J. Membrane Biol. 8, 109–132 (1972)

Fluorescence of Squid Axon Membrane Labelled with Hydrophobic Probes

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Received 17 September 1971

Summary. Extrinsic fluorescence changes in squid giant axons were examined under a variety of experimental conditions using 2-*p*-toluidinylnaphthalene-6-sulfonate (TNS) and other fluorescent probes. Measurements of the degree of polarization of the fluorescent light (with the axis of the polarizer parallel to the longitudinal axis of the axon) indicated that the class of the TNS molecules in the axon membrane which participate in production of fluorescence signals have a definite orientation with their absorption and emission oscillators directed parallel to the long axis of the axon. Rectangular depolarizing voltage pulses produced a transient decrease in the fluorescent intensity, of which the early component is correlated tentatively with the rise in the membrane conductance. In response to hyperpolarizing pulses, there was an increase in fluorescence intensity which may be explained in terms of increased incorporation of TNS into the ordered structure in the membrane. Hyperpolarizing responses in KCl depolarized axons were accompanied by a change in fluorescent intensity. Tetrodotoxin appeared to suppress the initial component of the fluorescence signal produced by depolarizing clamping pulses. The technique for detecting these fluorescence changes and the physico-chemical properties of TNS are described in some detail.

Changes in the extrinsic fluorescence of nerve associated with the action potential were reported three years ago [25] immediately after the successful demonstration of changes in light scattering and birefringence [5]. The phenomenon has now been recognized with a variety of dyes and with voltage clamp [8, 22]. Fluorescence changes have also been demonstrated to accompany excitation of muscle membrane [3]. Fluorescence changes of nerve membrane with voltage clamp have been verified by another laboratory [6]. Most of the initial effort had been devoted to showing that the phenomenon was real and not an artefact (*see* Discussion), but the significance of the intensity changes was not clear.

Recently, it has been possible to begin interpreting some fluorescence phenomena of the axon in terms of membrane structure [24]. To a large extent this success is a result of the use of the hydrophobic probe dyes. This

class of dyes, which includes 1-anilinonaphthalene-8-sulfonate (ANS) and 2-*p*-toluidinylnaphthalene-6-sulfonate (TNS), has been valuable in the study of protein conformation and mitochondrial and other membranes [4, 19, 26, 29]. Since the quantum yield and the peak wavelength of the spectral distribution of the emitted light of these dye molecules is related to the polarity (or more precisely, the *Z*-value; *see*, *e.g.*, ref. [1]) of their microenvironment, they can be used to detect conformational changes of macromolecules.

Recently, we analyzed the transient decrease in fluorescence of a TNS-stained squid axon which accompanies electrical excitation [24]. The light which is involved in the decrease is different from the major portion of the fluorescence in at least two ways. This light derives from a sub-set of the dye molecules located in (or near) the axon membrane (*see* Discussion). While the light from the whole axon has a broad spectrum, the optical signal has a narrow spectral distribution with a shorter wavelength maximum. Thus, in the resting state, the dye molecules which contribute to the optical signal have high quantum yield (*see* Appendix). In the excited state of the axon these molecules lose their high quantum yield producing a decrease in fluorescence of the axon. Whether this involves a conformational change of membrane macromolecules, a decrease in membrane binding sites or a change of the affinity of the membrane binding sites has not been clarified. However, we have suggested that it probably involves a change of the membrane sites from a hydrophobic state to a hydrophilic state [24].

Another characteristic of the TNS fluorescence signal is that it is highly polarized. This finding suggests that the axonal membrane has a rigid, crystalline structure which is capable of orienting the dye molecules (*see* Appendix and ref. [24]).

The present paper describes the results of further studies of the process of nerve excitation by the use mainly of TNS. It deals mostly with a detailed analysis of the optical signals obtained under voltage clamp.

A preliminary account of the results described in this paper has been presented at a Marine Biological Laboratory meeting in Woods Hole, Mass. [27]. Physico-chemical properties of the probe molecules employed are described in the Appendix of this paper.

