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LATERAL PRESSURES IN BIOMEMBRANES ESTIMATED FROM THE DYNAMICS OF FLUORESCENT PROBES

ANTHONY J.C. FULFORD and WILLIAM E. PEEL

Department of Cytochemistry, Imperial Cancer Research Fund, P.O. Box 123, Lincoln's Inn Fields, London WC2A 3PX (U.K.)

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Summary

A theoretical model is proposed which states that the time-independent fluorescence anisotropy of the rod-shaped molecule diphenylhexatriene incorporated into lipid bilayers is a direct result of forces constraining the diphenylhexatriene molecule. These forces are postulated as equating with the lateral pressure operating within the bilayer independently of the probe molecule.

Insertion into the model of experimental observations (recorded in the literature) on anisotropy of diphenylhexatriene in lipid bilayers as a function of temperature yielded values of lateral pressure, which decreased with temperature, and sharply at the temperature defining the transition from gel phase to fluid phase. The values so predicted for the mid-point of the transition and for the entirely fluid phase, respectively, compared favourably with estimates of the lateral pressures in these physical states, that have been reported elsewhere and arrived at either from theories describing lipid chain behaviour or from lipid monolayer compression experiments. Previously documented effects on anisotropy induced by incorporation of cholesterol into fluid lipid bilayers have been interpreted as reflections of rises in intramembranal lateral pressure.

Introduction

Fluorescence anisotropy data derived from the use of probe molecules incorporated into the model biomembranes and living cell membranes have for many years been interpreted in terms of microviscosity [1,2]. However, it has been shown that the same fluorescent probe incorporated into aliphatic oils, differing in chemical composition but having the same macroscopic viscosity,

yields different anisotropy values and hence different microviscosities [3]. Therefore, only relative membrane microviscosities, the values of which depend on the probe and calibration oil used, can be determined.

Interpretation at the molecular level requires consideration inter alia of constraints on a given probe molecule and how these may vary with temperature and the chemical nature of surroundings.

That there can be constraints has been shown for the rod-shaped molecule diphenylhexatriene by Chen et al. [4] and Kawato et al. [5] using the nanosecond fluorescence polarization technique. The latter authors visualised diphenylhexatriene as wobbling inside a cone (whereby the energy of the diphenylhexatriene molecule conforms to a simple square-well potential) and with a uniform diffusion constant. This enabled them to describe the motion of the probe in a bilayer by two parameters, namely the 'wobbling diffusion' constant (analogous to a rotational diffusion constant) and the cone angle (which determines the degree of orientational constraint).

However, Kawato et al. have specified reasons for regarding their model as too simplified, one of these being their adoption of the simple square-well potential. We have replaced this firstly by a weighted Gaussian function thereby producing a more likely distribution of spatial orientations of diphenylhexatrine. Having defined the mean orientation as perpendicular to the membrane plane we have calculated the angular standard deviation, which replaces the cone angle of Kawato et al. Our second approach has been to derive the orientational distribution using a Boltzmann energy distribution. This has enabled us to convert time-independent anisotropy data into pressure values. We believe that these equate with the lateral pressures operating within the lipid bilayers under the different experimental conditions. Since lateral pressure is dictated by the fluctuations in lateral forces between mobile lipid molecules, the dynamic behaviour of the probe molecule is considered to be governed by and to directly reflect these fluctuations.

The most probable angle of the long axis of diphenylhexatriene from the energetically preferred orientation has also been estimated.

Theoretical model

Order parameter

Data from fluorescence polarization experiments are frequently expressed as the anisotropy *, r, of the emitted light:

$$r = (I_{\parallel} - I_{\perp})/(I_{\parallel} + 2I_{\perp}) \tag{1}$$

where $I_{\parallel}$ = the vertical component of the fluorescence emission; $I_{\perp}$ = the horizontal component of the fluorescence emission.

