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Fluorescence Polarization Studies of Squid Giant Axons Stained with N-Methylanilinonaphthalenesulfonates

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Abstract. The polarized components of the extrinsic fluorescence of squid giant axons stained with 2,6-MANS or 1,8-MANS were studied. The polarization properties of the fluorescence changes associated with voltage-clamp pulses were found to be very different from those of the static fluorescence, supporting the notion that the optical changes involve highly oriented membrane adsorbed fluorophores. The theoretical expectations according to this hypothesis are discussed in detail. The experimental results are in good agreement with the theory assuming that possible probes reorientations are solely due to the action of the applied electric field upon the probes electric dipole. The quantitative analysis of the data for 2,6-MANS provides a fairly accurate determination of the orientation of the membrane bound 2,6-MANS molecules responsible for the fluorescence changes. Such orientation appears to be independent of the membrane face exposed to staining. The data for 1,8-MANS indicate a very different orientation of this isomer. The results suggest a profitable use of extrinsic fluorophores for studies of the structural organization of nerve membranes.

Key words: Fluorescence Polarization — Nerve.

Extrinsic fluorescence polarization measurements have been shown to yield valuable information about the tertiary structure of proteins and complex macromolecules (for a review, see Weber, 1972). Recently this technique has been applied to membranes, in order to gain an insight into their structural characteristics (Yguerabide and Stryer, 1971; Radda and Vanderkooi, 1972). This type of measurements was also thought to clarify the origin of the fluorescence intensity changes occurring at the nerve membrane during the excitation process (Tasaki *et al.*, 1971, 1972, 1973).

Recent comparative experiments on lipid bilayers and voltage-clamped squid axons, carried out with a large number of N-Arylaminonaphthalene derivatives (Conti and Malerba, 1972; Conti *et al.*, 1974) have shown that the fluorescence signals in both preparations are very similar and linearly related to the potential difference applied across the membrane. This suggested that a simple physical mechanism, rather than changes in the conformation of membrane macromolecules, might account for the observed changes. Among the several possible origins of the voltage dependent fluorescence signals, the interaction of the electric dipole of membrane adsorbed chromophores with the applied electric field, was indicated as the only simple mechanism which could account for all the experimental results.

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

Since measurements of polarized fluorescence contain additional information, they provide a further check of the validity of this model, allowing to extract more specific information about the structure of the nerve membrane from extrinsic fluorescence techniques. 2,6-MANS and 1,8-MANS were chosen for the present study because they yield relatively large optical signals when applied either intracellularly or extracellularly to squid giant axons (Conti *et al.*, 1974).

Materials and Methods

Preparation

Giant axons 500 to 700 μm in diameter were dissected from the squid *Loligo vulgaris* available in Camogli. After thorough removal of the small fibers and surrounding connective tissues, a 20 mm length of the axon was stained intracellularly or extracellularly with the dye under study, using the same chamber and following the same procedure described in detail previously (Conti *et al.*, 1974).

Intracellular staining was preceded by intracellular perfusion of the axon, following the technique of Tasaki *et al.* (1962). Extensive use of the proteolytic enzyme Pronase allowed to remove any visible trace of residual axoplasm. The standard perfusion solution was a K^+ phosphate buffer (pH = 7.3 ± 0.1) containing 300 meq/l K^+ and 0.6 M sucrose to reach an osmolarity of 1.1 M. The staining solution, containing 5×10^{-5} M-2,6-MANS or 1,8-MANS, was kept flowing for more than ten minutes before, and left into the axon during, the optical measurements. The external medium was artificial sea water (ASW) having the following composition: 450 mM-NaCl, 10 mM-KCl, 20 mM-$CaCl_2$, 40 mM-$MgCl_2$, 1 mM-Tris chloride buffer, pH = 8 ± 0.1.

In the experiments of extracellular staining the intact axons were kept immersed in ASW containing 5×10^{-5} M-2,6-MANS or 1,8-MANS.

All the fluorescence measurements were carried out at relatively low temperature (2° C ± 10° C), monitored by a small thermistor placed close to the axon.

Pronase was purchased from Calbiochem (Los Angeles, California). 2,6-MANS and 1,8-MANS, synthesized according to Cory *et al.* (1968), were a generous gift of Prof. A. Azzi (Un. Padova, Italy).

Optical and Electronic Apparatus

The voltage-clamp apparatus was identical to that used for previous similar studies in our laboratory (Conti *et al.*, 1971, 1974).

A slightly modified version of the optical set-up used for previous studies (Conti *et al.*, 1971, 1974) was adopted in the present work. The exciting light source was a 100 Watt quartz-iodine filament lamp. From this, a parallel beam of quasi-monochromatic light of 365 (± 10) nm wavelength was obtained by means of a quartz condenser and a narrow-band interference filter (Thin Film Inc.). The beam was then focused onto the axon by means of a cylindrical lens. To provide linearly polarized light, a polarizer (Polaroid HNP'B) was introduced in the pathway of exciting light before the cylindrical lens. The fluorescent light was detected through a cut-off filter (Corning CS 3-74), at right angles to the exciting beam and to the axon longitudinal axis, with a photomultiplier (RCA/4523). In order to limit the solid angle through which the fluorescent light was observed,

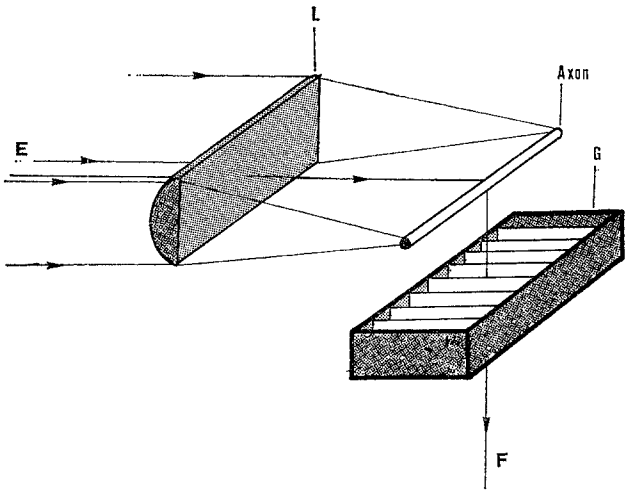

Fig. 1. Schematic drawing of part of the optical set-up used for polarized fluorescence measurements. The parallel beam of monochromatic exciting light *(E)* is usually polarized, with the electric vector either parallel or perpendicular to the axon longitudinal axis, by means of a polarizer not shown in the figure. The cylindrical lens, *L*, having a focal length of 26 mm, is 12 mm high and focuses the exciting light onto the axon. The fluorescent light *(F)* emitted perpendicularly to the axon longitudinal axis and to the exciting beam is selected by the grating system, *G*. The grating has external walls of black lucite and internal transverse walls of black-painted glass coverslips, 10 mm high and 2.5 mm apart from each other

