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LIPID STRUCTURAL ORDER PARAMETERS (RECIPROCAL OF FLUIDITY) IN BIOMEMBRANES DERIVED FROM STEADY-STATE FLUORESCENCE POLARIZATION MEASUREMENTS

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This paper presents an interpretation of fluorescence polarization measurements in lipid membranes which are labelled with the apolar probe 1,6-diphenyl-1,3,5-hexatriene. The steady-state fluorescence anisotropy, r_s, is resolved into a fast decaying or kinetic component, r_f, and an infinitely slow decaying or static component, r_∞. The latter contribution, which predominates in biological membranes, is exclusively determined by the degree of molecular packing (order) in the apolar regions of the membrane; r_∞ is proportional to the square of the lipid order parameter. An empirical relation between r_s and r_∞ is presented, which is in agreement with a prediction based on a theory of rotational dynamics in liquid crystals. This relation enabled us to estimate a lipid structural order parameter directly from simple steady-state fluorescence polarization measurements in a variety of isolated biological membranes. It is shown that major factors determining the order parameter in biomembranes are the temperature, the cholesterol and sphingomyelin content and (in a few systems) the membrane intrinsic proteins.

Introduction

In the last decade fluorescence polarization techniques have yielded valuable information about lipid structure and dynamics in membranes. In these techniques apolar fluorophores, such as 1,6-diphenyl-1,3,5-hexatriene (DPH) are embedded in the lipid regions and the fluorescence polarization of the labelled system is measured upon excitation with polarized monochromatic light. In the relatively simple 'steady-state' measurements, routinely performed now in many laboratories, the emission of the fluorophore is measured under continuous illumination. The steady-state fluorescence anisotropy (r_s) and polarization (P) are determined by the emission intensities through an analyzer oriented parallel ($I_\parallel$) and perpendicular ($I_\perp$) to the direction of polarization of the excitation light;

$$r_s = \frac{I_\parallel - I_\perp}{I_\parallel + 2I_\perp}, \quad P = \frac{I_\parallel - I_\perp}{I_\parallel + I_\perp}, \quad r_s = \frac{2P}{3 - P} \tag{1}$$

The extent of depolarization of the emission of the fluorophore in the membrane reflects the degree to which a population of photoselected (by the polarized light) excited fluorophores loses its initial selective orientation and becomes randomized.

Until recently, these steady-state fluorescence anisotropy measurements have been interpreted exclusively in terms of 'microviscosity' (Refs. 1 and 2, reviewed in Ref. 3), i.e. the rate of rotational diffusion of the probe, thought to reflect the viscous drag imposed by the local apolar environment of the

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Abbreviations: DPH, 1,6-diphenyl-1,3,5-hexatriene; DMPC, DPPC, DOPC, dimyristoyl-, dipalmitoyl-, and dioleoyl-L-α-phosphatidylcholine.

probe. The apparent 'microviscosity' of the membrane interior was estimated by comparing the fluorescence anisotropy in the membrane system with that observed in a macroscopic-isotropic system (a reference mineral oil) and applying classical hydrodynamic expressions of the Perrin type [4,5]. However, the implicit assumption that the freedom (isotropy) of the depolarizing rotations of the fluorophore in the two systems is similar, appeared to be wrong [6–16].

Recently, more information on the complex motions of the fluorophore in the lipid bilayer has been obtained from time-resolved fluorescence anisotropy decay measurements, within nanoseconds after a short pulse of polarized excitation light on a probed sample [7–16]. These studies revealed that it is mainly the degree to which the fluorophore rotations are restricted by the molecular packing of the lipids (a static factor), rather than its rotational rate (a dynamic factor), which determines the conventionally measured steady-state fluorescence anisotropy in lipid membranes. According to this new interpretation [14–16] (see also below), r_s is resolved into a static part, r_∞, and a dynamic part, r_f. The latter contribution is related to the rotational relaxation time of the fluorophore, which is in turn proportional to the 'microviscosity' [1–3], while r_∞ is proportional to the square of the lipid order parameter [15,16].

In this paper we show that for artificial and biological membranes the resolution of r_s into r_f and r_∞ is unique in the sense that if two membranes have the same r_s, they have about the same r_∞ as well, and therefore roughly the same order parameter. The empirical relation between r_s and r_∞ will be given and shown to be in good agreement with a theoretical one, which is based on a recent theory of rotational dynamics in liquid crystals [17]. Further, the relationship between r_s and r_∞ will be used to estimate the order parameter (S_{DPH}) in a variety of isolated biological membranes from steady-state DPH-fluorescence polarization data. Such estimations of lipid order parameters in biological membranes have not been presented as yet. It will be shown that the temperature, the amount of cholesterol, sphingomyelin and (in a few systems) membrane protein are major factors determining S_{DPH} in biomembranes.