This anisotropy is dependent upon the rotation of the probe as given by the general equation of Perrin [6]:

$$r = r_0 \frac{(3\,\overline{\cos^2\delta} - 1)}{2} \tag{2}$$

* or the related parameter, polarization, $P = (I_{\parallel} - I_{\perp})/(I_{\parallel} + I_{\perp}) = 3r/(2 + r)$

where δ is the angle through which the long axis of the probe rotates during the excited state and r_0 is the limiting anisotropy for which $\delta = 0$.

We now define an order parameter S_δ for rotation of the probe's long axis such that:

$$S_\delta = r/r_0 \tag{3}$$

Time-independent anisotropy

Using the nanosecond fluorescence polarization technique, Chen et al. [4], Kawato et al. [5] and, more recently, Hildenbrand and Nicolau [7] have followed the decay of anisotropy with time after excitation. When diphenylhexatriene is used to probe the lipid region of phospholipid liposomes the anisotropy falls towards a non-zero value, r_∞ (the time-independent anisotropy), indicating that there is some constraint on the molecule's orientational distribution within the bilayer. The order parameter $S_{\delta\infty}$ ($= r_\infty/r_0$) should yield information about the degree of this orientational constraint. Values of r_∞ can also be estimated from steady-state anisotropy values as outlined in the Appendix.

Relationship between $S_{\delta\infty}$ and the orientational distribution

We shall now define a director as the normal to the membrane plane. If the long axis of the probe starts at an angle α to the director and finishes at an angle β to the director with a rotation about the director through an angle ω, then, from simple geometry (see Fig. 1), the angle, δ, through which the long axis rotates, is given by:

$$\cos\delta = \cos\alpha \cdot \cos\beta + \sin\alpha \cdot \sin\beta \cdot \cos\omega \tag{4}$$

It is assumed that, as r approaches r_∞, β becomes completely independent of α and that all values of ω between 0 and 2π are equally probable. $\overline{\mathrm{Cos}^2\delta_\infty}$, the

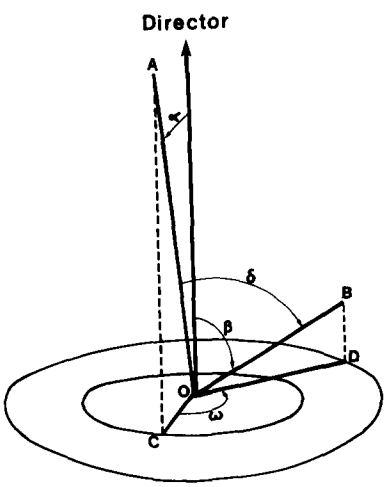

Fig. 1 Generalised representation of the motion of diphenylhexatriene during its excited state life-time. OA and OB are the initial and final directions of the long axis of diphenylhexatriene. OC and OD are the projections of OA and OB on to the plane of the bilayer.

time-independent mean value of $\cos^2\delta$, and hence $S_{\delta\infty}$, can be found by averaging over all possible values of α, β and ω:

$$\overline{\cos^2\delta_\infty} = \frac{\int_0^{\alpha_{MAX}} \int_0^{\beta_{MAX}} \Phi(\alpha) \cdot \Phi(\beta) \cdot (\cos^2\alpha \cdot \cos^2\beta + \tfrac{1}{2}\sin^2\alpha \cdot \sin^2\beta) d\alpha \cdot d\beta}{\int_0^{\alpha_{MAX}} \int_0^{\beta_{MAX}} \Phi(\alpha) \cdot \Phi(\beta) \cdot d\alpha \cdot d\beta} \quad (5)$$

where $\Phi(\alpha)$ and $\Phi(\beta)$ are the probability density functions of α and β, respectively.

If the relative probability of the long axis of the probe being at any angle θ to the director is $\Phi(\theta)$, then Eqn. 5, rewritten in terms of the order parameter is:

$$S_{\delta\infty} = \left[\tfrac{3}{2}\left(\int_0^{\theta_{MAX}} \Phi(\theta) \cdot \cos^2\theta \cdot d\theta \Big/ \int_0^{\theta_{MAX}} \Phi(\theta) \cdot d\theta\right) - \tfrac{1}{2}\right]^2 \quad (6)$$

Orientation distribution functions

It is now necessary to make some assumptions about the form of the constraint upon the probe orientation in order to derive $\Phi(\theta)$ and θ_{MAX}.

