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ELECTRIC BIREFRINGENCE AS A MEANS OF STUDYING THE EFFECT OF ANAESTHETICS ON LIPOSOMES

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Birefringence can be induced in liposome suspensions using electric fields. The fields interact predominantly with anisotropic electrical polarisabilities which give rise to induced dipole moments. Using pulsed electric fields, the optical and electrical polarisabilities and the geometrical size of the liposomes can be measured simultaneously. These parameters have been found to be very sensitive to the presence of small amounts of fluidising additives of polar and ionic nature. Illustrative data are presented for the influence of the amines ammonium chloride, methyl ammonium chloride and lignocaine and of benzyl alcohol on phosphatidylcholine /serine liposomes. Structural changes in the vesicle membranes were detected, which appeared to correlate with the biological functions, thus indicating that electric birefringence is a rapid and useful method for studying interactive phenomena in lipid membrane systems.

Introduction

Electric birefringence has become increasingly popular as a means of characterising the geometry, optical and electrical properties of macroparticles in suspension [1,2,3]. Recently we showed [4] that transient electrical birefringence can be used as a rapid and sensitive method of measuring the size and electrical polarisability anisotropy of liposomes in dilute suspension. Briefly stated, the principle is as follows. For particles which are either geometrically anisotropic or can be slightly deformed, application of a short-duration electric pulse induces optical birefringence in the medium. This property can be detected by having the sol in a cell placed between crossed polarisers. The ability to transmit light through such an assembly indicates birefringence in the active medium. By using a pulsed field, the light is transient in nature. A finite time is required for the particles to align, so that the rate of establishment and decay of the birefringence can be related to their size characteristics. The amplitude of the recorded birefringence is related to both the electrical dipoles and optical polarisability of the particles. In this way, changes in the size and in the structure of the particles, in so much as they influence the electrical and optical polarisabilities of the particles, can be measured with extreme rapidity.

In the present work, this method has been used to study the effect of four fluidising agents, including two clinical anaesthetics, on aqueous suspensions of liposomes. These additives are particularly interesting because of their diverse structures and electrical characteristics. The tertiary amine anaesthetic lignocaine has been compared with

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Abbreviations: PC, phosphatidylcholine; PS, phosphatidylserine.

ammonium chloride and the primary amine methyl ammonium chloride, because of the increasing awareness [5] that such weak bases share many modes of interaction with biological membranes. The measured changes in the vesicles may be directly related to their clinical function in producing a block in nerve conduction. The fourth compound is the non-polar fluidising agent benzyl alcohol.

Materials and Methods

Egg phosphatidylcholine (PC) (type V-E) and bovine brain phosphatidylserine (PS) were purchased from Sigma, London, U.K., and used without further purification. Suspensions of vesicles with 9 : 1 PC-to-PS composition were made by taking 50 mg of the lipid mixture and dissolving it in 5 ml of chloroform. The solvent was then removed, firstly by rotary evaporation and then by nitrogen blowing. The dry lipids were then resuspended in water. After slow, non-agitated hydration, vesicles were obtained which were highly polydisperse and, according to Reeves and Dowben [6], had a high population in the unilamellar form. The present work did not depend upon uniquely unilamellar material. A more nearly uniformly sized sample was achieved by the method of filtration under pressure as described by Olson et al. [7]. Using 0.6 μm 'Unipore' polycarbonate filters (Bio-Rad Labs., Richmond, CA), the sample was filtered twice under a nitrogen pressure of some 5 lb/in^2 to reduce the proportion of larger vesicles. Subsequent filtration through the same filter, but under gravity alone, left above the filter a retained sol of vesicles with dimensions close to the pore size. A further 10-fold dilution of the suspensions was made in water or the relevant concentration of additive, within 10 prior to the birefringence measurements. All of the additives were obtained from B.D.H. except for the lignocaine, which was purchased from Antigen International. The ammonium chloride was Analar grade. Electric birefringence data were recorded at room temperature ($\simeq 20\,^\circ$C) using the apparatus described elsewhere [8]. Each sample was subjected to a series of pulses of either d.c. or 15 kHz frequency sinusoidal voltage, and amplitudes within the range $0.5 < E\,(\mathrm{kV/cm}) < 3$. Pulse durations were of a few milliseconds as this gave sufficient time for the birefringence to attain a steady value during the pulse life.

Results

The forms of the optical responses were similar for all experiments. A typical transient is shown in Fig. 1, for which time runs from left to right. For such, analysis was made of the maximal birefringence amplitudes and the rates of decay of the phenomena. For a given r.m.s. field strength, little difference in response was noted using a.c. or d.c. voltages, thereby showing the frequency-independence of the birefringence and the essential absence of any permanent dipole moment for the liposomes. This is not surprising when their high structural symmetry is considered.