Materials and Methods

The isolation procedure and lipid analysis of human and bovine eye lens fibre membranes have been described [18–20]. Plasma membranes from human erythrocytes [21], rat (R/A) liver [22], 484A hepatoma [22], mouse thymocytes [23,24], leukemic GRSL cells and ascites extracellular vesicles [24,25] were isolated according to standard methods. Plasma membranes from Novikoff rat hepatoma ascites cells and human milk fat globules were isolated [26], and lipids were analysed [23,25] as described previously.

Steady-state fluorescence polarization (P) measurements were performed as described before [24–26] in an Elscint MV-1a apparatus (Elscint Ltd., Haifa, Israel) using DPH as a fluorescent probe. Correction for light scattering was carried out by successive dilutions of the suspended membranes, until a plateau value of P was obtained [2].

Theory

Interpretation of fluorescence depolarization in lipid bilayers

When the fluorescence anisotropy of a membrane

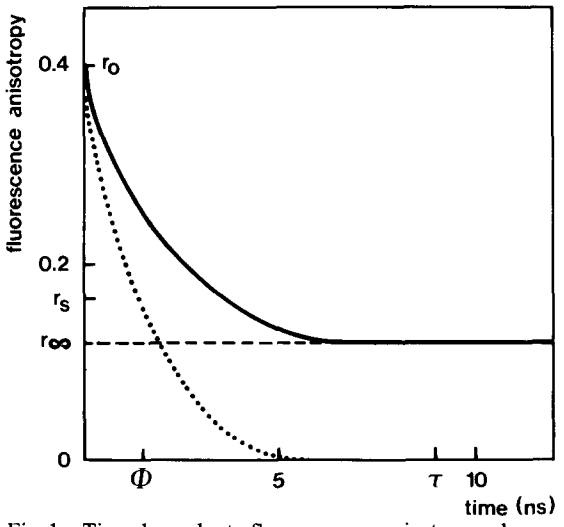

Fig. 1. Time-dependent fluorescence anisotropy decay in lipid membranes (solid line) and in an isotropic reference oil (dotted line). For symbols, see text. The relative positions of r_0, r_s, r_∞, ϕ and τ, as indicated in this graph, are determined by Eqn. 5. The indicated values are typical for a rather 'fluid' biomembrane, labelled with DPH.

probe, like DPH, is recorded as a function of time t, during nanoseconds after excitation with a short pulse of polarized light, the following picture emerges (Fig. 1): Initially, the emitting dipoles, laying along the long molecular axis of the probe, are oriented parallel to the direction of polarization of the excitation light, and the fluorescence anisotropy is large. Due to rotational (wobbling) diffusion of the probe, the orientation of the emitting dipoles becomes increasingly disordered and the fluorescence anisotropy decreases exponentially. In isotropic reference oils the fluorescence anisotropy decreases to zero (allowing application of Perrin's law), but in lipid membranes a finite level r_∞ is reached, because the probe does not assume all possible orientations with equal probability after a prolonged time period, i.e. the final distribution of emitting dipoles is anisotropic. This reflects the structural order in membranes. The fluorescence anisotropy decay, $r(t)$, can be expressed as

$$r(t) = (r_0 - r_\infty) \exp(-t/\phi) + r_\infty \tag{2}$$

in which r_0 is the maximal fluorescence anisotropy value in the absence of any rotational motion of the fluorophore, and ϕ the rotational correlation time for reorientations of the long molecular axis [10]. The total intrinsic fluorescence intensity is assumed to follow a simple exponential decay, according to $F(t) = (1/\tau) \exp(-t/\tau)$, in which τ is the fluorescence lifetime of the fluorophore. Then, the steady-state fluorescence anisotropy can be obtained by the time integral

$$r_s = \int_0^\infty r(t)\, F(t)\, dt \Big/ \int_0^\infty F(t)\, dt \tag{3}$$

yielding

$$r_s = \frac{r_0 - r_\infty}{1 + \tau/\phi} + r_\infty = r_f + r_\infty \tag{4}$$