The approach of Kawato et al. [5] was to assume that the direction of the probe's long axis was randomly distributed within a cone of half-angle θ_c. Thus, they used: $\Phi(\theta) = \sin\theta$ and $\theta_{MAX} = \theta_c$. Eqn. 6 then becomes

$$S_{\delta\infty} = [\tfrac{1}{2}\cos\theta_c \cdot (1 + \cos\theta_c)]^2 \quad (7)$$

Fig. 2 shows the relationship between θ_c and $S_{\delta\infty}$ predicted by this approach.

Our first extension of the theory was to assume a 'sine-Gaussian' distribution (as has been done previously in NMR [8] and ESR [9] studies) and to allow all angles θ between 0 and $\pi/2$. If α_0 is the standard deviation of the Gaussian distribution then:

$$\Phi(\theta) = \sin\theta \cdot \exp(-\theta^2/2\alpha_0^2) \quad (8)$$

By substituting the above expression for $\Phi(\theta)$, Eqn. 6 was solved by numerical integration giving the relationship between α_0 and $S_{\delta\infty}$ shown in Fig. 2.

Finally $\Phi(\theta)$ was derived from a Boltzmann energy distribution. This distribution can be written:

$$W_i/W_o = \exp(-\Delta H/\boldsymbol{k}T) \quad (9)$$

where W_i/W_o = the relative probability of an excited state, S_i, compared to the ground state, S_0, $\boldsymbol{k}$ = Boltzmann's constant, T = absolute temperature, ΔH = the difference in enthalpy between S_i and S_o.

It was then assumed that the work done by a rigid probe such as diphenylhexatriene on changing its orientation in the membrane is done against a lateral pressure, Π, exerted by the membrane components. The mean energy difference between orientations will then be proportional to the change in cross-

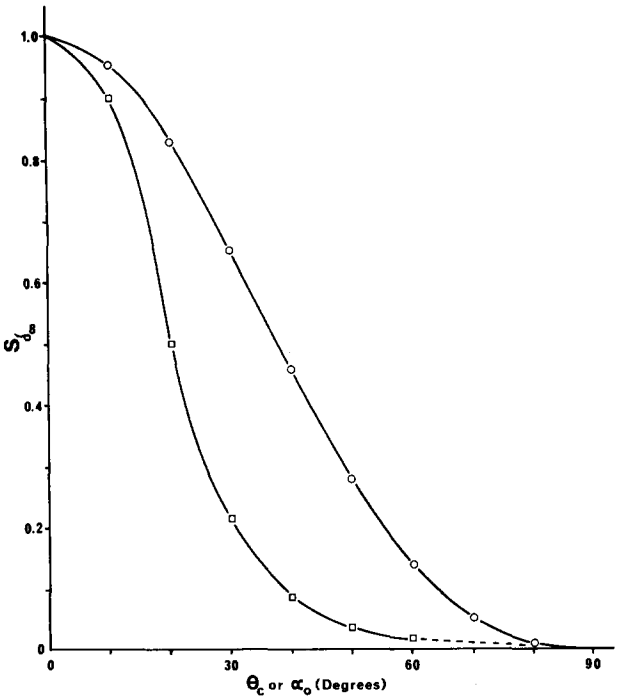

Fig. 2 Dependence of the order parameter $S_{\delta\infty}$ (for diphenylhexatriene undergoing either rapid wobbling diffusion inside a cone, or rapid reorientation with the angle of deviation normally distributed from the most preferred orientation) on the cone angle θ_c (○) or the standard deviation α_0 (□).

sectional area of the probe, as projected on to the membrane surface, ΔA. Thus

$$\Delta H = \Delta A \cdot \Pi \tag{10}$$

Diphenylhexatriene was taken to be a rod-shaped molecule with hemispherical ends whose dimensions L, the length excluding the hemispherical ends, and R, the radius of the hemispherical ends, were taken as 1.15 and 0.25 nm, respectively.