a set of parallel black painted coverslips, 1 cm high and 2,5 mm apart from each other, oriented perpendicularly to the axon longitudinal axis, were placed just in front of the photomultiplier tube. With this arrangement, only the fluorescent light emitted in a direction nearly perpendicular (within $\pm$ 14°) to the axon longitudinal axis reached the photocathode. The amount of detected fluorescence was reduced in this way by about a factor five, but the geometry of the measurement was better defined for theoretical analysis (see Fig. 1). The two cross components of the emitted fluorescence were analyzed using a KN-36 Polaroid sheet. By convention the "parallel-parallel" and "parallel-perpendicular" components of the fluorescence were defined as those measured with the optical axis of the polarizer parallel, and that of the analyzer either parallel or perpendicular, to the axon longitudinal axis. Similar definitions apply to the "perpendicular-parallel" and "perpendicular-perpendicular" components, measured with the optical axis of the polarizer perpendicular to the axon longitudinal axis. In the present work we shall in most occasions refer to the "parallel-parallel", "parallel-perpendicular", "perpendicular-parallel" and "perpendicular-perpendicular" components, in the order, using the indexes 1 to 4. Alternatively, the same somponents will be also indicated with the symbols: $\parallel\parallel$, $\parallel\perp$, $\perp\parallel$, $\perp\perp$.

The absence of systematic errors in the evaluation of the relative intensities of the various polarized fluorescence components was tested by measuring the fluorescence of solutions of 2,6-MANS in glycerol. A glass capillary 1.2 mm in diameter was placed instead of the axon in the chamber containing ASW. The

four polarized components of the light reaching the photocatode were measured with the capillary filled either with pure glycerol or with 2,6-MANS in glycerol. The first set of measurements allowed to subtract, from the second set of values, small contributions due to reflections or scattering of the incident light not blocked by the cut-off filter. A correct performance of the apparatus should yield the same intensities for the parallel-perpendicular, perpendicular-parallel and perpendicular-perpendicular component (for this and other basic comcepts of polarized fluorescence, see e.g. chapter 4 of Pesce *et al.*, 1971). This condition was satisfied in our measurements within 5%. Furthermore, the degree of polarization, p, for 2,6-MANS in glycerol was found to be about 0.35, a value which is very close to that obtained by other authors for a similar dye, 2,6-TNS (McClure and Edelman, 1966; Tasaki *et al.*, 1972). The measurements of p provided also an estimate of the angle, λ, between the absorption and emission transition moments of 2,6-MANS, through the relationship (see Pesce *et al.*, 1971):

$$p = \frac{3\cos^2\lambda - 1}{\cos^2\lambda + 3}.$$

We obtained $\lambda \sim 28°$, to be compared with the value of about 23° for 2,6-TNS, deducible from the data of other authors (Tasaki *et al.*, 1972; McClure and Edelman, 1966).

For measurements of fluorescence changes, the *d.c.* output of the photomultiplier, proportional to the background fluorescence intensity, was bucked off. *a.c.* components were fed into a unity gain neutralized amplifier (ELSA 2), then amplified with a variable bandwidth amplifier (TEK-122), and finally processed with an average computer (HP 4580 B).

In the present work we were not particularly interested in the time course or in the voltage dependence of the optical signals, which have been studied previously (Conti *et al.*, 1974). Thus, the signal to noise ratio could be considerably improved by reducing the time resolution of the recording apparatus, while only fluorescence changes associated with voltage-clamp hyperpolarizations of 100 mV amplitude were measured. For pulses of 0.6 ms duration an optimal filtering condition was achieved with a band-width of 80 to 1000 Hz. Such short hyperpolarizing pulses could be applied to the axon at a frequency of about 100 Hz, allowing to average over a sufficient number of trials, and to obtain a good signal to noise ratio, in a relatively short time. We could often perform up to ten measurements under various light polarization conditions, on a same axon stained with 2,6-MANS, and still observe action potentials higher than 100 mV at the end of the whole experiment.

Results

Background Fluorescence

The polarization properties of the background fluorescence, I, from axons stained with 1,8-MANS or 2,6-MANS, are given in Table 1. The results of various measurements are reported in terms of the ratios $I_{\perp\perp}/I_{\parallel}$, $I_{\perp\parallel}/I_{\parallel}$ and $I_{\perp\perp}/I_{\parallel}$. For a system of randomly oriented fluorophores it is expected that these ratios are equal. Therefore, the differences shown in Table 1 indicate the presence of some anisotropy in the system under study. However, since the background light

Table 1. Polarization properties of the background fluorescence from axons stained either extracellularly (out) or intracellularly (in) with 2,6-MANS and 1,8-MANS. Standard deviations of the means are enclosed in parentheses

	$I_{\perp\parallel}/I_{\parallel\parallel}$	$I_{\perp\parallel}/I_{\parallel\parallel}$	$I_{\perp\perp}/I_{\parallel\parallel}$
2,6 – MANS out (5 axons)	.78 (.03)	.56 (.04)	.66 (.04)
2,6 – MANS in (3 axons)	.74 (.03)	.53 (.05)	.60 (.06)
1,8 – MANS out (3 axons)	.65 (.05)	.47 (.05)	.55 (.03)
1,8 – MANS in (2 axons)	.66 (.06)	.36 (.07)	.47 (.06)

can originate from many different sources (probes in solution, probes bound to various membrane components, residual axoplasmic proteins, a little amount of scattered light transmitted by the cut-off filter), it is impossible to derive any more meaningful information from these data. The main purpose of presenting them here is to point out the qualitative differences between these data and the measurements of fluorescence changes under voltage-clamp, presented below.