Materials and Methods

Material and Nerve Chamber

Squid giant axons used in the present study were obtained from *Loligo pealii* available in Woods Hole, and were between 0.4 and 0.6 mm in diameter and about 40 mm in length. The major portion of the connective tissue and small nerve fibers around the

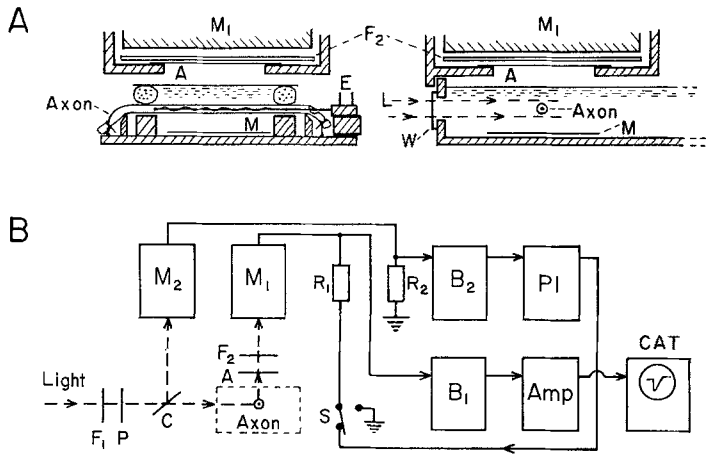

Fig. 1. *Top:* Diagram showing positions of multiplier phototube (M_1), analyzer (A), internal electrodes (E), incident light (L), quartz window (W) and mirror (M) relative to giant axon (not to scale). Both ends of the axon and the electrodes are fixed to the base of the nerve chamber made of black Lucite; the chamber is filled with sea water and sealed with vaseline and a cover glass. *Bottom:* Block diagram of compensating circuit for eliminating fluctuation in photo-current due to instability of xenon-mercury light source. M_1 and M_2 represent multiplier phototubes for detector and reference channel, respectively. B_1 and B_2 are Bak electrometer stages; *PI*, phase inverter; *Amp*, high-gain condenser-coupled amplifier; *R*'s, resistors; *S*, switch; CAT, computer for average transients. Other symbols are explained in the text

giant axon were removed under a low-power dissecting microscope during the course of dissection. The axon was then mounted horizontally in a nerve chamber made of black Lucite (*see* Fig. 1, top). The design of the chamber was similar to that used in previous studies [8, 24]; it was 10 mm deep, 18 mm wide and approximately 50 mm long. It was provided with two pairs of platinum wire electrodes (not shown in the figure): one pair near one end of the axon was used for external stimulation and the pair near the other end was used for extracellular recording of action potentials. The chamber was provided also with a quartz window (*W* in the figure) through which a beam of light could be focused on the axon. The distance between the quartz window and the axon was approximately 16 mm.

Intracellular injection of a solution containing probe molecules into the axon was carried out in the nerve chamber under a dissecting microscope. The method of introducing and fixing an internal electrode-assembly was the same as that used in a previous study [8]; this was accomplished by fixing the plastic base of the electrode-assembly (E) to the base of the nerve chamber by means of hard, sticky dental wax. The chamber was filled with modified artificial sea water containing 30 mM $CaCl_2$ and was sealed with a cover glass. [Expressed in mM, the modified sea water contained 423 NaCl, 9 KCl, 30 $CaCl_2$, 23 $MgCl_2$, 25.5 $MgSO_4$ and was buffered with Tris-(hydroxymethyl)-aminomethane-HCl, pH 7.9 to 8.1.] Precaution was taken not to leave any air bubbles in the chamber. Frequently, a mirror (M) was placed on the bottom surface of the chamber (to increase the intensity of light reaching the detector).

