Biophysical Chemistry 15 (1982) 73–86
Elsevier Biomedical Press

STEADY-STATE FLUORESCENCE POLARIZATION IN PLANAR LIPID MEMBRANES

EXPERIMENTAL AND THEORETICAL ANALYSIS OF THE FLUOROPHORES 8-ANILINO-1-NAPHTHALENESULFONATE, 1,6-DIPHENYL-1,3,5-HEXATRIENE, DANSYLLYSINE-VALINOMYCIN AND *n*-(9-ANTHROYLOXY) FATTY ACIDS

E. FREHLAND, R. KREIKENBOHM and W.G. POHL *

University of Konstanz, Fakultäten für Physik und Biologie, Postfach 5560, D-7750 Konstanz, F.R.G.

Received 13th May 1981
Revised manuscript received 17th November 1981

Key words: Fluorescence polarization; Fluorophore; Order parameter; Planar membrane; Fluorophore orientation; (Hindered rotation)

In continuation of earlier work, the steady-state fluorescence polarization in a globally oriented system of planar lipid membranes was analyzed experimentally and theoretically for the fluorophores 8-anilino-1-naphthalenesulfonate, 1,6-diphenyl-1,3,5-hexatriene, dansyllysine-valinomycin and *n*-(9-anthroyloxy) fatty acids. The theoretical analyses of experiments were mainly done in terms of the mean orientation of transition moments with respect to the membrane normal, an angle describing the region of hindered rotational diffusion and the coefficients of rotational diffusion perpendicular to the membrane and around the membrane normal. The nonvanishing angle between the moments of absorption and emission was taken into account. In the case of *n*-(9-anthroyloxy) fatty acids it was found that the orientational disorder increases significantly with the depth of the fluorophore within the membrane. In order to compare with recent results from time-dependent fluorescent polarization in globally isotropic membrane suspensions and with ^{2}H-NMR experiments, the second moment ('order parameter') of the steady-state orientational distribution of absorption dipoles was calculated. For all fluorophores the theoretical analysis indicates a preferred orientation of absorption moments within the membrane plane.

1. Introduction

In recent years, the so-called nanosecond fluorescence polarization technique has been used to analyze the orientation and mobility of fluorescent molecules in membranes [1–7]. The probes have been membrane suspensions, i.e., globally isotropic systems, however, locally the fluorescent molecule may be oriented with respect to the local membrane plane. Because of the increase in information obtainable from time-dependent fluorescence polarization these experiments must be regarded as great progress compared with the usual measurements of the steady-state anisotropy or degree of polarization with isotropic membrane suspensions. Similar progress has been achieved with differential phase fluorometry, a technique where the difference in lifetime between the parallel and perpendicular components of the emitted fluorescent light is measured and the sample is excited by polarized sinusoidally modulated light [8–11].

The results of steady-state experiments, containing only one independent piece of information, the fluorescence anisotropy or degree of polarization, have usually been interpreted in terms of a 'microviscosity' on the basis of a locally isotropic distribution of fluorophores (e.g., see ref. 14).

* Permanent address: Höhere Technische Bundeslehranstalt II, A-4040 Linz, Paul Hahnstr. 4, F.R.G.
Abbreviation: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

0301-4622/82/0000–0000/$02.75

However, it can be shown by simple theoretical arguments that the concept of 'microviscosity' must be regarded very sceptically because of the strong influence of local orientations of fluorophores on the steady-state fluorescence polarization [15]. Indeed, a significant result of the nanosecond experiments with the fluorescent probe 1,6-diphenyl-1,3,5-hexatriene has been the evidence that the motion of the fluorescent molecules is restricted and their anisotropic orientational distribution yields an essential contribution to the nonvanishing steady-state polarization.

In this connection, a very interesting theoretical result of Kinosita et al. [16] is that in the case of parallel transition moments of absorption and emission, the ratio of the values $r(\infty)$ and $r(0)$ of the time-dependent fluorescence anisotropy $r(t)$ depends only on the second moment of the stationary orientation distribution of the transition moments.

In ref. 16 this ratio is called the 'degree of orientational constraint', while in an earlier paper [3] the same authors remarked that it corresponds to the 'order parameter' commonly used in spin-label studies.

In two recent papers [17,18], a correlation was shown between the order parameter of the local orientation distribution of diphenylhexatriene, derived from the reported fluorescence polarization experiment, and the order parameter of lipids as determined by ^{2}H-NMR [19].

In contrast to steady-state experiments with globally isotropic membrane suspensions, it can clearly be shown by theoretical arguments that steady-state fluorescence polarization with globally oriented systems may yield an essentially greater amount of independent information about local orientation and mobility of fluorescent molecules [13]. Naturally, if one wishes to utilize this increase in the information obtainable one has to apply a theoretical analysis of increasing complexity [20,21]. It is not likely that general theoretical results (independent of a special model assumption), e.g., from the orientation distribution of transition moments similar to those of Kinosita et al. [16], for global isotropy are obtainable in global anisotropic systems apart from very special cases. In the special case of parallel transition moments of emission and absorption (i.e., fixed fluorescent molecules with parallel moments within the molecules), the steady-state fluorescence polarization experiment contains the information about the orientation distribution up to the fourth (hexadecapole) moments [13].

The experimental applications of steady-state fluorescence polarization to globally oriented biological membrane systems [22–28] have not been completely successful, mainly because of the lack of a satisfactory theoretical foundation for the analysis. A main point in this connection is the principal difficulty that the fitting of a very special theoretical model (concerning orientation and mobility properties) to experimental polarized intensities is rather problematic. In these cases the uniqueness of the fits cannot be estimated.

Because of these principal difficulties, some years ago we started to develop a systematic procedure for the theoretical analysis of the static polarized fluorescence experiment with planar lipid membranes (global axial symmetry) [20,21]. We calculated numerically the polarized intensities for a number of different model distributions, which on the one hand are as simple as possible but on the other may be expected to be sufficient for the analysis of a wide class of real cases. Then the analysis of measured polarized intensities for a special fluorescent probe has to be done by careful comparison with the corresponding theoretical polarized intensities for the different models. In this way one might expect to obtain information about the real behavior of the fluorescent molecules in the membranes.

Because in most of the fluorescent probes used in our experiments the angle Γ between the transition moments of emission and absorption within the molecules is about 30° and cannot be neglected, we extended the earlier model calculations in order to include this situation. The basis of the numerical calculations was the general formula [21] including the cases of rotational diffusion around the normal to the membrane and 'oscillatory diffusion' ('hindered rotation', 'wobbling diffusion') perpendicular to the membrane around a mean orientation.

The steady-state fluorescence polarization in planar lipid membranes has been measured and

analyzed for a number of fluorescent probes: 8-anilino-1-naphthalenesulfonate, 1,6-diphenyl-1,3,5-hexatriene, dansyllysine-valinomycin and *n*-(9-anthroyloxy) fatty acids ($n = 2, 6, 9, 12$ and 16).

With the *n*-(9-anthroyloxy) fatty acids we showed that the orientation and mobility of the fluorophores depend significantly on the position on the acyl chain.