in which r_f represents the fast decaying or kinetic component, and r_∞ the infinitely slow decaying component, determined by the membrane anisotropy (structural order) [14–16]. The Perrin formula, used in the past to calculate rotational correlation times and from these the 'microviscosities' of membranes [1–3], is obtained if the structural part, r_∞, is neglected. The relative weight of the two contributions is determined by r_∞ and the ratio τ/ϕ. In Fig. 1 the steady-state fluorescence anisotropy, r_s, lies in between the values for r_0 and r_∞, positioned by the ratio

$$\frac{r_s - r_\infty}{r_0 - r_s} = \frac{\phi}{\tau} = \gamma \tag{5}$$

Furthermore, r_f and r_∞ can be expressed as

$$r_f = \frac{\gamma}{1 + \gamma} (r_0 - r_\infty) = \gamma r_0 - \gamma r_s \tag{6}$$

$$r_\infty = (1 + \gamma)\, r_s - \gamma r_0 \tag{7}$$

The empirical relation between limiting (r_∞) and steady-state (r_s) anisotropy

Experimental data for the steady-state fluorescence anisotropy, r_s, and the limiting fluorescence anisotropy, r_∞ of a variety of artificial and biological membranes, obtained from the literature [10,12,14, 27,28], are plotted in Fig. 2. A definite trend emerges: the contribution of r_∞ to r_s is zero for very small r_s values, becomes larger with increasing r_s and approaches 100% for the highest r_s values. This trend is described by the empirical curve for r_∞ as a function of r_s, given in Fig. 2 (see also Fig. 3). It has a horizontal slope at $r_s = 0$ and touches the line $r_\infty = r_s$ at $r_s = 0.4$. In the region $0.13 < r_s < 0.28$ the curve is linear corresponding to the formula

$$r_\infty = \tfrac{4}{3} r_s - 0.10\,, \quad 0.13 < r_s < 0.28 \tag{8}$$

Especially the data for biological membranes, egg phosphatidylcholine containing liposomes and DMPC liposomes fit very well in the curve, data for other artificial membranes show somewhat more spreading (see also the following section).

The shape of the curve at its two ends is in complete agreement with what one would expect:

(a) In the region where r_s is very small, corresponding to high temperatures and vanishing orientational order, the Perrin relation must hold; therefore one obtains $r_\infty = 0$, $r_f = r_s$. In this region th viscosity and ϕ are very small, with the limiting value of $\phi = 0$ for $r_s = r_f = 0$.