The change in projected area on rotating from the director to any angle θ is

$$\Delta A = 2RL \sin\theta \tag{11}$$

Eqn. 9 now becomes

$$W_i/W_o = \exp(-2RL \sin\theta \cdot \Pi/kT) \tag{12}$$

Since the total number of orientations for a given value of θ is proportional to $\sin\theta$, then the probability density function, $\Phi(\theta)$, becomes

$$\Phi(\theta) = \sin\theta \cdot \exp(-2RL \sin\theta \cdot \Pi/kT) \tag{13}$$

Fig. 3 shows this function for various values of lateral pressure at 25° C.

The relationship between $S_{\delta\infty}$ and the reduced lateral pressure Π/T is shown in Fig. 4. $S_{\delta\infty}$ was again calculated by numerical integration of Eqn. 6 with respect to θ between the limits 0 and $\pi/2$.

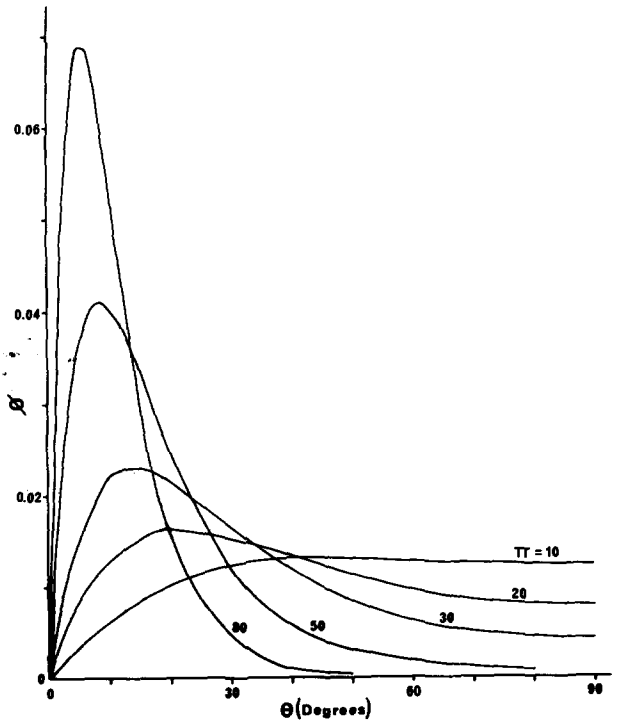

Fig. 3 Normalised angular distribution Φ of diphenylhexatriene shown as a function of the angular deviation from the director θ for various lateral pressures Π at 298 K.

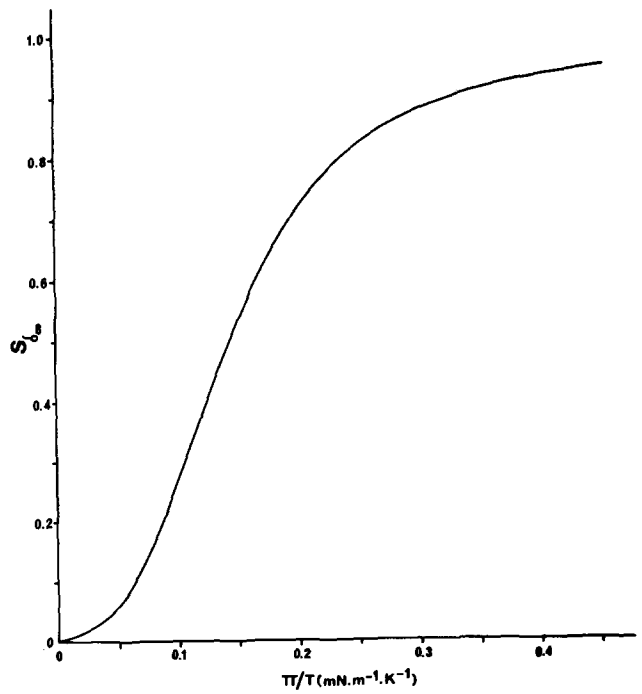

Fig. 4. Calculated relationship between the order parameter $S_{\delta\,\infty}$ for incorporated diphenylhexatriene and the reduced lateral pressure Π/T in a lipid bilayer.