With increasing depth of the position of fluorophores within the membrane, the orientational order decreases, a finding that is in qualitative agreement with the results of Sawyer and coworkers and ^{2}H-NMR experiments.

In section 5 an expression (eq. 23) for the second moment ('order parameter') of stationary orientational distribution of absorption dipoles as a function of the measured fluorescence polarization is derived. For all fluorophores used the order parameter tends to be negative, indicating a preferred orientation of absorption dipoles within the membrane plane. However, in the case of diphenylhexatriene, the order parameter is close to zero, thus indicating a nearly isotropic orientational distribution with respect to the membrane. This finding is in agreement with other results for diphenylhexatriene in membranes above the transition temperature.

Although a comparison with more complex biological membranes is problematic, we believe that steady-state fluorescence experiments with well defined oriented model membranes are a useful tool in the investigation of membrane structure, order, and dynamic and orientational behavior of macromolecules in membranes. Further progress could be made from time-dependent fluorescence polarization in globally oriented model membranes.

2. Theory

In this section we give a short description of the theoretical foundations as far as necessary for the following analysis. Further details may be found in previous papers [13,20,21].

Throughout this paper we make the assumptions that: (i) interactions between the fluorescent molecules can be neglected; (ii) their dynamic behavior is the same in the ground and excited state; (iii) the lifetime of fluorescence is independent of the orientation of the molecule; and (iv) the angle Γ between the moments of absorption and emission is fixed within the molecule.

The geometry of the experimental arrangement used is shown in fig. 1. The more general case where the planes of incidence and of measurement are not identical is applicable by a slight modification [21]. The plane of incidence is formed by the light beam and the normal $\vec{n}$ to the membrane.

The following angles, used in the theoretical analysis, are introduced:

α, angle between the incident light beam and the normal $\vec{n}$;

β, angle between $\vec{n}$ and the direction from the fluorescent sample to the analyzer (in our case $\alpha = \beta = 45°$);

θ, angle between the electric vector of the linearly polarized incident light and the plane of incidence;

ϵ, polar angle with respect to $\vec{n}$ as polar axis;

ϕ, azimuthal angle (axial symmetry of a distribution means independence of ϕ);

ρ, angle defining the direction of polarization of the analyzer;

ψ, angle between the direction of polarization

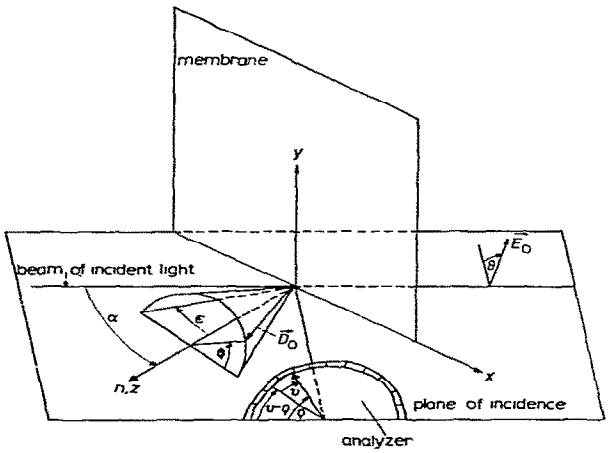

Fig. 1. Geometry of the fluorescence polarization experiment with planar membranes. The normalized vector $\vec{E}_0$ denotes the direction of polarization of exciting light. The normalized vector $\vec{D}_0$ indicates the direction of a transition moment on a cone of constant t (from ref. 20).

(electric vector) of incident light and a moment of absorption described by ϵ;

$\bar{\psi}$, angle between a moment of emission and the polarization direction of the analyzer;

μ, angle between a moment of emission and the plane of the analyzer;

ν, angle between the projection of the emission moment to the plane of the analyzer and the direction of polarization of the analyzer given by ρ.

Obviously, for the angle $\bar{\psi}$, the following expression holds

$$\cos^2\bar{\psi}=\cos^2\mu\cos^2\nu \tag{1}$$

ψ, μ and ν depend on the other angles α, β, θ, ϵ, ϕ and ρ in the following way [20]:

$$\cos\psi = -\sin\epsilon\cos\theta\cos\alpha\cos\phi+\sin\epsilon\sin\theta\sin\phi$$
$$=\cos\epsilon\cos\theta\sin\alpha \tag{2}$$

$$\sin\mu=\cos\beta\sin\epsilon\cos\phi+\sin\beta\cos\epsilon \tag{3}$$

$$\cot(\rho-\nu)=\frac{(\cos\beta\cos\epsilon-\sin\epsilon\cos\phi\sin\beta)}{\sin\epsilon\sin\phi} \tag{4}$$

The whole process taking place in the steady-state polarized fluorescence experiment may be schematically divided into absorption, rearrangement of the moments, emission and measurement. The absorption is proportional to $\cos^2\psi$ and the stationary orientational distribution of absorption moments.

From the absorption a steady-state intensity of the emission function $I_E(\epsilon,\phi)$ may be determined, taking into account the different processes causing a nonparallelism of moments of absorption and emission, e.g., rotatory and oscillatory diffusion during the lifetime of the excited state or a nonzero angle Γ between the moments. The measured polarized fluorescence intensity I_M for given polarization direction of polarizer and analyzer is in an arbitrary normalization [20,21] given by integration of I_E over all orientations

$$I_M=\int_{\phi=0}^{2\pi}\int_{\epsilon=0}^{\pi} I_E\cos^2\bar{\psi}\,\sin\epsilon\,d\epsilon\,d\phi \tag{5}$$

In ref. 21 we calculated the intensity of emission function for the case $\Gamma=0$ for different dynamic model situations. In the most general case we included a two-dimensional rotatory motion of the fluorophores: rotational diffusion in ϕ around the membrane normal $\vec{n}$ and oscillatory diffusion (hindered rotation) in ϵ, i.e., perpendicular to the membrane within an interval $[\epsilon_0-\Delta\epsilon,\epsilon_0+\Delta\epsilon]$ around a mean position ϵ_0. The lengthy result, which can be evaluated through eq. 5 only numerically, was