Fluorescence Changes during Voltage-Clamp

Records of fluorescence changes associated with voltage-clamp pulses, obtained from axons stained with 2,6-MANS, are shown in Fig. 2. Both the case of extracellular staining (A) and that of intracellular staining (B) are illustrated in the figure. The optical signals pertaining to each experiment were obtained with different positions of the polarizer and analyzer, as indicated. Notice that the calibrations in the figures relate to the ratio between the signal amplitude and one particular component of the background light rather than to each particular signal to background ratio. This is an important point to stress since, as discussed above, the background light depends on the polarization conditions explored.

A complete experiment on a single axon included usually the measurement of the fluorescence signals for all the four combinations of the polarizer and analyzer and a final control that the first signal observed could be still reproduced with the same amplitude. In many experiments the changes in the fluorescence intensity stimulated by unpolarized light and detected with the analyzer either in the parallel or in the perpendicular position, were also measured. These two signals correspond, after taking into account the optical transmission of the polarizer, to $(\Delta I_{\parallel\parallel} + \Delta I_{\perp\parallel})$ or $(\Delta I_{\parallel\perp} + \Delta I_{\perp\perp})$, respectively. They do not provide new information, but rather an independent experimental control.

Good signal to noise ratios in the detection of $\Delta I_{\parallel\perp}$, $\Delta I_{\perp\parallel}$ and $\Delta I_{\perp\perp}$ from axons stained with 2,6-MANS were usually obtained by averaging over 4000 trials,

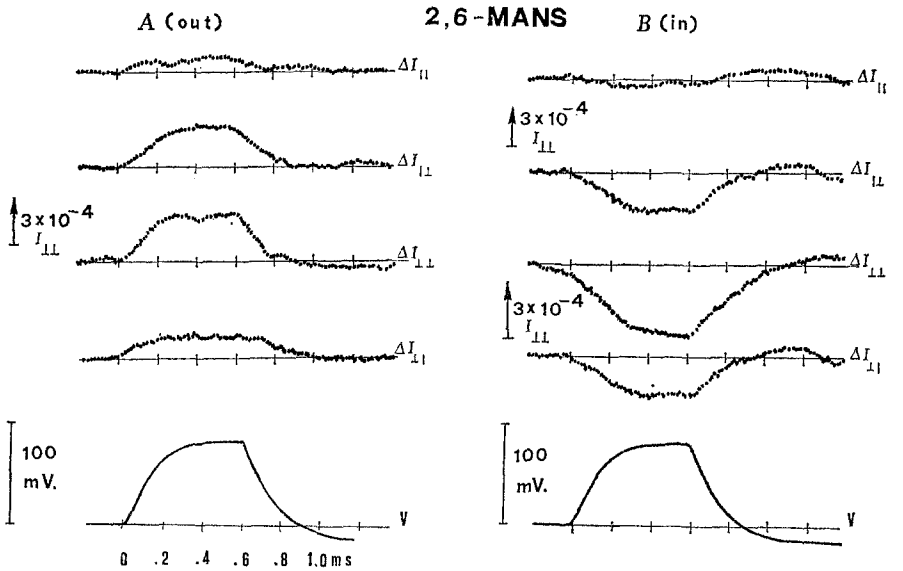

Fig. 2. Changes in fluorescence intensity, ΔI, associated with hyperpolarizing voltage-clamp pulses, in two axons stained with 2,6-MANS. The bottom traces show the applied voltage pulse (a square pulse with 100 mv amplitude) recorded with the same band-pass filter (80—1000 Hz) used to record the optical signals. For each axon and for all the components the arrows indicate a light increase of the same value stated in the figure, in units of the background light intensity measured with the "perpendicular-perpendicular" arrangement of polarizer and analyzer. *A:* extracellular staining; the record of $\Delta I_{||}$ is an average over about 16000 trials, all others averaged over about 8000 trials; $T = 6°$ C. *B:* intracellular staining; all records averaged over about 16000 trials; T = 2° C. The concentration of 2,6-MANS was 5×10^{-5} M in both experiments

while $\Delta I_{||}$ required al least 8000 trials to be revealed clearly. As a rule, no significant change in background light was observed during the buildup of each signal, indicating no significant photodegradation of the dye. Likewise, the fact that the axon was usually maintained in good physiological conditions throughout a complete experiment excluded important photodynamic effects of the UV radiation.

Full experiments such as those illustrated in Fig. 2 were performed successfully under constant conditions in five axons stained extracellularly and three axons stained intracellularly with 2,6-MANS. From the records obtained in each experiment the amplitudes of the various signals were measured as averages in the interval between 0.3 ms and 0.6 ms from the onset of the voltage-clamp pulse[1]. The polarization properties of the fluorescence changes were then characterized through the ratios between the amplitudes of $\Delta I_{||}$, $\Delta I_{|\perp}$ and $\Delta I_{\perp|}$, and that of the largest component, $\Delta I_{\perp\perp}$. These ratios should be independent of most variable experimental parameters, such as axon diameter, intensity of illumination and

[1] The bottom traces in Fig. 2 show the voltage-clamp command pulses filtered with the same bandwidth used to record the optical signals. It is seen that between .3 ms and .6 ms the distorted square wave is practically flat.

Table 2. Relative amplitudes of the extrinsic fluorescence changes observed under different polarizing conditions, in axons stained with 2,6-MANS extracellularly (out) or intracellularly (in). Standard deviations of the means are enclosed in parentheses. The values in the third row were derived according to theoretical analysis discussed on page 232

	$\Delta I_{\parallel}/\Delta I_{\perp\perp}$	$\Delta I_{\perp\parallel}/\Delta I_{\perp\perp}$	$\Delta I_{\parallel\perp}/\Delta I_{\perp\perp}$
2,6 - MANS out (5 axons)	.26 (.05)	.47 (.02)	.87 (.05)
2,6 - MANS in (3 axons)	.20 (.07)	.50 (.04)	.86 (.07)
THEORETICAL	.24	.48	.87

actual amplitude of the voltage-clamp pulse (provided the same pulse is used throughout a whole experiment). Table 2 summarizes the results obtained from complete experiments on axons stained with 2.6-MANS. The third row of Table 2 gives theoretical values according to the model discussed later in this paper.

