J. Membrane Biol. 21, 147–173 (1975)

Fluorescence Polarization in a Planar Array of Pigment Molecules: Theoretical Treatment and Application to Flavins Incorporated into Artificial Membranes

E. Frehland and H.-W. Trissl

Departments of Physics and Biology, University of Konstanz, D-7750 Konstanz, Postfach 733, Germany

Received 10 September 1974; revised 16 December 1974

Summary. A quantitative fluorescence polarization theory of molecules bound to two-dimensional plane layers has been developed when the electronic transition moments of absorption and emission are parallel within the fluorescent molecules. The transition moments are assumed to be in preferred orientation with respect to the normal to the plane and to be randomly oriented within the plane (rotational symmetry with the normal as axis of symmetry). Three basic model distributions of transition moments are investigated quantitatively. These model distributions represent a simplification but in most cases may be expected to describe reality with sufficient accuracy. For all distributions, two cases of different mobility of molecules are treated: (a) the lifetime of fluorescence is small compared with the characteristic relaxation time of the distribution, and (b) the lifetime of fluorescence is long, so that a complete reorientation of transition moments during the excited state can take place. From the quantitative calculations four characteristic quantities are derived, which are appropriate for the analysis of experimental data. Experiments are carried out with phosphatidylcholine bilayer membranes which contain three differently substituted amphiphilic flavins. All three flavins yield similar data. Their analyses predict free and fast mobility of the flavin chromophore.

Upon investigating the structure and dynamics of biological and artificial membranes, fluorescence polarization measurements turned out to be a very useful method (Radda & Vanderkooi, 1972). Fluorescent probes incorporated or bound to membranes give information about membrane mobility, surface structure and phase transitions (Vanderkooi & Chance, 1972; Haynes & Staerk, 1974). In the thylakoid membrane of the chloroplasts, in photosensitive membranes of the eye and in the light receptors of plants (phototropism, phototaxis), the pigments have a preferred orientation and their mutual orientation is of great importance. In past years, efforts were made to determine pigment orientation in artificial model membranes

(Badley, Schneider & Martin, 1971; Cherry, Kwan & Chapman, 1971; Yguerabide & Stryer, 1971; Steinemann, Stark & Läuger, 1972; Badley, Martin & Schneider, 1973).

In this paper amphiphilic flavins are used as fluorescent probes. The flavins are rendered lipid-soluble by substituting long hydrocarbon chains (C_{18}) at different parts of the nucleus. Thus, water solubility is hindered and the flavins are strongly bound to bilayer membranes. Since the flavin nucleus itself is polar and water-soluble, it is assumed to be located in the membrane-water interface while the hydrocarbon chain sticks into the hydrophobic interior of the membrane (*see* Fig. 12). This is shown by the fluorescence spectrum of plane bilayer membranes and of liposomes, which has a maximum at 520 nm corresponding to a spectrum of the flavin nucleus dissolved in water. More unpolar solvents cause a shift of the fluorescence maximum to shorter wavelength. Therefore these amphiphilic flavins can be used to probe the region of the membrane-water interface (Trissl, 1974).

In studying fluorescence polarization of planar bilayer membranes the special geometrical arrangement of the chromophores as well as their electronic transition moments have to be taken into account. Let us consider a rod-shaped chromophore which has a deep energetic minimum at a special angle to the membrane plane. The chromophore as well as the transition moments then have a preferred orientation with respect to the normal of the membrane. On the other hand, one expects random orientation within the membrane plane. This means that rotational symmetry of the distribution of chromophores exists with the normal to the plane as axis of symmetry.

In the last few years some work on the theoretical investigation of fluorescence polarization experiments for measuring preferred orientation has been done. Desper and Kimura (1967) have developed a mathematical theory under the restricting assumptions that the angle between the electronic transition moments of absorption and emission is zero, and the chromophoric group of the fluorescent molecule does not rotate appreciably during the lifetime of the excited state. For arbitrary directions of the linearly polarized incident light and the polarizing analyzer they could show that for biaxially orientated transition moments the measured polarized intensities can be expressed by a linear combination of five independent quantities. This number of independent quantities is reduced for distributions with special symmetries. In the case of rotational symmetry about a symmetry axis, which is the subject of this paper, the number of independent quantities is only two.

Polarized fluorescence experiments can provide us with more information than other techniques like dichroism or birefringence measurements. But from five (or less) independent quantities one cannot determine uniquely an orientation distribution function, i.e. an infinite set of independent quantities. Hence, the problem of inversion, given by the analysis of the polarized fluorescence experiment, is principally unsolvable. Therefore, the method applied in this paper is to install simple plausible model distributions which are dependent only on few parameters. Furthermore, the assumption that the pigment molecules do not reorient themselves during the lifetime of the excited state is an unrealistic restriction in membrane studies.

The first theoretical investigations, taking into account the mobility of molecules, have been done by Francis Perrin (1929) for the fluorescence depolarization by rotational diffusion of molecules in liquids. Investigations on the influence of anisotropic Brownian rotations were carried out among others by Tao (1969) and Weber (1971). The case of mobile molecules bound to artificial membranes has been treated by Badley *et al.* (1971, 1973), following the theory of Desper and Kimura (1967; *see* Discussion).

The method applied in our paper is as follows: We have developed three different, physically plausible model distributions of transition moments which, on the one hand are as simple as possible (rectangular distribution) but on the other hand may be expected to be sufficient for the quantitative analysis of a wide class of real cases. For each model distribution we have assumed the existence of a characteristic relaxation time determined by the mobility of molecules and have calculated two cases. In the first case, the lifetime of fluorescence is short, in the second it is long compared with this characteristic relaxation time.

Theory

(a) Basic Assumptions

The theory uses the following basic assumptions:

I. Intensity and directional dependence of absorption and emission are described by the classical theory of oscillating dipoles.

II. The reorientations of the dipole moments are governed by rotational and oscillatory diffusion.

III. The extension of the fluorescent area is small compared with the distance to the detecting system (far-field approximation).

IV. Interactions between the fluorescent molecules may be neglected. This includes that no energy transfer occurs.

V. The artificial membranes being used in the experimental part of this paper are planar and a rotational symmetry of dipole distributions with the membrane normal as axis of rotation is assumed.

Furthermore, we mention that in the calculated model distributions a dynamic behavior of fluorescent molecules is assumed which is equal in the ground and excited state.

(b) Geometry of the Fluorescent Process

A strongly simplified schematic description of the fluorescence process is

$$\text{absorption} \xrightarrow{\text{rearrangement}} \text{emission.}$$

The incident light beam of linearly polarized light excites a stationary orientation distribution $F_A(\varepsilon)$ of dipole moments of absorption. ε and φ are angles of spherical coordinates adapted to the plane of the membrane (*see* Fig. 1). Because the distributions of dipoles are assumed to have a rotational symmetry (basic assumption *V*), F_A is independent of φ. The intensity of absorption $I_A(\varepsilon, \varphi)$ is proportional to F_A and $\cos^2\psi$ where ψ is

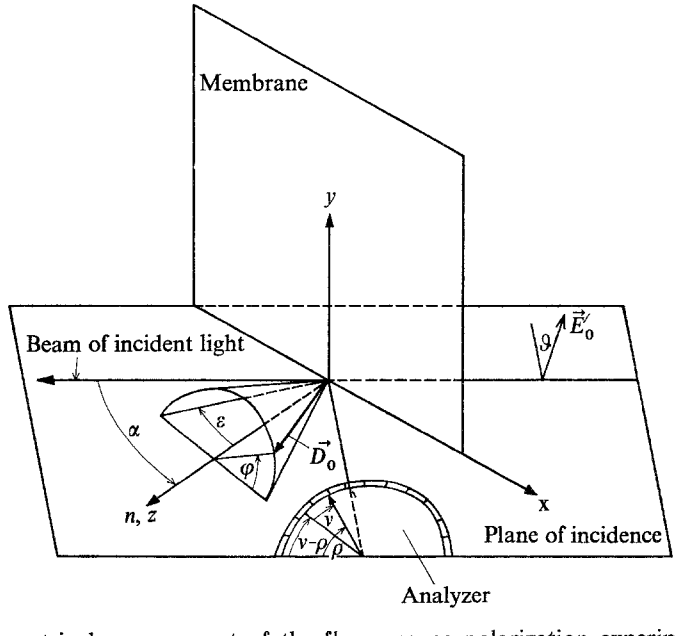

Fig. 1. Geometrical arrangement of the fluorescence polarization experiment and the introduction of adapted Cartesian coordinates x, y, z. $\vec{E}_0$ is a normalized vector denoting the direction of polarization of excitation. The normalized vector $\vec{D}_0$ indicates the direction of one special transition moment on a cone of constant ε

the angle between the electrical vector of the incident light and the dipole moment of absorption.

If the angle Γ between the electronic dipole moments of absorption and emission is zero and movements of the molecules during the excited state may be neglected, the distribution of the intensity of emission $I_E(\varepsilon, \varphi)$ is proportional to I_A. But in general the relation between I_A and I_E is more complicated. The rearrangement which may lead to a partial depolarization is determined by the angle Γ between dipole moments of absorption and emission and by the dynamics of the fluorescent molecules in the excited state (e.g. rotational and oscillatory diffusion). It should be emphasized that throughout this paper the angle Γ is restricted to be zero. This is valid for the flavins used in the experiments (Weber, 1950).