$$I_E=\frac{1}{\sin\epsilon}\frac{1}{2\pi}\left\{\frac{(1+\bar{B}^2)}{8\Delta\epsilon}-\frac{(1-3\bar{B}^2)}{8\Delta\epsilon}\cos(\epsilon_1+\epsilon_2)\frac{\sin 2\Delta\epsilon}{2\Delta\epsilon}\right.$$
$$-\left(\frac{1}{1+D_\phi\tau}\right)\frac{\bar{A}\bar{B}}{4\Delta\epsilon^2}\sin(\epsilon_1+\epsilon_2)\frac{\sin 2\Delta\epsilon}{2\Delta\epsilon}+\left(\frac{1}{1+4D_\phi\tau}\right)$$
$$\times\left(\frac{2\bar{A}^2+\bar{B}^2-1}{8\Delta\epsilon}\right)\left(1-\cos(\epsilon_1+\epsilon_2)\frac{\sin 2\Delta\epsilon}{2\Delta\epsilon}\right)$$
$$+\frac{2}{\pi^2}(1-3\bar{B}^2)\left[-\sin(\epsilon_1+\epsilon_2)\cos 2\Delta\epsilon\sum_{\nu=1}^{\infty}M_{2\nu-1}\frac{1}{\alpha_{2\nu-1}^2}\right.$$
$$\left.+\cos(\epsilon_1+\epsilon_2)\sin 2\Delta\epsilon\sum_{\nu=1}^{\infty}M_{2\nu}\frac{1}{\alpha_{2\nu}^2}\right]$$
$$+\frac{8}{\pi^2}\bar{A}\bar{B}\left[\cos(\epsilon_1+\epsilon_2)\cos 2\Delta\epsilon\sum_{\nu=1}^{\infty}M_{2\nu-1}\left(\frac{1}{\alpha_{2\nu-1}^2+D_\phi\tau}\right)\right.$$
$$\left.+\sin(\epsilon_1+\epsilon_2)\sin 2\Delta\epsilon\sum_{\nu=1}^{\infty}M_{2\nu}\left(\frac{1}{\alpha_{2\nu}^2+D_\phi\tau}\right)\right]$$
$$+\frac{2}{\pi^2}(2\bar{A}^2+\bar{B}^2-1)\left[-\sin(\epsilon_1+\epsilon_2)\cos 2\Delta\epsilon\right.$$
$$\times\sum_{\nu=1}^{\infty}M_{2\nu-1}\left(\frac{1}{\alpha_{2\nu-1}^2+4D_\phi\tau}\right)$$
$$\left.\left.+\cos(\epsilon_1+\epsilon_2)\sin 2\Delta\epsilon\sum_{\nu=1}^{\infty}M_{2\nu}\left(\frac{1}{\alpha_{2\nu}^2+4D_\phi\tau}\right)\right]\right\} \tag{6}$$

With the abbreviations

$$\bar{A}=\sin\theta\sin\phi-\cos\theta\cos\phi\cos\alpha,\quad \bar{B}=\cos\theta\sin\alpha \tag{7}$$

and

$$M_\nu=\cos\left[\nu\pi\left(\frac{\epsilon-\epsilon_1}{2\Delta\epsilon}\right)\right]\left(\frac{1}{\nu^2-(16\Delta\epsilon^2/\pi^2)}\right),$$
$$\alpha_\nu^2=1+\frac{\nu^2\pi^2}{4\Delta\epsilon^2}D_\epsilon\tau \tag{8}$$

where D_ϕ and D_ϵ are the coefficients of rotational diffusion in ϕ and ϵ, respectively. The dimensionless quantities $D_\epsilon\tau$ and $D_\phi\tau$ (τ, fluorescence lifetime) determine the magnitude of the influence of rotational diffusion on the steady-state polarization.

In eq. 6 different limiting cases are included

which correspond to different model situations, similar to our first numerical model calculations in ref. 20. For example, $D_\phi\tau = 0$ ($D_\epsilon\tau = 0$) means that during the lifetime of the excited state no rearrangement takes place, while $D_\phi\tau \to \infty$ ($D_\epsilon\tau \to 0$) means a complete rearrangement in ϕ or $[\epsilon_0 - \Delta\epsilon, \epsilon_0 + \Delta\epsilon]$.

Although eq. 6 is rather lengthy, it simplifies the numerical calculation in comparison with our previous calculations [20] because only the integration over the intensity of the emission function has to be performed. Furthermore, the cases $D_\phi\tau \approx 1$ and $D_\epsilon\tau \approx 1$, where one or both rotational relaxation times (in ϵ or ϕ) are comparable to the lifetime of fluorescence, can be treated and included for the interpretation of the experiments.

In most of the available fluorescent probes the nonzero angle Γ between the transition moments of absorption and emission cannot be neglected. This can be taken into account by using the intensity of the emission function and replacing in eq. 5 $\cos^2\bar{\psi}(\epsilon, \phi)$ by $\cos^2\bar{\psi}(\epsilon + \Gamma_\epsilon, \phi + \Gamma_\phi)$ in those cases where Γ leads to a displacement of the transition moment with fixed values Γ_ϵ and Γ_ϕ in ϵ and ϕ, respectively. We emphasize that this procedure, as used in our model calculations, must be regarded as a first approximation. In general, the problem of the nonvanishing angle Γ in globally oriented systems cannot be treated in such an elegant way as has been done by Perrin [12] for macromolecules in isotropic solutions.

Apart from the described method of theoretical analysis a calculation of the second moments of the stationary orientational distribution of absorption dipoles with respect to the membrane plane is done in section 5.

3. Materials and methods

3.1. Model membranes and probes

Black lipid membranes were formed from diacyl-L-α-phosphatidylcholines. Both dierucoyl- and diphytanoyl phosphatidylcholine, used throughout this study, were synthesized by K. Janko in our laboratory [32]. 1,2-Dipalmitoyl-*sn*-glycero-3-phosphorylcholine was used for vesicle preparations. 8-Anilino-1-naphthalenesulfonate was obtained from Serva as the magnesium salt. Dansyl-lysine-valinomycin was a gift from Dr. G. Stark [33]. The *n*-(9-anthroyloxy) fatty acids were a gift from Dr. W.H. Sawyer, Melbourne [29–31]. 2-(9-Anthroyloxy)stearic acid, 12-(9-anthroyloxy)stearic acid, and 16-(9-anthroyloxy)palmitic acid were obtained also from Molecular Probes, Plano, U.S.A. 1,6-Diphenyl-1,3,5-hexatriene was a product from Fluka. The electrolyte solution was either 0.1 M NaCl (Merck), pH $\approx$ 6, or 0.1 M NaCl and 5 mM Hepes (Sigma) buffered with NaOH (Merck) at pH 7.0 (table 2).

Membranes were formed, following the method of Mueller et al. [34], over a circular hole in the wall of a black Teflon cuvette. Its area was approx. 20 mm^2. The membrane-forming solution contained 1% (w/v) phospholipid in *n*-decane. The membranes reached the black state within 1 h or less.

8-Anilino-1-naphthalenesulfonate was added to the aqueous phase whereas all the other probes were dissolved in the membrane-forming solution. Vesicles were prepared following the method of Huang [35]. For separation of vesicles and unbound dye the vesicle solution was chromatographed on Sephadex G-50 in a column of 23 cm length and 1 cm inner diameter [36].

All absorption spectra of vesicle suspensions and dye solutions were measured with a Zeiss DMR-10 recording spectrophotometer. Fluorescence spectra were recorded with a Hitachi-Perkin-Elmer MPF-4 fluorometer.

3.2. Membrane fluorometer

Polarized fluorescence of thin lipid membranes and of vesicle suspensions was measured with the membrane fluorometer designed by Pohl [33].

Inserting two polarizers, one after the filters used for excitation and the other after the filters for emission, we were able to record the angle-dependent fluorescence values at about 25 positions. The illuminated spot in the middle of the membrane was about 7 ± 2 mm^2 so that the influence of the torus could be neglected. The filters for excitation and emission used for the probes examined in this paper are given in table 1.

Table 1

Optical properties of the filters used for the different fluorophores

ANS, 8-anilino-1-naphthalenesulfonate; Dns-val, dansyllysine-valinomycin; DPH, diphenylhexatriene; AS, (9-anthroyloxy)stearic acid; AP, (9-anthroyloxy)palmitic acid.