The nerve chamber carrying the giant axon was then transferred into a small refrigerator (with a capacity of approximately $30 \times 30 \times 40$ cm^3) of which the inner wall was

coated with black paint. The air in the refrigerator was maintained at temperatures between 6 and 10 °C. The refrigerator was provided with a quartz window through which a beam of quasi-monochromatic light could illuminate the axon in the nerve chamber. There was also a hole in the ceiling of the refrigerator through which a photomultiplier housing was introduced. The fluorescence of the axon was detected with a photomultiplier tube in the housing with its photosensitive head facing down toward the axon.

Optical Arrangement

The light source employed was a 200 w xenon-mercury lamp (Engelhard Hanovia, Inc.) operated with a stabilized power supply (Electro-Powerpacs Corp.). Such a lamp is necessary to provide adequate illumination for probes (such as TNS) which require excitation in the ultraviolet range. As in previous experiments [24] cylindrical quartz lenses with a focal length between 10 and 15 cm were used to condense the incident light on the 12-mm long portion of the giant axon. To convert the incident light into a beam of polarized, quasi-monochromatic light, an interference filter (F_1) and either a Glan-Taylor prism (Karl Lambrecht Corp.) or a HNP'B Polaroid sheet (P) were inserted into the pathway of the incident light in the manner illustrated diagrammatically in Fig. 1, bottom. The angle of convergence of the incident light was so small (less than about 6°) that the performance of the Glan-Taylor prism or of the interference filter remained undisturbed. When either TNS or ANS was used as a probe, the interference filter used (purchased from Thin Film Products; Infrared Industries, Inc.) had a central wavelength of 365 nm and a half band-width of 10 nm. In experiments with pyronin B, an interference filter of 550 nm (5 nm half band-width) was used.

The light arising from the giant axon under these conditions is a mixture of the fluorescent light emitted by the probe molecules and a portion of the incident light scattered by the axon. Since the wavelength at the emission maximum of fluorescence is more than 50 nm longer than that of the incident light under these conditions, the scattered light could be eliminated by the use of a secondary filter without reducing the intensity of the emitted light appreciably. The secondary filter used for this purpose (F_2 in the figure) was an absorption filter of the Corning CS series (either CS 3-73 or 3-74 in experiments with ANS or TNS or CS 3-66 with pyronin B). When measurements of the degree of polarization of the emitted light were required (*see* Figs. 2 and 3), a Polaroid sheet HN 38 (A in the figure) was inserted between the axon and the secondary filter.

The intensity of the xenon-mercury lamp used showed a fluctuation of about 1% at the frequency required for analyzing the fluorescent signals. Since the observed changes in the fluorescence intensity are far smaller than 1%, it is necessary to suppress the response of the detector system to this fluctuation. In the present series of experiments, a compensating circuit illustrated by the block diagram in Fig. 1, bottom, was used. With this circuit, it was also easier to compensate for changes in the level of fluctuation associated with "photodegradation" under constant illumination (*see* ref. [24]). The design and the mode of operation of this compensating circuit is as follows:

A small portion of the incident light was deflected by the use of a quartz cover slip (C) placed in front of the window of the nerve chamber at 45° to the incident light. By means of a fiber light guide for ultraviolet light, the deflected light beam was then led to a reference photomultiplier tube (M_2 in the figure). The output of M_2 was connected through a Bak preamplifier (Electronics for Life Sciences) to an operational amplifier (Philbrick P65A) used as a phase-inverter with a variable gain. By means of a switch (S in the figure) the output of the phase-inverter (PI) could be connected to the input of the Bak electrometer (B_1) through the load resistor (R_1) of the main photomultiplier tube (M_1). The load resistance was generally between 0.2 and 2 MΩ.

The following procedure was employed to eliminate voltage fluctuations at the output of B_1 caused by the instability of the light source: with switch S connected to ground and the output of B_1 to a cathode-ray oscilloscope, the potential drop across R_1 was determined. Then, switch S was thrown into the alternate position as shown in the figure, and the potential at the input of B_1 was adjusted to the ground level by varying the gain of phase-inverter *PI*. A large change in the intensity of the light from the source produced no detectable change in the output of the B_1 under these conditions. [Note that this compensating device is not needed if a dc-operated incandescent lamp is used as a light source; however, UV light emitted by an incandescent lamp is very weak.]