Fig. 3 shows the results of two complete experiments on axons stained with 1,8-MANS. The fluorescence changes observed with this dye were considerably smaller and averaging over at least 33000 trials was required to get a fair estimate of their amplitude. This made it rather difficult to collect a full set of records from a single axon under constant conditions. Besides those illustrated in Fig. 3 only one more complete experiment, with an axon stained extracellularly, was successful and yielded results qualitatively similar to those of Fig. 3A. In four axons, two stained extracellularly and two stained intracellularly, only the changes $(\Delta I_{\parallel} + \Delta I_{\perp\parallel})$ and $(\Delta I_{\parallel\perp} + \Delta I_{\perp\perp})$ were measured. In all cases these changes were found to have approximately the same amplitude, in qualitative agreement with the results of Fig. 3. However, these measurements do not allow to improve significantly the estimate of the relative values of the individual signal components. Thus, our present data provide only a qualitative picture of the polarization properties of 1,8-MANS signals.

A full discussion of the results presented in this section will be given later. We shall stress here only the main features of the fluorescence changes shown in Fig. 2 and Fig. 3. It is seen that in axons stained extracellularly, the hyperpolarizing voltage-clamp pulses produced an increase or a decrease of all fluorescence components, depending on whether the dye used was 2,6-MANS or 1,8-MANS. Furthermore, all fluorescence changes were reversed when the dyes were applied intracellularly. Both these observations are in agreement with previous measurements of changes in total extrinsic fluorescence intensity (Conti *et al.*, 1974). On the other hand, it should be noticed that both with 2,6-MANS and with 1,8-MANS the polarization properties of the fluorescence signals (as characterized by the relative amplitudes of the individual components) are fairly independent on the membrane side exposed to staining. In the case of 2,6-MANS this invariance

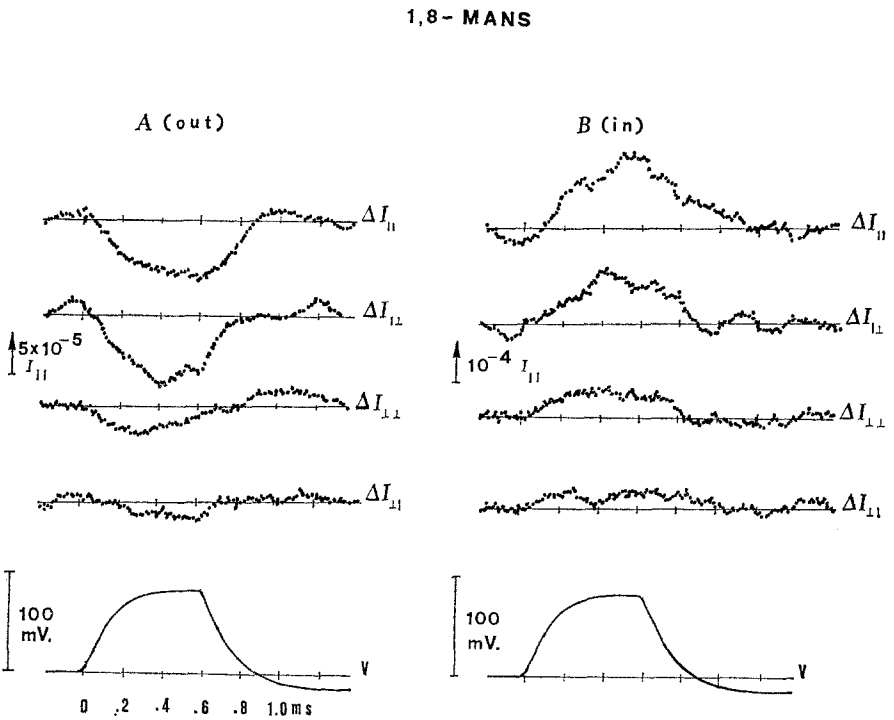

Fig. 3. Similar to Fig. 2, for two axons stained with 1,8-MANS 5×10^{-5} M. The calibrations of the fluorescence changes are given in units of the background light measured with the "parallel-parallel" configuration of the polarizer and analyzer. *A:* extracellular staining; all records averaged over about 33000 trials; T = 3° C. *B:* intracellular staining; all records averaged over about 33000 trials; T = 4° C

was verified rather accurately, as seen from Table 2. The most obvious differentiation between 2,6-MANS and 1,8-MANS, in terms of the polarization properties of their fluorescence changes, concerns the relative amplitudes of $\Delta I_{||}$ and $\Delta I_{\perp\perp}$. In axons stained with 2,6-MANS, $\Delta I_{\perp\perp}$ was the largest component and $\Delta I_{||}$ the smallest component of the observed fluorescence changes; while staining with 1,8-MANS yielded nearly the opposite result.

Theoretical Considerations

The main task of the present work is to test whether or not the results of polarized fluorescence measurements are compatible with the hypothesis of a particular physical mechanism of generation of the fluorescence signals, proposed on the basis of other experimental evidences (Conti and Malerba, 1972; Conti *et al.*, 1974). However, many of the theoretical considerations reported in this section are quite independent of such particular hypothesis, which will be used only at the end for specializing the discussion to the particular experimental situation explored in this work.