With conventional particle rotation, the field free decay of the birefringence (Δn) follows a single exponential for a monodisperse system, which is such that [9]

$$\Delta n = \Delta n_0 \exp(-t/\tau) \qquad (1)$$

with Δn the birefringence amplitude at any time t during the decay, and Δn_0 the birefringence at the commencement of the decay when $t = 0$. The rota-

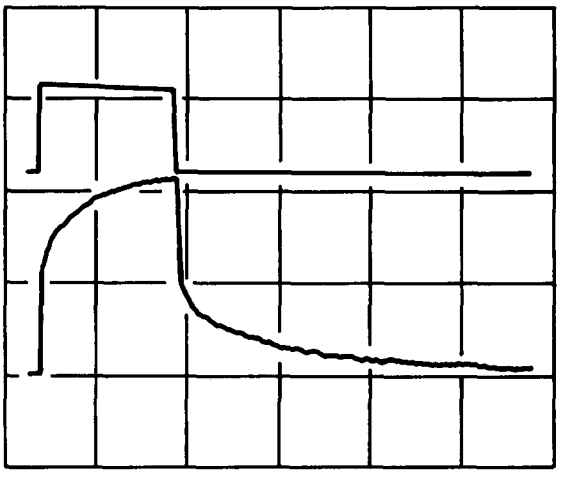

Fig. 1. Typical induced birefringence transient response. Top trace is the applied field pulse of 2.4 kV/cm and 6.7 ms duration; lower trace is the optical response for 9:1 PC/PS system in water with Δn_0 $2.55 \cdot 10^{-8}$. Time runs from left to right.

tory relaxation time has the form [10]

$$\tau = \frac{\pi \eta d^3}{6kT} \tag{2}$$

for spheres, or near spheres, such as would be encountered with liposomes, so that spherical diameters d can be determined directly from the slope of a plot of $\log(\Delta n/\Delta n_0)$ vs. t. In Eqn. 2, η is the medium viscosity and k and T are the Boltzmann constant and the absolute temperature, respectively.

Although spherical entities might not be expected to exhibit orientational birefringence, even if they contain charged phospholipids, measurements can be generally be made [8,11]. This is due to the fact that either small departures from sphericity or some geometrical deformation are encountered. For polydisperse systems, the semi-log plot is multi-component as each species contributes its exponential decline to the observed birefringence. Inspection of Fig. 1 reveals the existence of at least two major relaxation phenomena in both the establishment and the decay regions of the transient. The slower contribution, which is itself multiexponential, involves relaxation times in the millisecond time range, corresponding (via Eqn. 2) to particle sizes in the sub-micron region. This relates to the vesicle geometry and orientation (see below). The fast process, which is some four orders of magnitude quicker, probably relates to the rate of deformation. If due to rotational diffusion, it would correspond to liposomes of under 20 nm diameter, which is unrealistic. Both processes were due to the sample and not to the time constant of the apparatus, as this was independently measured to be as fast as 10 μs.

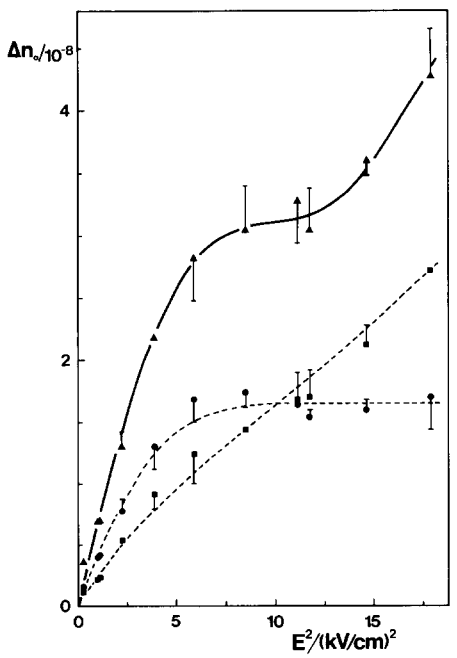

Fig. 2. Variation of the in-field equilibrium birefringence (▲) Δn_0 with field strength. Broken lines show the behaviour of the two contributing components: ●, the slow process; ■, the fast process. Bars represent the standard deviation.