As in previous experiments [24], the phototube used for detection of fluorescent changes was RCA C70109E tube used as a two-stage photomultiplier. The output of B_1 was led to a CAT computer (Technical Instruments Corp.) through a Tektronix 122 amplifier.

Preparation of Fluorescent Probes

ANS was obtained in most cases from Eastman Organic Chemicals as a Na-salt and was used as a Mg-salt after purification by the method described by Weber and Young [30]. In the later stage of the present study, ANS (practical grade) was purchased from the same firm in the acid form and was used after neutralization with K-phosphate (dibasic). TNS was purchased from Sigma Chemical Co. as a K-salt and was used without purification. Pyronin B was obtained from Allied Chemical and was used after removing impurities by dissolving the commercial product in ethanol and then evaporating the solvent following removal of the undissolved salt and carbohydrates.

The fluorescent probes were dissolved in K-phosphate solution at pH 7.3 ($\pm$0.1) containing 0.4 Equiv/liter K-ion. This solution also contained glycerol (4% by volume) to maintain tonicity. The concentration of the probe used was 0.5 mg/ml for TNS and ANS and 1 to 2 mg/ml for pyronin B. In the case of TNS, which has low solubility in injection fluid, the probe was first dissolved in a mixture of water and acetone at a high concentration and a small aliquot was added to the K-phosphate solution to obtain an injection fluid containing suspension of small crystals of TNS.

Injection of the probes into a giant axon was carried out with a capillary of approximately 70 μ diameter; the volume injected into one axon was of the order of 1 mm^3. The amount of the probe in one axon could be estimated by transferring the axon into a known amount of ethanol, waiting for equilibration of the dye and measuring the fluorescence of the ethanol solution of the probe with a spectrofluorometer.

Elimination of Disturbances Caused by Vibration and Extraneous Light

The optical setup described above is extremely sensitive to slight mechanical movements of the experimental table and of the electronic apparatus on the table. Even when there is no apparent source of vibration in the vicinity of the optical setup, slow oscillatory deflections are often observed in the output of the high-gain amplifier (AMP in Fig. 1). In the present study, mechanical disturbances of this type were suppressed by placing the entire optical setup on a "Serva Bench" (Barry Controls), namely, on a heavy granite slab (approximately 300 kg) supported by three rubber balloons filled with compressed nitrogen gas. Mechanical waves can be transmitted along electric wires connecting the apparatus on the table with the power supplies, cathode-ray oscillographs and computer; this transmission can be reduced by using flexible wires taped on the metallic top of the table. Additional precaution may be taken to place the giant axon at the most stable location in the center of the incident light beam; at the position where the axon is maximally illuminated by the beam, the photomultiplier output is least sensitive to vibratory movements of the experimental table.

To measure small changes in the intensity of the fluorescence of probe molecules, it is necessary to operate the photomultiplier at the level of supply voltage which yields the highest sensitivity and stability. Under these circumstances, weak extraneous lights reaching the detector can produce sizeable photocurrents. The nerve chamber has to be shielded, therefore, from all the extraneous light sources. When such shielding is accomplished, it is possible to demonstrate that the major portion of the observed photocurrent vanishes on removal of the axon from the chamber.

A different type of extraneous light can arise from imperfection or defect in the optical filters used. A simple test of the performance of the filter is to show that no detectable light passes through the secondary filter (F_2) superposed on the primary filter (F_1). Note that a change in the light scattering property of the axon can produce a spurious optical signal, if the light passing through F_1 is imperfectly blocked by F_2 (*see* Discussion).

Other possible artefacts encountered in fluorescence measurements of this type have been discussed previously [8] and will not be repeated here. In addition to the aforementioned complicating factors, injurious effects of strong ultraviolet light on squid giant axons can also produce undesirable complications [24]. Since the signal-to-noise ratio tends to increase with square root of the incident light intensity, a compromise intensity level (of the order of 10^{12} photons/sec at the photomultiplier input) has to be chosen so that a sizeable optical signal can be obtained before physiological properties of the axon are seriously altered by the applied ultraviolet light.