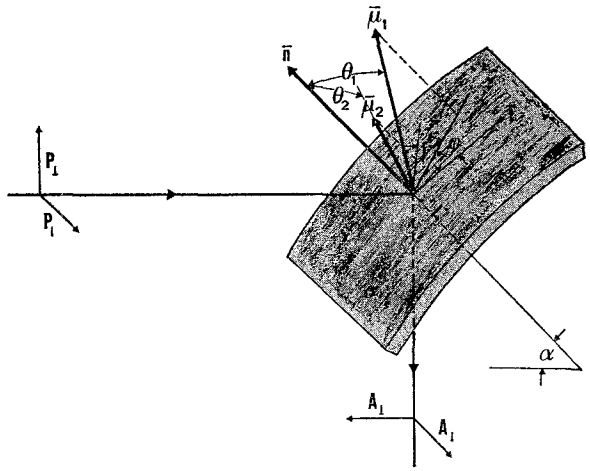

Fig. 4. Schematic drawing of an element of axon membrane, σ, whose normal, $\vec{n}$, forms an angle α with the direction of the exciting beam. A chromophore adsorbed on σ is represented by its absorption and emission oscillators, $\vec{\mu}_1$ and $\vec{\mu}_2$, forming angles θ_1 and θ_2 with $\vec{n}$. t is a local axis of reference on the plane of σ. φ and γ refer to the projections on this plane of $\vec{\mu}_1$ and $\vec{\mu}_2$, *at any given time*. $P_\parallel$ and $P_\perp$ indicate the directions of the electric vector for exciting light polarized either parallel or perpendicular to the axon longitudinal axis. With the same meaning, $A_\parallel$ and $A_\perp$ refer to the poalrized components of the fluorescence, emitted at right angles to the exciting beam and to the axon longitudinal axis

Let us assume that the optical changes produced by voltage-clamp pulses applied to a stained axon involve only one type of membrane bound probes[2], and that the probes fluorescence in solution is negligible with respect to that in the membrane-bound state. Any particular component, F_i, of that portion of the background fluorescence which is susceptible to changes, can then be written as;

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

where: K is a constant; N is the surface density of the probes involved in optical changes; Q is the average product (over the spectra of the exciting light and of the sensitivity of the detection apparatus) of the absorption efficiency for excitation with the electric vector parallel to the absorption transition moment, times the fluorescence quantum yield; Φ_i is a quantity which depends on the orientation of the bound probes with respect to the membrane surface.

In order to derive expressions for the relative values of the quantities Φ_i, we shall refer to Fig. 4. This shows a schematic picture of a small area of axonal membrane, σ, illuminated by an exciting light beam perpendicular to the axon longitudinal axis and forming an angle, α, with the normal to the membrane, $\vec{n}$. A fluorescent probe adsorbed on σ is represented in the figure by its absorption

[2] The justification of this assumption relies purely upon the fact that it is sufficient to account for the experimental results. However, the possibility that the optical changes originate from probes bound to n different types of membrane sites, cannot be ruled out. In this latter case the theoretical problem would not be conceptually more complicated, but it would involve n times as many parameters.

and emission oscillators, $\vec{\mu}_1$ and $\vec{\mu}_2$, respectively. θ_1 and θ_2 indicate the angles that these oscillators form with $\vec{n}$, and γ indicates the angle between their projections on the membrane surface *at any given time*. Since there is no evidence of particular structures in the nerve membrane which make its surface macroscopically anisotropic, we must assume that $\vec{n}$ is a local axis of symmetry for σ and that all values of the angle ϕ in Fig. 4 have the same probability. On the other hand, it seems reasonable to assume that the angles θ_1, θ_2, and γ are fixed (apart from small thermal fluctuations) by the interaction of the probe with its binding site. Thus, the extrinsic fluorescence of the elemental area σ is that of a system of fluorophores, of the type shown in Fig. 4, having fixed orientation parameters θ_1, θ_2 and γ, a random distribution of ϕ values, and, possibly, undergoing Brownian rotations around $\vec{n}$. The relative intensities of the four polarized components of the fluorescence emitted by σ can then be evaluated following standard procedures (see e.g. chapter 4 of Pesce *et al.*, 1971), and the properties of the whole axon are obtained after integrating over its cylindrical surface (i.e. over all values of α):

$$\Phi_{||} = \Phi_1 = \langle \int_0^{2\pi} \int_0^{2\pi} (\sin\theta_1 \sin\phi)^2 \, [\sin\theta_2 \sin(\phi + \gamma')]^2 \, d\phi \, d\alpha \rangle \tag{2a}$$

$$\Phi_{|\perp} = \Phi_2 = \langle \int_0^{2\pi} \int_0^{2\pi} (\sin\theta_1 \sin\phi)^2 \, [\sin\theta_2 \cos(\phi + \gamma') \sin\alpha - \cos\theta_2 \cos\alpha]^2 \, d\phi \, d\alpha \rangle \tag{2b}$$

$$\Phi_{\perp|} = \Phi_3 = \langle \int_0^{2\pi} \int_0^{2\pi} [\cos\theta_1 \sin\alpha + \sin\theta_1 \cos\phi \cos\alpha]^2 \, [\sin\theta_2 \sin(\phi + \gamma')]^2 \, d\phi \, d\alpha \rangle \tag{2c}$$

$$\Phi_{\perp\perp} = \Phi_4 = \langle \int_0^{2\pi} \int_0^{2\pi} [\cos\theta_1 \sin\alpha + \sin\theta_1 \cos\phi \cos\alpha]^2 \, [\sin\theta_2 \cos(\phi + \gamma') \sin\alpha - \cos\theta_2 \cos\alpha]^2 \, d\phi \, d\alpha \rangle , \tag{2d}$$

where γ' is the angle between the projection on the membrane surface of the absorption oscillator and that of the emission oscillator at the time of absorption and at the time of emission, respectively (γ' coincides with γ when Brownian rotations are absent); and the brackets, $\langle\rangle$, indicate a stochastic average over the distribution of γ'. After performing the integrations and the stochastic averages, Eqs. (2) become:

$$\Phi_1 = h \sin^2\theta_1 \sin^2\theta_2 \tag{3a}$$

$$\Phi_2 = 2\,(1 - h \sin^2\theta_2/4) \sin^2\theta_1 \tag{3b}$$

$$\Phi_3 = 2\,(1 - h \sin^2\theta_1/4) \sin^2\theta_2 \tag{3c}$$

$$\Phi_4 = 1 + (\sin^2\theta_1 + \sin^2\theta_2)/2 + (h - 16) \sin^2\theta_1 \sin^2\theta_2/8 - 2 \cos\gamma \sin\theta_1 \sin\theta_2 \cos\theta_1 \cos\theta_2 \tag{3d}$$

with:

$$h = 2 + \varrho\,(2\cos^2\gamma - 1)/(\varrho + 2\tau) , \tag{4}$$

ϱ being the relaxation time for the Brownian rotations around $\vec{n}$, and τ the fluorescence lifetime. The stochastic average of $\cos\gamma'$, intervening in the determination of Eqs. 3, was evaluated using a Soleillet's equation for one dimensional rotations and following classical arguments (see chapter 4 of Pesce *et al.*, 1971), according to:

$$\langle 2\cos^2\gamma' - 1 \rangle = (2\cos^2\gamma - 1)\,\varrho/(\varrho + 2\tau) . \tag{5}$$

Eqs. (3) show that the static fluorescence from a system of fluorophores adsorbed on nerve membranes may have in general polarization properties very different from those of an isotropic bulk phase. In the latter case we expect $\Phi_2 = \Phi_3 = \Phi_4$ and no dependence from any angular parameter other than the fixed characteristic angle, λ, between $\vec{\mu}_1$ and $\vec{\mu}_2$. This general consideration *per se* should warn against straightforward applications to stained nerve preparations of concepts and theoretical results which concern the fluorescence of bulk phases.