Probe	Excitation filters	Wavelength of maximum (nm)	Maximum transmission (%)	Half-width (nm)	Emission filters	Wavelength of maximum (nm)	Maximum transmission (%)	Half-width (nm)
ANS	UG 11/6	333	57	60	KV 450 NAL 447	473	55	50
Dns-val	WG 305/3 UG 11/6	335	53	60	KV 399 KV 470 NAL 520	515	41	45
2-AS, 6-AS, 9-AS, 12-AS, 16-AP	WG 280 UG 1	361	54	60	KV 389 KV 399 NAL 451	447	52	60
DPH	UG 2	359	59	60	KV 418 NAL 429	433	51	45

4. Results

4.1. Experiments

For the analysis of the recorded intensities we used the following degrees of polarization defined by Frehland and Trissl [20]

$$P_\rho = \frac{I_M(\rho=0°) - I_M(\rho=90°)}{I_M(\rho=0°) + I_M(\rho=90°)} \tag{9}$$

where ρ gives the position of the analyzer. P_ρ was determined for two angles between the electric vector of the linearly polarized incident light and the plane of incidence ($\theta = 0°$ and $\theta = 90°$).

Furthermore, we calculated P_t, defined by

$$P_t = \frac{I_t(\theta=0°) - I_t(\theta=90°)}{I_t(\theta=0°) + I_t(\theta=90°)} \tag{10}$$

where I_t is given by integration of I_M over ρ.

$$I_t = \frac{1}{\pi}\int_0^\pi I_M \, d\rho \tag{11}$$

For all probes, within the experimental error for $\theta = 0°$ and $\theta = 90°$, the measured intensities $I_M(\rho)$ were found to be symmetrical according to

$$I_M(\rho) = I_M(\pi - \rho)$$

$$I_M(\rho) + I_M\left(\tfrac{1}{2}\pi - \rho\right) = I_M(0°) + I_M\left(\tfrac{1}{2}\pi\right) \tag{12}$$

for $0 \leq \rho \leq \frac{1}{2}\pi$, I_t is simply

$$I_t = I_M(0°) + I_M(90°) \tag{13}$$

Additionally, the angle ρ_{min} of minimum fluorescence intensity at $\theta = 45°$ was determined to yield more information about the mobility of the probes (cf. the arguments in ref. 20). All signals from the membrane-bound probes were corrected by the signals obtained after breaking the membrane. These values served as a rough estimate of background intensity of the unbound dye during the membrane experiment. It was also checked that the scattering from the membrane without probe was negligible. All parameters used for the theoretical interpretation are listed in table 2. It also contains the ratio of dye to lipid (D/L) in the membrane-forming solution or the concentration of the dye in the aqueous phase at the beginning of the experiment, respectively. The relatively high dye concentration was necessary for intensity reasons. Membranes were formed from dierucoylphosphatidylcholine in all cases except for measurements with dansyllysine-valinomycin and diphenylhexatriene where diphytanoylphosphatidylcholine was used.

Although the thinning of the membrane had finished 1 h before the polarization measurement started, there might still have been an amount of solvent left [42,43]. Therefore, the possibility of formation of lenses cannot be excluded.

For the interpretation of the data presented we

Table 2

The parameters $P(\theta = 90°)$, $P(\theta = 0°)$, P_t and ρ_{min} resulting from the measurement of fluorescence polarization of different probes in planar lipid membranes
Abbreviations as in table I.

Dye	Concentration	Conditions	$P(\theta = 90°)$	$P(\theta = 0°)$	P_t	ρ_{min} (°)	Number of experiments
ANS	$c_{aq} = 2 \times 10^{-6}$ M	0.1 M NaCl	−0.333±0.022	−0.123±0.025	−0.222±0.010	173±4	3
Dns-val	D/L = 1:40	0.1 M NaCl	−0.230±0.037	−0.079±0.022	−0.112±0.009	178±7	4
2-AS	D/L = 1:50	0.1 M NaCl, 5 mM Hepes, pH 7	−0.305±0.010	−0.154±0.011	−0.218±0.031	176±5	4
6-AS	D/L = 1:50	0.1 M NaCl, 5 mM Hepes, pH 7	−0.260±0.035	−0.086±0.020	−0.229±0.032	170±5	5
9-AS	D/L = 1:50	0.1 M NaCl, 5 mM Hepes, pH 7	−0.208±0.019	−0.043±0.015	−0.186±0.022	178±5	4
12-AS	D/L = 1:50	0.1 M NaCl, 5 mM Hepes, pH 7	−0.154±0.018	−0.033±0.012	−0.174±0.015	174±5	4
16-AP	D/L = 1:50	0.1 M NaCl, 5 mM Hepes, pH 7	−0.135±0.046	−0.027±0.016	−0.128±0.025	177±5	6
DPH	D/L = 1:50	0.1 M NaCl, 5 mM Hepes, pH 7	−0.155±0.013	−0.036±0.016	−0.099±0.022	168±8	4

took into account the nonzero angle Γ between the emission and the absorption moments: For 8-anilino-1-naphthalenesulfonate Γ was determined in two ways. First, a solution of 8-anilino-1-naphthalenesulfonate marked DPL-vesicles was cooled down to a temperature far below that of the transition point. In this temperature region, the probe was assumed to be rigidly held during the lifetime of the excited state, and the degree of polarization P

$$P=\frac{I_{\parallel}-I_{\perp}}{I_{\parallel}+I_{\perp}} \tag{14}$$

should take the characteristic value P_0.

From P_0 one can calculate Γ according to [12]

$$P_0=\frac{3\cos^2\Gamma-1}{\cos^2\Gamma+3} \tag{15}$$

The other approach was to cool down isotropic glycerol/water solutions with several concentrations of the dye, measuring the degree of polarization P as a function of temperature T and viscosity η. By plotting the reciprocal of the degree of polarization against the coefficient of temperature and viscosity we determined P_0 according to the well known equation [12]

$$\frac{1}{P}-\frac{1}{3}=\left(\frac{1}{P_0}-\frac{1}{3}\right)\left(1+\frac{KT\tau}{V\eta}\right)$$

For dansyllysine-valinomycin we conducted a vesicle experiment like that described above [38]. In this way a value of $\Gamma = 33°$ was determined. In the case of the *n*-(9-anthroyloxy) fatty acids, published values [29–31] of 30° were taken. For diphenylhexatriene we used $\Gamma = 10°$ as resulting from the limiting polarization P_0 [3,11] accordings to refs. 4 and 5.

Some remarks concerning experimental difficulties with diphenylhexatriene should be made: As shown by Mason and Cehelnik [39], diphenylhexatriene photolyzed in a variety of solvents, similar to those we used as membrane-forming solutions. In agreement with their findings, we observed a loss of fluorescence intensity during our experiments. By measuring all intensities $I_M(\rho,\theta)$ twice we minimized the effect of this loss from about 25 to 10%. Therefore, the position of minimum intensity ρ_{min} is only a rough estimate of the real value and our fit of the experimental data is based only on the degree of polarization.