Repetitive Voltage Clamping

The electric circuit employed to apply rectangular voltage pulses to the axon membrane was similar to that used previously (*see* Appendix C in ref. [7]). To maintain the membrane current in the intervals between pulses close to zero, a source of adjustable voltage was added to the input for commanding pulses.

During the course of the present study, it was found necessary to compare the sizes of optical signals observed at two or three different levels of clamping voltage. Since repetitive application of depolarizing clamping pulses to the axon membrane gradually changes the state of the axon, it is difficult to compare two optical signals obtained in two successive trials. A more reliable comparison could be made by alternating two voltage pulses in such a way that each pulse followed the other at a constant interval. The detail of this "alternation procedure" may be illustrated by the following example (*see* Fig. 5, right): the analysis time of the CAT computer was chosen to be 125 msec and one voltage pulse, 5 msec in duration and 70 mV in amplitude, was delivered 20 msec after the triggering pulse to the CAT. Then another voltage pulse, 5 msec in duration and 105 mV in amplitude, was delivered 75 msec after the onset of the first pulse. The triggering pulses to the computer were repeated at a regular interval of 150 msec and the optical signals produced by these two pulses were averaged over 5,580 trials. Under these conditions, the effect of a gradual change in the state of the axon influences the two optical signals to the same extent. Consequently, a comparison of the two signals is more reliable than in previous experiments where comparison was made by recording one signal at a time.

Results

Fluorescence Polarization During Action Potential

Squid giant axons were injected with approximately 0.2 μg of 2-*p*-toluidinylnaphthalene-6-sulfonate (TNS). TNS fluorescence was produced

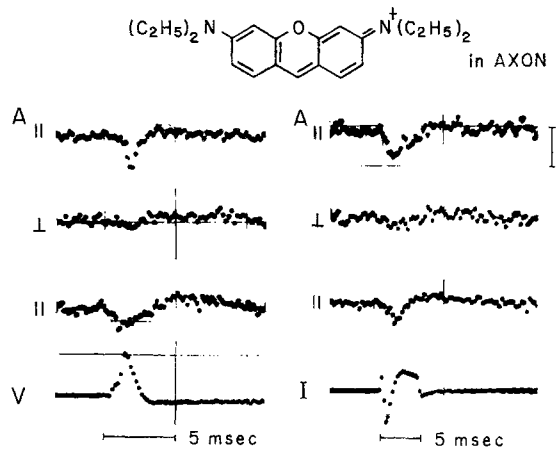

Fig. 2. Changes in polarized fluorescent light observed in axons with pyronin B internally. The records in the left column were obtained under current-clamp and those in the right column under voltage-clamp. The current pulse used was 0.5 msec in duration and roughly 60 μamp/cm^2 in intensity. The voltage pulse was 5 msec in duration and 53 mV in amplitude. The directions of the analyzer (*A*) are indicated. The action potential observed was approximately 106 mV in amplitude and the peak inward current was about 0.8 mamp/cm^2. The vertical bar represents 1.6×10^{-4} times the resting fluorescence intensity for the left column and 1.2×10^{-4} times for the right column

by illumination with quasi-monochromatic light of 365 nm in wavelength. A slight decrease in fluorescence (called the optical signal) of about 10^{-4} times the background light intensity can be demonstrated when such an axon is stimulated electrically [22, 24]. When the polarizing axes of the polarizer and analyzer are both parallel to the longitudinal axis of the nerve, the optical signal is practically unaffected. However, when the analyzer was rotated by 90° so that its polarizing axis is perpendicular to the nerve, an optical signal was never observed [24]. From considerations of signal-noise ratio, we estimate that if a signal is present with the analyzer perpendicular, it would be at most 10% of the signal with the analyzer parallel. The physical chemistry of such highly polarized emission is discussed in the Appendix. The result suggests that the TNS molecules which give rise to the optical signal (when the axis of the polarizer is parallel to the axon) have a definite orientation with their absorption and emission oscillators parallel to the long axis of the axon.

