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A Comparative Analysis of Extrinsic Fluorescence in Nerve Membranes and Lipid Bilayers

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Abstract. Changes in extrinsic fluorescence intensity, associated with step changes in membrane potential, have been studied in intracellularly or extracellularly stained squid axons, and in lipid bilayers, using six different aminonaphthalene dyes: 1,8-TNS; 2,6-TNS; 1,8-MANS; 2,6-MANS; 2,6-ANS and NPN*. In all preparations the optical signals were found to be roughly proportional to the voltage applied. All signals had a very fast initial component, which was followed in some case by a slower change in the same direction. The slow component was observed only in intracellularly stained axons, and not for all chromophores studied. 1,8-TNS, 1,8-MANS and 2,6-MANS yielded the largest fluorescence signals in all preparations. The sign of these signals was independent of the type of membrane studied. However, the fluorescence changes of 2,6-MANS were opposite to those of 1,8-TNS and 1,8-MANS. Staining of both sides of the axolemma with 1,8-MANS or 2,6-MANS showed that these dyes yield larger signals when applied to the extracellular face. The changes in fluorescence light intensity of 2,6-TNS, 2,6-ANS and NPN were smaller and their sign depended on the membrane preparation studied. The comparison of the extrinsic fluorescence signals from the nerve membrane and the phosphatidylcholine bilayer suggests strong similarities between the basic structures of the two systems. The variety of observed signals cannot be easily interpreted in terms of changes in membrane structure. A possible alternative interpretation in terms of electrically induced displacements, rotations and changes in partition coefficient of bound chromophores, is discussed.

Key words: Fluorescence — Nerves — Bilayers.

The technique of extrinsic fluorescence offers one of the most sensitive tools for probing the structure of biological systems. First introduced for studies of protein structures (Weber, 1952; see *e.g.* Stryer, 1968), this technique has been applied in recent years to the investigation of many biological membranes (for a review, see Radda and Vanderkooi, 1972).

The first successful observations of changes in extrinsic fluorescence in nerves (Tasaki *et al.*, 1968; 1969 [1]; 1969 [2]) and muscles (Carnay and Barry, 1969) during activity, were tentatively interpreted as direct evidences for changes in conformation of membrane macromolecules (Carnay and Tasaki, 1971). It is now clear that such interpretation cannot account for the changes in fluorescence observed in 1,8-ANS stained squid giant axons under voltage-clamp (Conti and Tasaki, 1970; Cohen *et al.*, 1970; Conti *et al.*, 1971; Cohen, 1973), which are not abolished by local anaesthetics and little affected by the drugs TTX and TEA

* *Abbreviations:* 1,8-TNS, 1-toluidinonaphthalene-8-sulfonate, and similarly, 2,6-TNS; 1,8-MANS, 1-N-methylanilinonaphthalene-8-sulfonate, and similarly, 2,6-MANS; 1,8-ANS, 1-anilinonaphthalene-8-sulfonate, and similarly, 2,6-ANS; NPN, N-phenyl-1-naphthylamine.

(Conti and Wanke, 1971). In addition to 1,8-ANS it has been reported that 38 other dyes, when applied to squid axons, yield fluorescence signals which are largely dependent on the voltage applied across the axolemma, rather than being directly related to the structural changes responsible for nerve excitation (Davila *et al.*, 1972; Cohen, 1973).

The aim of the present work is the understanding of the mechanisms underlying the voltage dependent extrinsic fluorescence signals, and of the possible informations that these signals can provide about the structure of the nerve membrane. We focused our attention on a particular class of dyes, the aminonaphthalene derivatives. Our approach is the comparative study of squid axons under voltage-clamp, and lipid bilayers. These two preparations, when stained with 1,8-ANS, have already been shown to yield very similar fluorescence signals (Conti and Malerba, 1971; 1972). We report here the results of extrinsic fluorescence measurements from voltage-clamped squid giant axons stained intracellularly or extracellularly with six different 1,8-ANS analogs, together with similar studies of lipid bilayers stained with the same fluorescent probes. An interpretation of the origin of the fluorescence signals, in terms of the interaction of the electric dipole of membrane adsorbed chromophores with the applied electric fields, has been already suggested (Conti and Malerba, 1972). Such interpretation, which can account also for the present experimental observations, will be discussed in further detail.

The results of the experiments on intracellularly stained axons and on lipid bilayers have been briefly reported elsewhere (Conti, 1972).

Materials and Methods

Extrinsic Chromophores

The structure of the six chromophores which have been used in the present study is shown in Table 1. All these dyes belong to the same family of 1,8-ANS and are known to have fluorescence properties particularly sensitive to the polarity of their microenvironment (see *e.g.* Turner and Brand, 1966; Cory *et al.*, 1968; Brand *et al.*, 1971).

The dyes were administered intracellularly to squid giant axons by adding them to the standard intracellular perfusion solution (see below) at the desired concentration. In the case of NPN, which is practically insoluble in water, the dye was introduced as a very fine suspension. The concentration at which all the other dyes were administered varied between 5×10^{-6} and 5×10^{-5} M.

In the experiments with extracellularly stained axons the extracellular medium consisted of artificial sea water (ASW; see below) containing the dye under study at concentrations between 5×10^{-6} and 5×10^{-5} M. For NPN the supernatant of a fully saturated solution in ASW was used. For studies of combined intracellular and extracellular staining, identical intracellular and extracellular concentrations were used.

The staining of lipid bilayers was obtained by adding small amounts of concentrated ethanol solutions of the various dyes to one side of the black film. The final concentrations ranged between 5×10^{-6} and 5×10^{-5} M, for 1,8-TNS, 1,8-MANS and 2,6-MANS, or were very close to saturation for 2,6-TNS, 2,6-ANS and NPN.

Experiments with Giant Axons

Giant axons, 500—700 μm in diameter, dissected from the hindmost stellar nerve of the squid *Loligo-Vulgaris*, have been used. The length of intact axon which was completely cleaned of small fibers and exposed to voltage-clamping and optical studies, was 20 mm.