(b) In the region where r_s approaches the theo-

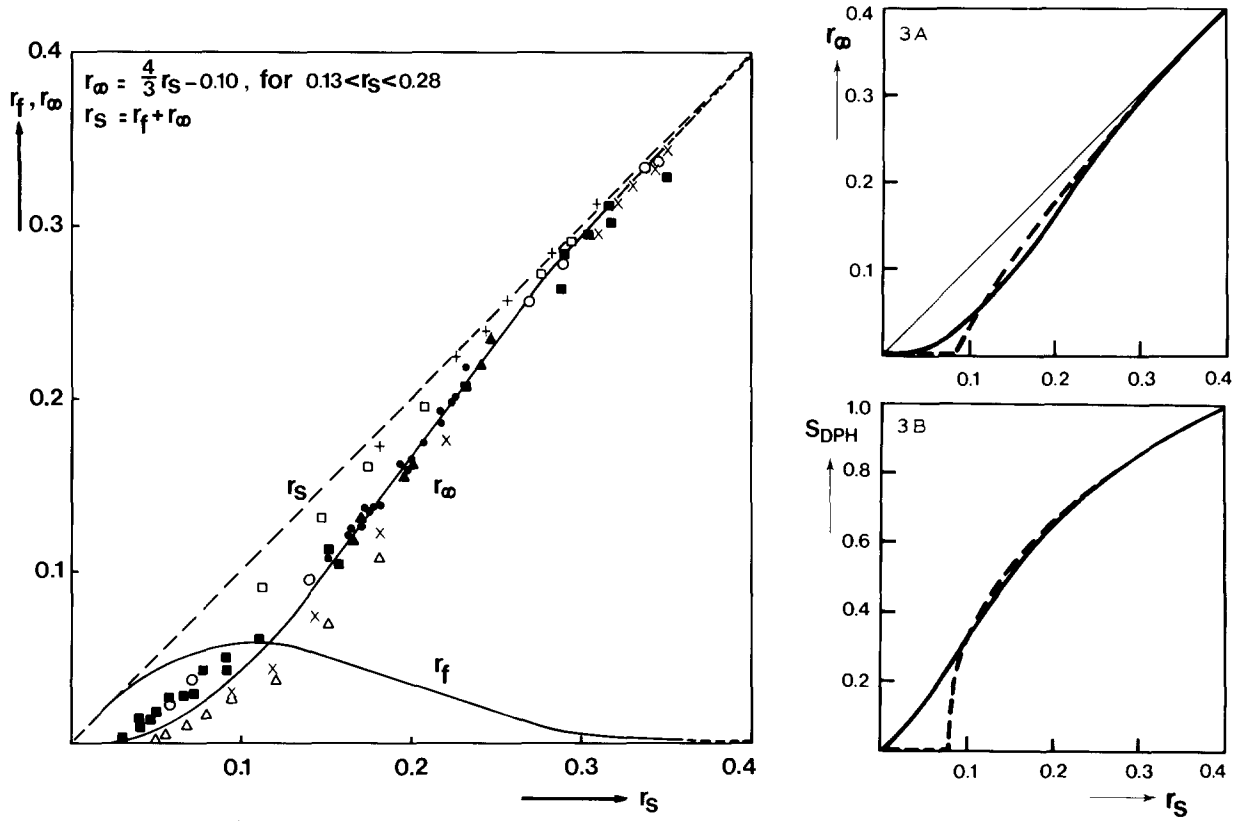

Fig. 2. Empirical resolution of the steady-state fluorescence anisotropy (r_s) of DPH-labelled artificial and biological membranes into a kinetic (r_f) and a structural (r_∞) component, within the theoretically limiting values of 0.0 and 0.4. A best-fitting curve for r_∞ as a function of r_s was constructed from the following experimental data. From Hildenbrand and Nicolau [14], at 25°C: ▲, various biological membranes; ●, liposomes composed of egg yolk phosphatidylcholine/cholesterol/phosphatidylethanolamine in varying molar ratios. From Lakowicz et al. [27,28], at temperatures ranging from 5 to 65°C and at 30 MHz: ■, DMPC vesicles; ○, DPPC vesicles; □, DMPC/cholesterol (3/1) vesicles; △, DOPC vesicles. From Kawato et al. [10,12], at temperatures ranging from 5 to 60°C: X, DPPC vesicles; +, DPPC/cholesterol (3/1) vesicles. The r_s values for DPPC vesicles were obtained from the data for ϕ and τ in Ref. 10 using Eqn. 4, with $r_0 = 0.4$; the r_∞ values for DPPC/cholesterol (3/1) vesicles were obtained from cone angle (θ) data in Ref. 12 using Eqn. 9, with $r_0 = 0.4$. The curve for r_f was derived from that of r_∞, according to $r_f = r_s - r_\infty$.

Fig. 3. A. Empirical and theoretical relation between r_s and r_∞. The empirical (solid) curve was obtained from Fig. 2. The theoretical (dashed) curve was constructed using Eqn. 13. B. Empirical (solid line) and theoretical (dashed line) relation between r_s and the structural order parameter, S_{DPH}, obtained from the curves in Fig. 3A, using Eqn. 10, with $r_0 = 0.4$.

retically maximum value of 0.4 ($=r_0$), orientational order is nearly complete. Here the excitation and emission dipoles of the fluorophore have a minimal freedom of rotation (minimal 'cone angle', see below), and the rotations are of very short duration ($\phi \ll \tau$ in Eqn. 4), with the limiting value of $\phi = 0$ at $r_s = r_0$ [17,29]; here one obtains $r_\infty = r_s$, $r_f = 0$.

Estimation of order parameters (S_{DPH})

It is generally assumed that the orientational distribution of the elongated apolar DPH probe reflects that of the surrounding lipid chains, that is axially symmetric around the normal to the membrane [15,30]. Kinosita et al. [11] adopted a 'cone model' to describe the wobbling diffusion of the probe. According to this model the probe rotations

are restricted to a 'cone', formed by the surrounding fatty acyl and cholesterol entities or other apolar structures in the membrane. The opening angle θ of this cone around the membrane normal reflects the degree of molecular packing (order) in the membrane and is related to r_∞ as

$$\frac{r_\infty}{r_0} = [\tfrac{1}{2}\cos\theta(1+\cos\theta)]^2 \qquad (9)$$

This ratio, called the 'degree of orientational constraint' by Kinosita et al. [11], has only very recently been recognized as the square of the orientational order parameter S [15,16,29] of the lipid bilayer, known from deuterium magnetic resonance (DMR) and spin label (ESR) studies. For DPH, the relation

$$S^2 = r_\infty/r_0\,, \quad 0 \leqslant S \leqslant 1 \qquad (10)$$

was derived from theoretical considerations on fluorescence depolarization in liquid crystals, independent of any model [15,16,29].