Measurements were also made with the incident light polarized in the direction perpendicular to the axon. When the analyzer was parallel to the axon, optical signals were never observed. However, when the analyzer was perpendicular to the axon (similar to the polarizer), a small increase in

the fluorescence was frequently seen. Under illumination with the same intensity, the magnitude of this positive signal was about one-third or less of the size of the negative signal obtained with the analyzer and polarizer both parallel to the axon. This finding may be interpreted that there is a set of TNS molecules in or near the membrane with their oscillators oriented perpendicularly to the long axis of the axon (that is, radial or annular) during the action potential.

Similar studies with 1-anilinonaphthalene-8-sulfonate (ANS) did not show such highly polarized fluorescence. Signals obtained with all four possible configurations of analyzer and polarizer showed only small quantitative differences. TNS was not, however, the only probe that emits highly polarized fluorescence. Pyronin B was found to yield results somewhat similar to TNS (*see* Fig. 2, left). As has been pointed out previously [8], optical signals observed in axons stained with pyronin B are diphasic, an initial negative component followed by a slow positive component. (The existence of diphasicity was not recognized when pyronin B signals were first described [22].) With the polarizer in parallel, the negative component was present when the analyzer was parallel and absent, or very small, when the analyzer was perpendicular. The positive component of pyronin B signals was not polarized.

Fluorescence Polarization during Voltage Clamp

Emission of highly polarized fluorescence from TNS molecules in the axon membrane could be demonstrated under voltage clamp conditions (*see* Fig. 3). When rectangular voltage pulses were delivered to the axon membrane, small changes in fluorescence were observed. These changes remain unaffected by inserting a polarizer and an analyzer parallel to the axon. When the analyzer was rotated to a perpendicular position (with the polarizer parallel to the axon), no optical signal was seen.

As seen in Fig. 3, a hyperpolarizing voltage pulse applied to the axon membrane produced a transient increase in fluorescence and a depolarizing pulse a transient decrease. The time course of the change produced by hyperpolarizing pulse was very different from that brought about by a depolarizing pulse. Nevertheless, the changing portion of the fluorescent light was found to be almost completely polarized irrespective of the sign of applied voltage pulses. The difference in time course between the optical signals obtained with hyperpolarizing and depolarizing pulses will be discussed later.

Pyronin B also emits highly polarized light under these conditions (Fig. 2, right). The negative optical signal, which was present with the

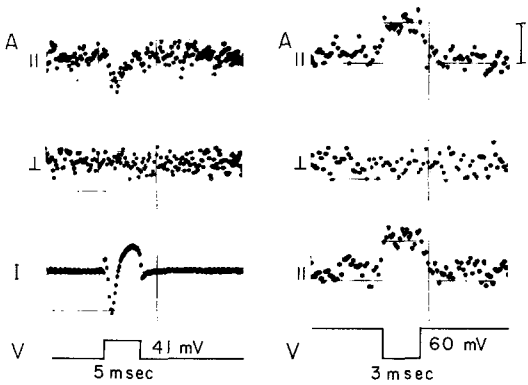

Fig. 3. Changes in polarized fluorescent light observed in TNS-treated axons under voltage-clamp. The amplitude and duration of the voltage pulses applied are shown by the tracings marked *V*. The electric vector of the incident light was parallel to the axon; the direction of the polarizing axis of the analyzer (*A*) is indicated. The trace marked *I* represents the membrane current; the peak inward current observed was approximately 0.8 mamp/cm^2. The left and right columns were taken from two different axons. The bar represents a fluorescence change of 8×10^{-5} times the resting light intensity for the left column and 6×10^{-5} times for the right column. Temperature, 6 °C. Note that fluorescence signals were absent when the analyzer was in the perpendicular position

polarizer and analyzer parallel to the axon, was not observed when the analyzer was rotated to the perpendicular position.