In the experiments of intracellular staining the giant axons were previously internally perfused for 15 to 20 min with the technique developed by Tasaki *et al.*, 1962. Extensive use was made of the proteolytic enzyme Pronase, to obtain the most extensive removal of axoplasm compatible with no appreciable change in the electrophysiological properties of the nerve membrane. This was done in order to reduce as much as possible the amount of background fluorescence from chromophores bound to axoplasmic proteins. The standard perfusion solution was a potassium phosphate buffer containing 300 meq. K^+, added with sucrose to reach a final osmolarity of 1.1 M. The pH was 7.2 ± 0.1. When the removal of axoplasm was considered sufficient, the perfusion fluid was switched from the standard perfusate to one containing the dye under study. This new solution was kept flowing for few minutes and was left into the axon after the perfusion was stopped. After withdrawal of the perfusion pipettes, the internal stimulating and recording electrodes were inserted into the axon and fixed to the chamber, and the whole preparation was moved to the final position in the set-up, following the procedure described elsewhere (Conti and Tasaki, 1970; Conti *et al.*, 1971). In most experiments with internally stained axons the extracellular medium was ASW with the following composition: 450 mM-NaCl; 10 mM-KCl; 20 mM-$CaCl_2$; 40 mM-$MgCl_2$; 1 mM-Tris buffer at pH 8. In many experiments we used natural sea water (NSW), to which 10 mMoles per liter of $CaCl_2$ were added.

For studies of extracellular staining, the axons were kept, during the experiments, in ASW or NSW containing fluorescent dyes. To avoid excessive absorption of exciting light by chromophores in solution, the optical path in the extracellular medium was reduced to 2 mm.

All the measurements of fluorescence signals from squid axons were performed at relatively low temperatures (3 °C÷10 °C), as monitored by a small thermistor placed close to the axon.

The chamber in which the axons were mounted, the optical arrangement, the voltage-clamp system and the set-up for recording optical signals have already been discussed elsewhere (Conti *et al.*, 1971). A 100 W quartz-iodine incandescent lamp and a narrow band interference filter (Thin Film, Inc.) were used to provide quasi-monochromatic exciting light around 365 nm wavelenght. The fluorescent light was recorded, through a cut-off filter (Corning CS 3-74), with a photomultiplier (RCA 4523). The changes in fluorescence light intensity were extracted from the generally much higher background noise with the use of an average computer (HP 5480 B).

Experiments with Lipid Bilayers

Lipid bilayers were formed from a 15 mg/ml solution in *n*-decane of soy bean lecithin, purified with standard chloroform:methanol extraction procedures (Szabo *et al.*, 1969). The black films, 3÷4 mm in diameter, were formed on a black lucite septum separating two identical solutions of 0.5 M-KCl, $pH = 5.7 \pm 0.1$.

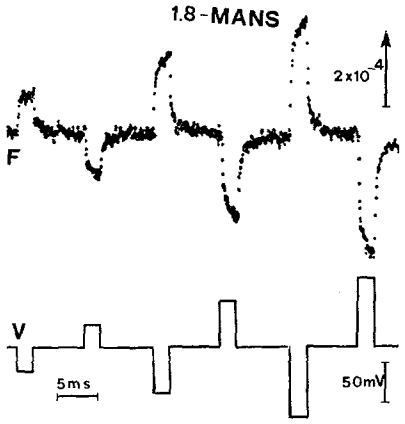

Fig. 1

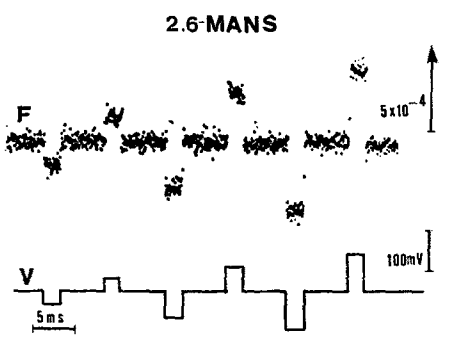

Fig. 2

Fig. 1. Changes in 1,8-MANS fluorescence intensity (F) associated with voltage-clamp pulses (V) in an internally stained squid axon. The optical record is an average over about 10,000 trials. In this and all other records from squid axons, the arrow indicates a fluorescence increase of stated value times the background light intensity; positive voltage pulses indicate depolarizations. The high cut-off frequency of the optical recording was 2 kHz, and the 1,8-MANS concentration in the perfusate was 10 μM. The interval between two sampling points was 50 μsec. Temperature: 4 °C

Fig. 2. 2,6-MANS fluorescence changes in an internally stained axon. High cut-off frequency of the optical recording: 10 kHz. 2,6-MANS concentration in the perfusate: 30 μM. Temperature: 8 °C. 1,000 trials averaged

The method for recording extrinsic fluorescence signals from lipid bilayers has been described previously (Conti and Malerba, 1972). The optical set-up for exciting and recording fluorescent light was very similar to that used for experiments with squid axons. Careful control measurements of the absence of light scattering signals (Conti and Malerba, 1971; 1972), were usually performed on the black films before staining. Long aging of the bilayers usually removed the major cause of light scattering artifacts, due to the presence of trapped solvent droplets (Andrews and Haydon, 1968). The smallest fluorescence signals (with 2,6-ANS, NPN and 2,6-TNS) could be recorded, without serious artifacts, only using short voltage pulses (< 1 msec) of small amplitudes ($\leqslant 100$ mV), more than two hours after the formation of the bilayers. All the measurements were performed at room temperature (18 °C÷20 °C).

Results

Intracellular Staining

Fig. 1 shows a record of fluorescence signals, associated with voltage-clamp pulses, from an axon perfused with a solution containing 10^{-5} M — 1,8-MANS. The top record is an average over about 10.000 trials. At each trial, a train of 6 different voltage-clamp pulses of alternating polarities was applied to the axon. The time course of the membrane potential is shown in the bottom trace. The changes in fluorescence light intensity were recorded with a high cut-off frequency