Having characterized the properties of the static fluorescence through Eqs. (1) and (3) we can now proceed to the evaluation of the expected properties of the fluorescence changes which might be produced by perturbations of our system of membrane adsorbed probes. It is seen from Eq. (1) that these changes may have in general three different origins (Conti, 1975): changes in the density of adsorbed fluorophores, N; changes in the fluorescence properties of membrane bound probes, characterized by Q; changes in Φ_i due to probes' reorientations.

For small perturbations, disregarding second order terms in the variations ΔN, ΔQ and $\Delta\Phi_i$, any particular component, δ_i, of the fluorescence change can be written as:

$$\Delta I_i = \Delta F_i = \delta_i = K\,(Q\,\Phi_i\,\Delta N + N\Phi_i\,\Delta Q + NQ\,\Delta\Phi_i) \quad (i = 1, 2, 3, 4)\,. \tag{6}$$

The separate contribution of the first term on the right hand side of Eq. (6) might be estimated on the basis of its characteristic time course, as discussed elsewhere (Conti *et al.*, 1974; Conti, 1975). However, both this term and the next one have the same polarization properties of the background fluorescence, F. On the other hand, since the quantities Φ_i have different functional dependence on θ_1 and θ_2, the relative contribution of the third term on the right hand side of Eq. (6) is expected to vary from one component to another. Therefore, in the presence of changes in the angles θ_1 and θ_2, the polarization properties of the total fluorescence changes are expected to be generally different from those of the background, F. The relation between ΔF and the total background light, I, is further remote.

When the major cause of probes' reorientations is the interaction of the probes' electric dipole with an applied electric field, Eq. (6) may be further simplified. For fluorophores such as 2,6-MANS it has been shown that the dipole moment in the first excited singlet state is much larger than in the ground state (Seliskar and Brand, 1971). Therefore, it appears justified to assume that these probes undergo the most significant rotations only after having absorbed a light quantum. Thus:

$$\Delta\Phi_i = \left(\frac{\partial\Phi_i}{\partial\theta_1}\right)\Delta\theta_1 + \left(\frac{\partial\Phi_i}{\partial\theta_2}\right)\Delta\theta_2 \sim \left(\frac{\partial\Phi_i}{\partial\theta_2}\right)\Delta\theta_2 \qquad (i = 1, 2, 3, 4)\,. \tag{7}$$

With the above approximation Eq. (6) yield:

$$\delta_i/\delta_j = (\alpha\Phi_i + \partial\Phi_i/\partial\theta_2)/(\alpha\Phi_j + \partial\Phi_j/\partial\theta_2)\,, \quad (i, j = 1, 2, 3, 4) \tag{8}$$

where

$$\alpha = (\Delta N/N + \Delta Q/Q)/\Delta\theta_2\,. \tag{9}$$

For any particular value of α, taking into account Eqs. (3) and (4), Eq. (8) give the expected relative values of the various components of the fluorescence changes as a function of the parameters θ_1, θ_2, γ and ϱ, which specify the interaction of the probe molecules with the membrane.

Conversely, it is possible in many ways to extract from Eqs. (8) two independent equations relating the interaction parameters to the experimental quantities δ_i/δ_j. A possible choice is given by the following Eqs. (10) and (11):

$$\sin\theta_1 = 2\,h^{-1/2}\,(1+2\,\delta_3/\delta_1)^{-1/2}\,, \tag{10}$$

$$\frac{\partial}{\partial\theta_2}\ln\left|(1-\delta_4\Phi_1/\delta_1\Phi_4)/(1-\delta_4\Phi_2/\delta_2\Phi_4)\right| = 0\,. \tag{11}$$

In Eq. (11) the explicit expressions of Φ_1, Φ_2 and Φ_4 [Eqs. (2a), (2b), (2d)] have been avoided for simplicity.

Finally, a third independent equation can be extracted from Eq. (8), yielding an explicit expression for α; e.g.:

$$\alpha = \frac{2\sin\theta_2\cos\theta_2}{4/h\,(1+2\,\delta_2/\delta_1)-\sin^2\theta_2}\,. \tag{12}$$

Analysis of Experimental Results

The Case of 2,6-*MANS*

For 2,6-MANS, quite a number of independent experimental evidences support the model and the assumptions underlying the theoretical results of the previous section.

Direct spectroscopic studies, on the interaction of 2,6-MANS with lipid bilayers, assign to this probe a well defined location and orientation upon adsorption at lipid-water interfaces (Barker *et al.*, 1974). These studies justify the model underlying Eqs. (1) to (4) in the case of the extrinsic fluorescence of lipid bilayers. On the other hand, the identity of the fluorescence signals in lipid bilayers and squid axons stained with 2,6-MANS strongly suggests that the optical changes in nerves originate from probe molecules bound to structures very similar to the lipid-water interfaces studied by Barker *et al.* (1974) (Conti *et al.*, 1974).

The hypothesis that possible probes reorientations are due mainly to the direct interaction with the applied electric field, rather than to changes in the structure of the binding site (or of the microenvironment), is supported by the studies of the characteristics of the 2,6-MANS fluorescence changes, both in aquid axons and in lipid bilayers (Conti *et al.*, 1974). In particular, it is worth mentioning that the rise time of these changes is much shorter than that of the birefringence or light scattering changes, following voltage-clamp steps in squid axons (Cohen *et al.*, 1971, 1972). Furthermore, 2,6-MANS is one of the compounds which shows the largest difference between the dipole moments of the first excited singlet state and that of the ground state (Seliskar and Brand, 1971). The assumption that the most significant electric-field-induced rotation of this probe occurs just before the fluorescence emission [see Eq. (7)], seems therefore also justified.