Since in general actual S values may depend on the technique, the type and location (label position) of the probe, we prefer to use the designation S_{DPH} in the experimental part of this paper. S_{DPH} refers to a mean position of the DPH probe along the lipid acyl chains [30], as opposed to the local order parameters of a particular acyl chain segment, as determined by DMR and ESR, which decrease towards the end of the chain [31]. Heyn [15] and Jähnig [16] have calculated S_{DPH} values from nanosecond fluorescence anisotropy (r_∞) data of DPPC containing liposomes, and have shown that these values agree with the DMR values of the 10–12th carbon-atom segment of the acyl chains, suggesting that this may be the average location of the DPH molecule.

Order parameters, S_{DPH} in lipid membranes can thus be derived from steady-state fluorescence anisotropy measurements by using the empirical relation between r_s and r_∞ (Fig. 2) and Eqn. 10. The error in S_{DPH} due to the scattering of the data around the empirical curve in Fig. 2 may be rather high at low r_s values, but decreases strongly when r_s increases. The relative error is estimated as

$$100\,\Delta S/S\,\% \approx 50(1-\tfrac{5}{2}r_s)^2\,\% \qquad (11)$$

The error corresponds to the maximal deviation from the empirical curve, taking all membrane systems presented in Fig. 2 into account. It should be noted, however, that the scattering of the data for biological membranes is much smaller than for the artificial membranes.

Theoretical prediction of the relationship between r_s and r_∞ (or S_{DPH})

The empirical relation between r_∞ and r_s (Fig. 2) is in good agreement with a theoretical prediction of Nordio and Segre [17], who have calculated the rotational correlation times for molecules undergoing rotational diffusion in a model of an orientational potential. The rotational correlation time ϕ for reorientations of the long molecular axis of DPH (τ_{00}^2 in their notation), is a function of the order parameter S. It has a maximum for $S \approx 0.25$ and is zero for $S = 1$. We can approximate the dependence of ϕ on the order parameter by a parabolic function. This approximation deviates less than 4% from the numerical results of Nordio and Segre [17]. The fluorescence lifetime of DPH, τ, may range from about 5.5 ns, for high temperatures where S is nearly zero, to 11 ns, for low temperatures where S approaches unity [2,12,27,28]. This variation is taken into account here by assuming, as a rough approximation, that τ is proportional to the factor $1 + S$. Consequently, we obtain the following expression for $\gamma = \phi/\tau$,

$$\gamma = \gamma_0(1+2S)(1-S)/(1+S) \qquad (12)$$

where γ_0 is the value of γ at $S = 0$.

In order to derive a relation between r_s and r_∞ the following assumptions are made:

(a) The variation of γ in membranes is entirely due to the variation in the order parameter S, and γ_0 is therefore constant, whereas γ_0 is temperature dependent for systems that do not exhibit orientational order, e.g. mineral oils in the fluid phase.

(b) $\gamma_{max} = 1/4$, for $S = 0$; this assumed maximum value of γ is consistent with published data, e.g. of Hildenbrand and Nicolau [14].

Consequently, $\gamma_0 = 1/4$ for $0 < S \leqslant 1$, and $\gamma_0 \leqslant 1/4$ for $S = 0$. Substitution of Eqn. 11 in Eqn. 4 gives a theoretical relation between r_s and r_∞,

$$\begin{cases} r_\infty = \frac{2}{5}S^2 \quad (r_0 = \frac{2}{5} \text{ in Eqn 10}) \\ r_s = \dfrac{2(1+2S)(1-S)(1-S^2)}{20(1+S)+5(1+2S)(1-S)} + r_\infty \\ \qquad\qquad \text{for } 0 < S \leqslant 1 \qquad (13) \\ r_s \leqslant 0.080 \quad \text{for } S = 0 \end{cases}$$

Both the empirical and the theoretical relation between r_∞ and r_s are plotted in Fig. 3A. Note that there is an appreciable discrepancy only for $r_s < 0.1$. For biological membranes, however, the r_s values are generally larger than 0.1 (Fig. 2, Table I). Using the formulas derived above, the results of Fig. 3A can be converted in an empirical and a theoretical relation between S and r_s (Fig. 3B). The discrepancy between the curves for $r_s < 0.1$ is probably due to our assumptions concerning the variation of ϕ and τ (γ and γ_0), which may be more intricate in reality and may depend on the chemical composition of the system. Especially the transition from nonordered into ordered (the break point in the theoretical curves, at $r_s = 0.080$) may conceivably be more gradual in lipid membranes, as actually found empirically.