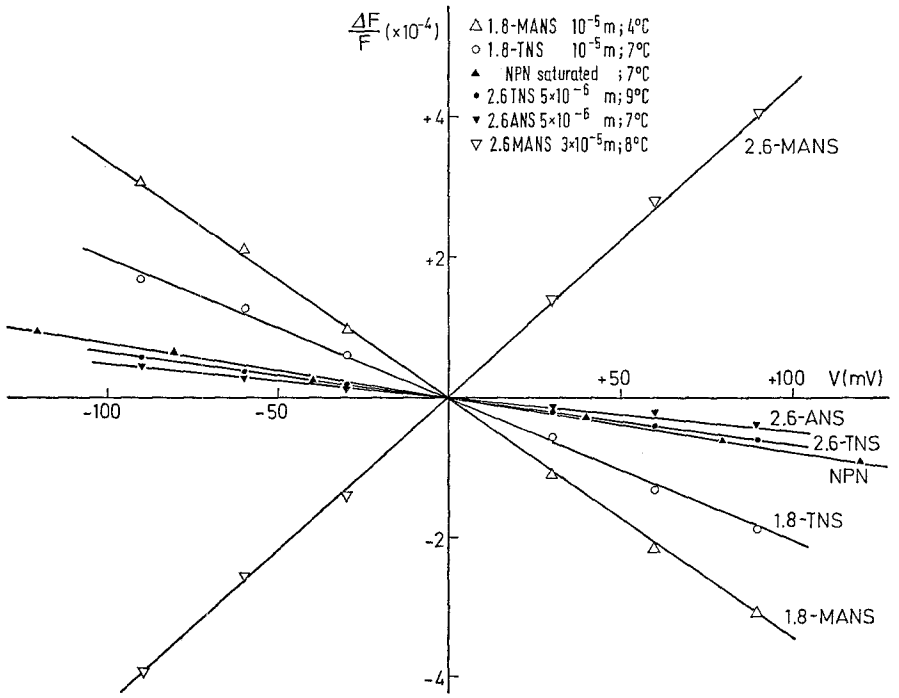

Fig. 3. Fluorescence intensity change versus displacement from resting potential (depolarizations positive) in squid axons stained intracellularly with different dyes. The fluorescence changes were measured at the end of voltage-clamp pulses lasting 2 ms. Dye concentration and temperature, in each particular experiment, as indicated

of 2 kHz. Fig. 2 shows the result of a similar experiment on an axon perfused with 3×10^{-5} M— 2,6-MANS. In this case the optical signals were averaged over about 1,000 trials, and the high cut-off frequency was 10 kHz. From records such as those shown in Figs. 1 and 2, the dependence of the fluorescence changes on potential can be determined in a single experiment.

Plots of the amplitude of the fluorescence signals, at the end of a voltage-clamp pulse lasting 2 msec, against voltage, are shown in Fig. 3. Each series of experimental data was obtained from a single axon, stained with one of the six dyes studied. It is seen that for all chromophores the experimental points fall near a straight line through the origin[1]. The slope of this straight line (in V^{-1}) will be taken as the measure of the fluorescence changes. The average values (in 3—6 axons) of the signals obtained using the various dyes are given in Table 1. Varia-

[1] In some experiments with 1,8-TNS and 1,8-MANS, the decrease in fluorescence associated with a depolarization was found to be clearly larger (up to 30 %) than the increase produced by a hyperpolarization of the same amplitude. These asymmetries may represent an important aspect of the 1,8-TNS and 1,8-MANS signals in connection with the excitability of the axolemma. However, these effects were not reproducible quantitatively and represent a minor detail of the overall optical responses. We have at present no elements for discussing them in any detail.

tions around the average were usually within ± 20%[2]. Except for 2,6-MANS, all dyes explored yielded negative signals, or, equivalently, a decrease in fluorescence intensity in response to a depolarization. 2,6-MANS yielded the largest fluorescence changes ($\sim 4 \times 10^{-3}$ V^{-1}) in the opposite direction.

Some qualitative features of the time course of the fluorescence changes in axons stained with 1,8-MANS, 1,8-TNS and 2,6-MANS could be easily revealed. As seen from Fig. 1, the monotonic change in 1,8-MANS fluorescence, following a step change in membrane potential, cannot be described as a single exponential. At 4 °C, at the end of a voltage-clamp pulse of 2 msec duration the signals have not reached a plateau, while 60% of the final fluorescence change occurs in about 150 μsec (3 sampling intervals, corresponding to the rise time of the recording apparatus). A similar time course was observed for the 1,8-TNS signals. From the results of the experiments described in this work, it is not possible to obtain a more quantitative description of the fast and slow components of 1,8-MANS and 1,8-TNS signals. The fluorescence signals from an axon stained intracellularly with 2,6-MANS (see *e.g.* Fig. 2) had a much simpler time course. They invariably appeared as square waves with a rise time determined by the response time of our recording apparatus. This indicated that the entire change in 2,6-MANS fluorescence occurs in much less than 30 μsec. In two axons stained with NPN the presence of a slow component in the signals obtained from double pulse (± 80 mV) experiments, could also be detected. The signals from axons stained with 2,6-TNS or 2,6-ANS were always too small to be analyzed with a high time resolution.

Extracellular Staining

Extrinsic fluorescence signals were obtained from squid axons stained extracellularly with 1,8-MANS, 2,6-MANS, 2,6-ANS and NPN. In all cases the fluorescence changes during voltage-clamp were found to be linearly related to the voltage applied.

Fig. 4 shows a record of optical signals from an axon stained with 1,8-MANS. It is seen that the changes in fluorescence intensity are opposite in sign to those observed, for the same voltage-clamp pulses, when this dye is applied intracellularly. The sign reversal was also observed by comparing the extracellular and intracellular staining with 2,6-MANS. However, in axons stained with 2,6-ANS or NPN, the sign of the fluorescence changes was independent of the side to which the dyes were applied. The average values of the signals obtained with various dyes are given in Table 1.

A comparison of Fig. 4 with Fig. 1 shows that the time course of the 1,8-MANS fluorescence changes is quite different when the dye is applied to opposite sides of

[2] Most of the fluctuations in the signal amplitude reflect variations in the background light intensity due to the different amount of residual axoplasm from one preparation to another. All the results presented in this work were obtained with axons in which all traces of axoplasm, visible with a normal dissecting microscope (50 *X*), were removed. It should be noticed that the signals reported here for 1,8-TNS and 1,8-MANS are roughly one order of magnitude higher than in axons intracellularly stained by injection of 1,8-ANS (Conti and Tasaki, 1970; Conti *et al*,, 1972; Cohen, 1973). We have verified that also 1,8-ANS yields signals of much larger size when administered to extensively perfused axons.

Table 1. Fluorescence signals in squid axons and lecithin bilayers stained with various N-aryl-aminonaphthalene dyes. The direction of the arrow indicates the direction of the fluorescence changes associated with depolarizations (squid axons) or with an increase in the electric potential of the stained solution (bilayers). The signal amplitude is given as the average value, from at least three different measurements, of the slope of the signal to voltage relationship. The fluorescence changes from squid axons were measured at the end of a 2 ms voltage-clamp pulse. The signals from lipid bilayers were measured with voltage pulses less than 1 ms in duration. For each membrane preparation, the lengths of the arrows give an idea of the relative amplitude of signals from various dyes. The signals marked with an asterisk were square waves, with a rise time shorter than 30 μs. The signals marked with two asterisks contained an additional slow component. For all other signals, the presence or not of slow components was not ascertained

CHROMOPHORE	FLUORESCENCE SIGNALS ($\times 10^{-3}$ V^{-1})		
	AXON INT. STAINING	AXON EXT. STAINING	BILAYERS
1.8-MANS	(* *) ↓ −3.2	(*) ↑ +.6	↓ −3.0
1.8-TNS	(* *) ↓ −1.7	—	↓ −2.0
2.6-ANS	↓ −.3	↓ −.1	↓ −.8
2.6-TNS	↓ −.8	—	↑ +.5
2.6-MANS	(*) ↑ +4.0	(*) ↓ −2.0	↑ +6.0
NPN	(* *) ↓ −.7	↓ −.3	↓ −.6

the axolemma. When applied extracellularly, both 1,8-MANS and 2,6-MANS yielded square fluorescence signals, with a rise time determined by the response time of the recording apparatus. The time course of the signals from axons stained with 2,6-ANS or NPN could not be analyzed.

