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Orientation and Rotational Freedom of Fluorescent Probes in Lecithin Bilayers

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Summary. The fluorescence polarization properties of lecithin bilayers stained with 2,6-MANS and 1,8-ANS under applied potential steps have been studied. The fluorescence signal components of both dyes were found to have different sign and relative amplitude, suggesting that 1,8-ANS and 2,6-MANS behave differently when bound to black lipid membranes. In order to determine the location and the extent of rotational brownian motions of the bound chromophores, the experimental data were analyzed by using a simplified physico-mathematical model. According to it 2,6-MANS appears to have a ratio ϱ/τ higher than 1,8-ANS (ϱ being the rotational relaxation of in plane rotations and τ the lifetime of the excited singlet state of the bound molecules), suggesting that the former chromophore is more tightly held inside the bilayers. Furthermore, 2,6-MANS is found to possess the absorption and emission oscillators more closely oriented to the normal of membrane surface, while 1,8-ANS has both oscillators almost near the plane of the bilayers. The results furnish also a fair estimate of the random molecular motion own by the phospholipid molecules at room temperature. The comparison of the present data with those obtained from squid axon membranes confirms the validity of the proposed physical model, yielding a rough estimate of the axon membrane-area covered by integral protein macromolecules. These preliminary results derived from lecithin model membranes suggest that fluorescence polarization techniques can provide valuable informations if applied to study the macromolecular organization of in vitro reconstituted membranes.

Key words: Fluorescent probes — Polarization — Bilayers.

Introduction

Early investigations over the nature of the fluorescence changes in stained lipid bilayers under applied potentials were initiated with the aim of obtaining indirect informations about the physico-chemical phenomena responsible for the fluores-

Abbreviations: 2,6-MANS, 2-N-methylanilinonaphthalene-6-sulfonate; 1,8-ANS, 1-anilinonaphthalene-8-sulfonate

cence signals in stained nerve membranes under voltage-clamp steps (Conti and Malerba, 1972). Their similar time courses and voltage dependencies strongly suggested that common types of physical mechanisms were at the origins of the fluorescence signals from both, nerves and lipid membranes (Conti et al., 1974). In particular, it was emphasized the existence of at least three types of interactions occurring between membrane bound chromophores and the electric field applied across the membrane. Two of them, depending on the dye's molecular configuration, were found to be characterized by a fast kinetics (probe's orientation and probe's displacement under the action of the electric field). The third, related to changes in the partition coefficient of membrane-bound and free chromophores, was found to depend upon the diffusional properties of the space near the membrane.

In spite of its utility in identifying the causes of the fluorescence signals, the method yielded little informations about the overall molecular array of the adsorbed chromophores inside the excitable membranes structure. In order to gain further evidences, experiments over the fluorescence polarization properties of stained squid axon membranes under applied voltage-clamp steps were recently carried out (Carbone et al., 1975). From those measurements it could be possible to determine the average orientation of the absorption and emission oscillators and the extent of the parameters characterizing the rotational brownian motion of membrane-bound chromophores subjected to the action of external electric fields. The theoretical analysis of the phenomena was facilitated a great deal by making reasonable hypotheses whose validity we have verified to hold true also for identical dye molecules that interact with lecithin bilayers. However, the lack of data concerning the fluorescence polarization properties of probe molecules adsorbed to model membranes prevented us from drawing out definite conclusions about the structural properties of the axon membrane regions responsible for the fluorescence signals.

To overcome this serious limitation and to get further supports for the validity of the proposed physico-mathematical model we have initiated a comparative analysis of the fluorescence polarization properties of axonal and lipidic membranes. This allows us to acquire specific informations about the degree of freedom of phospholipid molecules and a rough estimate of the axon membrane area covered with proteins.

Materials and Methods

Stained Bilayers

Bilayers membranes were formed across a 3.5 mm hole in a chamber made of black lucite by means of a very fine sable brush following the procedure of Mueller et al., 1962. The chamber was divided in two compartments with a volume ratio of roughly 1 : 2 by a septum (at 45° degree) also made of black lucite (see Fig. 1). Both compartments contained identical electrolyte solutions (0.1 M KCl) and their water level was continuously balanced by adding or sucking from the large compartment few drops of bathing solution in order to eliminate any curvature of the lipid bilayers. Narrowing of lecithin membranes was controlled either by electrical monitor or by