For particles with no permanent dipole moment, the field strength dependence of the maximal birefringence amplitude can be interpreted to give the electrical polarisability, then [9],

$$\Delta n_0 = \frac{2\pi c}{n} G\phi \tag{3}$$

where c is the volume concentration of the particles with optical anisotropy per unit volume G in a medium of refractive index n. The factor ϕ is the orientation function which at high fields is unity, whilst at low field amplitudes (E) has the form

$$\phi = \frac{\alpha E^2}{15kT} \tag{4}$$

with α the electrical polarisability. The general applicability of these equations to macromolecular solutions and particulate suspensions has been well verified, as has the temperature-dependence of the phenomena [1,2,9]. Theoretically, the field dependence of Δn_0 thus follows a linear E^2 dependence at low fields, and reaches an independent value at high fields from which G is determined. Substitution of the high-field data into those for the E^2 region enables α to be found.

Fig. 2 shows a representative field-dependent set of data. Surprisingly, a further increase in Δn_0 is exhibited beyond the initial field-independent plateau region. In the light of two contributions to the relation decay process, it was thought that Fig. 2 indicated a two-component origin. This was confirmed by extracting the amplitude contributions to the birefringence corresponding to the

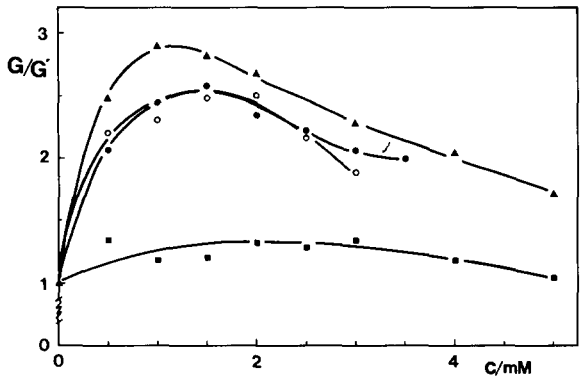

Fig. 3. Variation of the optical polarisability anisotropy G with concentration of various agents. G' is G in the absence of additive. ■, benzyl alcohol; ▲, lignocaine; ●, methyl ammonium chloride; ○, ammonium chloride.

'fast' and 'slow' contributions as recorded in the transient decay. These contributions are shown independently in Fig. 2. Only the 'slow' component satisfies the orientation theory (Eqns. 3 and 4) and thus confirms the assignment of this contribution to geometrical rotary diffusion. The fast contribution demonstrates a continuing growth with E^2, as might be expected from a deformation mechanism. At very high field strengths ($E > 6$ kV/cm), the birefringence decay curves started to indicate an increased breadth in vesicle size distribution, indicating that such fields possibly damage the liposomes.

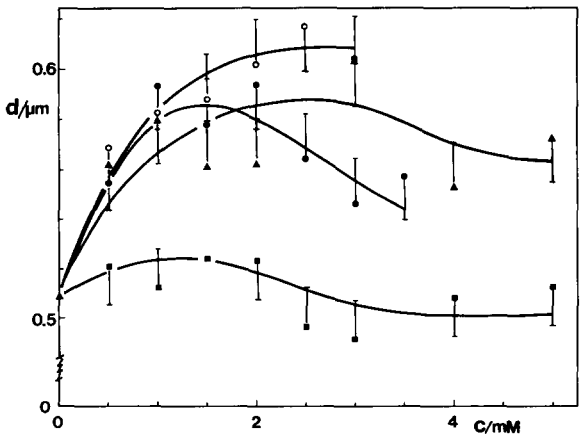

Fig. 4. Variation of the equivalent spherical diameter with concentration of the various agents. ■, benzyl alcohol; ▲, lignocaine; ●, methyl ammonium chloride; ○, ammonium chloride.

In this work, the 'slow' process has been isolated and analysed to obtain values of τ, size, G and α. Figs. 3 and 4 show the effect of the additives on these properties. The optical polarisability was always greater in the presence of the additives but passed through a maximum at an ill-defined concentration. The charged and larger molecules showed a much more substantial effect than the uncharged benzyl alcohol. For all systems, changes in the electrical polarisability were insignificant and within the experimental error. The average spherical diameter also increased upon addition of each of the compounds and passed through maximum values.

Discussion

During recent years [12–14] evidence has accumulated that, with aqueous colloid and biocolloid sols, the electrical polarisability has its major origin in the field-induced displacement of charge in the surface electrical double layer. This parameter is generally very sensitive to additives if they have a surface-active influence. The negligible change in this parameter during the present experiments indicates that the anaesthetics' primary action does not appear to be at the external liposome surface. Since those agents which act as anaesthetics penetrate the membrane, their interaction is principally with the inner surface, with the result that the external surface may be minimally effected. Resulting structural changes would not then be influential on α. This appears to be the case.

Variation in G is more interesting. It depends upon the inherent structure and the size of the vesicles. Size changes are not sufficient to account for the 3-fold variation in G (see below). Compounds which reversibly block the formation of an action potential, but have little effect on the resting potential of the target cell, induce anaesthesia. The extraordinary range of chemically different structures that can act in this way suggests that anaesthesia is induced not through a specific reaction, but rather by means of an induced structural perturbation in the cell membrane [15]. In the present study, no significant change in the electrical polarisability was observed but the anaesthetics did produce changes in the optical polarisability, indicating that structural changes in the liposome

membrane do occur. This is an important observation.