Combined Intracellular and Extracellular Staining

In the attempt of finding quantitative differences between the intracellular and extracellular fluorescence signals of 1,8-MANS and 2,6-MANS, the following experiments on bilaterally stained nerve membranes were performed.

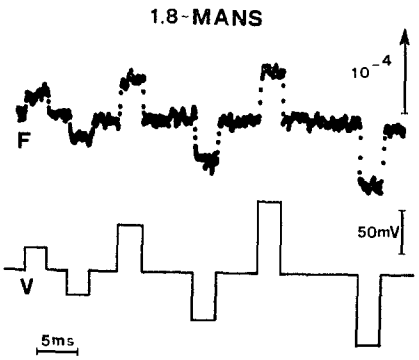

Fig. 4. 1,8-MANS fluorescence changes from an axon stained extracellularly. High cut-off frequency of optical recording: 1 kHz. 1,8-MANS concentration in sea water: 50 μM. Temperature: 4 °C. 8,200 trials averaged

Squid axons were first internally stained with the usual procedure and the amplitude of the fluorescence signals measured from low time resolution records. These preliminary measurements could be performed in less than 30 sec leaving the preparation physiologically unaffected. The external bath was then substituted with ASW containing the same dye concentration of the internal perfusate, and fluorescence signals from the latter preparation were recorded at time intervals of about 2 min. Thus, a gradual disappearance of the original signals was observed and in less than 10 min the fluorescence changes became of opposite sign and reached new steady values. In four axons, two stained with 1,8-MANS and two with 2,6-MANS, this type of experiments yielded the same qualitative results. Records from one of the experiments with 2,6-MANS are shown in Fig. 5.

The measurements described above can give an absolute estimate of the ratio between the amplitudes of extracellular and intracellular signals. In practice the accuracy of these measurements is reduced by two factors: 1. the absorption of incident light by chromophores in solution decreases the intensity of the exciting light actually reaching the axolemma in the second phase of the experiment; 2. the effective intracellular dye concentration may be smaller than in the original perfusate, if a significant amount of chromophores is bound to residual protoplasmic proteins. Considering these possible errors, which may compensate each other to some extent, the ratio between extracellular and intracellular signals was estimated to range between 1.2 and 1.5 for both 1,8 and 2,6-MANS.

Lipid Bilayers

All the chromophores studied in the present work yielded voltage induced fluorescence signals, when applied to one side of lipid bilayers. The fluorescence changes were always reversed by a change of polarity, and proportional to the amplitude of the voltage pulse which produced them. Optical signals from a bilayer stained with 2,6-MANS, recorded with a high cut-off frequency of 10 kHz, are shown in Fig. 6.

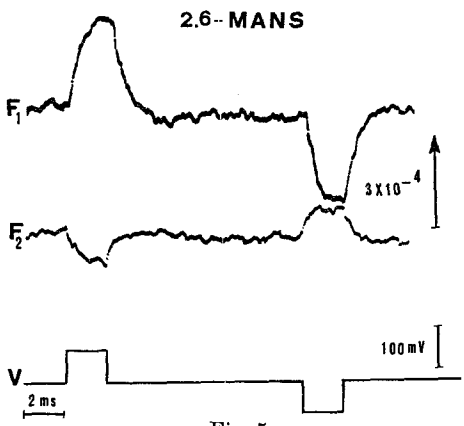

Fig. 5

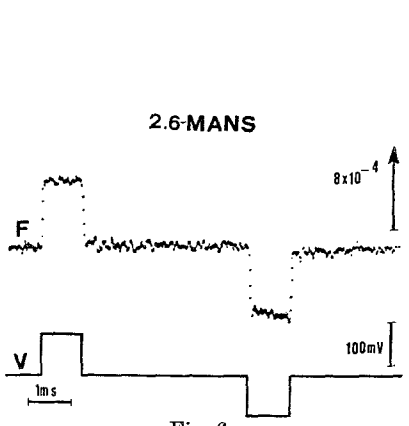

Fig. 6

Fig. 5. Records of fluorescence signals from an experiment of combined intracellular and extracellular staining of a giant axon. F_1: average record, over 128 trials, of fluorescence changes after internal staining. F_2: average record, over 512 trials, of fluorescence changes after staining the same axon also extracellularly. Both for F_1 and F_2 the arrow indicates a fluorescence increase of stated value times the background light during the measurements of F_1. High cut-off frequency of optical recording: 250 Hz. 2,6-MANS concentration was 50 μM, both in the perfusate and in sea water. Temperature: 5 °C

Fig. 6. 2,6-MANS fluorescence changes in a lecithin bilayer, stained on one side, under applied voltage pulses. A positive voltage indicates an increase in the electric potential of the stained solution. 2,6-MANS concentration in solution was 50 μM. High cut-off frequency: 10 kHz. Interval between two sampling points: 10 μsec. Temperature: 20 °C

Similarly to what observed in squid axons, stained either intracellularly or extracellularly, the largest optical signals were obtained with the dyes 2,6-MANS, 1,8-MANS, and 1,8-TNS. If a positive voltage indicates conventionally an increase in the electric potential of the solution containing the dye, the fluorescence changes of 1,8-TNS, 1,8-MANS, 2,6-MANS, 2,6-ANS and NPN in lipid bilayers, had the same sign as in intracellularly stained squid axons. The smallest signals from lipid bilayers were obtained after staining with 2,6-TNS. These signals were opposite to those observed in intracellularly stained axons. The average values of the signal amplitude for various dyes are given in Table 1.

For all chromophores the rise time of the fluorescence changes was determined only by the response time of the recording apparatus. Within 1 msec, significant contributions from slow components could be excluded in the signals of 1,8-TNS, 1,8-MANS, and 2,6-MANS. This is clearly shown in Fig. 6 for 2,6-MANS. The slow light scattering contaminations prevented any analysis of the late time course of the signals in bilayers stained with 2,6-ANS, 2,6-TNS or NPN.