The contribution of one single oscillating dipole to the intensity of fluorescence light passing through the analyzer can be derived from the dipole radiation characteristics to be proportional to $\cos^2\mu \cdot \cos^2 v$. μ is the angle between the dipole and the plane of the analyzer and v is the angle between the projection of the dipole to this plane and the direction of polarization ρ of the analyzer.

Hence the fluorescence intensity I passing through the analyzer is given by integration over all possible orientations with the weighting function I_E:

$$I \propto \int I_E \cdot \cos^2\mu \cdot \cos^2 v \cdot \sin\varepsilon \cdot d\varepsilon d\varphi. \tag{1}$$

The intensity of emission as well as the other distribution functions used in this paper are defined as densities on the unit sphere. The proportionality factor between the left- and right-hand side of Eq. (1) is determined by a number of different quantities such as intensity of incident light, quantum yield of fluorescence, the area concentration of the fluorescent molecules and an apparatus constant. But it is not necessary to specify this factor because the following analysis is based on quantities which are independent of it.

The geometry of the experimental arrangement is shown in Fig. 1. The angle between the beam of incident light and the direction from the fluorescent spot to the analyzer is 90°. For clarity the introduced angles are summarized:

α: Angle between incident light beam and the normal n of the membrane.

ϑ: Angle between the electric vector of the linearly polarized incident light and the plane formed by n and the incident light beam (plane of incidence).

ε: Angle between a dipole moment and the normal n.

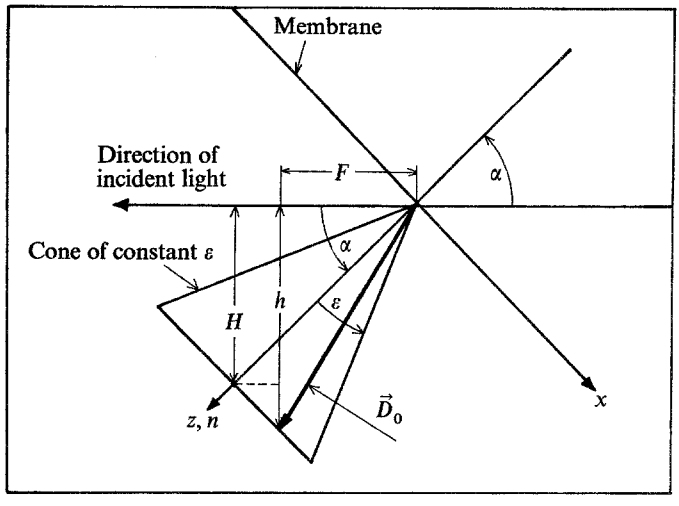

Fig. 2. Projection to the plane of incidence (x, z-plane)

φ: Azimuthal angle. Rotational symmetry of a distribution means independence of φ.

ρ: Direction of polarization of the analyzer.

In addition we need the three angles ψ, μ, ν which have been introduced above.

The rotational symmetry of the distribution of the dipole moments of absorption as well as emission is indicated in Fig. 1 by a cone of constant ε.

According to Fig. 1, ψ, μ and ν depend on the experimental parameters α, ϑ and ρ and the coordinates ε and φ in the following way:

$$\begin{aligned} \psi &= \psi(\alpha, \vartheta, \varepsilon, \varphi) \\ \mu &= \mu(\alpha, \varepsilon, \varphi) \\ \nu &= \nu(\varepsilon, \varphi, \rho, \alpha). \end{aligned} \tag{2}$$

The explicit dependence for $\cos\psi$ is derived by the scalar product between the normalized vectors $\vec{E}_o$ and $\vec{D}_o$ introduced in Fig. 1. The result reads (*cf.* Steinemann *et al.*, 1972):

$$\begin{aligned} \cos\psi = \vec{E}_0 \cdot \vec{D}_0 = &-\sin\varepsilon \cdot \cos\vartheta \cdot \cos\alpha \cdot \cos\varphi \\ &+\sin\varepsilon \cdot \sin\vartheta \cdot \sin\varphi - \cos\varepsilon \cdot \cos\vartheta \cdot \sin\alpha. \end{aligned} \tag{3}$$

For the determination of an explicit relation for the angle μ between $\vec{D}_o(\varepsilon, \varphi)$ and the plane of the analyzer, regard Fig. 2 which shows a pro-

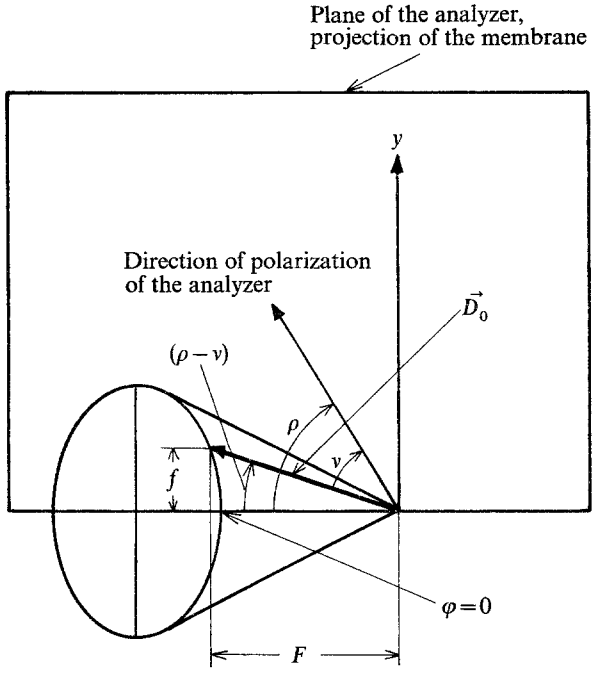

Fig. 3. Projection to the plane of the analyzer

jection to the x, z-plane. For simplicity the plane of the analyzer is parallely displaced to the origin of the x, y, z coordinates. From Fig. 2 it is easily seen that $H = \sin\alpha \cdot \cos\varepsilon$ and $h - H = \cos\alpha \cdot \sin\varepsilon \cdot \cos\varphi$. Hence with $h = \sin\mu$ we get:

$$\sin\mu = \cos\alpha \cdot \sin\varepsilon \cdot \cos\varphi + \sin\alpha \cdot \cos\varepsilon. \tag{4}$$

The derivation of a relation for the angle ν between the projection of $\vec{D}_o$ to the plane of the analyzer and the polarization direction of the analyzer is facilitated by Fig. 2 and Fig. 3, the latter showing a projection to the plane of the analyzer. From Fig. 2 we see that: $F = \cos\alpha \cdot \cos\varepsilon - \sin\varepsilon \cdot \cos\varphi \cdot \sin\alpha$. From Fig. 3: $f = \sin\varepsilon \cdot \sin\varphi$ and $\operatorname{cotg}(\rho - \nu) = \frac{F}{f}$. Hence:

$$\operatorname{cotg}(\rho - \nu) = \frac{\cos\alpha \cdot \cos\varepsilon - \sin\varepsilon \cdot \cos\varphi \cdot \sin\alpha}{\sin\varepsilon \cdot \sin\varphi}. \tag{5}$$

(c) Two Special Cases

First we discuss two especially simple examples. From the study of these examples a method of analyzing and fitting experimental and theoreti-

cal data is derived. In both examples the stationary distribution $F_A(\varepsilon)$ of the dipole moments is assumed to be sharp in ε:

$$F_A(\varepsilon)=\delta(\varepsilon-\varepsilon_0)\cdot\frac{1}{2\pi\cdot\sin\varepsilon} \tag{6}$$

where δ is the usual delta function. In Eq. (6) the factor $1/(2\pi\cdot\sin\varepsilon)$ occurs because all distribution functions in this paper are defined as densities on the unit sphere. In the first case the lifetime τ of the fluorescent state is short compared with the equipartition time T in φ, which is determined by the one-dimensional rotational diffusion of fluorescent molecules around the normal n of the membrane. In this case the intensity of absorption $I_A(\varepsilon, \varphi)$ is proportional to the intensity of emission I_E:

$$\underline{\tau\ll T}\text{: } I_E\propto I_A\propto F_A\cdot\cos^2\psi=\delta(\varepsilon-\varepsilon_0)\cdot\frac{1}{2\pi\cdot\sin\varepsilon}\cdot\cos^2\psi \tag{7}$$

with $\cos^2\psi$ given by Eq. (3).