In this respect, these studies support the NMR studies of Metcalfe and Burgen [16], who showed that, in cyto-membranes, several anaesthetics at concentrations of about 100 mM induced disorder which seemed to be correlated with physiological changes found in anaesthetic action. The changes found here in the optical polarisability in the mM concentration range indicate that the disturbances produced in the cell membrane and related to the anaesthetic phenomena, occur at much lower concentrations, and do so via the lipid bilayer. It is this ability to infer changes at such low anaesthetic concentrations which supports the work on lipid membranes by Ashcroft et al. [17], and particularly recommends the electric birefringence method.

Changes in the average spherical diameter of the vesicles are also interesting. From Fig. 4, three factors are noticed: (i) diameter changes of up to 20% were recorded for the amines; (ii) changes in d showed similar variation to those exhibited by G; (iii) the amines again had similar influences and a greater influence on the vesicles than did the alcohol.

From the first of these, a 20% change in d corresponds to a volume increase of 1.7, which is insufficient to account for the recorded change in G. From the second observation, there appears to be both a size and a structural change for the vesicles in the presence of the anaesthetics. It is evident that some correlation exists between the behaviour of G and d. Owing to the experimental uncertainty in the values of d, it is difficult to compare the additive concentrations at which both G and d peak. This may prove to be a rewarding study in the future when the origin of the peak, reminiscent of interaction experiments with erythrocytes and several cytomembranes, [18,16], is better understood.

The third factor is interesting when compared with the work of Dean et al. [5], who showed that amines significantly affect biological membranes in a similar manner. This would appear to be true for the vesicles studied here. Finally, it is interesting to note that the increase in liposome size is similar in behaviour to that found in erythrocyte ghosts in the presence of several anaesthetics [19]. In the present study, the changes were of greater magnitude.

In conclusion, the results presented in this work show how the birefringence method can be used in studying a diversity of interaction phenomena in vesicle systems. The parameters thus obtained offer the possibility of following size and structural changes associated with these interactions. The measurements can be very quick, and thus provide a rapid monitor of chemically induced changes in cells, organelles and vesicles.

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References

1 Fredericq, E. and Houssier, C. (1973) in Electric Dichroism and Electric Birefringence, Clarendon Press, Oxford
2 O'Konski, C.T. (ed.) (1978) in Molecular Electro-Optics, Marcel Dekker Inc., New York
3 Krause, S. (ed.) (1981) in Molecular Electro-Optics, Plenum Press, New York
4 Ruderman, G., Jennings, B.R. and Lyle, I.G. (1984) Int. J. Biol. Macromol. 6, 99–102
5 Dean, R.T., Jessup, W. and Roberts, C.R. (1984) Biochem. J. 217, 29–42
6 Reeves, J.P. and Dowben, R.M. (1969) J. Cell Physiol. 73, 49–60
7 Olson, F., Hunt, C.A., Szoka, F.C., Vail, W.J. and Papahadjopoulos, D. (1979) Biochim. Biophys. Acta 557, 9–23
8 Oakley, D.M. and Jennings, B.R. (1983) J. Coll. Interface Sci. 91, 188–198
9 Benoit, H. (1951) Ann. Phys. (Paris) 6, 561–607
10 Perrin, F. (1934) J. Phys. Radium 5, 497–510
11 Jennings, B.R. and Oakley, D.M. (1982) Appl. Optics 21, 1519–1524
12 Stoylov, S.P. (1967) in Chemistry, Physics and Applications of Surface Active Substances, (Overbeek, J.T.G., ed.), p. 171, Gordon and Breach, New York
13 Dukhin, S.S. and Shilov, V.N. (1976) Dielectric Phenomena and the Double Layer in Disperse Systems and polyelectrolytes, J. Wiley and Son, New York
14 Morris, V.J. and Jennings, B.R. (1975) J. Chem. Soc. Faraday Trans. II 71, 1948–1957
15 Houslay, M.D. and Stanley, K.S. (1982) Dynamics of Biological Membranes, John Wiley and Son, New York
16 Metcalfe, J.C. and Burgen, A.S.V. (1968) Nature 220, 587–588
17 Ashcroft, R.G., Coster, H.G.L. and Smith, J.R. (1977) Nature 269, 819–820
18 Seeman, P. (1966) Biochem. Pharmacol. 15, 1753–1766
19 Roth, S. and Seeman, P. (1972) Biochim. Biophys. Acta 255, 190–198