Discussion

General Remarks

Our present results, summarized in Table 1, do not show significant differences in the fluorescence signals from squid axons or phosphatidylcholine bilayers. Thus there is no indication that the mechanisms underlying the changes in extrinsic

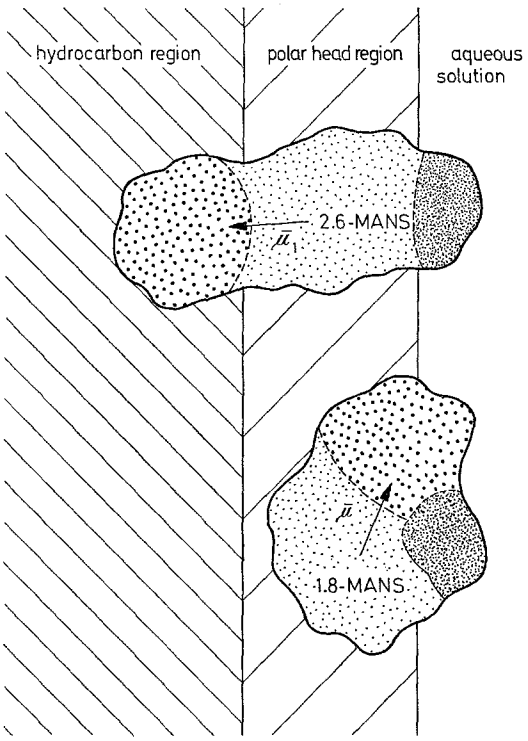

Fig. 7a. Model representation of the location and orientation of 1,8-MANS and 2,6-MANS at a membrane-solution interface. Compare Fig. 5 of Gulik-Krzywicki *et al.*, 1970; and Fig. 6 of Barker *et al.*, 1974. The silhouettes of the chromophores were obtained from space filling models. Vectors $\vec{\mu}$ and $\vec{\mu}_1$, represent the electric dipoles of the first excited singlet state. The orientation chosen in the figure is roughly that expected for a transition, from the ground to the first excited state, involving a charge transfer (see *e.g.*: Brand *et al.*, 1971)

fluorescence of artificial membranes are different from those operating in nerve membranes. The slow component of some intracellular signals cannot be considered a distinctive characteristic of the axolemma, since for 1,8-MANS we found that it disappeared when the dye was applied extracellularly. We shall show below that this component may be interpreted in simple physical terms.

The simplest, albeit tentative suggestion from this first general remark, is that the major part of the extrinsic fluorescence signals in squid axons derives from chromophores adsorbed on either sides of the inert, phospholipid matrix of the axolemma. This conclusion goes along with, and reinforces, the widely accepted notion that the simple lipid bilayer structure is largely present in the nerve membrane, while the physiologically responsive sites, involving proteins or lipoproteins complexes, cover only a very small fraction of the total membrane area (see *e.g.*: Keynes, 1972). According to this view, extrinsic fluorescence measurements such as those presented here yield relevant informations only about the passive nerve membrane matrix. Some simple contributions along these lines, from the present experimental results, will be considered at the end of this discussion.

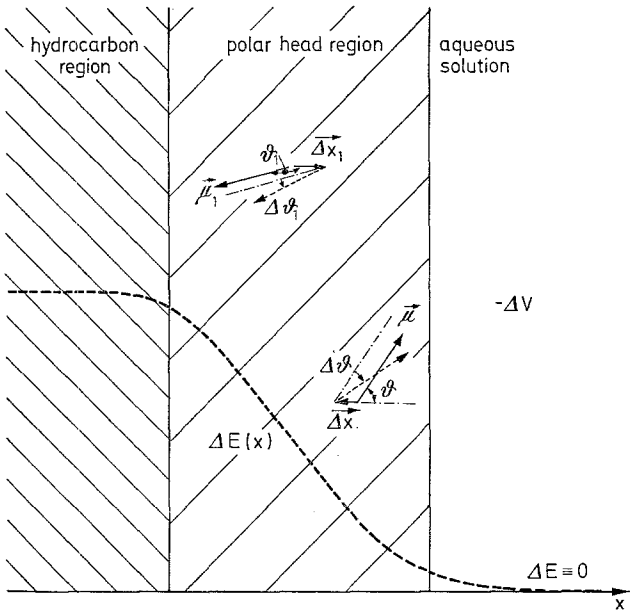

Fig. 7b. Schematic drawing of the effects produced by a negative voltage step across the membrane, $-\Delta V$, on the system of Fig. 7a. The particular profile of the electric field ΔE, within the polar head region, is arbitrary. The displacements, $\vec{\Delta x}$ and $\vec{\Delta x}_1$, and the reorientations, $\Delta\vartheta$ and $\Delta\vartheta_1$ are in the right directions, but their size is exaggerated for the sake of illustration

A second remark concerns the possible physical mechanisms underlying the observed fluorescence signals. It seems unlikely that the effects of the electric field on the orientation and conformation of the phospholipid membrane matrix, play a major role in the generation of the fluorescence changes. First of all, simple phenomena like electrostriction, which vary as the square of the applied voltage are excluded by the linear dependence of the fluorescence signals over a wide range of voltages. On the other hand, it is difficult to imagine a type of electrically induced structural modification of the membrane producing opposite effects on the fluorescence intensity of two different adsorbed chromophores, like *e.g.* 1,8-MANS and 2,6-MANS. Due to their amphypatic nature these dyes are expected to be located in a similar microenvironment, at the membrane-solution interface (Gulik-Krzywicki *et al.*, 1970; Lesslauer *et al.*, 1971; Barker *et al.*, 1974). Since the fluorescence properties of 1,8-MANS and 2,6-MANS are known to be affected qualitatively in the same way by modifications of the microenvironment (Turner and Brand, 1968; Cory *et al.*, 1968; Brand *et al.*, 1971), any membrane structural change should produce effects in the same direction for both chromophores, in contrast with what is observed.