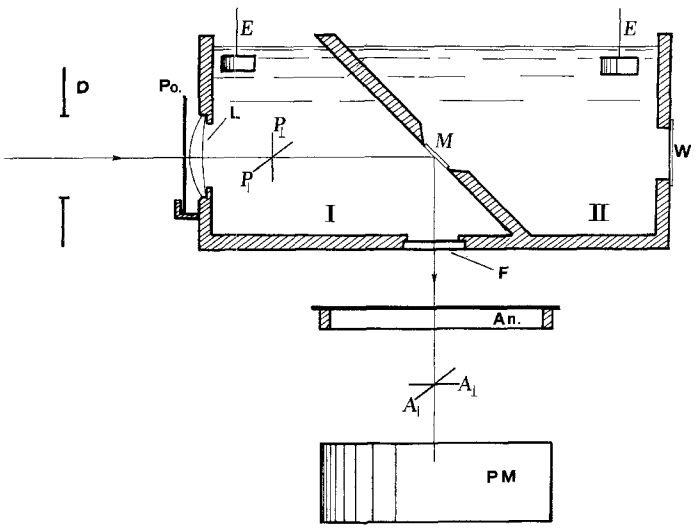

Fig. 1. Schematic diagram showing the chamber and the optical set-up used for the polarization studies of stained lecithin membranes. The monochromatic parallel beam of light (*P*) from the diaphragm, *D*, is polarized using a HNP'B polaroid sheet, *Po*, and then focused into the bilayers, *M*, by means of the spherical lens, *L*. The emitted light (*A*) passing through the cut-off filter, *F*, is analyzed using a KN-36 polaroid sheet, *An*, and then collected by the phototube, *PM*. *W* represents the side window used to check the formation of the "black film", *E* the stimulating potential electrodes

visual observation until light reflections from the membrane disappeared. "Black" membranes were stained as a rule 10 min later the bilayers were formed by adding to compartment II a small amount of concentrated dye's solution in distilled water (1 ml of 10^{-3} M) by means of a stop flow device. To allow a complete diffusion of the chromophore through out the bathing compartment, the optical measurements were initiated about twenty minutes after addition of the staining solution. In this way the optical signals were found to be large and quite reproducible.

Fresh lipid solutions obtained by dissolving 50 mg of purified egg-lecithin (gently supplied by Vevy Fund., Genoa) into 1 ml n-decane (BDH, England), were prepared almost daily and stored at low temperature. Stock solutions of 2,6-MANS (10^{-3} M) in distilled water were obtained by previously dissolving 2,6-MANS crystals in pure ethanol (Carlo Erba, Milano) to a final concentration of 10^{-3} M, and evaporating the solutions in air at room temperature. The obtained fine crystals were then easily dissolved into an equal amount of distilled water. Concentrated solutions of 1,8-ANS (10^{-3} M) were prepared by directly dissolving the purified chromophores in distilled water at room temperature. Solution's containers of both dyes were refrigerated in the dark at low temperature to avoid dye photo-degradation.

2,6-MANS, purified following the procedure of Cory et al. (1968), was a generous gift of prof. A. Azzi (Padova Univ.); 1,8-ANS, purchased from Eastman Organic Chemicals was purified following the procedure of Weber and Laurence (1954).

Electrical and Optical Apparatus

Most of the electrical set-up used was similar to that emploied by Conti and Malerba in earlier studies. Substantial changes have been made to their optical arrangement. The light source was a 150 W-Xenon lamp (Engelhard Hannovia, Inc.) operated with an Electro Powerpacs (Cambridge, Mass.) power supply. Line-fluctuations of the background light intensity (3% of the DC level) due to the lamp power supply were practically reduced at the size of the phototube output shot-noise by using a series of two low-pass RC network with time constants of 150 and 400 ms.

Light filtering was accomplished in two different ways, yielding similar results. For some experiments it was used a narrow band interference filter 365 ± 10 nm (Thin Film, Inc.) while for others was employed a combination of wide-band Reichert filters, UG-1 and BG-38. The latter choice was preferred because of the larger amount of exciting UV light obtainable from the lamp. The normalized light spectrum in this case was peaked at $\lambda_{max} = 365$ nm with an half peak bandwidth of ± 20 nm.

The parallel beam of light, provided by a spherical quartz condenser placed in front of the lamp, was linearly polarized using a HNP'B Polaroid sheet and then focused into a wide portion of lipid membrane by means of a spherical lens (∅ = 1 cm, f = 25 mm in water). The fluorescence light detected through the cut-off filter Corning Cs 3–74 (at 45° degree to the plane of membrane) was analyzed using a KN-36 Polaroid sheet before entering the photomultiplier tube (RCA 4523).

To limit the solid angle through which the emitted light was observed, a small hole (8 mm in diameter) was made at the bottom of the chamber. This was done to better satisfy the theoretical requirements of the mathematical model described in the next paragraph, although the amount of light reaching the phototube was considerably reduced. As a consequence, in order to confine the photocathode current density within acceptable values, we increased the photocathode illuminated area by placing it 3 cm apart from the bottom-hole of the chamber (see Fig. 1).

In Figure 1 are illustrated the directions of the polarizing axis adopted for the polarizer and analyzer. For the sake of clarity, we will often refer to the "parallel-parallel", "parallel-perpendicular", "perpendicular-parallel" and "perpendicular-perpendicular" components either using the symbols: ∥∥, ∥⊥, ⊥∥, ⊥⊥ or the indexes in order from 1 to 4.

Theoretical Background

The most probable orientation and molecular motion of 2,6 and 1,8-MANS bound to axonal membranes under externally applied electric fields has been already analyzed in full details in preceding works (Carbone et al., 1975; Conti, 1975). Since the early general hypotheses made to solve the "squid axon problem" were independent of the particular type of membrane there are good reasons to think that they will hold true also in the case of 2,6-MANS and 1,8-ANS adsorbed to lecithin bilayers. Therefore, it seems reasonable to begin our theoretical analysis by defining the background fluorescence light from membrane bound chromophores at rest as done previously.