In the second example the lifetime of the excited state is long compared with the equipartition time in φ. The intensity of emission is now independent of φ but proportional to the mean value $F_A\cdot\overline{\cos^2\psi}$ of $I_A(\varepsilon, \varphi)$:

$$\underline{\tau\gg T}\text{: } I_E\propto F_A\cdot\frac{1}{2\pi}\int_0^{2\pi}\cos^2\psi\cdot d\varphi. \tag{8}$$

With $\cos^2\psi$ according to Eq. (3) the evaluation of the integral yields

$$I_E\propto\frac{1}{\sin\varepsilon}\,\delta(\varepsilon-\varepsilon_0)\cdot\left\{\frac{\sin^2\varepsilon}{2}(\cos^2\vartheta\cdot\cos^2\alpha+\sin^2\vartheta)+\cos^2\varepsilon\cdot\cos^2\vartheta\cdot\sin^2\alpha\right\}. \tag{9}$$

From I_E one can calculate the (measured) intensity I passing through the analyzer at a polarization direction according to the basic relation (1). Generally the integration in Eq. (1) cannot be performed analytically. Fig. 4 shows the results of numerical evaluation of Eq. (1) for three different polarization angles ϑ of the incident light beam. The first example is given in Fig. 4*a* and the second in Fig. 4*b*. The intensity I is drawn as a function of the direction of the polarization angle ρ of the analyzer. I is plotted in arbitrary units. Throughout this paper we have dispensed with the presentation of results for further directions (for example $\vartheta=135°$). As may easily be seen from symmetry considerations for all possible distributions with rotational symmetry about the normal n and with the angle $\Gamma=0$, the intensity $I(\vartheta)$ is symmetric to $I(180°-\vartheta)$ with respect to $\rho=90°$:

$$I(\vartheta, \rho)=I(\pi-\vartheta, \pi-\rho). \tag{10}$$

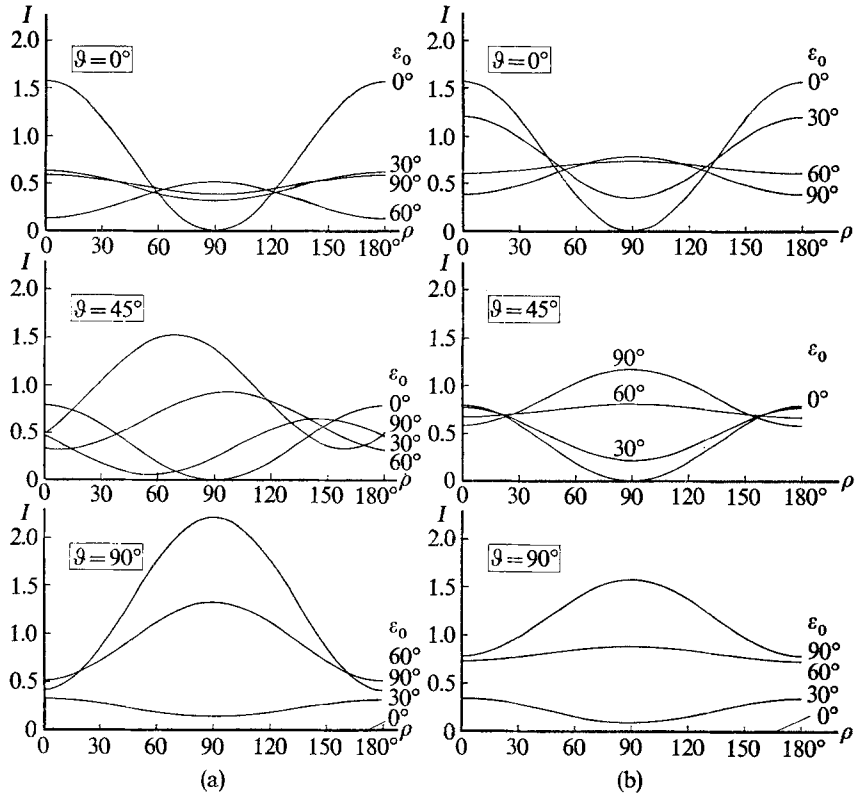

Fig. 4. Numerical results for distributions of dipole moments of transition which, according to Eq. (8), are sharp in ε_0. The intensities I are plotted in arbitrary units as functions of polarization angle ρ of the analyzer for different polarization angles δ of the incident light and different orientations ε_0 of dipoles. (*a*) The lifetime of the excited state τ is small compared with the characteristic relaxation time T of equipartition in φ: $\tau \ll T$. (*b*) $\tau \gg T$

In Fig. 4 the intensity I is symmetric with respect to $\rho = 90°$ for $\vartheta = 0°$ and $\vartheta = 90°$ in both cases, whereas for $\vartheta = 45°$ this symmetry is found only for the second case. From this we see that for the given geometrical arrangement of experiments, measurements with directions of polarization of incident light between $\vartheta = 0°$ and 90° may be important. The above result is a consequence of a more general fact, which may easily be derived from symmetry considerations for arbitrary distributions of dipole moments of transition in ε with rotational symmetry about the normal n: If the lifetime τ of the fluorescent state is long compared with the characteristic equipartition time T in $\varphi(\tau \gg T)$, the intensity of emission I_E is independent of φ for all directions of polarization angles ϑ of incident light. Then

the measured intensity I as a function of ρ is symmetrical with respect to $\rho = 90°$. Therefore the position of the minimum (or maximum) of I for $0° < \vartheta < 90°$ contains important information about the relaxation in φ (e.g. by rotational diffusion). Hence the position $\rho_{\min}$ of the minimum of I at $\rho = 45°$ is introduced. In the following $\rho_{\min}$ serves as a relevant quantity for the analysis of experimental data. $I(\rho)$ is symmetrical with respect to the position of its minimum (or maximum). The course of $I(\rho)$ is determined by three parameters: The minimum and maximum values of I and the position of the minimum. Because I is calculated in arbitrary units, from one curve $I(\rho)$ for one special ϑ it is possible to derive only two different pieces of information.

We use the degree of polarization p_ρ which is defined by

$$p_\rho = \frac{I(\rho=0°) - I(\rho=90°)}{I(\rho=0°) + I(\rho=90°)}. \tag{11}$$

p_ρ is independent of the normalization of I. For $\vartheta = 0°$ as well as for $\vartheta = 90°$, p_ρ already contains two pieces of information, because $I(\rho)$ must be symmetrical with respect to $\rho = 90°$. First the sign of p_ρ yields the position of the minimum. For $p_\rho > 0$ the minimum is at $\rho = 90°$ (maximum at $\rho = 0°$, 180°) and for $p_\rho < 0$ at $\rho = 0°$ (180°). Second the absolute value of p_ρ determines the ratio of the maximal and minimal value of I. For $\vartheta = 45°$ one needs in addition to p_ρ the position of the minimum $\rho_{\min}$.

The total intensity I_t (again in arbitrary units) is defined by integration over ρ:

$$I_t = \frac{1}{\pi} \int_0^\pi I \cdot d\rho. \tag{12}$$

We see from Fig. 4 that for a given ε_0, I_t varies for different ϑ (I_t is proportional to the area under the $I(\rho)$ curves). This change contains further important information. Hence, the degree of polarization of total intensity p_t is introduced:

$$p_t = \frac{I_t(\vartheta=0°) - I_t(\vartheta=90°)}{I_t(\vartheta=0°) + I_t(\vartheta=90°)}. \tag{13}$$

p_t is also independent of the normalization of I. Generally, for the analysis of experiments it will be sufficient to compare only the total intensities for $\vartheta = 0°$ and $\vartheta = 90°$.

Consequently, we will use four quantities for the following study of more complicated distributions of dipole moments: The degrees of polari-

zation p_ρ for $\vartheta = 0°$ and $\vartheta = 90°$, the position $\rho_{\min}$ of the minimum of I for $\vartheta = 45°$ and the degree of polarization of the total intensity p_t.

(d) Different Model Distributions

This section deals with the numerical results for some model distributions. On the one hand we could choose from the infinite number of possible distributions only some examples under the aspect of simplicity and clarity. On the other hand we believe that with the use of the calculated model distributions a great number of possible and realistic cases may be represented with sufficient accuracy though in general the dipoles may be arranged in other distributions (for example Gaussian). Therefore the following results should be useful also for the analysis of fluorescence polarization with other molecules but with a geometrical arrangement as shown in Fig. 1. The models which we will investigate include the two examples treated above as special cases.

We assume the dipole moments to be in some preferred orientation which however is described not necessarily by a delta shape distribution function. If we regard a single dipole, such a stationary distribution function determines the probability that the dipole points in a certain direction. For a great number of molecules it is the density of dipole orientations. The distributions are assumed to be homogeneous within a given region as defined below (rectangular distribution).

Fig. 5 presents the different distributions which have been calculated. It shows the distribution about some mean position given by the polar angles ε_0, φ_0 in a projection from the unit sphere to the plane of the membrane.

Let us first consider dipoles which are homogeneously distributed between the angles $(\varepsilon_0 - \Delta\varepsilon)$ and $(\varepsilon_0 + \Delta\varepsilon)$. Reorientation in φ is assumed to be zero. Then the normalized distribution $F_\Delta(\varepsilon)$ is

$$F_\Delta(\varepsilon) = \begin{cases} \dfrac{1}{4\pi\Delta\varepsilon} \cdot \dfrac{1}{\sin\varepsilon} & \text{for } (\varepsilon_0 - \Delta\varepsilon) \leqq \varepsilon \leqq (\varepsilon_0 + \Delta\varepsilon) \\ 0 & \text{else} \end{cases}. \tag{14}$$

For $\Delta\varepsilon \to 0$ we get the delta shape distribution Eq. (6). Within the interval $[\varepsilon_0 - \Delta\varepsilon, \varepsilon_0 + \Delta\varepsilon]$ the dipoles may change their positions. This movement may be caused by the motion of the fluorescent molecules as a whole or of the fluorescent part of them. We introduce the characteristic time $T_{\Delta\varepsilon}$ for equipartition of the dipoles within the interval $[\varepsilon_0 - \Delta\varepsilon, \varepsilon_0 + \Delta\varepsilon]$.