Origin of the Fluorescence Changes

Apart from changes in membrane structure, the most obvious physical mechanisms which may generate fluorescence changes are the effects, produced by the

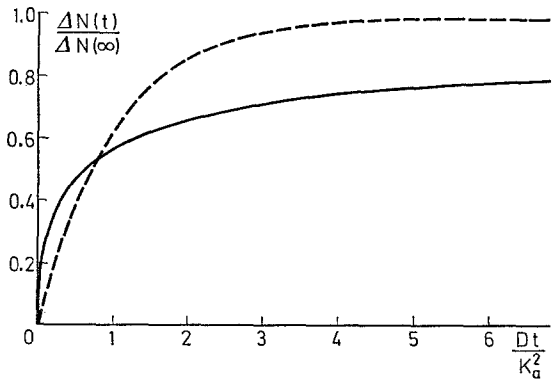

Fig. 8. Theoretical time course of the change in concentration of adsorbed chromophores, $\Delta N(t)$, following a step change in membrane adsorption coefficient (solid line), as compared with a simple exponential (dashed line). The solid line is a plot of the function: $1 - \exp\{Dt/K_a^2\}$ erfc $\{Dt/K_a^2\}$. See Appendix, Eq. (8). The dashed line is a plot of the function: $1 - \exp\{Dt/K_a^2\}$

applied electric fields directly on the probe molecules. The simple electrophoretic effect is ruled out by the absence of any direct correlation between the net charge of the fluorochromes (all anions, except for the neutral NPN) and the direction of the fluorescence changes. On the other hand, all the dyes of Table 1 have large electric dipole moments particularly in the excited states (see *e.g.*: Brand *et al.*, 1971), and the position and orientation of the electric dipole at any particular membrane-solution interface is expected to be generally different for different chromophores. A primary role of the interaction of the probes dipole with the electric field has been suggested for the interpretation of 1,8-ANS signals in lipid bilayers (Conti and Malerba, 1972). The effects which can be produced by this interaction are discussed below.

Fig. 7a shows the general picture, supported by direct spectroscopic studies (compare: phase 1 in Fig. 5 of Gulik-Krzywicki *et al.*, 1970; Fig. 6 of Barker *et al.*, 1974), for the adsorption of 1,8-MANS and 2,6-MANS on neutral bilayers[3]. The average positions and orientations, with respect to the membrane solution interface, of the electric dipoles drawn in the figure were chosen on merely speculative grounds, for illustrative purposes. Anyhow, it should be stressed that, in each particular experimental situation, these parameters are fixed essentially by the hydrophobic interaction of the probes with the membrane surface. The consideration of the remaining four degrees of freedom (two translations parallel to the membrane surface, and two rotations, *e.g.* around the axis of the dipole and around the normal to the membrane surface) is irrelevant to our discussion. Each one of these motions can be thought as being totally free (Radda and Vanderkooi, 1972; Yguerabide and Stryer, 1971), or strongly hindered, without affecting the following analysis.

[3] It is assumed that 1,8-MANS behaves similarly to 1,8-ANS, which is the dye actually studied by Gulik-Krzywicki *et al.*, 1970 and Lesslauer *et al.*, 1971. This assumption is indirectly supported by the strong similarity of the fluorescence signals obtained with these two dyes. The qualitative differences between the fluorescence signals of 2,6-ANS and 2,6-TNS and those obtained with 2,6-MANS, warn against a similar generalization of the results of Barker *et al.*, 1974, for 2,6-MANS, to the case of 2,6-ANS or 2,6-TNS.

Fig. 7b illustrates schematically two of the possible effects that a change in membrane potential, ΔV, can produce on the binding parameters of adsorbed chromophores. The size of these effects, and of the changes that they may produce in the fluorescence properties of the probe molecules, cannot be evaluated quantitatively without making particular assumptions about the potential energy profile of the adsorption energy well. We can only state here their qualitative features. 1. the displacements and rotations shown in Fig. 7b will take place in a time comparable with the period of the oscillations of the probe molecules in their adsorption energy well, probably shorter than 10^{-10} sec. Therefore the electric dipole moment which is relevant to the evaluation of the fluorescence changes produced by these effects is that of the first excited singlet state. 2. The effects are linearly related, in amplitude and sign, to the change of electric field, ΔE, in the region where the probe dipole is located. 3. Let $\theta^{(s)}$ be the average angle between the dipole of an adsorbed chromophore in its first excited singlet state and the outwardly directed normal to the membrane. Fig. 7b shows that, for $\cos\theta^{(s)} < 0$, a negative ΔV will tend to dissociate the probes from the membrane, with a consequent decrease in their fluorescence. Thus, with the sign convention used in Table 1 for lipid bilayers, this effect will produce positive fluorescence signals in those dyes for which $\cos\theta^{(s)} < 0$, and negative signals for $\cos\theta^{(s)} > 0$. The direction of the reorientations illustrated in Fig. 7b will also depend on the sign of $\cos\theta$. For $\Delta V < 0$, the axis of the dipole will approach the normal to the membrane for $\cos\theta^{(s)} > 0$, or it will deviate more from it, for $\cos\theta^{(s)} < 0$. These reorientations do not necessarily produce changes in the intrinsic fluorescence properties of the probes. However, they can alter significantly the polarization properties of the fluorescence emission in any particular direction, and change consequently the intensity of the fluorescence detected at right angles to the exciting light. Since the results reported in this paper do not concern studies of fluorescence polarization, this latter effect cannot be discussed here any further.

A third consequence of the interaction of adsorbed dipoles with the applied electric fields is a change in the adsorption properties of the membrane. This effect can be analyzed in greater detail, without need of particular assumptions about the hydrophobic interaction of the chromophores with the membrane. Let K_a, in cm, be the membrane adsorption coefficient, defined as:

$$K_a = \frac{N}{C}, \tag{1}$$

where N, in moles $\cdot$ cm^{-2}, is the surface concentration of adsorbed chromophores which is in equilibrium with the volume concentration, C, in moles $\cdot$ cm^{-3}, of chromophores solution. The contribution, ΔU, to the free energy of an adsorbed dipole, $\vec{\mu}$, due to the electric field, ΔE, is given by:

$$\Delta U = \mu \Delta E \cos\theta\,. \tag{2}$$

Therefore, the change in the adsorption coefficient, ΔK_a, due to ΔE, can be derived from the equation:

$$\frac{K_a + \Delta K_a}{K_a} = \exp\{\mu^{(g)} \Delta E \cos\theta^{(g)}/KT\}\,; \tag{3}$$

or, for $|\mu^{(g)} \Delta E \cos \theta^{(g)}| \ll KT$:

$$\frac{\Delta K_a}{K_a} = \mu^{(g)} \Delta E \cos \theta^{(g)} / KT \ . \tag{4}$$