The analysis of our present experimental results according to Eqs. (10), (11) and (12) is simplified further if we take into account the molecular structure of 2,6-MANS. This should not be very different from that of 2-p-toluidinylnaphthalene-6-sulfonate (2,6-TNS), which is known to have an almost planar configuration (Camerman and Jensen, 1970). Thus the transition moments of absorption and emission are both expected to lie on the plane of the aromatic rings of the molecule, which is set perpendicular to the membrane surface when the probe

is adsorbed at the water-membrane interface (Barker *et al.*, 1974). It follows that, if Eqs. (10) and (11) provide a correct theoretical description of the 2,6-MANS fluorescence changes, they must admit a solution either for $\cos\gamma = 1$ and $\theta_2 = \theta_1 + \lambda$, or for $\cos\gamma = -1$ and $\theta_1 + \theta_2 = \lambda$ (see Fig. 4).

Conversely, if we impose the above conditions on the angles γ, θ_1, and θ_2, Eqs. (10) and (11) contain only two unknown interaction parameters, ϱ and (e.g.) θ_1, since λ can be evaluated from independent measurements. Solving these equations would lead to the determination of these parameters, from our present data of polarized fluorescence changes.

The existence of a solution of Eqs. (10), (11), (12), satisfying the above requirements and correctly describing our data, can be verified directly by evaluating the various δ_i/δ_j from Eqs. (8) and (3), for the following values of the parameters: $\theta_1 = 31.2$; $\theta_2 = 56.2$ ($\lambda = 25°$); $h = 3$ ($\varrho/\tau \to \infty$); $\alpha = -1.75$. The results are reported in Table 2 for direct comparison with the experimental data and it is seen that the agreement is quite good. For the above setting of the parameters, the theoretical contributions to the total fluorescence changes from the first two terms on the right hand side of Eq. (6), or from the third one, would stay in the ratios: —1.31 to 1, for δ_1; 1.22 to 1, for δ_2; —1.31 to 1, for δ_3; —3.39 to 1, for δ_4. It is also worth stressing that the size of the rotations, required by the theoretical model to account for the observed absolute amplitude of the fluorescence changes, is indeed quite small. In the above example, for $\Delta\theta_2 = -8 \times 10^{-3}$ ($\sim -.5°$) and $\Delta Q/Q + \Delta N/N = \alpha\Delta\theta_2 \sim 1.4 \times 10^{-2}$, we expect from Eqs. (6), (9) and (3d): $\delta_4/F_4 \sim 10^{-2}$. This figure would be large enough, even if the probes involved in the generation of the signals contributed only to 1/30 of the total background light, I_4.

For values of the experimental variables δ_1/δ_4, δ_2/δ_4 and δ_3/δ_4 ranging within the standard error from their mean, the solutions of Eqs. (10) and (11), satisfying the above conditions for γ, θ_1 and θ_2, were found to fall in a very restricted range. The solutions were obtained by plotting both Eqs. (10) and (11) as explicit expressions of h as a function of θ_1 and looking for the intersections. For $\lambda = 25°$ we found solutions only for $30.4 < \theta_1 < 31.8$ ($\theta_2 = \theta_1 + \lambda$, $\gamma = 0$) and $2.5 < h < 3.7$. Since the permissible values of h range between 2 and 3 [see Eq. (4) with $\cos^2\gamma = 1$], $h = 3$ is estimated to be the most likely value compatible with our data. For slightly different values of λ ($\pm 3°$) these results would not be modified significantly. Only the estimates of θ_1 and θ_2 would be slightly alterated by the same amount. The value of 25° for λ was chosen merely as a compromise between our estimate of 28°, from the fluorescence polarization measurements described in the Methods, and the estimate of 23° for 2,6-TNS derived from data (probably more accurate) of other authors (McClure and Edelman, 1966; Tasaki *et al.*, 1972).

The Case of 1,8-MANS

The direct comparison between Fig. 2 and Fig. 3 shows a large difference in the behaviour of 1,8-MANS and 2,6-MANS, suggesting that the two chromophores are adsorbed on the axolemma with completely different orientations. This possibility had already been suggested in order to account for the opposite sign of the fluorescence changes yielded by two dyes (Conti *et al.*, 1974).

Our present data for 1,8-MANS are not susceptible to the same quantitative analysis described above for the case of 2,6-MANS. We have already pointed out the low accuracy of the data and the lack of an adequate number of complete experiments with 1,8-MANS. Besides, some of the particular hypothesis used for the analysis of the 2,6-MANS data are not justifiable as well in the case of 1,8-MANS. Thus, the difference between the dipole moment of the first excited singlet state and that of the ground state may not be large enough to fully justify Eq. (7). For N-phenyl-1-naphthylamine, a compound which is expected to have a structure similar to that of 1,8-MANS, this difference is estimated to be only about 2.5 Debye (Radda, 1971). Also, there is no evidence that 1,8-MANS molecules have a planar configuration, so that no trustworthy hypothesis can be made about the angle γ and the relationship between θ_1 and θ_2.

Despite the above reservations it can at least be shown that the observed polarization properties of the 1,8-MANS signals are not inconsistent with the theoretical model presented in this work. For $\theta_1 = 75°$, $\theta_2 = 45°$, $\gamma = 0$, $h = 3$, $\alpha \to \infty$, Eqs. (8) and (3) yield: $\delta_2/\delta_1 = 0.83$, $\delta_3/\delta_1 = 0.21$, $\delta_4/\delta_1 = 0.51$. Alternatively, for $\theta_1 = \theta_2 = 75°$, $\gamma = 31°$, $h = 2.46$, $\alpha = -1.28$, the same equations yield: $\delta_2/\delta_1 = 1$, $\delta_3/\delta_1 = 0.37$, $\delta_4/\delta_1 = 0.39$. Comparing these values with the results shown in Fig. 3, it is seen that these two hypothetical sets of parameters are both capable of giving a fair description of the experimental observations. In the first example ($\alpha \to \infty$) there would be no significant contribution to the fluorescence changes from probe's reorientations and the adsorption and emission oscillators would lye both on a plane perpendicular to the membrane surface. In the second example ($\alpha = -1.28$) some contribution from probes' reorientations would be present, but $\vec{\mu}_1$, and $\vec{\mu}_2$ would lie on a cone with axis $\vec{n}$. In both examples it is assumed that ϱ/τ is much larger than unity [see Eq. (4)] and that $\lambda = 30°$. This last value was chosen assuming a close similarity of 1,8-MANS to 1,8-ANS, a dye for which the estimate of λ can be deduced from published data of fluorescence polarization in highly viscous media (Stryer, 1965).