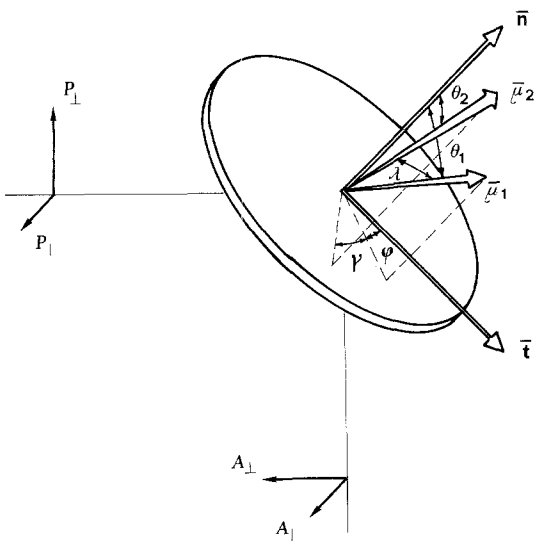

Fig. 2. Schematic diagram of the membrane bilayers forming $\pi/4$ radiants with the direction of the exciting light beam. Vectors $\vec{\mu}_1$ and $\vec{\mu}_2$ represent respectively the adsorption and the emission oscillators associated with the adsorbed chromophores; θ_1 and θ_2 the angles they form with the normal to the membrane surface, $\vec{n}$; λ is the spatial-angle formed by the two oscillators and γ its projection on the plane of the membrane. The angle φ represents the integration variable of the system. $\vec{t}$ is a reference axis normal to $\vec{n}$ laying on the plane of the membrane. The symbols $P_\parallel$, $P_\perp$ and $A_\parallel$, $A_\perp$ indicate the direction of the electric vector corresponding to the parallel and perpendicular direction of the polarizer and analyzer

Called N the surface density of probes involved in the optical signals, Q the average fluorescence quantum yield and Φ_i the quantity depending on the orientation of the emission and absorption oscillators; each polarized component of the resting background fluorescence, F_i, can then be written as:

$$F_i = K\ N\ Q\ \Phi_i \qquad (i = 1, 2, 3, 4) \tag{1}$$

K being a proportionality constant depending on the experimental conditions. Following classical procedures, the four Φ_i components of a stained bilayers systems can be easily derived as a particular case of the more general squid axon membranes (see Carbone et al., 1975; p. 228 and following). The axon's cylindrical symmetry requires an integration, over the possible orientations of a membrane elementary area with the direction of light propagation, that is not needed for planar lecithin bilayers. In our case, the fixed angle formed by the bilayers surface with the direction of light propagation will equally influence any given infinitesimal area of lecithin membranes. Hence, with reference to Figure 2 the quantitiy Φ_i can be derived by substituting $\alpha = 3\pi/4$ and integrating over all values of the variable φ. The results are then averaged out over a gaussian distribution of emission oscillators having fluorescence lifetime, τ, and undergoing brownian movements.

The final expression of each Φ_i component obtained after performing the above integrations, will be the following:

$$\Phi_{\parallel} = \Phi_1 = \frac{\pi}{4} h \sin^2 \theta_1 \sin^2 \theta_2 \tag{2a}$$

$$\Phi_{\parallel\perp} = \Phi_2 = \frac{\pi}{2}(1 - h \sin^2 \theta_2/4) \sin^2 \theta_1 \tag{2b}$$

$$\Phi_{\perp\parallel} = \Phi_3 = \frac{\pi}{2}(1 - h \sin^2 \theta_1/4) \sin^2 \theta_2 \tag{2c}$$

$$\Phi_{\perp\perp} = \Phi_4 = \frac{\pi}{2}[1 - (\sin^2 \theta_1 + \sin^2 \theta_2)/2 + h \sin^2 \theta_1 \sin^2 \theta_2/8 - 2 g \sin \theta_1 \sin \theta_2 \cos \theta_1 \cos \theta_2] \tag{2d}$$

where $$h = 2 + (2 \cos^2 \gamma - 1) \frac{\varrho/\tau}{2 + \varrho/\tau} \tag{3a}$$

and[1] $$g = \cos \gamma \frac{2 \varrho/\tau}{1 + 2 \varrho/\tau} \tag{3b}$$

ϱ being the rotational relaxation time of the membrane incorporated dye molecules; θ_1 and θ_2 the angle formed by the emission and absorption oscillators with the normal to the membrane surface; γ the projection over the membrane surface of the angle $\lambda = |\theta_2 - \theta_1|$ at the time of light quantum absorption.