Our relation between r_∞ and r_s differs from that of Jähnig [16], who proposed a linear dependence of r_∞ as a function of r_s, setting $\gamma = \phi/\tau$ equal to 0.125. Although his result is in agreement with data for DPPC, the assumption that γ is a constant, cannot be correct in general. In fact, the theoretically predicted variation of γ with S is in agreement with a great

TABLE I

FLUORESCENCE POLARIZATION (P_{DPH}, AT 25°C), LIPID ORDER PARAMETERS (S_{DPH}, AT 25°C) AND CHOLESTEROL AND SPHINGOMYELIN-PHOSPHOLIPID MOLAR RATIOS IN A VARIETY OF BIOLOGICAL MEMBRANE SYSTEMS

The types of biomembranes are listed by decreasing P_{DPH} and S_{DPH} values; mean values are presented from at least three experiments [25,26]. S_{DPH} is calculated according to Eqn. 10, with $r_0 = 0.4$. n.d., not determined.

Type of biomembrane	P_{DPH}	r_s	r_∞	S_{DPH}	Chol./PL (molar ratio)	Sphingomyelin/total PL (mol %)
Plasma membranes, purified from:						
Human eye lens fibre *						
nucleus	0.403	0.310	0.304	0.87	2.6	72
cortex	0.400	0.308	0.302	0.87	1.8	63
Bovine eye lens fibre nucleus	0.395	0.303	0.297	0.86	1.6 **	23
Rat Novikoff ascites hepatoma	0.351	0.265	0.253	0.80	1.09	31
Rat hepatoma 484A (solid tumor)	0.337	0.253	0.237	0.77	0.89	24
Human erythrocytes	0.332	0.249	0.232	0.76	0.85	20
Mouse GRSL leukemic (ascites) extracellular vesicles	0.327	0.245	0.226	0.75	1.10	9
Rat (R/A) liver	0.322	0.240	0.220	0.74	0.65	23
Human milk fat globules	0.310	0.230	0.207	0.72	n.d.	n.d.
Mouse (GR/A) thymocytes	0.306	0.227	0.203	0.71	0.79	8
Mouse GRSL leukemia (ascites)	0.269	0.197	0.163	0.64	0.32	0.8
Other systems:						
Rat liver endomembranes ***						
Human milk fat globules	0.208	0.149	0.099	0.50	0.08	n.d.
(non disrupted; including lipid content)	0.120	0.083	0.028	0.26	n.d.	n.d.

* Data presented refer to a 70-year-old eye lens; chol./PL molar ratios of human eye lens fibre membranes may range from 1.0 (cortex) up to 3.2 (nucleus) by increasing age [18,20].

** Chol./PL molar ratios may range from 1.0 to 2.9, depending on the age [19].

*** Consisting of all cellular membranes (except for nuclei and the bulk of plasma membranes) precipitable between 1500 × g (10 min) and 105 000 × g (1 h) in 10^{-3}M $NaHCO_3$.

number of data for various biomembranes and liposomes. Consequently, taken over the whole fluorescence anisotropy range, r_∞ is a non-linear function of r_s, as is shown above. The conclusion of Heyn [15], that r_∞ and thus also S can be determined from steady-state measurements alone if r_s is large or γ is small, is confirmed and extended here. We have thus shown how the order parameter can be derived from steady-state fluorescence anisotropy, not only if r_s is large, but from lower r_s values as well.