Dependence of Magnitude of Fluorescence Signal on Amplitude of Applied Voltage Pulse

In axons labelled with ANS the magnitude of fluorescence signal was found to increase with increasing amplitude of the hyperpolarizing voltage pulse applied across the axon membrane [8]. By the use of the alternation technique described under Methods, the relationship between the magnitude of ANS fluorescence signal and the amplitude of hyperpolarizing voltage pulse was examined. An example of the results obtained by this technique is found in Fig. 4.

The time course of the optical signal produced by a rectangular hyperpolarizing pulse was roughly rectangular, its rise time being slightly longer than that of the clamping pulse. The magnitude of the signal is seen to increase roughly linearly with the size of the applied voltage pulse.

The results obtained from axons treated with TNS (and using a parallel polarizer) were very similar to those obtained with ANS, except for the amplitude of the signals observed at the same voltage. At the clamping

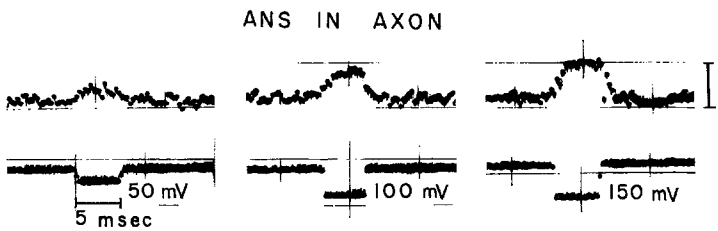

Fig. 4. Computer records showing dependence of fluorescence signals on amplitude of hyperpolarizing voltage pulses applied across axon membrane labelled with ANS. The three records were obtained by averaging the signals simultaneously by the alternation method. The duration and the amplitude of clamping pulses are indicated. The bar indicates a change of 7.6×10^{-5} times the resting fluorescence intensity

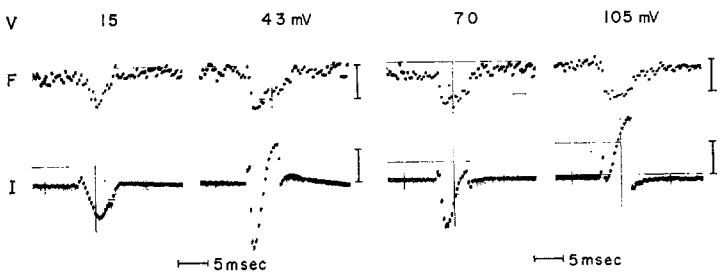

Fig. 5. Records showing the relationship between the amplitude of depolarizing voltage pulses (V) and the size of fluorescence signals (F) in TNS-treated axons. The records for clamping and pulses of 15 and 43 mV in amplitude were taken from one axon by the alternation method; the records for 70 and 105 mV were taken from a different axon. The vertical bar for the fluorescence trace represents a change of 3.5×10^{-5} times the resting intensity for the left-hand records and 4.5×10^{-5} for the right-hand records. The bar for the current trace (I) represents 1 mamp/cm^2

level of -50 mV, the amplitude of the signals obtained from axons treated with TNS was approximately one-third of the signal amplitude at a clamping level of $+50$ mV. With ANS, the amplitude of the signals at -50 mV was about two-thirds of the amplitude at $+50$ mV [8].

In TNS-stained axons (under illumination with the polarizer parallel to the axon), the relationship between the magnitude of fluorescence signal and the pulse amplitude was found to be non-linear (*see* Fig. 5). In the range of depolarization between 10 and 45 mV, there was a sharp increase in the peak amplitude of the TNS signal with increasing pulse amplitude; there was also a continuous decrease in the time interval between the onset of applied rectangular pulse and the peak of the optical signal. An aproximate parallelism was seen between the initial phase of the optical signal

and the early phase of the inwardly directed membrane current. The duration of the optical signal was found to increase as the amplitude of the applied pulse was increased.