Differentiation of Equation 1 with respect to N, Q and θ_2 disregarding terms higher than the first-order, furnishes four independent equations of the type:

$$\Delta I_i = \Delta F_i = \alpha \Phi_i + \dot{\Phi}_i \qquad (i = 1, 2, 3, 4)$$
$$\text{with } \alpha = (\Delta N/N + \Delta Q/Q/\Delta \theta_2 \tag{4a}$$

which form the basic system of our problem. After normalization with respect to the fourth component and substitution of the quantities Φ_i and $\dot{\Phi}_i$, Equation 4 can be further reduced to three equations having the following expressions:

$$h = 4/x_1(1 + 2 a_3/a_1) \tag{5a}$$

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[1] g represents the average cosine of the angle γ' ($\langle \cos \gamma' \rangle$) that the projection on the membrane surface of the emission oscillator forms with the reference axis $\vec{t}$ at the time of light quantum emission. According to classical works (Weber, 1952) $\langle \cos \gamma' \rangle$ is proved to be equal to the product $\langle \cos \gamma \rangle \langle \cos \gamma_\beta \rangle$, γ_β being the average rotation undergone by the bound chromophore under the action of thermal agitation. The brackets $\langle\ \rangle$ represent the stochastic average over a gaussian distribution of emission oscillators mediated by the exponential probability function of the fluorescence decay. In our case γ is constant, while γ_β depends upon ϱ and τ according to the formula $\frac{2 \varrho/\tau}{1 + 2 \varrho/\tau}$.

Introduction of Equation (3b) makes the analytical treatment of the problem more rigorous. As will be shown in "discussion" the model does not require any further assumption about the variable ϱ/τ to furnish reliable solutions of the problem. The best fitting value of ϱ/τ will be determined together with the other unknowns by solving simultaneously Equations (5), (3a) and (3b).

$$h = (2\,a_1/a_2)\,[y_2 + 2\,gy_1(1 - 2\,x_2)]/ \\ [(2 + a_1/a_2)\,(y_2 - gx_2y_1) - x_1y_2\,(1 + 2/a_2)] \tag{5b}$$

$$h = (2/x_1)\,[x_1y_2(2 + a_2 + a_3) + 2\,gy_1(a_3x_1 + a_2x_2 + 2\,a_3x_1x_2) - 2\,a_2y_2]/ \\ [(a_3 - a_2)\,(y_2 - gx_2y_1) + x_1y_2(1 + a_2/2)] \tag{5c}$$

$$x_1 = \sin^2\theta_1 \qquad y_1 = \sin\theta_1 \cos\theta_1$$
$$x_2 = \sin^2\theta_2 \qquad y_2 = \sin\theta_2 \cos\theta_2$$

relating the normalized experimental fluorescence signals $a_i = \frac{\Delta I_i}{\Delta I_4}$ $(i = 1, 2, 3)$ with the unknown quantities: θ_1, $\theta_2 \cos\gamma$ and ϱ/τ.

Solutions of Equations (5) can be univocally determined only if reasonable assumptions are made about two of the unknown quantities of the system. This argument will be further developed in the following paragraphes.

Results

Bilayers Stained with 2,6-MANS

Typical fluorescence signals detected from lecithin bilayers stained with 2,6-MANS during application of brief potential steps are shown in Figure 3. The records were obtained sequentially from the same lipid membrane under different positions of the polarizer and analyzer. According to Conti et al. (1974) signals corresponding to negative and positive voltage pulses were found to have identical shape and opposite sign than those reported. To obtain fluorescence signals as those of Figure 3, alternated voltage pulses of equal amplitude and duration (100 mV; 0.6 ms) were applied

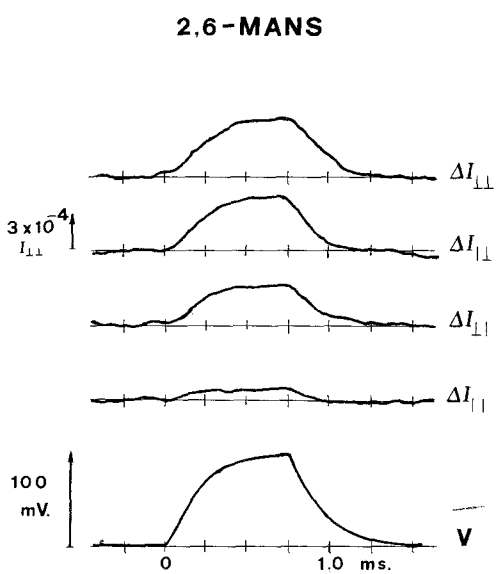

Fig. 3. Fluorescence polarization signals associated with square voltage pulses of 100 mV, from 2,6-MANS stained lecithin membranes. The arrow on the left of the records indicates the size of the light intensity variation referred to the perpendicular-perpendicular background light component. The bottom trace illustrates the time course of the square voltage pulse measured through the same recording apparatus used for detecting the optical signals Bandwidth 0.8 Hz–1 kHz. The final concentration of the staining solution was 5×10^{-4} M. T = 12° ÷ 18° C

Table 1. Experimental values of the polarized signal components obtained from nine different lipid membranes stained with 2,6-MANS. On the right of each component, in parenthesis, are reported the standard deviation. In fourth column are indicated the theoretical values calculated by substituting the values: $\theta_1 = 32.2°$, $\theta_2 = 57.2°$, $h = 2.45$ and $\alpha = -1.99$ into Equation (4) described in theoretical section. In first and third column are reported, for comparison, the values obtained from the squid axon membranes externally stained with 2,6-MANS