Results and Discussion

By considering theoretically in energy terms the orientational fluctuations of the rod-shaped (fluorescent) probe molecule diphenylhexatriene incorporated into bilayered lipid membranes, the constraints on rotation, which would be detected experimentally as residual anisotropy by nanosecond fluorescence polarization technique, emerge as pressures that should equate with the lateral pressures operating within the bilayers (due to forces between mobile lipid molecules) independently of the probe molecule. To test this postulate, the experimentally observed anisotropy data of Kawato et al. [5] for diphenylhexatriene inserted into dipalmitoyl phosphatidylcholine liposomes have been converted to order parameters, adopting 0.362 as the value for r_0, and read off as lateral pressures from the theoretical relationship (Fig. 4). The results have been examined as a function of temperature (Fig. 5). A sudden drop in pressure of about 40 mN · m^{-1} is observed between 34.5°C and 43.5°C, which presumably occurs on passing through the transition temperature, 41.5°C. The pressure at the mid-point of the transition is approx. 50 mN · m^{-1}. This is in good agreement with the value of 47 mN · m^{-1} which Hui et al. [10] calculated as the external lateral pressure under which a dipalmitoyl phosphatidylcholine monolayer would have the same transition temperature as a bilayer of the same material under no external lateral pressure. Their calculation assumed that no internal lateral pressure had developed within the compressed monolayer. Nagle

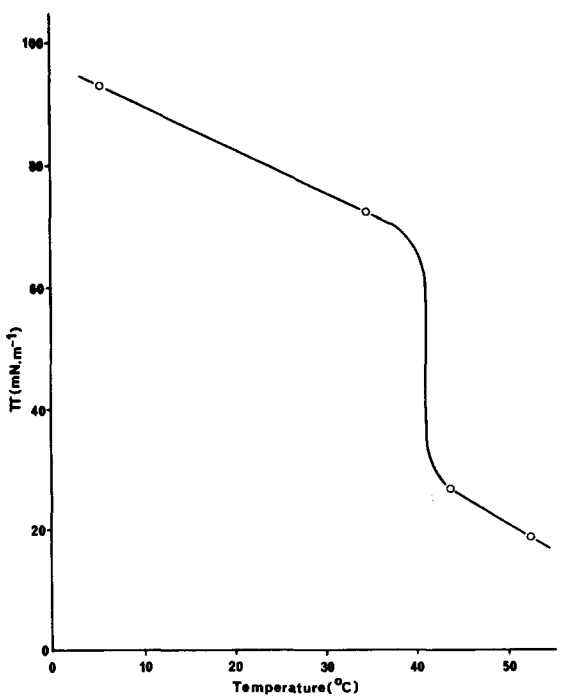

Fig. 5 Dependence of the lateral pressure Π for diphenylhexatriene liposomes on temperature, calculated from fluorescence anisotropy data [5].

[11] has estimated a similar value (50 mN · m^{-1}) using a model-independent thermodynamic calculation.

Also, our estimates of lateral pressure in the fluid phase are close to the value of 20 mN · m^{-1} required by Marčelja [12] for consistency between his molecular field model and published NMR order parameter data for fluid bilayers [13].