4.2. Theoretical analysis

The theoretical analysis of the experimental results listed in table 2 was done by numerical evaluation of eq. 5 with the use of the general expression (eq. 6) for the intensity of emission function and taking into account the nonvanishing angle Γ between the moments of absorption and emission as described in section 2. From the intensity function $I_M(\rho,\theta)$ the parameters $P(\theta=0°)$, $P(\theta=90°)$, P_t and ρ_{min} were calculated. The fitting procedure of the theoretical values of these quantities with the experimental ones was done by variation of the model parameters ϵ_0, $\Delta\epsilon$, $D_\phi\tau$ and $D_t\tau$.

Table 3

The parameters ϵ_0, $\Delta\epsilon$, $D_\phi\tau$, $D_t\tau$ fitted to experimental parameters $P(\theta=90°)$, $P(\theta=0°)$, P_t and ρ_{min} in the table for the different fluorescent probes

Abbreviations as in table 1.

	ϵ_0 (°)	$\Delta\epsilon$ (°)	$D_\phi\tau$	$D_t\tau$
ANS	73.0±1.2	19.3±4.6	1.12±0.28	>0.05
Dns-val	69.8±1.2	40.0±2.0	1.24±0.40	>1.50
2-AS	73.1±1.2	22.0±2.6	1.48±0.21	>0.11
6-AS	77.6±2.6	27.7±9.3	1.20±0.28	>0.28
9-AS	78.7±2.2	39.4±3.4	0.90±0.18	>0.85
12-AS	81.0±1.9	43.0±2.7	1.14±0.16	>1.10
16-AP	78.9±2.6	49.3±4.2	1.05±0.28	>1.05
DPH	68.5±1.4	55.5±2.1	1.3 ±0.4	>0.15

The results of this procedure are listed in table 3. All fits for those problems with $\Gamma \approx 30°$ could be done with $\Gamma_\epsilon = +30°$, $\Gamma_\phi = 0°$ (cf. the remarks in section 2). $\Gamma_\phi \neq 0$ resulted in poorer fits. This consideration of the angle Γ between the moments of absorption and emission must be regarded as a first rough approximation.

The following important remarks concerning the results listed in table 3 must be made:

(a) From simple symmetry considerations, regarding the axial symmetry of the fluorescent sample it was shown that each set of parameters ϵ_0, $\Delta\epsilon$, $D_\phi\tau$, $D_\epsilon\tau$ cannot be distinguished from the equivalent set $180° - \epsilon_0$, $\Delta\epsilon$, $D_\phi\tau$, $D_\epsilon\tau$ if one replaces Γ_ϵ by $-\Gamma_\epsilon$. It is not possible to decide which set describes the actual situation. For our results, this fact is of minor importance because for approx. 75° replacement of ϵ_0 by $180° - \epsilon_0$ and Γ_ϵ by $-\Gamma_\epsilon$ leads only to an exchange of the absorption and emission moments preserving the main orientation of both moments approximately parallel to the membrane. In the discussion of the results in section 6 we will not mention this point further.

(b) The errors given in table 3 were determined by slight variation of the parameters ϵ_0, $\Delta\epsilon$, $D_\phi\tau$, $D_\epsilon\tau$ around the fit resulting in a variation of the experimental parameters P_ρ, P_t, ρ_{min}, respectively, which should not be greater than the experimental errors. Naturally, these errors in the theoretical analysis must not be regarded as a strong measure for the possible deviations of the theoretical fit from the actual situation: First, there may be systematic experimental errors, which cannot be estimated and second, it is clear that the theoretical models used are a simplification and idealization of the real situation (cf. the remarks in ref. 20).

(c) The uniqueness of the theoretical fits cannot be proven. Nevertheless, a variation of the model parameters, especially the mean orientation ϵ_0, and comparison with the extensive numerical results presented previously [20] for $\Gamma = 0$ showed that there is no evidence for another set of model parameters fitting equally well.

In figs. 2 and 3, for the two examples, 8-anilino-1-naphthalenesulfonate and 6-(9-anthroyloxy)-stearic acid, the agreement between the measured polarized intensities $I_M(\rho, \theta)$ and the theoretical fits is demonstrated.

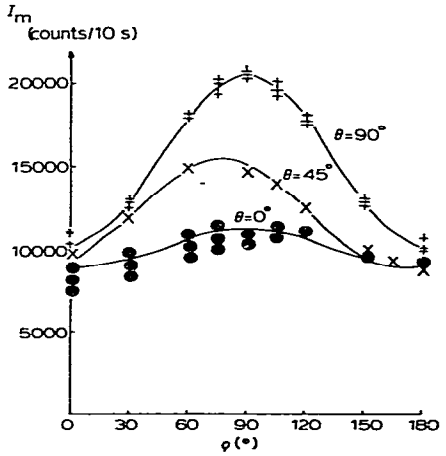

Fig. 2. Measured polarized fluorescence intensities $I_M(\theta, \rho)$ for the fluorophore 8-anilino-1-naphthalenesulfonate and theoretical fit (full lines) according to table 3.

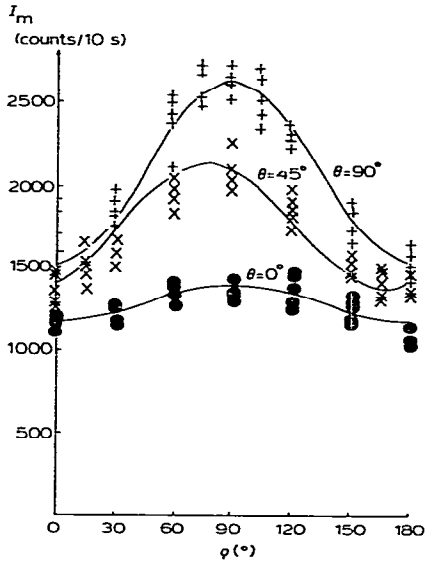

Fig. 3. Measured polarized fluorescence intensities $I_M(\theta, \rho)$ for the fluorophore 6-(9-anthroyloxy)stearic acid and theoretical fit (full lines) according to table 3.

5. The order parameter

The information obtained from the steady-state fluorescence polarization experiments with the described method of theoretical analysis is essentially contained in the mean orientation ϵ_0, the regime of hindered rotation $\Delta\epsilon$ around ϵ_0 and the dimensionless quantities $D_\delta\tau$ and $D_\epsilon\tau$ as a measure for the rotational mobilities within and perpendicular to the membrane plane, respectively.

As reported in section 1, a result of recent theoretical investigations [16] on time-dependent fluorescence polarization with isotropic membrane suspensions has been that under special assumptions (e.g., parallel transition moments of absorption and emission) the second moment (quadrupole moment) of the orientation distribution of absorption dipoles is given by the ratio $r(\infty)/r(0)$.

It has been argued by Heyn [18] and Jähnig [17] that for experiments with diphenylhexatriene, a correlation exists between the second moment or 'order parameter' of the local orientation distribution of the moments and the order parameter of lipids derived from ^{2}H-NMR [19].