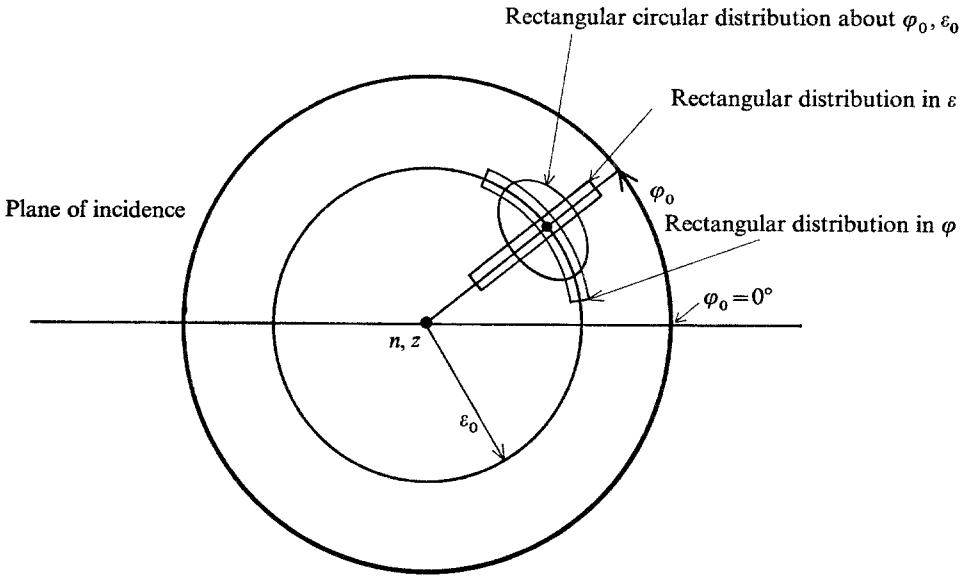

Fig. 5. Different model distributions of transition moments around a mean position ε_0, φ_0 presented in a perpendicular projection from the unit sphere to the plane of the membrane

Similarly to *(c)* two special cases are distinguished by comparison of $T_{\Delta\varepsilon}$ with the lifetime τ of the excited state. Then we get as generalizations of Eq. (7) for the intensity of emission:

$$\underline{\tau \ll T_{\Delta\varepsilon}}: I_E \propto \begin{cases} \dfrac{1}{4\pi\Delta\varepsilon} \cdot \dfrac{1}{\sin\varepsilon} \cdot \cos^2\psi & \text{for } \varepsilon \text{ in } [\varepsilon_0 - \Delta\varepsilon, \varepsilon_0 + \Delta\varepsilon] \\ 0 & \text{else} \end{cases} \tag{15}$$

where no rearrangement between absorption and emission can take place. If a total rearrangement of dipoles within $[\varepsilon_0 - \Delta\varepsilon, \varepsilon_0 + \Delta\varepsilon]$ during the fluorescent lifetime is possible, the equation reads

$$\underline{\tau \gg T_{\Delta\varepsilon}}: I_E \propto \begin{cases} \dfrac{1}{4\pi\Delta\varepsilon} \cdot \dfrac{1}{\sin\varepsilon} \left\{ \dfrac{1}{2\Delta\varepsilon} \cdot \int\limits_{\varepsilon_0 - \Delta\varepsilon}^{\varepsilon_0 + \Delta\varepsilon} \cos^2\psi(\varepsilon') \cdot d\varepsilon' \right\} & \text{for } \varepsilon \text{ in } [\varepsilon_0 - \Delta\varepsilon, \varepsilon_0 + \Delta\varepsilon]. \\ 0 & \text{else} \end{cases} \tag{16}$$

In the second model a similar linear rectangular distribution in the angle φ about a mean value φ_0 is considered. The distribution in ε is assumed to be delta shaped according to Eq. (6). This model distribution describes a situation different from those discussed in *(c)*: Consider a single

dipole in position φ_0. In addition to rotatory diffusion in φ (causing a change in φ_0) the dipole can carry out an independent (i.e. oscillatory) motion about φ_0. This motion may be a torsional one of the whole molecule or an independent motion of a part of the fluorescent molecule containing the chromophore. Again we introduce an equipartition time $T_{\Delta\varphi}$. But now one has to calculate only the case $\tau \gg T_{\Delta\varphi}$. The other case $\tau \ll T_{\Delta\varphi}$ leads to the same intensity of emission as relation (7) for $\tau \ll T$ because now as before we assume rotational symmetry of distribution around n. The intensity of emission I_E is for

$$\underline{\tau \gg T_{\Delta\varphi}}: I_E \propto \frac{1}{2\pi}\delta(\varepsilon-\varepsilon_0)\cdot\frac{1}{\sin\varepsilon}\cdot\left\{\frac{1}{2\Delta\varphi}\cdot\int_{\varphi_0-\Delta\varphi}^{\varphi_0+\Delta\varphi}\cos^2(\varphi')\cdot d\varphi'\right\}. \tag{17}$$

For $\Delta\varphi \to 0$ Eq. (17) describes also the first example discussed in (*c*) [*see* Eq. (7)] and for $\Delta\varphi = \pi$ the second one [Eq. (8)]. The conditions $\tau \ll T$ and $\tau \gg T$ are limiting cases with pure one-dimensional rotational diffusion in φ. Hence, though relation (17) describes a rectangular distribution, it is expected that this equation for $0 < \Delta\varphi < \pi$ is a good quantitative approximation to the case $\tau \approx T$, where the lifetime of the excited state τ is comparable with the relaxation time T.

Apart from these two linear rectangular distributions we consider now the third model in Fig. 5, a circular rectangular distribution. This means a homogeneous distribution on the unit sphere with a solid angle $2\pi(1-\cos\Delta\gamma)$ around a mean position ε_0, φ_0. For convenience a set of polar angles $\bar{\varepsilon}$, $\bar{\varphi}$ with the mean dipole direction ε_0, φ_0 as polar axis is introduced; i.e., the point ε_0, φ_0 on the unit sphere is $\bar{\varepsilon}=0$. The transformation from angles ε, φ to $\bar{\varepsilon}$, $\bar{\varphi}$ is given by the relations:

$$\begin{aligned}
\sin\varepsilon\cdot\sin(\varphi-\varphi_0) &= \sin\bar{\varepsilon}\cdot\sin\bar{\varphi}\\
\cos\varepsilon &= \cos\bar{\varepsilon}\cdot\cos\varepsilon_0-\sin\bar{\varepsilon}\cdot\cos\bar{\varphi}\cdot\sin\varepsilon_0\\
\sin\varepsilon\cdot\cos(\varphi-\varphi_0) &= \cos\bar{\varepsilon}\cdot\sin\varepsilon_0+\sin\bar{\varepsilon}\cdot\cos\bar{\varphi}\cdot\cos\varepsilon_0.
\end{aligned} \tag{18}$$

The stationary distribution F_A belonging to a mean position ε_0, φ_0 is

$$\bar{F}_A(\varepsilon_0,\varphi_0,\bar{\varepsilon}) = \begin{cases} \dfrac{1}{2\pi(1-\cos\Delta\gamma)} & \text{for } \bar{\varepsilon} \leqq \Delta\gamma \\ 0 & \text{else} \end{cases}. \tag{19}$$

Again a characteristic time $T_{\Delta\gamma}$ for equipartition within the spherical segment around ε_0, φ_0 with the solid angle $2\pi(1-\cos\Delta\gamma)$ is introduced.

In the case $\tau \ll T_{\Delta\gamma}$ the intensity of emission I_E is proportional to the intensity of absorption I_A and hence given by integration over φ_0.

$$\underline{\tau \ll T_{\Delta\gamma}}: I_E \propto \frac{1}{2\pi} \int_{\varphi_0=0}^{2\pi} \overline{F_A} \cdot d\varphi_0. \tag{20}$$

In the case $\tau \gg T_{\Delta\gamma}$ we first calculate the mean value of absorption in the spherical segment around φ_0, ε_0 and define the mean distribution $\overline{\overline{F_A}}$:

$$\overline{\overline{F_A}}(\varepsilon_0, \varphi_0, \bar{\varepsilon}) = \begin{cases} \left\{\frac{1}{2\pi(1-\cos\Delta\gamma)}\right\} \cdot \int_{\bar{\varphi}=0}^{2\pi} \int_{\bar{\varepsilon}=0}^{\Delta\gamma} \sin\bar{\varepsilon} \cdot \cos^2\psi \cdot d\bar{\varepsilon} \cdot d\bar{\varphi} & \text{for } \bar{\varepsilon} \leqq \Delta\gamma \\ 0 & \text{else} \end{cases}. \tag{21}$$

Then the intensity of emission is

$$\underline{\tau \gg T_{\Delta\gamma}}: I_E \propto \frac{1}{2\pi} \int_{\varphi_0=0}^{2\pi} \overline{\overline{F_A}} \cdot d\varphi_0. \tag{22}$$

In Figs. 6–10 the quantitative results of numerical computer calculations for the described model distributions are presented. Each Figure is correlated to one model. The quantities p_ρ for $\vartheta = 0°$ and 90°, p_t and $\rho_{\min}$ for $\vartheta = 45°$ are drawn as a function of ε_0. Parameters are $\Delta\varepsilon$, $\Delta\varphi$ and $\Delta\gamma$, respectively.

Materials and Methods

The experiments were carried out with (black) bilayer membranes made from a solution of lecithin (5 mM) and different amphiphilic flavins in *n*-decane. Into this membrane-forming solution 10% (v/v) *n*-butanol was added in order to increase membrane stability. The membranes were formed either with dioleoyllecithin obtained from Supelco or with dierucoyllecithin synthesized by K. Janko (Benz, Stark, Janko & Läuger, 1973). Both lecithins form membranes of similar properties.