Although it appears that a situation intermediate between the two examples given above might yield the best description of our data, it is also clear that such a quantitative description would be inappropriate to the low accuracy of the data themselves. The only trustworthy conclusion which might be derived from the present analysis concerns perhaps the approximate estimate of 75° for θ_1, far apart from the value of 31° for 2,6-MANS.

Discussion

The large difference, between the polarization properties of the static fluorescence and those of the fluorescence signals, confirms the notion that these signals originate from chromophores which contribute only to a small fraction of the total background fluorescence. In further support of this notion we notice that, while the background fluorescence of axons stained with 1,8-MANS or 2,6-MANS have very similar characteristics, the polarization properties of the fluorescence changes yielded by the two dyes are very different.

While it is impossible to ascertain the relative contributions to the total background fluorescence from various possible sources (probes in solution, probes bound to connective tissues and Schwann cells or to axoplasmic proteins, probes

bound to the nerve membrane), we can be reasonably confident that the fluorescence changes associated with voltage-clamp pulses involve only probes in close interaction with the axolemma (but not necessarily all of them). The advantage of this better localization is obtained, however, at the expenses of a much more difficult interpretation of the experimental results. In fact, while relevant information from static fluorescence measurements could be obtained merely on the basis of the empirical calibration of the probes' fluorescence properties in various media (see e.g. Turner and Brand, 1968), the interpretation of our extrinsic fluorescence signals requires also particular assumptions about the possible changes, produced by the applied voltage pulses, both on the axolemma and on the probe molecules themselves.

We have presented in this work a theoretical analysis of the fluorescence changes, based on a simple model which had been suggested previously (Conti and Malerba, 1972; Conti *et al.*, 1974). The major assumptions of the model are that only one type of membrane bound probes is involved in the changes, and that possible probes' reorientations are solely due to the direct interaction of the applied electric fields with the probes' electric dipole. Perhaps, the most interesting result of our analysis is the realization that small rotations (0.5° or less) of membrane bound probes may yield relatively large fluorescence changes independently of any actual change in the fluorescence properties of the probes themselves. This type of effect is not new and it has been exploited, for exampie, to measure the electrical polarizability of rodlike DNA fragments stained with Acridine Orange (Weill and Hornick, 1971).

The results of all our measurements of polarized fluorescence signals were found to be fairly consistent with the theoretical expectations of the simple model, provided 1,8-MANS and 2,6-MANS are adsorbed on the axolemma with very different orientations of their absorption and emission oscillators with respect to the normal to the membrane surface. In the particular case of 2,6-MANS this consistency provides a rather strong support to the model, since the independent available informations about the structure of this probe and about its interaction with model membranes reduce the number of parameters involved in the theoretical equations. Conversely, assuming that the model is correct, our present experimental data would provide some nontrivial information about the interaction of 2,6-MANS with the axolemma.

The most striking prediction of the model is the high value of the parameter h, implying a rotational relaxation time much higher than the fluorescence lifetime. Studies of extrinsic fluorescence polarization indicate that at least some hydrophobic probes have rotational relaxation times around the normal to the surface of an adsorbing lipid bilayer of the order of the fluorescence lifetime (Yguerabide and Stryer, 1971). The high value of ϱ predicted from our present analysis appears, therefore, in contradiction with the basic notion, underlying this and similar previous works (Conti and Malerba, 1972; Conti *et al.*, 1974), that the fluorescence changes originate from probes adsorbed on lipidic regions of the axolemma. This might imply that the model is in some respects too simple to allow accurate predictions about the nerve membrane. However, the possibility that 2,6-MANS and 1,8-MANS behave differently from the probes studied by Yguerabide and Stryer (1971), or that the phospholipids of the axolemma have a strongly hindered

mobility along the plane of the membrane, due to their interaction with membrane proteins, cannot be excluded.

Although the experimental results which we have reported here concern only the dyes 1,8-MANS and 2,6-MANS, we believe that the simple theoretical model discussed in this work may also account for similar results of other authors obtained with different aminonaphthalene-sulfonate derivatives (Tasaki *et al.*, 1971, 1972, 1973; Davila *et al.*, 1974). In particular the opposite sign of $\Delta I_{||}$ and $\Delta I_{\perp\perp}$, in axons stained intracellularly with 2,6-TNS, can be accomodated within the framework of the model, without need of *ad hoc* hypotheses on the existence of two different 2,6-TNS binding sites undergoing different dynamic changes (Tasaki *et al.*, 1973; Davila *et al.*, 1974). For example, for $\theta_1 = 75°$, $\theta_2 = 100°$ ($\lambda = 25°$, $\gamma = 0$), $h = 3$ and $\alpha = -0.36$, Eqs. (8) yield: $\Delta I_{\perp\perp}/\Delta I_{||} = -0.33$; $\Delta I_{|\perp}/\Delta I_{||} = -0.15$; $\Delta I_{\perp|}/\Delta I_{||} = 0.2$. Should the above set of parameters give a fair picture of the adsorption of 2,6-TNS on the inner face of the axolemma, the discrepancy with the behaviour of 2,6-MANS could suggest an important role of the amino group (which might be protonated in 2,6-TNS and not in 2,6-MANS) in the interaction with the polar heads of membrane phospholipids.

The main purpose of the present study was to improve our understanding of the physical mechanisms underlying extrinsic fluorescence signals in nerves. We believe that such understanding will allow us to extract some useful information about the nerve membrane structure. One important step in this direction should be the comparison of the data presented in this work with the results of similar studies on artificial lipid bilayers.

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