In a paper concerning the general theory of the steady-state polarized fluorescence experiment [13], it was shown that this experiment quite generally, apart from information about reorientation (mobility) and higher (fourth) moments, yields information about the second (quadrupole) moments of the global stationary orientation distribution of absorption moments.

Thus, in our case where the global and local (with respect to the membrane plane) orientation distributions are identical, the derivation of the second moments of absorption dipoles should be possible without further assumptions and conditions such as parallel absorption and emission moments.

We now briefly outline how an expression for the dependence of the second moment on the experimental parameters $P_\rho(\theta=0°)$, $P_\rho(\theta=90°)$ and P_t can be derived. For further details of the theoretical background we refer to ref. 13.

In cartesian coordinates, the measured polarized fluorescence intensity I_M for a direction of polarization of the analyzer given by the normalized vector $\boldsymbol{M}$ with components $M_a(M_x, M_y, M_z)$ and a direction of polarization of the exciting light correspondingly given by the normalized vector $\boldsymbol{E}$ is quite generally determined by the fluorescence tensor $\boldsymbol{F}$ of fourth order with at most 36 independent components F_{abcd} through the relationship

$$I_M = \sum_{a,b,c,d=1}^{3} M_a M_b F_{abcd} E_c E_d \tag{16}$$

On the other hand, in the general steady-state polarized absorption experiment, the absorbed intensity A for an arbitrary direction $\boldsymbol{E}$ of excitation is completely determined by the second-order tensor $\boldsymbol{A}$ of absorption with components A_{ab}:

$$A = \sum_{a,b=1}^{3} A_{ab} E_a E_b \tag{17}$$

If F_A is the stationary orientation distribution function of absorption dipoles, normalized to unity by integration over the unit sphere

$$\int F_A \, d\Omega = 1, \tag{18}$$

then A_{ab} is given by F_A through

$$A_{ab} = P_A \int F_A D_a D_b \, d\Omega, \quad A_{ab} = A_{ba} \tag{19}$$

$$P_A = \sum_{c=1}^{3} A_{cc}$$

where D_a and D_b are components of a normalized vector $\boldsymbol{D}$ in cartesian coordinates.

The factor P_A depends on different quantities, e.g., the intensity of incident light, concentration of fluorophores and apparatus constants.

The tensor of second (quadrupole) moments $\boldsymbol{Q}^A$ of the absorption dipole distribution is contained in $\boldsymbol{A}$:

$$2Q^A_{ab} = \frac{(3A_{ab} - \delta_{ab} P_A)}{P_A},$$

$$\delta_{ab} = \begin{cases} 1 & \text{for } a = b \\ 0 & \text{for } a \neq b \end{cases} \tag{20}$$

On the other hand, $\boldsymbol{A}$ may be derived from the fluorescence tensor $\boldsymbol{F}$ according to

$$A_{cd} = \sum_{a=1}^{3} F_{aacd} \tag{21}$$

In our case of axial symmetry with a known axis of symmetry ($\epsilon = 0$), F_A is independent of ϕ and the situation may be drastically simplified, e.g., if the normal to the membrane is chosen

parallel to the z-axis of the cartesian coordinates. Then the second moment $\langle P_2^A \rangle$ of the absorption dipole distribution is

$$\langle P_2^A \rangle = Q_{zz}^A = \frac{\left(\frac{3}{2}A_{zz} - \frac{1}{2}\sum_{\alpha=1}^{3} A_{\alpha\alpha}\right)}{\sum_{\alpha=1}^{3} A_{\alpha\alpha}} \qquad (22)$$

Bearing in mind that the cartesian coordinates are the principal axes of A, A is in diagonal form and $A_{xx} = A_{yy}$. With further use of the condition of axial symmetry and of eq. 13 one obtains by lengthy but elementary geometric considerations an approximate representation of P_2^A by four intensities $I_M(\rho, \theta)$.

$$\langle P_2^A \rangle = [2I_M(90°, 0°) + 4I_M(0°, 0°) - 2I_M(90°, 90°) - 4I_M(0°, 90°)] / [2I_M(90°, 0°) + 4I_M(0°, 0°) + I_M(90°, 90°) + 2I_M(0°, 90°)] \qquad (23)$$

Eq. 23 is valid only approximately, because in an exact treatment of eq. 21 the nondiagonal component F_{xzxz} of the fluorescence tension still occurs, if one wants to represent P_2^A by the four intensities $I_M(90°, 0°)$, $I_M(0°, 0°)$, $I_M(90°, 90°)$, $I_M(0°, 90°)$. However, an estimation of F_{xzxz} by a closer inspection of the experimental data in table 2 shows that in all cases the error in eq. 23 is close to zero. A publication, where the model-independent analysis of fluorescence polarization with planar membranes based on the fluorescence tensor is extensively discussed including its nondiagonal components, is in preparation.

The intensities $I_M(\rho, \theta)$ in eq. 23 may be replaced by the parameters $P(\theta = 90°)$, $P(\theta = 0°)$, P_t (cf. eqs. 11, 12 and table 2) used for the theoretical analysis. The result is

$$\langle P_2^A \rangle = \frac{(\alpha - \beta)}{\left(\alpha + \frac{\beta}{2}\right)},$$

$$\alpha = \left[\frac{(1+P_t)}{(1-P_t)}\right][3 + P(\theta = 0°)], \quad \beta = [3 - P(\theta = 90°)] \qquad (24)$$

For control of the consistency of the theoretical analysis, we can calculate the order parameter from the fitted parameters ϵ_0 and $\Delta\epsilon$ in table 3. According to the definition of $\langle P_2 \rangle$

$$\langle P_2 \rangle = \tfrac{1}{2} \int F_A (3\cos^2\epsilon - 1)\, d\Omega \qquad (25)$$

and of the model distribution, a simple integration yields the alternative model-dependent expression

$$\langle P_2^A \rangle (\epsilon_0, \Delta\epsilon) = \frac{1}{4} + \frac{3}{16\,\Delta\epsilon}[\sin 2(\epsilon_0 + \Delta\epsilon) - \sin 2(\epsilon_0 - \Delta\epsilon)] \qquad (26)$$

The results according to eqs. 24 and 26 for the different fluorophores are listed in table 4 and show good agreement between both quantities.

For most fluorophores negative values were obtained. For diphenylhexatriene the deviation from zero is not significant within the relatively large experimental error. From the definition, eq. 25

Table 4

Calculated second moments $\langle P_2^A \rangle$ of the stationary orientational distributions of the absorption dipoles for the different fluorescent probes used

The 'corrected' order parameter $\bar{S}$ was derived from $\Delta\epsilon$ and referred to a mean orientation around the membrane normal (see text). In the fourth column, for comparison, the corresponding outer parameters S_ν from ^{2}H-NMR experiments of Seelig and Seelig [19] depending on the deuterated position ν are listed. Abbreviations as in table 1.