7,8,10-trimethyl-3-octadecyl-isoalloxazine (flavin 1), 3,7,8-trimethyl-10-[2-(octadec(9c)enoyloxy)-ethyl]-isoalloxazine (flavin 2) and 3-methyl-10-octadecyl-isoalloxazine (flavin 3) were synthesized by Dr. W. R. Knappe. All three compounds were finally purified by column chromatography on neutral aluminia and gave correct elemental analysis for C, H and N. Flavin 2 turns out to be slightly soluble in water up to a concentration of 3×10^{-6} M whereas flavin 1 and flavin 3 are insoluble in water ($< 5 \times 10^{-11}$ M).

The membranes were formed on a teflon frame with a circular hole of 8 mm diameter. The blackness of the membranes has been controlled by a telescope. They were illuminated by a single line (456.9 nm) of a CW-Ar-Laser (Cryophysics, model 252) under an angle of 45°. The light was plane polarized. Perpendicular to the laser beam and in the plane which is given by the beam and the normal of the membrane plane, fluorescence is measured by a photographic technique described in more detail elsewhere (Trissl, 1974). In all experiments the fluorescence light of the membrane passes a narrow band interference filter (Balzers, Filtraflex B 40) with a maximum of transmission at

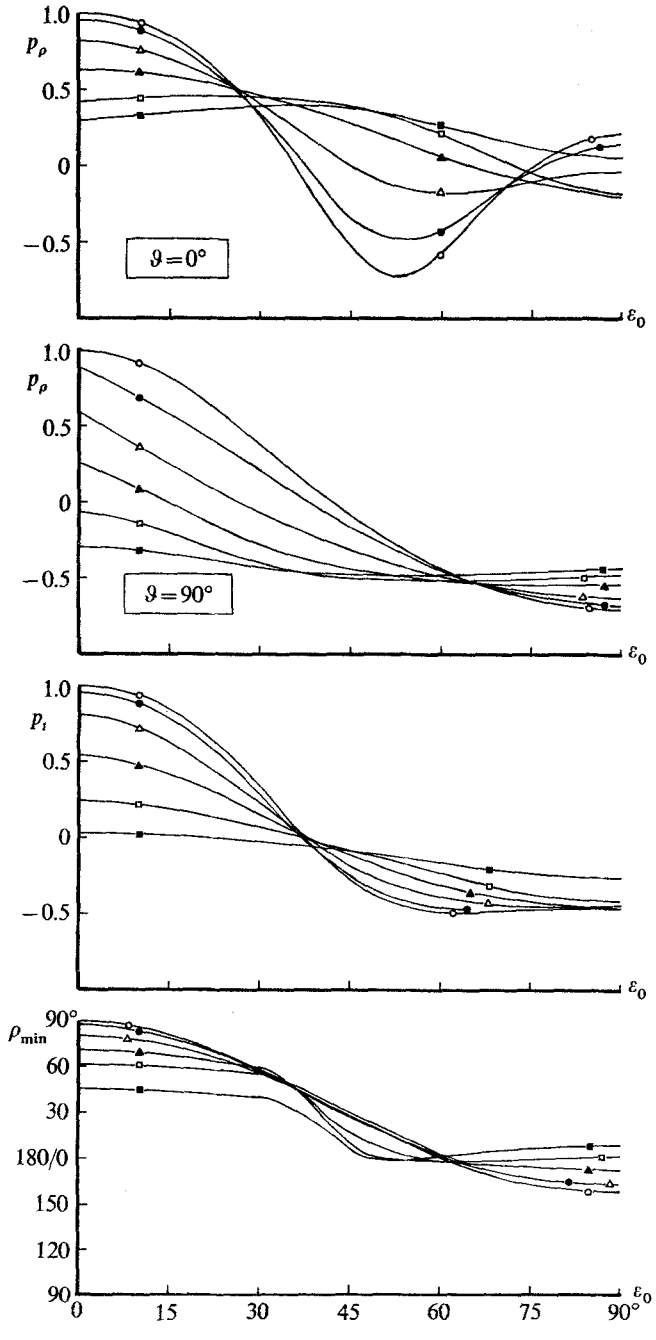

Fig. 6. Quantitative results for a rectangular distribution in ε around ε_0 of transition moments in the case $\tau \ll T_{\Delta\varepsilon}$. The different curves are parametered by $\Delta\varepsilon$. $\Delta\varepsilon = 0° \triangleq \circ$; $15° \triangleq \bullet$; $30° \triangleq \triangle$; $45° \triangleq \blacktriangle$; $60° \triangleq \square$; $75° \triangleq \blacksquare$

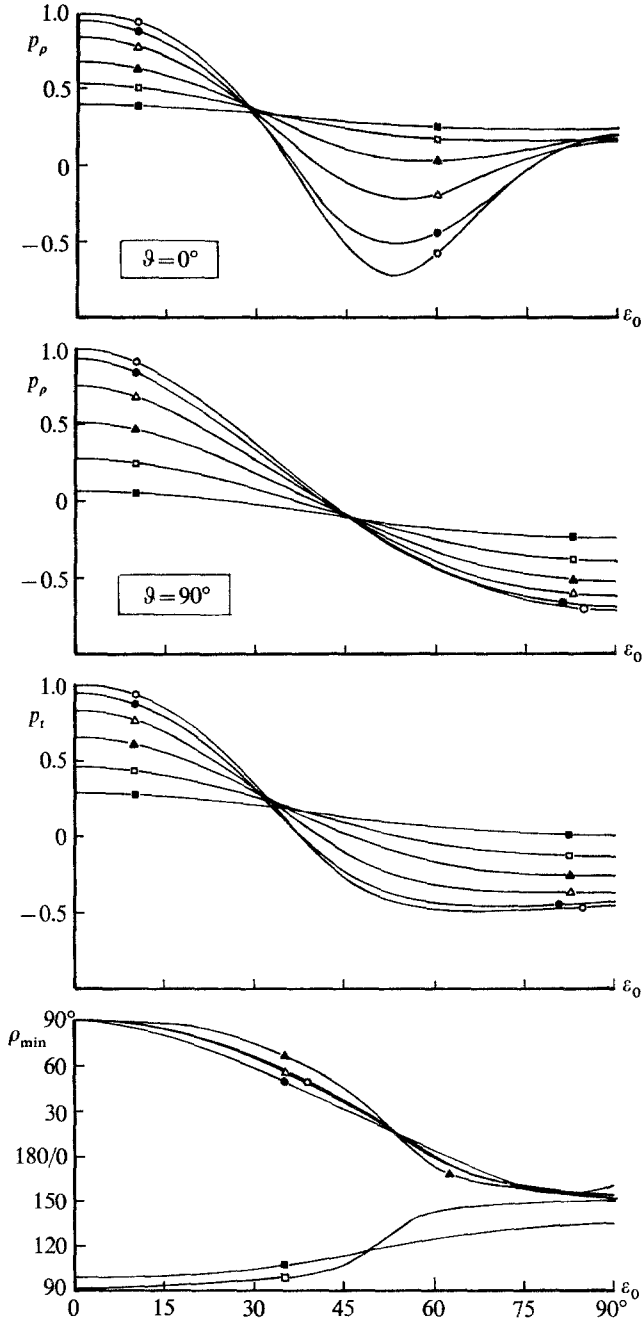

Fig. 7. Quantitative results for a rectangular distribution in ε around ε_0 of transition moments in the case $\tau \gg T_{\Delta\varepsilon}$. The different curves are parametered by $\Delta\varepsilon$. $\Delta\varepsilon = 0° \triangleq \circ$; $15° \triangleq \bullet$; $30° \triangleq \triangle$; $45° \triangleq \blacktriangle$; $60° \triangleq \square$; $75° \triangleq \blacksquare$

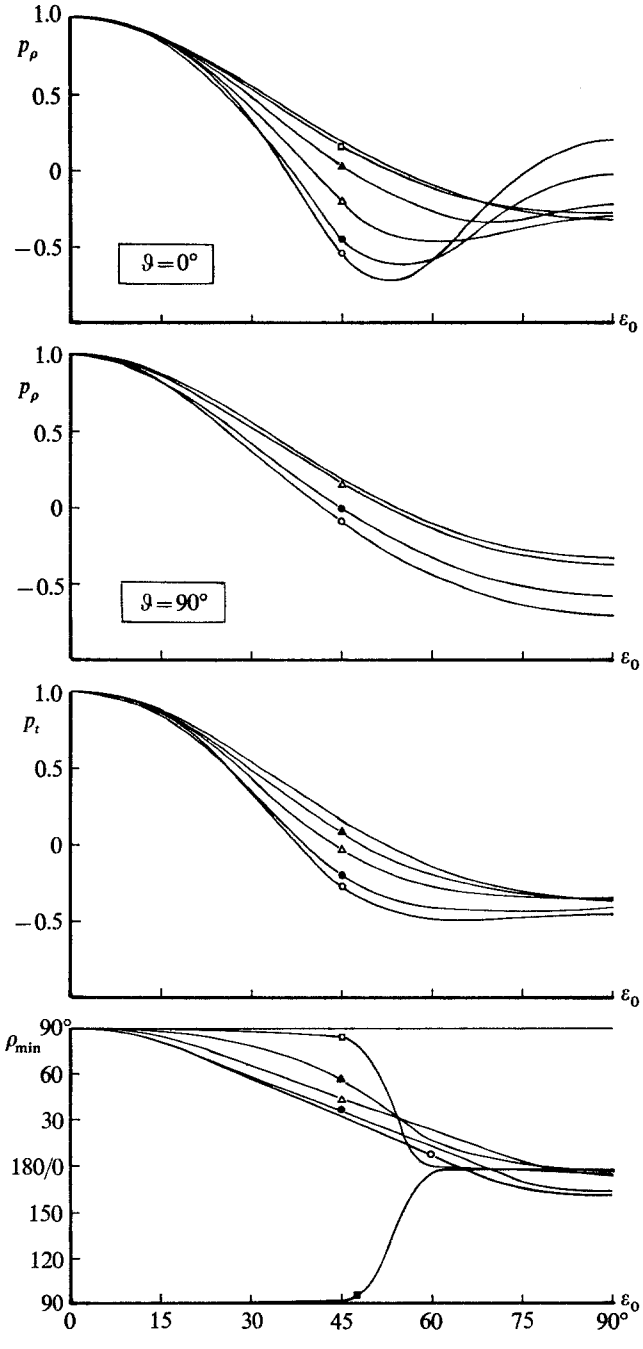

Fig. 8. Quantitative results for a rectangular distribution in φ around φ_0 of transition moments in the case $\tau \gg T_{\Delta\varphi}$. The different curves are parametered by $\Delta\varphi$. $\Delta\varphi = 0° \triangleq \circ$; $30° \triangleq \bullet$; $60° \triangleq \triangle$; $90° \triangleq \blacktriangle$; $120° \triangleq \square$; $150° \triangleq \blacksquare$; $180°$ not marked

