellar phase (L_α of Figure 1), support the disc-like concept for L_1 phase micelles. Furthermore, symmetrical electric birefringence transients as recorded herein generally arise from induced rather than permanent electric dipole moments[10]. In all aqueous colloidal systems studied to date, the principal electric polarisation direction has been associated with the major molecular axis, which thus aligns predominantly parallel to the electric field. Disc-like micelles, in which the individual molecules align parallel to the minor (thin) micellar axis could then account for the observed negative electric birefringence. This explanation is consistent with flow birefringence data on sodium lauryl polyoxyethylene sulphate[13].

(i) *Interpretation for discrete micelles:* For rotating thin discs[14],

$$\tau = \frac{2\eta d^3}{9kT} \qquad (3)$$

where d is the disc diameter in an environment of viscosity η at a temperature T with k the Boltzmann constant. Using the viscosity of water in equation (3), approximate values in the range from 40 nm to 120 nm can be calculated for d from the τ data. This is discussed below.

The simultaneous increase of B and decrease in τ which accompanies the heating of the sample of constant composition (Figure 3) could be compatible with a change in micelle size and shape. The micelles would be smaller in major dimension (descreased τ) and yet of a more anisotropic structure (increased B). This could occur by a reduction in both the major and minor axes of discs, with the reduction in disc thickness drastically affecting the axial ratio and hence the anisotropy. The mechanism of this is a mystery, since the expected reduction in head group hydration with increase in temperature might be expected to lead to an increase in the disc size.

The increases of both B and τ which accompany isothermal enrichment of the surfactant content (Figure 4) could be compatible with a radial growth of micellar discs under these conditions. This growth would be expected to result eventually in the formation of very large discs which spontaneously become ordered — i. e. the formation of L_α phase, as is observed (Figure 1).

(ii) *Micelle interactions:* Apart from the difficulties associated with the aforementioned discussion, certain additional factors cause apprehension. The first relates to the size of the micelles so obtained. Analysis of the NMR data published elsewhere[15] for this same system indicated that a fast relaxing species exists with a size of a few nm. This was thought to be the value for the individual micelles. Secondly, the discrete single τ values for each experimental transient are surprising for micelle solutions where such large micelles are currently expected to be somewhat polydisperse. Thirdly, a crude estimate based on surfactant content and micelle size, indicates that for TGE content in excess of 40% by weight, the micelles must be in very close proximity, with a mean path of less than the major micelle dimension. Strong intermicellar interactions must then be evidenced, so that the electric birefringence data may indicate this predominantly.

The exact significance of the parameters B and τ is unclear with such a hypothesis, although an explanation for the data of Figure 3 can be proffered. Any temperature increase would be accompanied by reduced head group hydration resulting in decreased micelle interactions. Reduced interaction would be reflected in a reduction in τ. In addition, a tendency towards more isolated entities is likely to be accompanied by increased optical anisotropy and hence an increase in B. It is the data of Figure 4 which present the difficulty. An increase of TGE composition must increase micelle interactions which would expand the relaxation time. How such enhanced interactions could cause an increase in B is not at all clear. This factor must await further investigation.

CONCLUSIONS

Current studies are being undertaken to differentiate between the individual micellar or micellar interaction origins of the parameters B and τ for the TGE/water system. In the interim the following conclusions are deduced:

(i) The L_1 micellar phase of the TGE/water system is not homogeneous in micelar morphology.

(ii) The region is characterised by oblate rather than rod-like micelles.

(iii) The Kerr constant (B) and rotational relaxation time (τ) vary with both the temperature and the surfactant content of the phase.

(iv) Inter-micellar interactions may be a significant factor in the experimental birefringence phenomena.

(v) Electric birefringence has potential as an investigative method for these complicated systems.

Acknowledgement. — The authors wish to thank the S.E.R.C. for a CASE Studentship for P.G.N.

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SAŽETAK

Proučavanje tetraetilenglikol-dodecileterskih micela u vodenom mediju s pomoću električnog dvoloma

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Metoda pulsnoga električkog dvoloma upotrijebljena je za proučavanje izotropne micelarne faze u sistemu tetraetileneglikol-dodecileter/voda. Povišenje temperature (uz stalni sastav) i povećanje udjela površinski aktivne tvari (uz stalnu temperaturu) dovodi do znatne promjene Kerrove konstante i relaksacijskog vremena rotacije. Nalazi su objašnjeni uzimajući u obzir promjenljivost oblika micela i micelarne interakcije. Ni jedna od te dvije pretpostavke potpuno ne zadovoljava dobivene podatke.