Where the dipole moment of the ground state, $\mu^{(g)}$, has been used, since the probability for a chromophore to be in an excited state is usually very low[4]. For $\mu^{(g)} = 2$ Debye, $|\Delta E| = 5 \times 10^4$ V · cm^{-1} and $|\cos \theta^{(g)}| = 1$, Eq. (4) gives: $|\Delta K_a / K_a| \sim 8 \times 10^{-3}$. Assuming that 10% of the total background light is due to the fluorescence of adsorbed chromophores, this effect can give rise to relative changes in light intensity of 8×10^{-3} V^{-1}. These changes would have the same sign of those due to the dissociation effect discussed above, if $\cos \theta^{(g)}$ and $\cos \theta^{(s)}$ have the same sign. Positive signals are expected for $\cos \theta^{(g)} < 0$, and negative signals for $\cos \theta^{(g)} > 0$. The time course of the change in the number of adsorbed chromophores, ΔN, following a change in K_a, will depend both on K_a and on the diffusion properties of the space adjacent to the membrane. For free diffusion, this time course is shown by the solid line in Fig. 8. The derivation of the theoretical expression for $\frac{\Delta N(t)}{\Delta N(\infty)}$ is given in the Appendix. In extracellularly stained axons the diffusion is constrained by the presence of the Schwann cell layer (Frankaenheuser and Hodgkin, 1956). As shown in the Appendix, in this case $\frac{\Delta N(t)}{\Delta N(\infty)}$ is expected to follow a simple exponential behaviour, with a time constant: $\tau \sim K_a / P$; P being the permeability, in cm · sec^{-1}, of the Schwann layer to the dye molecules.

Finally, we can consider the effect of the applied electric fields, directly on the energy levels of the electronic states of the fluorochromes. If the major contribution to the quenching of the fluorescence is due to intersystem crossing from the singlet to the triplet state, the quantum yield, Q, is expected to vary according to the relationship (Seliskar and Brand, 1971):

$$\frac{\Delta Q}{Q} = 2 \frac{Q_0 - Q}{Q} \frac{\Delta \varepsilon}{\varepsilon} , \tag{5}$$

where Q_0 is close to unity ($Q_0 \sim 1{,}3$ in Fig. 9 of Seliskar and Brand, 1971), and ε is the energy difference between the first excited singlet and triplet states. From the energy of a dipole in an electric field, Eq. 2, we obtain:

$$\frac{\Delta Q}{Q} = - \frac{2(\mu^{(s)} \cos \theta^{(s)} - \mu^{(t)} \cos \theta^{(t)}) \Delta E}{\varepsilon} \cdot \frac{Q_0 - Q}{Q} , \tag{6}$$

where the superscripts s and t refer to the first excited singlet and triplet state, respectively. For $|\mu^{(s)} \cos \theta^{(s)} - \mu^{(t)} \cos \theta^{(t)}| = 5$ Debye, $|\Delta E| = 5 \times 10^4$ V · cm^{-1}, $\varepsilon \sim 1$ eV, $Q = 0.5\, Q_0$, Eq. (6) gives: $|\Delta Q/Q| = 4 \times 10^{-3}$. For these values of the parameters appearing in Eq. (6), this last effect could produce very fast fluorescence changes of the order of 4×10^{-3} V^{-1}, large enough to account for the fast components of the observed optical signals. However, there is a fundamental reason to rule out the interpretation of the observed fast signals on the basis of Eq. (6) alone. In fact, since $\mu^{(s)} > \mu^{(t)}$ (Seliskar and Brand, 1971; Brand *et al.*, 1971), we generally expect the sign of $\frac{\Delta Q}{Q}$, for a fixed ΔE, to be determined by

[4] The validity of this condition for our exponential situation was prooved indirectly by the linearity between the amplitude of the fluorescence signals and the intensity of the exciting light.

$\vec{\mu}^{(s)}$, and Eq. (6) would predict positive signals for $\cos\theta^{(s)} > 0$, or negative signals for $\cos\theta^{(s)} < 0$; just the opposite of what is expected from the first effect discussed in this section. Therefore, if the electric dipoles of the first excited singlet states of 1,8-MANS and 2,6-MANS are oriented as in Fig. 7, this last effect would induce in both dyes fluorescence changes opposite to those observed. The type of dipole orientation shown in Fig. 7, from the naphthalene ring towards the nitrogen, seems, on the other hand, justified on both theoretical and experimental grounds (see *e.g.*: Brand *et al.*, 1971).

Conclusions

In the framework of the preceeding analysis, the fast fluorescence signals, seen for all dyes and for all membrane preparations, are attributed to changes in position and/or orientation of bound chromophores, and possibly to changes in the first excited singlet-triplet energy interval. In all cases, the sign and amplitude of the fluorescence changes would be essentially determined by the position and orientation of the electric dipole of the first excited singlet state. According to this interpretation, the observation that 2,6-MANS yields fluorescence changes in the same direction and of the largest size, independently of whether it is adsorbed to the inner, or to the outer face of the axolemma[5], or to a phosphatidylcholine membrane, indicates that the average position and orientation of 2,6-MANS are independent of the membrane solution interface to which the dye is bound. They seem to be mainly determined by the amphipatic nature of the probe and little sensitive to the particular polar head groups of the membrane phospholipids. The same statement is valid for 1,8-MANS and 1,8-TNS. On the other hand, the whole pattern of optical responses obtained with the various dyes of Table 1, particularly with 2,6-ANS, 2,6-TNS and NPN, might represent a characteristic property of the phospholipid composition of any particular membrane. In this respect our present results indicate significant differences between the inner and outer face of the axolemma, while the former appears to be similar to the neutral phosphatidylcholine bilayer.

The slow components of the fluorescence signals may be attributed to changes in the number of adsorbed chromophores and their time course could provide a direct determination of the partition coefficient, K_a. The consideration of the possible range of values for K_a shows, however, that these effects may be even too slow to yield any significant contribution in the millisecond time range. From measurements of surface potentials, produced by the adsorption of 1,8-ANS on neutral phosphatidylethanolamine bilayers (McLaughlin *et al.*, 1971), K_a for this system is estimated to be about $1{,}7 \times 10^{-3}$ cm. Assuming that this value is a good approximation for K_a also in our experiments with lecithin bilayers, only 10% of the asymptotic change in the number of bound chromophores, consequent to a change in K_a, is expected to occur in about 3 msec ($Dt/K_a^2 = 10^{-2}$, D $\sim 10^{-5}$ cm^2

[5] It should be stressed that, since all the observed fluorescence changes are reversed by a change in the voltage polarity, any voltage step must produce signals of opposite sign when the same dye is applied to opposite sides of a symmetric membrane. Squid axons showed this symmetry property when stained with 1,8-MANS or 2,6-MANS, but not when stained with 2,6-ANS or NPN. The latter two dyes seem therefore more directly suitable to reveal membrane asymmetries.