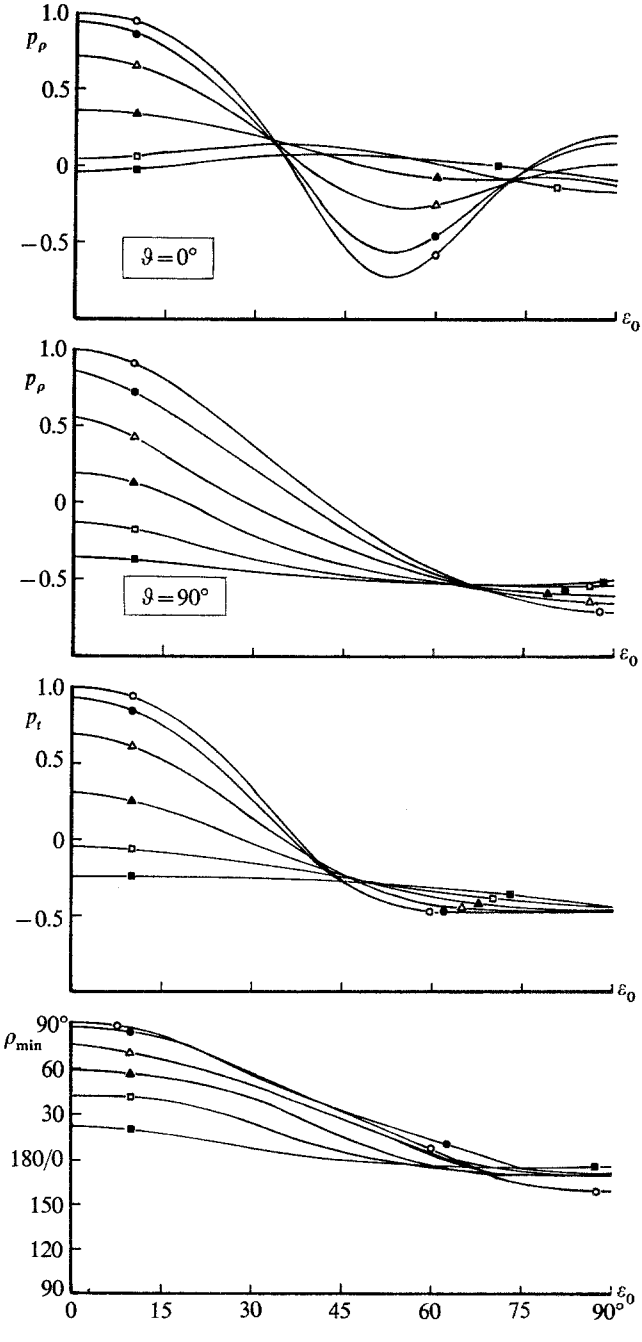

Fig. 9. Quantitative results for a rectangular circular distribution in ε around ε_0, φ_0 of transition moments in the case $\tau \ll T_{\Delta\gamma}$. The different curves are parametered by $\Delta\gamma$. $\Delta\gamma = 0° \triangleq \circ$; $15° \triangleq \bullet$; $30° \triangleq \triangle$; $45° \triangleq \blacktriangle$; $60° \triangleq \square$; $75° \triangleq \blacksquare$

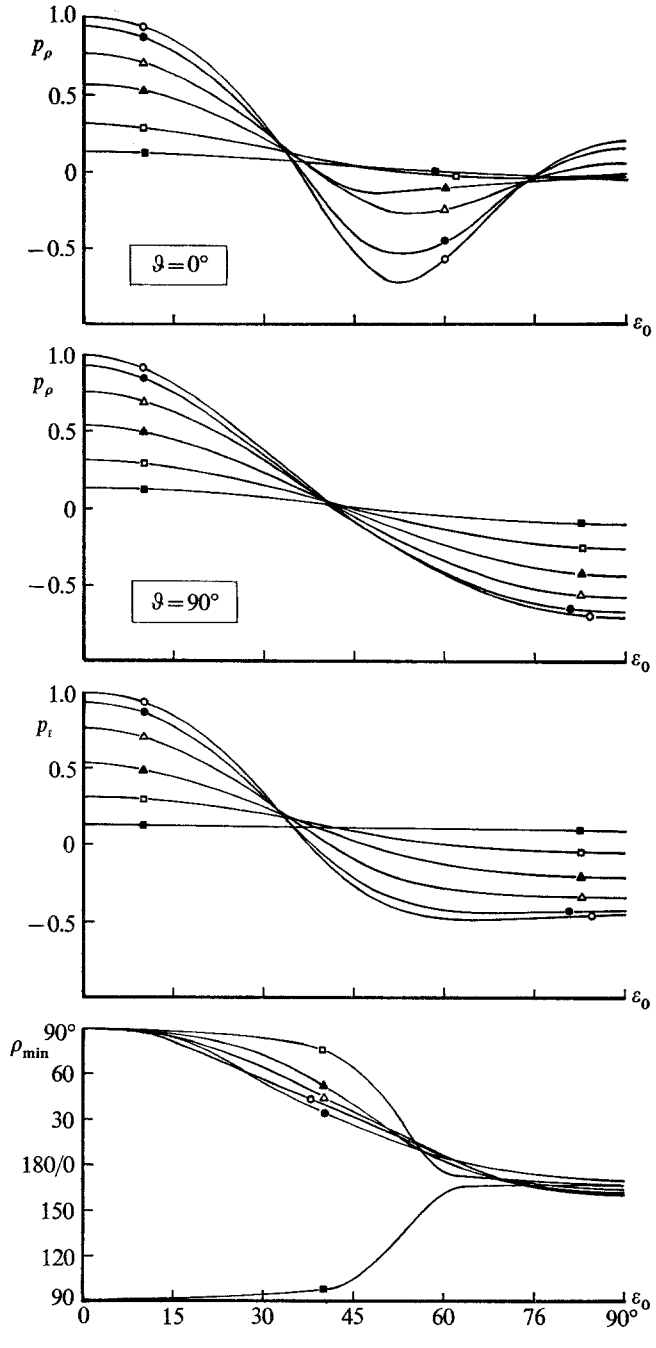

Fig. 10. Quantitative results for a rectangular circular distribution in ε around ε_0, φ_0 of transition moments in the case $\tau \gg T_{\Delta\gamma}$. The different curves are parametered by $\Delta\gamma$. $\Delta\gamma = 0° \triangleq \circ$; $15° \triangleq \bullet$; $30° \triangleq \triangle$; $45° \triangleq \blacktriangle$; $60° \triangleq \square$; $75° \triangleq \blacksquare$

520 nm and is then focussed by a photographic objective (Tessar 2.8/50) onto a sensitive PIN silicon photodiode (Motorola MRD 500). The photovoltage of the diode is measured by an electrometer (Keithley model 610 B), using an input impedance of 10^{10} Ω. When polarization of fluorescence is measured a polarizer is fixed between membrane cuvette and interference filter. All experiments were carried out at room temperature.

Results

The following control experiments were carried out. Using an aqueous solution of 3-methyl-lumiflavin in an optical microcell which is placed in the same geometrical arrangement as the membrane; no residual polarization of fluorescence is observed (*see* Table 1). From this experiment the minimal error of the detecting system is estimated to be $\pm 3.5\%$ in determining the photovoltage. The error in polarization degree is then $p_\rho = \pm 0.02$. The flavin concentrations were chosen as high as possible, but so that a linear relationship between the concentration of pigment in the membrane-forming solution and the fluorescence signal of the bilayer membrane still holds. As described elsewhere (Trissl, 1974) the area concentrations of flavins in the membrane are about 0.9×10^{13} molecules/cm^2.

The polarization properties of the apparatus were tested by reflecting the laser beam (intensity strongly reduced) through the polarizer onto the photodiode under identical experimental conditions. It was found that the measured fluorescence intensity for polarization direction of laser light $\vartheta = 90°$ as a function of the polarizer angle ρ satisfied the theoretically predicted $\sin^2\rho$-dependence within an experimental error of $\rho < 1°$ and of intensity $\pm 1.5\%$.