Probe	$\langle P_2^A \rangle$	$\langle P_2^A(\epsilon_0, \Delta\epsilon) \rangle$	$\bar{S}$	S_ν (50°C)
ANS	−0.26±0.02	−0.31±0.08	≈0.9	
Dns-val	−0.12±0.03	−0.15±0.04	≈0.7	
2-AS	−0.27±0.05	−0.31±0.05	≈0.9	0.43 (C-2)
6-AS	−0.29±0.05	−0.33±0.1	≈0.85	0.41 (C-5)
9-AS	−0.22±0.05	−0.23±0.05	≈0.7	0.38 (C-9)
12-AS	−0.21±0.05	−0.22±0.03	≈0.65	0.28 (C-12)
16-AP	−0.15±0.05	−0.14±0.04	≈0.6	0.16 (C-15)
DPH	−0.1 ±0.1	−0.03±0.04	≈0.4	

follows a possible range of values for $\langle P_2 \rangle$ between +1 (complete forward orientation, i.e., $\epsilon_0 = 0$, $\Delta\epsilon = 0$) and $-1/2$ (orientation within the membrane plane, i.e., $\epsilon_0 = 90°$, $\Delta\epsilon = 0$). Positive values correspond to a preferred forward orientation of the absorption dipoles, negative values to a preferred orientation within the membrane plane. Thus, negative values indicate a preferred orientation of absorption dipoles within the membrane plane, a result which is confirmed by the obtained mean orientations ϵ_0 between 70 and 90° according to table 3. The interpretation of the results in terms of an 'order' of the lipid surrounding of the fluorophores must be done very carefully and will be a point of discussion in section 6.

6. Discussion

The discussion of our results is organized as follows: First, we present a critical interpretation mainly in terms of our model parameters ϵ_0, $\Delta\epsilon$, $D_\phi\tau$, $D_\epsilon\tau$. Second, we make a comparison with the statements about order and structure in lipid bilayers gained from the recent time-dependent fluorescence polarization experiments and from ^{2}H-NMR.

Regarding the experimental results listed in table 2, we see that the probes 8-anilino-1-naphthalenesulfonate and 2-(9-anthroyloxy)stearic acid, which may be assumed to be located in the outer parts of the membrane, show similar behavior and exhibit relatively large degrees of polarization resulting, according to table 3, in a small angle $\Delta\epsilon \approx 20°$, i.e., a sharp distribution around the mean orientation $\epsilon_0 \approx 73°$. For those probes which are assumed to be located in the interior regions of the membranes (12- and 16-(9-anthroyloxy)stearic acid, diphenylhexatriene), the degrees of polarization decrease strongly, resulting in a greater angle $\Delta\epsilon \approx 45$–$55°$, i.e., a more disoriented behavior. The results for the different *n*-(9-anthroyloxy)stearic acid probes clearly show an increasingly disoriented behavior depending on the position of the fluorophores on the fatty acid molecules. This is in agreement with the results of Tilley et al. [30] for the steady-state degree of polarization observed with multilayered liposomes (globally isotropic!) of DL-α-dipalmitoylphosphatidylcholine in the liquid-crystalline phase, which decreased by a factor of approx. 3 from the outer to the inner position of attachment of fluorophores to the acyl chain.

We obtained a similar factor for $P(\theta = 90°)$, naturally with greater absolute values because of the global anisotropy of the planar bilayer. From the experiments of Sawyer and co-workers [29–31], it is evident that the polarization gradient across the lipid bilayer cannot be explained by differences in the fluorescence lifetime of the probes. Our results clearly show that the strong differences in the orientational behavior, i.e., varying regions of hindered rotational diffusion, are decisive for the observed differences in fluorescence polarization, while the differences in the velocity of rotational motions in ϵ and ϕ, expressed by the dimensionless quantities $D_\phi\tau$ and $D_\epsilon\tau$, are less significant.

We must emphasize that our analysis of global anisotropic systems is based upon a larger amount of experimental information compared to measurements on globally isotropic membrane systems, which yield a higher degree of certainty of the theoretical conclusions. It can clearly be shown that varying degrees of polarization measured in globally isotropic membrane suspensions can always be explained by several completely different underlying mechanisms [15]. The attempts at interpretation within the concept of microviscosity [14] should be abandoned as they are misleading even in the case of diphenylhexatriene [37]. The membrane cannot be regarded as a standard solution.

Before we make a comparison with the results of time-dependent fluorescence polarization and ^{2}H-NMR concerning the order parameter, we want to make some general remarks on the use of the concept of the order parameter for fluorophores. First, all the theoretical parameters, e.g., ϵ_0, $\Delta\epsilon$ or the order parameter, are related to properties of transition moments. A conclusion on the properties of the fluorescent molecules must be based on a more or less accurate knowledge of the position of transition moments of absorption and emission for the special frequencies of excitation and emission used in the experiments. Second, the interpretation in terms of mean orientation ϵ_0 and range $\Delta\epsilon$ of hindered rotation is more appropriate and

precise. If we accept that a small $\Delta\epsilon$ corresponds to high orientational order of the fluorophores, the value of the order parameter may vary between $+1$ and $-1/2$ depending on the mean orientation ϵ_0, thus for small values, approx. 0, yielding a measure of the order of the fluorophore orientation only in connection with additional information about mean orientation. This information about higher moments (higher than the second) of the orientational distribution can be derived from steady-state polarized fluorescence in a globally oriented system as shown in ref. 13.

While the order parameters given in table 4 were derived without any model assumption and are related to the real steady state, the order parameters from time-dependent fluorescence polarization are related to an average over a time scale of approx. 10^{-8} s and that from ^{2}H-NMR to approx. 10^{-4} s. It might be interesting to try a comparison with the results for the different *n*-(9-anthroyloxy)stearic acid probes with the ^{2}H-NMR results of Seelig and Seelig [19] on liposomes for lipids deuterated at varying positions of the fatty acids. Because the planar artificial bilayers used in our experiments are in the liquid-crystalline phase, a comparison must be made with the ^{2}H-NMR experiments above the phase transition temperature.

The qualitative behavior for both methods is in agreement and shows a decreasing order from the outer to the inner parts of the membrane. The quantitative comparison is problematic because the absorption moments of the fluorophores have a preferred orientation within the membrane plane. This results in negative values of the order parameter, while the fatty acids can be assumed to have a mean orientation normal to the membrane. In order to make possible some reasonable comparisons, we have taken the fitted values for $\Delta\epsilon$ in table 3 and calculated an order parameter $\bar{S}$ for a mean orientation (with cone angle $\Delta\epsilon$) around the membrane normal according to the relationship

$$\bar{S} = \tfrac{1}{2}\cos\Delta\epsilon(1+\cos\Delta\epsilon) \qquad (27)$$

following from eq. 25. The underlying idea is that $\Delta\epsilon$ is the measure for orientational order, and if the order of fluorophore orientation is influenced by the surrounding lipids it should be related to the mean orientation of the acyl chains. The calculated values of $\bar{S}$ are listed in the third column of table 4. Comparison with the corresponding S_z values from ^{2}H-NMR shows a higher order of the *n*-(9-anthroyloxy)stearic acid fluorophores. The reasons for this difference might be that (a) because of its relative magnitude the mobility of the fluorophores is more restricted than that of segmented mobility of the acyl chains, or (b) the fluorophores strongly influence the rigidity of their environment.