· sec^{-1}; see Fig. 7). Even slower effects are expected from extracellularly stained axons. In fact as shown in the Appendix, the time constant of the exponential uptake or release of bound chromophores in this preparation is at least of the order of 200 msec. The fluorescence signals from axons stained intracellularly with 1,8-TNS, 1,8-MANS and NPN, show slow components. According to our analysis, this indicates that the adsorption coefficient of the inner face of the axolemma for these dyes, is lower than 10^{-3} cm. More accurate estimates of K_a cannot be inferred from the fluorescence signals, associated with short voltage-clamp pulses, which have been presented in this work.

The measurements of fluorescence signals in axons stained both intracellularly and extracellularly indicated that the outer and inner face of the axolemma have approximately the same adsorption capacity for anionic hydrophobic probes. This appears to be in contrast with the evidences of a high negative fixed charge density on the extracellular axonal membrane (Gilbert and Ehrenstein, 1969). The contradiction might be partly explained by the screening effect of the divalent cations present in sea water (Vanderkooi and Martonosi, 1969). An alternative, or additional, possible suggestion is that the outer face of the nerve membrane contains a larger phospholipid to protein ratio than the inner face.

Appendix

Time Course of the Changes in Concentration of Membrane Adsorbed Chromophores

a) *Free Diffusion in Acqueous Solution,* Let the x axis be perpendicular to the membrane surface, and its origin be placed at the membrane-solution interface. Let: $c(x,t)$ be the concentration of chromophores in solution, $N(t)$ the concentration on the membrane, and D the diffusion coefficient in free solution. We first look for the solution of the diffusion equation:

$$\frac{\partial C(x,t)}{\partial t} = D\,\frac{\partial^2 C(x,t)}{\partial x^2}\,,\ (x>0)\,, \tag{A 1}$$

with the boundary conditions:

$$c(\infty,b)=0;\quad N(t)=KC(0,t);\ K\frac{dc(0,t)}{dt}=D\,\frac{\partial C(x,t)}{\partial x}\bigg|_{x=0}; \tag{A 2}$$

and the initial conditions:

$$C(x,0)=0,\ (x>0);\ C(0,0)=\Delta C_0 \tag{A 3}$$

The solution for the system of Eqs. (A 1), (A 2) and (A 3), which can be obtained using *e.g.* the method of Laplace transforms, is given by:

$$C(x,t)=\Delta C_0 \exp\{x/K+Dt/K^2\}\ \mathrm{erfc}\,\{x/2\sqrt{Dt}+\sqrt{Dt}/K\}\,, \tag{A 4}$$

where:

$$\mathrm{erfc}\,\zeta = 1-\frac{2}{\sqrt{\pi}}\int_0^{\zeta} e^{-z^2}\,dz\,. \tag{A 5}$$

Let us now consider a membrane facing a solution in which the concentration of chromophores is C_0, and undergoing at $t=0$, a sudden change of adsorption coefficient, from K_a' to K_a. If the adsorption reaction at the membrane surface is

very fast this situation is described by Eqs. A1 and A2, with $K = K_a$, and by the following modified boundary and initial conditions:

$$C(x, 0) = C_0,\ x > 0;\ C(0, 0) = \frac{N_0}{K_a} = C_0 - C_0\left(1 - \frac{K_a'}{K_a}\right); \tag{A 6}$$

where $N_0 = K_a \cdot C_0$ is the surface concentration of bound chromophores for $t \leqslant 0$. The solution of this problem is easily obtained from the previous one, and is given by:

$$C(x, t) = C_0\left[1 - \left(1 - \frac{K_a'}{K_a}\right)\exp\{x/K_a + Dt/K_a^2\}\ \mathrm{erfc}\ \{x/2\sqrt{Dt} + \sqrt{Dt}/K_a\}\right]. \tag{A 7}$$

For $x = 0$, multiplying both sides of Eq. (A 7) by K_a we obtain, after rearrangements:

$$\Delta N(t)/\Delta N(\infty) = [N(t) - N_0]/[N(\infty) - N_0] = 1 - \exp\{Dt/K_a^2\}\mathrm{erfc}\{\sqrt{Dt}/K_a\}, \tag{A 8}$$

where $N(\infty) = KaC_0$. A plot of $\frac{\Delta N(t)}{\Delta N(\infty)}$ is shown in Fig. 8. For $\sqrt{Dt}/K_a \ll 1$, Eq. (A 8) becomes:

$$\Delta N(t)/\Delta N(\infty) = 2\sqrt{Dt}/\sqrt{\pi}K_a,\ (\sqrt{Dt}/K_a \ll 1); \tag{A 9}$$

showing that, for $t = 0$, the rate of change in concentration of adsorbed chromophores is infinitely fast. For $t = 10^{-2}K_a^2/D$, $\Delta N(t)$ is already 11.3% of its final value, $\Delta N(\infty)$. However, for $t > K_a^2/D$, the rate of change of $\Delta N(t)$ becomes progressively slower, and, for $t = 16K_a^2/D$, $\Delta N(t)$ is still only about 86% of its final value.

b) *Thin Diffusion Barrier Adjacent to the Membrane.* This case describes the experimental situation for extracellularly stained axons (Frankenhaeuser and Hodgkin, 1956). Following the simple picture of Frankenhaeuser and Hodgkin, 1956, the concentration of chromophores in the narrow acqueous space between the axolemma and the Schwann layer, $C(0, t)$, is governed by:

$$\theta\frac{dC(0, t)}{dt} + K_a\frac{dC(0, t)}{dt} = -P[C(0, t) - C_0], \tag{A 10}$$

where θ is the thickness of the space in cm, P is the permeability of the Schwann layer to the fluorochromes in cm $\cdot$ sec^{-1}, and C_0 is the concentration of fluorochromes in the extracellular medium in mole $\cdot$ cm^{-3}. Solving Eq. (A 10), with the last of Eq. (A 6) as initial condition, we get:

$$C(0, t) = C_0\left[1 - \left(1 - \frac{K_a'}{K_a}\right)e^{-t/\tau}\right], \tag{A 11}$$

with:

$$\tau = (\theta + K_a)/P. \tag{A 12}$$

Multiplying both sides of Eq. (11) by K_a, we obtain:

$$\Delta N(t)/\Delta N(\infty) = [N(t) - N_0]/[N(\infty) - N_0] = 1 - e^{-t/\tau} \tag{A 13}$$

where $N(0) = K_a'C_0$, and $N(\infty) = K_aC_0$. The process described by Eq. (A 13) is, in practice, very slow. Even for K_a values as small as 10^{-5} cm (two orders of magnitude smaller than in phosphatidylethanolamine bilayers; see p. 41), and using for P the value of about 5×10^{-5} cm sec^{-1}, measured for potassium ions (Frankenhaeuser and Hodgkin, 1956), τ would be of the order of 200 msec.

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