The angle between the electronic transition moments of absorption and emission Γ was determined for all three flavins by measuring the polarization degree as a function of the viscosities of isotropic glycerol-water

Table 1. The degree of fluorescence polarization p at $\vartheta = 0°$ and 90°, of total intensity p_t and the polarizer angle of minimal fluorescence intensity $\rho_{\min}$ at $\vartheta = 45°$ for different flavins in lecithin bilayer membranes [a]

Flavin	p_ρ ($\vartheta = 0°$)	p_ρ ($\vartheta = 90°$)	p_t	$\rho_{\min}$	ε_0	$\Delta\varepsilon$
1	0.35	0.15	0.33	101°	26°	60°
2	0.38	0.25	0.31	100°	30°	55°
3	0.40	0.20	0.35	94°	20°	60°
3-methyl-lumiflavin	±0.02	±0.02	±0.02	—	—	—

[a] In addition, some data for 3-methyl-lumiflavin in water are given. In the last two columns the angles ε_0 and the $\Delta\varepsilon$ angles are listed, determined by application of the theory of a rectangular distribution around ε_0 with $\tau \ll T_{\Delta\varepsilon}$.

solutions. These experiments were carried out with the apparatus at hand ($\vartheta = 0°$). The degrees of fluorescence polarization of the solutions is defined by:

$$p_s = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + I_{\perp}} \tag{23}$$

where $I_{\parallel}$ is the intensity of the polarized wave with its electric vector parallel to that of the exciting light, whereas $I_{\perp}$ is the intensity of the polarized wave with its electric vector normal to that of the exciting light. Plotting the reciprocal polarization degree against the reciprocal viscosity straight lines result at high viscosities. By extrapolating to infinite viscosity all three flavins yield $p_{s,\infty} = 0.5$. Using a formula derived by Perrin (1929)

$$p_{s,\infty} = \frac{3\cos^2 \Gamma - 1}{\cos^2 \Gamma + 3} \tag{24}$$

the angle Γ between the transition moments is calculated to be zero. These results are comparable with those by Weber (1950) who found the same polarization degree $p_{s,\infty} = 0.5$ for riboflavin and flavin-adenine-dinucleotide.

The dependence of the relative fluorescence intensity on the analyzer angle ρ for flavin 1 incorporated into a dioleoyllecithin membrane is shown in Fig. 11 for $\vartheta = 0°$, 45° and 90°. When the polarization plane of excitation light ϑ is 0° and 90° the curves are symmetric to $\rho = 90°$ within the experimental error. At $\vartheta = 45°$ the fluorescence intensity is not symmetrical to $\rho = 90°$ but the minimum is shifted to $\rho \approx 100°$. The solid lines in Fig. 11 are obtained from a computer calculation of Eq. (17) (for $\varepsilon_0 = 26°$ and $\Delta\varepsilon = 60°$) using a proportionality constant which leads to the best adaption to the experimental points. When ϑ is chosen to be 135° the minimum lies at $\rho \approx 80°$.

It should be noted that different membranes formed from identical membrane-forming solution show slightly different fluorescence intensities. A total error of at least $\pm 10\%$ must be given for the experimental intensity values. One series of measurements for a fixed angle ϑ took about 20 min. In order to avoid a systematic error, a statistical sequence of analyzer angles ρ for the single points of the curves was chosen.

Flavins 2 and 3 show similar experimental curves as those for flavin 1 in Fig. 11. Experimental data of flavin 1, flavin 2 and flavin 3 are summarized in Table 1. At the polarization angles of incident light, $\vartheta = 0°$ and $\vartheta = 90°$, the polarization of fluorescence intensity is symmetrical to $\rho = 90°$ for all three flavins with the minimum at $\rho = 90°$. When ϑ is chosen between

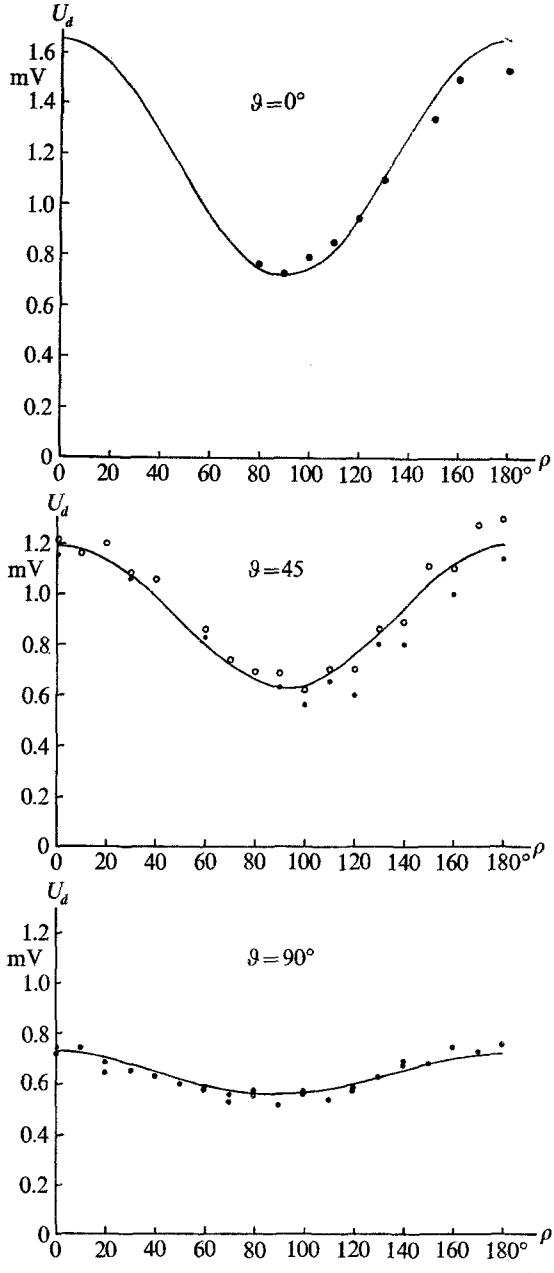

Fig. 11. Measured fluorescence intensity of a membrane containing flavin 1 as a function of the analyzer angle ρ for $\vartheta=0°$, 45° and 90°. The bimolecular film was formed from a solution of 5 mM dioleoyllecithin and 0.2 mM flavin 1 in decane. The measuring points of two different membranes are entered into one graph marked by the open and the full points. The theoretical fit (full lines) was done with the model of rectangular distribution of transition moments in ε for $\tau \gg T_{\Delta\varepsilon}$ (*see* Fig. 7). The fitting parameters are $\varepsilon_0=26°$ and $\Delta\varepsilon=60°$. The arbitrary normalization of intensity was adapted to the experimental values

$\vartheta = 0°$ and $\vartheta = 90°$ the minimum is shifted to $\rho_m > 90°$ whereas for $90° < \vartheta < 180°$ the minimum lies at angles $\rho < 90°$. The shift is always smaller than 10° and is most pronounced at $\vartheta = 45°$. Though a considerable error of ρ_{min} should be assumed, the observed asymmetry itself is significant for all amphiphilic flavins at $\vartheta = 45°$ as well as $\vartheta = 135°$. Furthermore the symmetry relation (10) could be verified.

It is possible to determine experimentally the total fluorescence intensities by two different procedures. Either by integrating graphically the $I(\rho)$-curves [*see* Fig. 11 and Eq. (12)] or by measuring the fluorescence intensity without the analyzer. It was checked that these two methods lead to the same values of p_t.

In order to show whether fluorescence quenching and/or energy transfer influence the measurements, control experiments with flavin 1 were carried out at a fivefold lower area concentration resulting in the same polarization data.

Discussion

As has been pointed out in the introduction, the investigation and interpretation of the polarized fluorescence experiments represent an inversion problem, which is principally unsolvable even in the most simple case of immobile molecules in which the electronic transition moments of absorption and emission are parallel. Hence, in most cases a satisfactory analysis can be done only by the aid of additional information. If this additional information makes it likely that one of the treated model distributions may be a sufficient approximation to the real situation, one can try to fit experimental data to the quantitative results given in Figs. 6–10. If a fit is not possible, the analysis of Figs. 6–10 may help to find out possible intermediate distributions.

We do not know whether the procedure which we have developed makes an optimal use of all information obtainable from the polarized fluorescence experiments. Some critical remarks should be made on the work of Badley *et al.* (1971, 1973) who have also investigated mobility and orientation of pigment molecules in plane layers by fluorescence polarization experiments. In line with the theory of Desper and Kimura (1967) they claim, without proof, that generally all information is contained in a set of nine intensities. But, as mentioned in the introduction, the mathematical theory of Desper and Kimura is strongly based on the restrictions $\Gamma = 0$, biaxial symmetry and immobility of molecules. There is no real evidence that in the most general case of arbitrary Γ, mobility, orientation and energy transfer, all

information is contained in nine quantities. At least the mathematical theory of this general case has not been developed.

The analysis of our experiments leads to similar results for all three flavins. A fit to the experimental data (Table 1) can be done only by the rectangular distribution in ε when the lifetime τ of the excited state is long compared with the equipartition time $T_{\Delta\varepsilon}$. A fit with the other model calculations turns out to be completely impossible. Fig. 11 shows that theoretical curves $I(\rho)$ yield a good quantitative fit to the experimental data.