Finally, we discuss the results for diphenylhexatriene as a commonly used fluorescent probe in time-dependent experiments. While in the literature a preferred orientation of diphenylhexatriene parallel to the membrane normal is always assumed, this assumption is not confirmed by our results because of the relatively large experimental error and thus definite conclusions should be made very carefully. Nevertheless, we give some arguments showing that a preferred orientation of diphenylhexatriene in the membrane plane would not be in contradiction to the results obtained by the other methods. First, we should emphasize that the order parameter derived from the ratio $r(\infty)/r(0)$ in time-dependent measurements according to the theory of Kinosita et al. [16] is based on special model assumptions, such as parallelism between absorption and emission moments within the fluorophore, and represents an average over 10^{-8} s while $\langle P_2^A \rangle$ in the first column of table 4 is independent of model assumptions and represents the real steady state. Second, the possibility cannot be completely excluded that the measured fluorescence polarization is generated by different orientational distribution populations of the fluorophores. This problem has also been discussed recently by Steiner [40] who, in an analogous geometrical arrangement, measured the fluorescence polarization of diphenylhexatriene incorporated into flat in vitro cell cultures and whose results, apart from a slight difference in $P_\rho(\theta = 0°)$, are in agreement with ours.

Furthermore, depending on the generation of lipid membranes as described above, the diphenylhexatriene molecules might be preferentially located within special regions (lenses) of the membranes. Nevertheless, we must stress that a dis-

crepancy would exist only with the usual assumption about the mean orientation of diphenylhexatriene, while the experimental results are in good agreement. For example, Kawato et al. [3] calculated a cone angle of approx. 70° limiting the hindered rotation, which is comparable with our result $\Delta\epsilon \approx 55°$. The results of Andrich and Vanderkooi [41] indicating a preferred orientation of diphenylhexatriene parallel to the membrane normal are related to the system below the phase transition temperature. From experiments with membranes above the transition temperature these authors concluded a random orientation of diphenylhexatriene relative to the membrane plane. Thus, all methods agree as to the conclusion that above the phase transition the orientation of diphenylhexatriene with respect to the membrane is nearly random.

Acknowledgements

This work has been done in connection with and in continuation of the Diplom thesis of one of us (R.K.). The help and assistance of H.A. Kolb in the successful completion of this thesis are gratefully acknowledged. The greatest part of the experiments with diphenylhexatriene was performed by Imme Stiebler. A number of numerical calculations were done by Beatrix Kleutsch. We thank G. Adam, P. Läuger, G. Stark and U. Steiner for valuable discussions. The *n*-(9-anthroyloxy)stearic acid probes were a generous gift from W.H. Sawyer, Australia, the dansyllysine-valinomycin probe from G. Stark. This work was financially supported by the Deutsche Forschungsgemeinschaft (SFB 138).

References

1 L.A. Chen, R.E. Dale, S. Roth and L. Brand, J. Biol. Chem. 252 (1977) 2163.
2 R.E. Dale, L.A. Chen and L. Brand, J. Biol. Chem. 252 (1977) 7500.
3 S. Kawato, K. Kinosita and A. Ikegami, Biochemistry 16 (1977) 2319.
4 S. Kawato, K. Kinosita and A. Ikegami, Biochemistry 17 (1978) 5026.
5 P. Glatz, Anal. Biochem. 87 (1978) 187.
6 C. Senê, D. Genest, A. Obrenovitch, P. Wahl and M. Monsigny, FEBS Lett. 88 (1978) 181.
7 K. Hildenbrand and C. Nicolau, Biochim. Biophys. Acta 553 (1979) 365.
8 G. Weber, J. Chem. Phys. 66 (1977) 4081.
9 J.R. Lakowicz and F.G. Prendergast, Science 200 (1978) 1399.
10 J.R. Lakowicz and F.G. Prendergast, Biophys. J. 24 (1978) 213.
11 J.R. Lakowicz, F.G. Prendergast and D. Hogen, Biochemistry 18 (1979) 508.
12 F. Perrin, Ann. Phys. 10 (1929) 169.
13 E. Frehland, Z. Naturforsch. 30a (1975) 1241.
14 M. Shinitzky and Y. Barenholz, Biochim. Biophys. Acta 515 (1978) 367.
15 E. Frehland, Biophys. Struct. Mech. 2 (1976) 243.
16 K. Kinosita, S. Kawato and A. Ikegami, Biophys. J. 20 (1977) 289.
17 F. Jähnig, Proc. Natl. Acad. Sci. U.S.A. 76 (1979) 6351.
18 M.P. Heyn, FEBS Lett. 108 (1979) 359.
19 A. Seelig and J. Seelig, Biochemistry 15 (1974) 4839.
20 E. Frehland and H.W. Trissl, J. Membrane Biol. 21 (1975) 147.
21 E. Frehland, Biophys. Chem. 4 (1976) 65.
22 J. Yguerabide and L. Stryer, Proc. Natl. Acad. Sci. U.S.A. 68 (1971) 1217.
23 R.A. Badley, H. Schneider and W.G. Martin, Biochem. Biophys. Res. Commun. 45 (1971) 174.
24 R.A. Badley, W.G. Martin and H. Schneider, Biochemistry 12 (1973) 268.
25 F. Conti, R. Fiovaranti, F. Malerba and E. Wanke, Biophys. Struct. Mech. 1 (1974) 27.
26 F. Conti, Annu. Rev. Biophys. Bioeng. 4 (1975) 287.
27 E. Carbone, F. Conti and R. Fiovaranti, Biophys. Struct. Mech. 1 (1975) 221.
28 E. Carbone, F. Malerba and M. Poli, Biophys. Struct. Mech. 2 (1976) 251.
29 K.R. Thulborn and W.H. Sawyer, Biochim. Biophys. Acta 511 (1978) 125.
30 L. Tilley, K.R. Thulborn and W.H. Sawyer, J. Biol. Chem. 254 (1979) 2592.
31 K.R. Thulborn, L.M. Tilley, W.H. Sawyer and F.E. Treloar, Biochim. Biophys. Acta 558 (1979) 166.
32 R. Benz, G. Stark, K. Janko and P. Läuger, J. Membrane Biol. 14 (1973) 339.
33 W.G. Pohl, W. Knoll, B.F. Gisin and G. Stark, Biophys. Struct. Mech. 2 (1976) 119.
34 P. Mueller, D.O. Rudin, H. Ti Tien and W.C. Wescott, Nature 194 (1962) 979.
35 C. Huang, Biochemistry 8 (1969) 344.
36 W.G. Pohl, Z. Naturforsch. 31c (1976) 575.
37 F. Hare and C. Hussan, Biochim. Biophys. Acta 467 (1977) 262.
38 R. Kreikenbohm, Diplom Thesis, Universität Konstanz, (1979).
39 R. Mason and E.D. Cehelnik, J. Photochem. 9 (1978) 219.
40 U. Steiner, Doctoral Thesis, Universität Konstanz (1980).
41 M.P. Andrich and J.M. Vanderkooi, Biochemistry 15 (1976) 1257.
42 R. Benz, O. Fröhlich, P. Läuger and M. Montal, Biochim. Biophys. Acta 394 (1975) 323.
43 R. Benz, O. Fröhlich and P. Läuger, Biochim. Biophys. Acta 464 (1977) 465.