Pol. signal components	Exper.				Theor.	
	Axon (ext.)		Bilayers		Axon (ext.)	Bilayers
$\Delta I_{\parallel} / \Delta I_{\perp\perp}$	0.26	(0.05)	0.29	(0.02)	0.24	0.29
$\Delta I_{\perp\parallel} / \Delta I_{\perp\perp}$	0.47	(0.02)	0.69	(0.03)	0.48	0.70
$\Delta I_{\perp\parallel} / \Delta I_{\perp\perp}$	0.87	(0.05)	0.85	(0.03)	0.87	0.81

across the stained bilayers at a repetition rate of 11 ms. As a rule, records with good signal to noise ratio could be achieved averaging out over about 16,000 trials.

In order to check the reproducibility of the optical signals, the measurements of the four polarized components were repeated two or three times, performing as a whole more than ten measurements over the same lecithin bilayers. This possibility allowed us to study how signals evolve with time. However, the investigations of this interesting aspect of stained bilayers were prevented by the presence of two contrasting phenomena influencing to a different extent the size of the signals. One tending to decrease the signal amplitude together with the background fluorescence (photodegradation of the dye molecules) and the other enhancing only the signal size. This latter most likely being related to the narrowing of the black lipid thickness with time (Benz et al., 1975). It is clear that a reliable evaluation of the fluorescence signals amplitude should take into consideration the effects of both contrasting phenomena. For this reason the value of each polarized component was corrected by compensating phenomena influencing to a different extent the size of the signals. One tending ments.

To compare the results of fluorescence measurements from different membranes, the signal amplitude of each record was normalized with respect to the largest component, in our case $\Delta I_{\perp\perp}$. In this way the measured values become independent of the experimental conditions under examination (background light, staining-solutions concentration, temperature, etc...). The average values of the three normalized components obtained from nine different membranes are summarized in Table 1. Together are reported the theoretical values predicted by the mathematical analysis and the results from stained axon membranes (Carbone et al., 1975). These findings are confirmed by the measurements of the four partially polarized components performed without analyzer or polarizer sheet over 6 membranes. As already stressed these measurements do not provide any further information about the unknowns of the problem but only an independent control for the totally polarized components.

Finally, we should mention the results of the background fluorescence measurements from lecithin membranes at rest which were found very similar to those of the squid axon. For instance, the ratio between $I_{\perp\perp}$ and $I_{\parallel}$ was found to be 0.75 against

the value 0.78 and 0.73 obtained for extra- and intra-cellularly stained squid axon membranes, indicating that the polarization properties of the bulk phases are quite similar in both systems. As in the case of stained axons, the main contribution to the background light intensity comes from fluorescent molecules not interacting with the thin lipid bilayer; for instance, free dye molecules in solution or molecules bound to the thick toroidal edges of the black film.

Bilayers Stained with 1,8-ANS

Characteristic fluorescence signal components obtained from lecithin membranes labelled with 1,8-ANS are illustrated in Figure 4. According to Conti and Malerba (1972) fluorescence signals corresponding to negative voltage pulses (100 mV amplitude, 1.2 ms duration, 11 ms repetition rate), were found to have similar shape and size but different sign than those corresponding to positive pulses. The signals amplitudes were found to be smaller than those from bilayers stained with 2,6-MANS. On the average, records with good signal to noise ratio could be achieved after repetition of 33,000 trials. Because of the long averaging-time required to detect good records, the number of polarized components measured from the same membrane was reduced. Therefore, to get a reliable statistical estimate of the size of each signal component we had to make measurements over more than fifteen lecithin membranes.

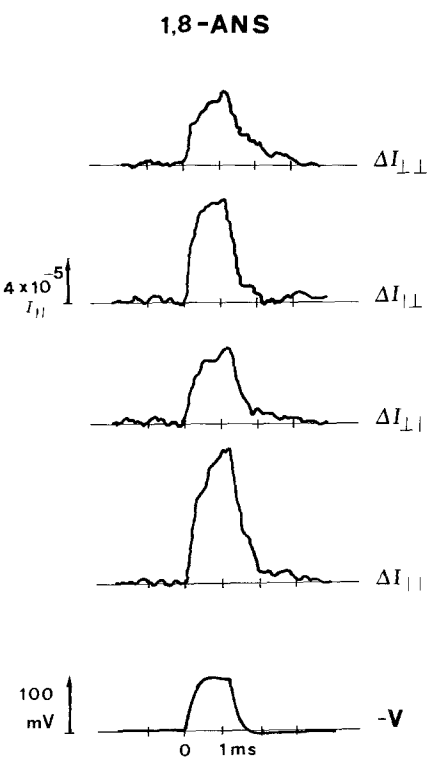

Fig. 4. Changes in fluorescence light intensity from 1,8-ANS stained bilayers, during applied voltage pulses. The bottom trace represents the time course of the square voltage pulse as appears at the output of the Tek-122 amplifier used for recording the optical signals. Band width 8 Hz—1 kHz. The concentration of the staining solution was 5×10^{-4} M. T = 12° ÷ 18° C