Experimental results and discussion

Table I compiles the steady-state fluorescence polarization data of a variety of DPH-labelled biomembranes at 25°C. From these data order parameters (S_{DPH}) are derived using the empirical relation between r_s and r_∞ (Fig. 2), and Eqn. 10, taking $r_0 = 0.4$. For DPH, r_0 values of 0.395 [10], 0.390 [28] and 0.362 [2] have been reported. The latter value would increase the S values listed in Table I about 5%. The various membrane types from normal as well as tumour cells are listed in Table I by decreasing fluorescence anisotropy and S values, ranging from the highly structurally ordered (rigid) eye lens membranes to the very fluid leukemic cell membranes and liver endomembranes. Intact milk-fat globules are included in this table, in addition to the purified globule surface membranes, to illustrate how the non-polar lipid (triacylglycerols) content of these globules, which obviously exhibits a very low structural order, affects the overall S_{DPH}. Likewise, but to a lower extent, non-membrane lipid droplets which may occur in the cytoplasm of cells, have a lowering effect on P_{DPH} and S_{DPH} values of intact cells [26]. Such values of intact cells can be considered to represent a weighted average of all lipid pools, i.e. plasma- and endomembranes and lipid droplets, present in the cell [26].

Effect of membrane cholesterol and sphingomyelin

It has been demonstrated by a variety of physical techniques [12,32] that cholesterol has a (small) fluidizing effect below the phase transition temperature (gel phase) of phospholipids and a (large) rigidizing (ordering) effect above this temperature (liquid crystalline phase). In biological membranes at ambient temperatures, cholesterol thus imposes on the heterogenous population of phospholipids a condition of 'intermediate fluidity' [32], and causes a smoothening or disappearance of phase transitions. In general, the overall effect of cholesterol in these membranes is rigidization (increased order or molecular packing, decreased 'cone angle' θ [12,14]).

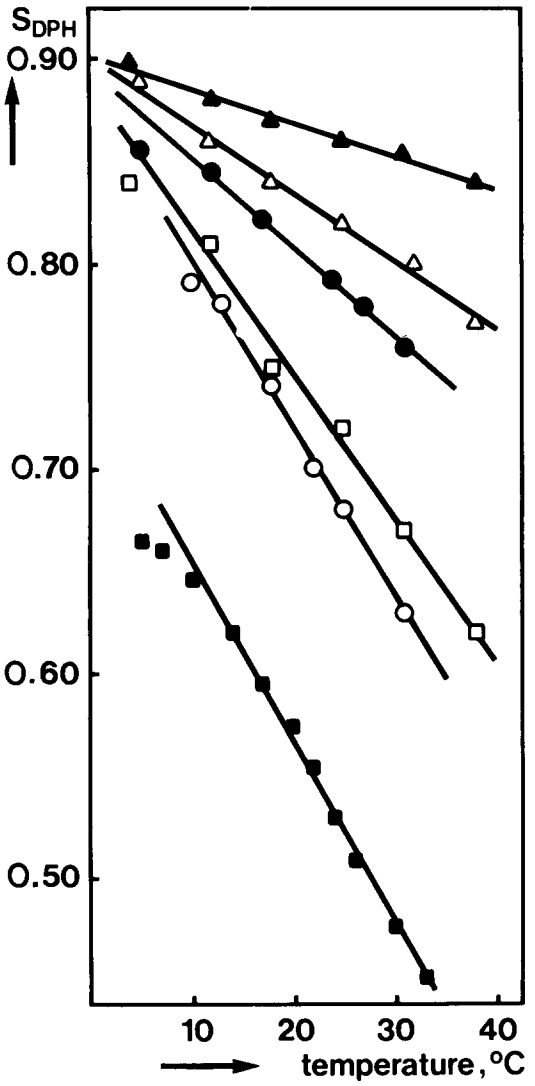

Fig. 4. Structural order parameter, S_{DPH}, as a function of the temperature for various types of biological membranes. The slopes of the curves are inversely related to S_{DPH} of the membranes at a given temperature. ▪, rat liver endomembranes; ○, plasma membranes from GRSL leukemia; □, rat liver plasma membranes; ●, GRSL extracellular vesicles; △, calf eye lens outer cortex membranes; ▲, calf eye lens nucleus membranes.

This is illustrated by the experimental data in Table I: An increase in fluorescence anisotropy and S_{DPH} values in biomembranes is generally correlated with an increased cholesterol-phospholipid molar ratio, which confirms previous reports [24,33]. It is, however, also shown that in a few cases, i.e. murine leukemic extracellular vesicles and thymocyte plasma membranes, the decreasing order of S_{DPH} values listed in Table I is not accompanied by decreasing cholesterol-phospholipid ratios. This may be due to the very low sphingomyelin content of these membranes, which confirms the notion that the amount of sphingomyelin is the second important parameter determining structural order in biomembranes [34]. Interestingly, there is much evidence supporting the existence of a strong preferential interaction between cholesterol and sphingomyelin among the various lipids in biomembranes (reviewed in Ref. 34), which could be instrumental in the formation of rigid membrane domains [24,25] or, in extreme cases (human lens membranes, Table I), entirely rigid membranes.