It was a little surprising to us that all three amphiphilic flavins yield similar kinetic behavior although the hydrocarbon chains are bound in quite a different way to the flavin nucleus. We do not venture to give a definite explanation for this behavior before more and other experimental data are available. A rather speculative explanation is that the motion of the flavin nucleus is governed mainly by the motion of the polar lecithin heads and obviously the kind of binding to the membrane interface is of minor importance. Lhoste (1971) has shown experimentally that the angle between the absorption transition moment at 445 nm and the direction of the longer molecule axis is about 30°. Thus the geometrical arrangement of flavins 1, 2 and 3 shown in Fig. 12 seems to be not unrealistic, if one regards this picture only as a rough approximation of reality.

In our experiments, energy transfer between neighboring flavins should not occur since no fluorescence quenching could be detected and polarization data remained unchanged for lower flavin concentrations. An additional theoretical argument against depolarization effects by energy transfer is the small overlapping area of less than 5% between the excitation and emission spectrum.

The experimentally verified symmetry relation (10) for $I(\vartheta, \rho)$ provides support for the correctness of the theoretical assumptions.

It is possible to use the information about the orientation distribution and the mobility of the transition moments for determining the absolute area concentration c_m of flavins in the membrane. This can be done by comparison of the total fluorescence intensity I_t defined by Eq. (12) with that of 3-methyl-lumiflavin in aqueous solution in a flat microcell (Trissl, 1974) where the membrane is replaced by this cell. In the latter case the fluorescence light is completely depolarized. Hence, the fluorescence intensity can be calculated as follows: Because the angle between the excitation light beam and the normal to the microcell plane is $\alpha = 45°$ (*see* Fig. 1) the light intensity per membrane unit area is reduced by the factor $\cos^2\alpha$. Since the orientation is isotropic and the relaxation time for rotational diffusion is short (complete depolarization), the intensity of emission I_E is

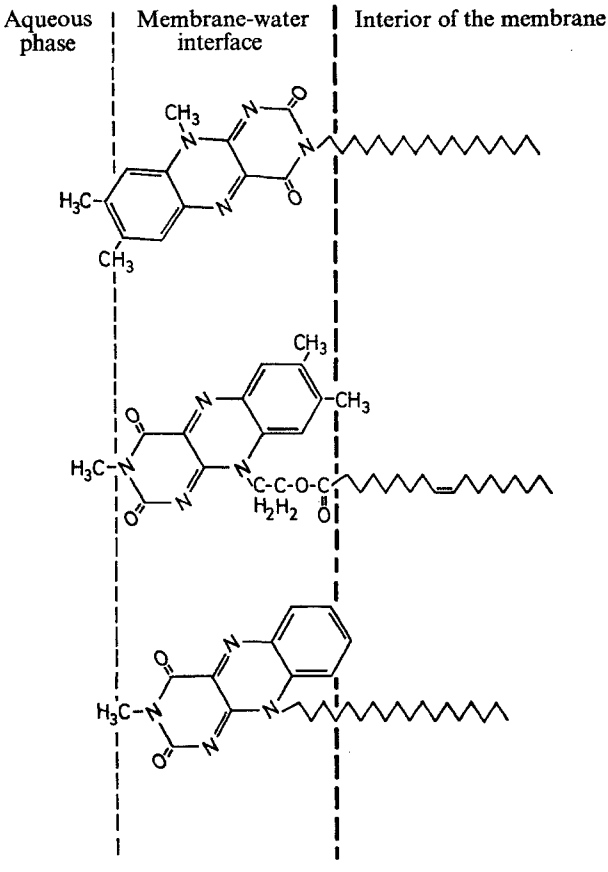

Fig. 12. Graphic formulae and possible binding geometry of the three used amphiphilic flavins

proportional to the mean value of $\cos^2\psi$, which is obtained by integration of $\cos^2\psi$ over the unit sphere. From this the fluorescence intensity I_{aqueous} may easily be determined by a second integration over the unit sphere. I_{aqueous} is independent of the polarization direction of incident light ϑ.

We have calculated the fluorescence intensities I_{aqueous} and $I_t(\vartheta)$ for the model distribution being relevant for flavins 1, 2 and 3. The ratios given in Table 2 are independent of the normalization. Since the area concentration c_{aqueous} of the calibration solution is known, the area concentration c_m of flavins in the membranes can be calculated using the ratios $[I_{\text{aqueous}}/I_t(\vartheta)]_{\text{observed}}$ and $[I_{\text{aqueous}}/I_t(\vartheta)]_{\text{calculated}}$:

$$c_m = c_{\text{aqueous}} \frac{(I_{\text{aqueous}}/I_t)_{\text{calculated}}}{(I_{\text{aqueous}}/I_t)_{\text{observed}}}. \tag{25}$$

Table 2. Quotient $[I_{\text{aqueous}}/I_t(\delta)]_{\text{calculated}}$ for rectangular distribution in ε around ε_0 in the case $\tau \gg T_{\Delta\varepsilon}$

Flavin	ε_0	$\Delta\varepsilon$	$[I_{\text{aqueous}}/I_t(\delta)]_{\text{calculated}}$	
			$\vartheta = 0°$	$\vartheta = 90°$
1	26°	60°	0.49	0.93
2	30°	55°	0.51	0.92
3	20°	60°	0.47	1.03

The numerical values are selected for the ε_0 and $\Delta\varepsilon$ data given in Table 1.

Since the experimental determination of the flavin concentration in the membrane described above was carried out at $\vartheta = 90°$ in a previous paper (Trissl, 1974), it may be seen from Table 2 that the chosen correction factor of 1 causes an error of less than 10%, which lies within the other experimental errors.

The fluorescence lifetime τ of the flavin chromophore is 4.5 nsec (Lasser & Feitelson, 1973). The analysis of our experiments has shown that the relaxation time $T_{\Delta\varepsilon}$ is short compared with τ. This result could make a rough estimation possible of the microviscosity η of the membrane-water interface. We introduce a rotational diffusion coefficient $D_{\text{rot},\varepsilon} \approx 1/T_{\Delta\varepsilon}$ for rotation in ε. $D_{\text{rot},\varepsilon}$ is dependent on η:

$$D_{\text{rot},\varepsilon} \approx \frac{kT}{V\eta} \tag{26}$$

where k is the Boltzmann constant, T the absolute temperature and V the volume of the flavin nucleus ($V \approx 3 \times 10^{-23}$ cm^3). From this one gets an upper limit for η of about 1 Poise.

The authors wish to thank Prof. P. Hemmerich and Prof. P. Läuger for their interest in this work and for stimulating discussion, and Mr. L. Carls for excellent technical assistance. This work has been financially supported by the Deutsche Forschungsgemeinschaft (Sonderforschungsbereich 138). The numerical calculations have been done on the TR 4 of the Rechenzentrum der Universität Konstanz.

References

Badley, R. A., Martin, W. G., Schneider, H. 1973. Dynamic behavior of fluorescent probes in lipid bilayer model membranes. *Biochemistry* **12**:268

Badley, R. A., Schneider, H., Martin, W. G. 1971. Orientation and motion of a fluorescent probe in model membranes. *Biochem. Biophys. Res. Commun.* **45**:174

Benz, R., Stark, G., Janko, K., Läuger, P. 1973. Valinomycin-mediated ion transport through neutral lipid membranes: Influence of hydrocarbon chain length and temperature. *J. Membrane Biol.* **14**:339

Cherry, R. J., Kwan, H., Chapman, D. 1971. Polarized absorption spectroscopy of chlorophyll-lipid membranes. *Biochim. Biophys. Acta* **267**:512

Desper, C. R., Kimura, I. 1967. Mathematics of the polarized-fluorescence experiment. *J. Appl. Physiol.* **38**:4225

Haynes, D. H., Staerk, H. 1974. 1-Anilino-8-naphthalenesulfonate: A fluorescent probe of membrane surface structure, composition and mobility. *J. Membrane Biol.* **17**:313

Lasser, N., Feitelson, J. 1973. Excited state pK values from fluorescence measurements. *J. Phys. Chem.* **77**:1011

Lhoste, J.-M. 1971. Flavin cations: Ground and excited states properties. *Proc. First Europ. Biophys. Congr.* E. Broda, A. Locker and H. Springer-Lederer, editors, Verlag der Wiener Medizinischen Akademie

Perrin, F. 1929. La fluorescence des solutions. *Ann. Phys., Paris* **10**:169

Radda, G. K., Vanderkooi, J. 1972. Can fluorescent probes tell us anything about membranes? *Biochim. Biophys. Acta* **265**:509

Steinemann, A., Stark, G., Läuger, P. 1972. Orientation of the porphyrin ring in artificial chlorophyll membranes. *J. Membrane Biol.* **9**:177

Tao, T. 1969. Time-dependent fluorescence depolarization and Brownian rotational diffusion coefficients of macromolecules. *Biopolymers* **8**:609

Trissl, H.-W. 1974. Studies on the incorporation of fluorescent pigments into bilayer membranes. *Biochim. Biophys. Acta* **367**:326

Vanderkooi, J. M., Chance, B. 1972. Temperature sensitivity of fluorescence probes in the presence of model membranes and mitochondria. *FEBS Letters* **22**:23

Weber, G. 1950. Fluorescence of riboflavin and flavin-adenine dinucleotide. *Biochem. J.* **47**:114

Weber, G. 1971. Theory of fluorescence depolarization by anisotropic Brownian rotations, discontinuous distributions approach. *J. Chem. Phys.* **55**:2399

Yguerabide, J., Stryer, L. 1971. Fluorescence spectroscopy of an oriented model membrane. *Proc. Nat. Acad. Sci.* **68**:1217