Kawato et al. [14] and Hildenbrand and Nicolau [7] have also published nanosecond fluorescence polarization technique data derived from diphenylhexatriene incorporated into dipalmitoyl phosphatidylcholine and egg-yolk phosphatidylcholine liposomes containing increasing amounts of cholesterol. Using these data we have calculated that the lateral pressure at 50° C increases from 20 mN · m^{-1} for pure dipalmitoyl phosphatidylcholine liposomes to 56 mN · m^{-1} for dipalmitoyl phosphatidylcholine liposomes containing 50 mol% cholesterol. If dipalmitoyl phosphatidylcholine is replaced by egg-yolk phosphatidylcholine the corresponding figures at 25°C are approx. 25 mN · m^{-1} and 46 mN · m^{-1}. One possible explanation for this increase is that the probe senses an additional lateral pressure caused by interactions between cholesterol molecules and lipid chains, which interactions result in the well-known 'condensing' and 'ordering' effects of cholesterol on fluid lipid chains in monolayers and bilayers.

Despite the encouraging agreement between our lateral pressure estimates and others, caution is required in equating the observed lateral pressure experienced by a fluorescent probe with the actual, macroscopic lateral pressure within a bilayer. Some reasons why the theoretical equivalence may break down in reality will now be outlined:

(i) The lateral pressure against which work must be done in order to increase the probe's projected area probably changes with θ (even assuming no lateral pressure gradient across the bilayer). At small values of θ steric hindrance to collision will be approximately equal on all sides. This effectively produces zero lateral pressure across the perimeter of the probe's projected area.

(ii) A lateral pressure gradient probably exists across the membrane. Hard-disc repulsion will not be the same for the head groups as for the hydrocarbon chain region due to the difference in excluded area. There is possibly also a gradient along the length of the hydrocarbon chains, the change in order parameter along the length of the chains observed in NMR and ESR studies [13] would support this notion. It is quite probable that the probe would not be evenly distributed across any lateral pressure gradient present.

(iii) Diphenylhexatriene and other probes probably disrupt the phospholipid packing around them. This may affect the lateral pressure felt by the probe. However, diphenylhexatriene a rigid, symmetrical, hydrophobic, rod-shaped molecule with a diameter intermediate between those of rigid and fluid lipid chains, was chosen partly because it would be expected to cause minimal disruption to both the lipid chain and polar head group packing [1,2].

(iv) The assumption that the energetically favoured orientation for diphenylhexatriene is perpendicular to the bilayer may not be valid, especially for the gel phase ($L_{\beta'}$), where phospholipid chains are known to be tilted [15].

(v) When the probe is partitioned inside a heterogeneous system such as a

cell, the anisotropy value will represent the average for many different environments, membranal or otherwise, and each with its own lateral pressure and/or local viscosity. The probe may even become tightly bound to molecules such as proteins and thereby reflect the motile behaviour of the host molecule.

Conclusions

The model presented here enables values of the time-independent anisotropy of diphenylhexatriene incorporated in lipid bilayers to be converted into lateral pressure values. The agreement between these and previously reported estimates using alternative methods suggests that our model provides a reasonable description of the orientational distribution of rod-shaped fluorescent probe molecules in membranes.

Appendix

The theoretical relationship between the steady-state anisotropy, $\bar{r}$, and r_∞ for a probe in a given environment, assuming exponential decays for both fluorescence and anisotropy, is [7]:

$$r_\infty = (1 + \Upsilon_r/\Upsilon_F)\bar{r} - (\Upsilon_r/\Upsilon_F)r_0$$

where Υ_r = the anisotropy relaxation constant for the probe in the given environment; Υ_F = the fluorescence relaxation constant for the probe in the given environment.

Data published by Hildenbrand and Nicolau [7] for diphenylhexatriene in a range of liposomes and biomembranes at 25°C show a strong correlation between $\bar{r}$ and r_∞.

$$r_\infty = 1.324\bar{r} - 0.099 \qquad \text{(correlation coefficient = 0.993)}$$

This allows rough estimates of lateral pressure to be made using the steady-state fluorescence polarization technique.

Hildenbrand and Nicolau advocate caution over use of this empirical relationship. The approximate linearity is observed because variations of Υ_F and Υ_r with environment are not large and tend to compensate for one another. Also, both decays are only approximately mono-exponential [4].

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