Our data suggest that there may be an upper limit to the fluorescence anisotropy and S_{DPH} values measurable in any membrane system at a given temperature. This limiting value, $S_{DPH} = 0.87$ at 25°C (Table I) or 0.90 at 4°C (Fig. 4), is reached by the eye lens membranes, for which large variations in cholesterol and sphingomyelin content invariably yield the same limiting fluorescence anisotropy values. These values were also measured in liposomes of DPPC (in the gel phase) and in liposomes of cholesterol/sphingomyelin, in various molar ratios ranging from 0 to 1.5 (unpublished data).

Effect of membrane proteins

The contribution of membrane proteins to the DPH steady-state fluorescence anisotropy can be estimated by comparing isolated native membranes with liposomes prepared from their lipid extracts. In this manner virtually no positive contribution is detectable in the lymphoid- [24], liver-, hepatoma-, and erythrocyte plasma membranes, listed in Table I, the difference in S_{DPH} values being less than 4%. However, we have found an appreciable protein contribution of 7–12% to the S_{DPH} values of the eye lens membrane. The extent to which the proteins contribute may thus depend on the type of membrane, probably on the type and concentration of proteins in the membrane.

Others have found virtually no protein effect for rhabdovirus membranes [35,36] and erythrocyte ghosts [37], and a positive effect for the membranes of retinal rod outer segment discs [38], togaviruses [35,39], intestinal microvilli [40,41] as well as artificial lipid-protein vesicles [42,43]. In order to obtain a positive contribution of protein to the fluorescence anisotropy, the probe must be assumed to partition into highly structurally ordered regions of lipids, surrounding the apolar parts of membrane intrinsic proteins [43]. Such proteins may influence the S_{DPH} in gel phase or fluid phase of lipid bilayers, in the same way as does cholesterol [15,16]. It should finally be noted that our results disagree with the proposition of Mély-Goubert and Freedman [44] that membrane-associated proteins generally make a dominant contribution to the fluorescence anisotropy values in biomembranes.

Effect of the temperature

Fig. 4 shows that the order parameter, S_{DPH} for the various types of membranes is almost linearly related to the temperature, within the experimental error. Below 10°C the measured data have a tendency to deviate from this linearity, except for the eye lens membranes. Apart from this deviation, no clear break points, indicative of lipid phase transitions, can be observed in any of the membranes. Fig. 4 shows clearly that the slope of the curve for a given type of membrane is inversely related to the S_{DPH} of that membrane at any temperature.

Concluding remarks

One may criticize the use of the term 'lipid fluidity' (a dynamic concept) with the connotation of structural order [15]. However, the structural order of membrane lipids is related to the degree of molecular packing and this suggests its inverse relationship to the lateral diffusion of membrane lipids and proteins, possible interactions with the cytoskeleton not taking into account. Therefore, the heuristic term 'lipid fluidity', used throughout the literature, seems justified and may in light of the present paper conveniently be defined as the reci-

procal of the lipid structural order parameter (S_{DPH}), rather than of the 'microviscosity'. The 'microviscosity' concept [1–3] should be abandoned because it only considers the rate of rotational reorientation, and erroneously neglects the major contribution to the steady-state fluorescence anisotropy (r_s), i.e. the limiting long-time fluorescence anisotropy (r_∞), determined exclusively by the membrane structural order.

In this paper we have shown how the order parameter, S_{DPH} in biological membranes can be quantitatively estimated from steady-state fluorescence anisotropy data, by making use of an empirically and a theoretically founded relationship between r_s and r_∞. It would be very useful to extend this approach to other, more polar fluorescent probes, such as *trans*-parinaric acid or the (9-anthroyloxy) fatty acids, which have a confined localization in the membrane bilayer. However, the time-resolved fluorescence anisotropy decay data for these probes, which are needed for such an approach, are not available as yet.

We have illustrated that S_{DPH} in biomembranes is mainly determined by the cholesterol- and sphingomyelin-phospholipid molar ratios, the temperature and, occasionally, the membrane intrinsic proteins.

Finally, it should be realized that these measurements in unfractionated membrane preparations only yield weighted-average order parameters, i.e. they give no resolution into particular lipid domains, which may exist in a biological membrane.

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