The procedure to obtain optical records was identical to that followed for 2,6-MANS. Similar phenomena were observed during a whole experiment. Striking differences could be noticed between the relative amplitudes of the polarized signal components. For instance, in membranes stained with 2,6-MANS, $\Delta I_{\parallel}$ and $\Delta I_{\perp\perp}$ stay in the ratio of 1 to 3 while in the case of 1,8-ANS their ratio increases about six fold. With reference to the parallel-parallel component the calculated average values of the three normalized components are: $\Delta I_{\perp\parallel}/\Delta I_{\parallel} = 0.82 \pm 0.02$; $\Delta I_{\perp\parallel}/\Delta I_{\parallel} = 0.59 \pm 0.02$ and $\Delta I_{\perp\perp}/\Delta I_{\parallel} = 0.53 \pm 0.02$ (mean $\pm$ S.D.).

Discussion

General Remarks

It has already been stressed elsewhere (Conti, 1975) that the fluorescence polarization properties of non-isotropic distribution of chromophores, such as stained lecithin membranes, are not easy to understand unless specific assumptions are made about the geometry and physico-chemical properties of the system under examination. On the other hand anisotropic perturbations to the system such as electric fields applied across the membrane, will create enormous difficulties to the analysis of the problem if the overall effects produced by the perturbing cause are not taken into proper consideration. A rigorous treatment of the problem, hence, would require the use of a specialized mathematical model whose physical and geometrical parameters are derived either from direct experimental data or from reasonable assumptions in harmony with the experimental conditions.

In our case a great deal is known about the molecular structure of the dyes employed. Spectroscopic and X-ray studies of a compound similar to 2,6-MANS (McClure and Edelman, 1966; Turner and Brand, 1968; Camerman and Jensen, 1970) have shown that the molecule has an almost planar configuration with the emission and absorption oscillators roughly separated by 25°. Furthermore, according to the studies of Barker et al. (1974) 2,6-MANS assumes a well defined orientation when incorporated into the membrane matrix, with its longitudinal axis almost perpendicularly alligned to the membrane surface. In normal conditions the sulfonate group of the molecule is faced toward the surrounding polar medium while the naphthalene and aniline rings are burried inside the lipid hydrocarbon chains. Quite different is the case of 1,8-ANS. Because of its compact molecular structure the orientation of the probe inside the membrane is not as well defined as 2,6-MANS. According to Gulik-Krzywicki et al. (1970), Haynes and Staerk (1974), 1,8 ANS binds the membrane at the water-lipid interface but its hydrophobic part (the naphtalene ring) does not reach the hydrocarbon region. The molecule benefits in this case of a wide possibility of movements inside the bilayers while the 2,6 compound is expected to be held more tightly at the binding site, i.e., 2,6-MANS adsorbed molecules are less free of undergoing rotational relaxations. In contrast, however, 1,8-ANS would not fully satisfy the condition that cos γ is equal 1 ($\gamma = 0$), as could be reasonably assumed for 2,6-MANS. Such condition simplify remarkably the analytical procedure of solving Equations (5), making the results easier to be interpreted on pure geometrical basis. Incidentally, the behavior of 2,6-MANS and 1,8-ANS is

representative of the chromophores belonging to the family of 2,6 and 1,8 N-aryl-aminonaphthalenesulfonates derivatives (Tasaki et al., 1973).

The main difficulty arising from the theoretical analysis of the problem concerns the interpretation and justification of several hypotheses made about the kinetics of the phenomena responsable for the fluorescence changes. An accurate study reveals that probes orientations are mainly due to the direct interaction of the dye-dipole moment with the applied electric field, rather than to changes in the structure of the dye binding sites (Conti et al., 1974). Along with this the amount of energy involved in such interaction is proportional to the scalar product of the electric dipole $\vec{\mu}$ associated to the absorption (or emission) oscillators and the electric field variation, $\Delta\vec{\mathbf{E}}$, acting over the bound chromophore. Consequently to the free energy change the adsorbed dipole is expected to vary almost instantaneously its average position and orientation, the extent of movements being limitated by the surrounding macromolecular array of the probe binding site.

For compounds like 2,6-MANS and 1,8-ANS (having the emission dipole moment larger than the absorption), displacements and orientations will be more intenses when molecules are in the excited singlet state rather than in the ground state. Then, the change of the Φ_i quantities can be assumed to depend mainly on the variation of the angle θ_2 formed by the emission oscillator and the normal to the membrane, $\vec{\mathbf{n}}$, justifying the assumption that $\dot{\Phi}_i$ depends merely on the variable θ_2. The assumption is fully satisfied, at least in the case of 2,6-MANS which is known to own largely different dipole moments in the excited and in the ground state.

Orientation and Motion of Membrane Adsorbed Probes

The possibility of assuming the angle γ equal zero in the case of 2,6-MANS allows us to solve uniquely the mathematical system of Equations (5), simplifying enormously the relationship between the angles θ_1 and θ_2. Under these conditions both oscillators would lay on the same plane, separated by $\lambda = |\theta_2 - \theta_1|$. According to the in vitro studies of Mc Clure and Edelman, 1968, on the degree of fluorescence polarization of 2,6-TNS (2p-toluidinylanaphtalene-6-sulfonate) and those on 2,6-MANS of Carbone et al., 1975, a good estimate of the angle λ is found to be 25° [2]. By substituting $\theta_2 = \theta_1 + 25°$ and $\cos\gamma = 1$ into Equations (5), the mathematical system will reduce the number of unknown variables requiring only two equations rather than three, to be solved. The third Equation (5c), however, is employed for both verifying the validity of the found solutions and limiting the range of scattered mathematical solutions of the problem.

A theoretical solution satisfying the experimental values of Table 1 can be derived by solving directly Equations (5a), (5b) and (5c). This can be done in many ways. One way consists of plotting Equations (5) as a function of θ_1 and g, looking for the intersection point of the three curves. Among the intersections (one for each value of g), we will choose those with a real physical meaning, i.e., those

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[2] Recent studies on the fluorescence polarization properties of 2,6-TNS incorporated into oriented poly (vinyl alcohol) sheets confirm that the angle formed by the emission and the absorption oscillators is very close to 25° (Tasaki et al., 1974)

satisfying simultaneously Equations (3a) and (3b). In the case of 2,6-MANS, the best fitting of the experimental results is obtained for the following values of the unknown parameters: $\theta_1 = 32.2°$, $\theta_2 = 57.2°$, $\varrho/\tau = 1.67$ ($h = 2.45$) and $\alpha = -1.99$. For experimental values ranging in the limits of the standard deviation the mathematical results of θ_1, θ_2 and ϱ/τ are found to vary slightly from the mean. If the same procedure is followed to compute the theoretical solutions from the squid axon data (see Table 1), one obtains similar results as those already published in early works (Conti, 1975; Carbone et al., 1975), indicating that the correct use of the g term does not introduce any further modification to the analytical interpretation of previous experimental results.

Quite different from 2,6-MANS is the case of 1,8-ANS. These latter molecules do not own a planar configuration. Furthermore they do not possess a dipole moments difference enough large to justify the assumptions made for the 2,6 compound. This implies that in the case of 1,8-ANS the angle γ is no longer equal zero and that the quantities $\dot{\Phi}_i$ are in part influenced also by the variation of θ_1, restricting the validity of our working hypotheses. However, in spite of this difficulty it is possible to show that Equations (5) can provide solutions with trustworthy meanings also in the case of 1,8-ANS stained lecithin bilayers.

Let us start from the observation that γ is no longer equal zero. This would imply that θ_1 and θ_2 do not lay on the same plane normal to the membrane surface. By writing the trigonometrical relationship between θ_1, θ_2 and γ, assuming $\lambda = 30°$ (the value is derived from the experimental data of L. Stryer, 1968), from Equations (5) we can get the solutions of the problem by proceeding as done for 2,6-MANS.

The values of the parameters better fitting the experimental findings shown in Figure 4 are: $\theta_1 = 107.8°$, $\theta_2 = 83.8$, $\gamma = 17.5°$, $h = 2.01$ and $\alpha = -1.58$. As can be noticed the value of h is remarkably small in comparison with the value of 2,6-MANS. This in part confirms our expectations that 1,8-ANS is more loosely bound to lecithin bilayers than the 2,6 compound. However, the value seems to us excessively small to represent the real physical situation, reflecting the limits of the model mentioned above. In fact $h = 2.01$ implies that the ratio ϱ/τ is much smaller than unity, being more appropriate for free molecules in solutions rather than for molecules bound to macromolecular structures. Another important observation should be made about the value of the angle θ_2 which is very similar to the angle found for 1,8-MANS bound to axon membranes ($\theta_2 = 75°$), indicating that the molecules are located in a very similar way in both, lecithin bilayers and squid axon membranes. This would suggest that both dyes are bound to corresponding regions of the two membranes.

2,6-MANS and 1,8-ANS as a Probe for Membrane Structure

The most relevant finding of our study is the small value of the parameter h, suggesting that the rotational relaxation time of membrane-bound chromophores is far shorter in lecithin bilayers than in axonal membranes. This occurs although dye molecules are adsorbed to black lipid films in similar fashion as to squid axon membranes. The values of θ_1 and θ_2 differ slightly from one case to the other (about

1° degree for 2,6-MANS), reflecting the existence of identical phospholipid structures in both, lecithin and axon membranes. Examples of natural membranes containing phosphatidylcholine molecules are quite common in the literature (Ansell, 1973). A second implication concerns the absolute value $h = 2.45$, found for the 2,6 isomer. If, as we think, 2,6-MANS is deeply hindered inside the phospholipid bilayers, it would be difficult to believe that planar molecules are free of rotating around their longitudinal axis. Hence, the rotational relaxations that chromophores undergo during the fluorescence lifetime would reflect the brownian motion of membrane phospholipid molecules. We noticed that the effects of the two motions are in any case undistinguishable, i.e., both tend to depolarize the fluorescence light coming from the adsorbed chromophores. Conversely, the high value of h found in stained axon membranes might suggest the existence of restricted motions of the phospholipid molecules, probably due to the presence of large proteins incorporated into the lipid matrix.

According to the aforementioned results a qualitative evaluation of the membrane area covered by proteic macrostructures can be done. An accurate analysis would be only matter of speculation, requiring specific notions about the geometry of membrane-macromolecules and the molecular array of surrounding phospholipids. Besides, a direct comparison of the results from the two types of membranes would not be quite adequate since both measurements were carried out at slightly different temperature: $12° \div 18°$ C for lipid bilayers and $4° \div 8°$ C for the axon membrane. Nevertheless, if we accept the idea of a fluid mosaic type of membrane the results can be easily explained by assuming that a large area of axonal membrane is occupated by integral proteins and that the remaining phospholipid portion is in part strongly coupled to the embedded membrane-proteins. Then, according to this picture only a small fraction of phospholipid molecules will be free of undergoing fast diffusional motions along the plane of membrane during the fluorescence lifetime (1—100 ns), causing a reduced depolarization of the total light coming from the bound chromophores responsable for the fluorescence changes. It is worth to recall that integral proteins incorporated in the membrane fluid phase have an effective viscosity 10^3—10^4 times that in water (Singer and Nicolson, 1972). Hence a large portion of axonal membrane area is likely to be immobile with respect to fast events such as the lifetime of excited dye molecules.

Similar conclusions can also be reached from the data analysis of the more popular fluorescent dye, 1,8-ANS. Comparing the value $h = 2.01$ from lecithin bilayers with the one found for 1,8-MANS applied to axonal membranes ($h = 2.46$) we will notice a strong analogy between the behaviour of the 2,6 and 1,8 compounds. A part from any other consideration about the absolute value of the parameter h, we can infer that both dyes have more rotational freedom when incorporated in lecithin bilayers than in squid axon membranes, reflecting a reduced exposure of the number of free phospholipid molecules to the staining solution.

Conclusions

As far as the fluorescence polarization studies are concerned many questions about the overall structural organization of the axonal membrane remain to be answered. In the present work we have investigated only a restricted number of physico-chemi-

cal parameters of a membrane-chromophores system. Our attention has been focused on the molecular orientation and rotational freedom own by the bound dye molecules. Certainly a lot of work remains to be done in order to get a clear picture of what is going on, when stained membranes are subjected to external electrical perturbations. However, up to this point several remarkable conclusions dealing with the structure and properties of axonal and model membranes can be drawn out from our studies.

The strong similarity between the two family of fluorescence polarization components from lecithin bilayers and squid axon membranes is with no doubt proving that signals are originated in both systems by identical mechanisms acting on chromophores located to corresponding regions of the membrane structure. On the other hand, it is most unlikely that same molecules bound with different orientations to separate membranes may yield equal fluorescence polarization characteristics. To prove how sensitive the method is to the orientation of the dye molecules is sufficient to compare signal components from membranes stained with 2,6-MANS and the positional isomer 1,8-MANS (Carbone et al., 1975). Relevant differences can also be observed between signals and background light components of the same stained preparation, proving that the method is highly sensitive for measuring the anisotropy of the system. All these theoretical considerations seem to us very convincing arguments for concluding that fluorescence signals from squid axons are originated by a small fraction of dye molecules bound to the accessible phospholipid regions of the membranes. Besides that, our findings reinforce the experimental evidences against any easy criticism about the nature of extrinsic fluorescence changes in stained lipid bilayers during application of potential steps.

The small value of the parameter h derived from both dyes agrees qualitatively well with the conclusions of Badley et al. (1971) and Yguerabide and Stryer (1971) even though different dyes and model membranes have been used. All the probes employed by those authors showed an appreciable rotational mobility parallel to the plane of the bilayers, which is substantially the conclusion we have reached from the study of 2,6-MANS and 1,8-ANS in lecithin bilayers. Of course black lipid films are, for our purpose, more appealing than any other kind of membrane. Unfortunately they require the application of small external perturbations for obtaining suitable results. A different approach to this problem has been recently undertaken from Zingsheim and Haydon (1973), by studying the *static* fluorescence properties of 1,8-ANS bound to black lipid films.

Quite interesting are the implications arising from the different value of the ratio ϱ/τ found in lecithin and axonal membranes. According to the proposed model, membrane bound chromophores have equal orientation and less freedom in axonal than lecithin membranes, indicating that molecules responsable for the fluorescence changes in squid axons are embedded into membrane regions having similar molecular organization but less mobility than the phosphatidylcholine molecules of lecithin bilayers. To explain this, it has been suggested that signals in squid axons might arise from those regions of phospholipid tightly bound to integral proteins of the membrane. This in turn would imply that large areas of squid axon membrane might be covered with protein macromolecules surrounded by strongly attached phospholipid molecules. Of course, up to now we are not able to infer anything about the extent of protein regions involved in the ionic-conductance changes occurring during nerve

excitation. Infact there are no experimental evidences that these regions are contributing in a consistent manner to the total fluorescence signal from stained nerves during electric stimulation.

As a final remark we stress the great utility that polarization techniques can have in studying the structure and dynamic behavior of lipid membranes, especially if applied to the study of the structural organization of in vitro reconstituted membranes.

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