In the range of applied voltage between 50 and 60 mV, the inward membrane current reached its maximum. In this range, the magnitude of TNS fluorescence signal produced by a depolarizing voltage pulse was approximately 3 times that brought about by a hyperpolarizing pulse of the same amplitude. The time interval between the onset of the clamping pulse and the negative peak of the optical signal was about 0.6 msec at 7 °C. During the following period of maintained voltage, the optical trace approached the resting level very gradually.

In the range of depolarizing voltage between 65 and 110 mV, the fluorescence signal was found to reach its peak after the peak of the inward membrane current. There was a gradual increase in the magnitude of this peak with higher voltages. However, when comparison was made at the moment when the inward membrane current reached its peak, the amplitude of the optical signal was found to be roughly independent of the depolarizing voltage. Unlike the current trace, the optical trace showed no discontinuity at the end of the applied clamping pulse. (The behavior of the optical signals with ANS using the depolarizing voltage steps is reported elsewhere.)

Based on the experimental results described in the preceding paragraphs, it was possible to construct a curve relating the magnitude of the optical signal to the applied voltage. Since the present method of fluorescence measurement with TNS requires averaging over 3,000 to 6,000 trials, the data obtained are not very precise. Nevertheless, significant conclusions could be drawn from analyses of such a curve. It is important to note that a polarizer parallel to the axon was used in all of the present experiments with TNS.

The broken line in Fig. 6 represents an averaged current-voltage relationship obtained from TNS-treated axons. The continuous line represents the amplitude of the TNS fluorescence signal measured at the moment of the peak of inward current. The two curves were normalized to unity at +70 mV. It is noted that, on the positive side of the voltage, the amplitude of the optical signal indicated by the solid line is seen to increase sharply between the origin (0 mV) and 50 mV. Between 50 and 100 mV, there was only a small increase in the amplitude of the optical signal. If the amplitude of the optical signal were measured at the peak of the signal (without regard to timing), then the relationship between voltage and amplitude would be monotonically increasing and roughly linear.

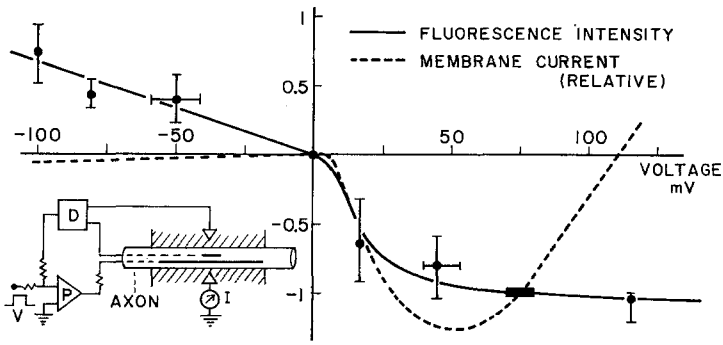

Fig. 6. Fluorescence-voltage relationship (solid line) constructed from the results of measurements on 12 axons. The current-voltage relationship is shown by the dotted line. Both curves are normalized to unity at 75 mV. The inset is a simplified diagram of the electric setup used to determine the current (I) as a function of the voltage (V) applied across the axon membrane. P represents a feed-back amplifier and D a differential amplifier

Further Analysis of Fluorescence Change Associated with Membrane Hyperpolarization

The alternation technique described under Materials and Methods was applied to the following analysis of the effect of hyperpolarization upon fluorescence changes associated with all-or-none action potentials. It was found by this technique that the fluorescence signal associated with an all-or-none action potential under membrane hyperpolarization is much larger than that observed in the absence of hyperpolarization. Two examples of experiments showing this effect are presented in Fig. 7, one example using TNS and the other using ANS.

In these experiments, hyperpolarizing current pulses were 80 msec in duration. The current intensity was adjusted so that there was a negative potential shift of 30 to 50 mV in the steady state. No break excitation was observed at the end of hyperpolarizing pulses under these conditions. To evoke action potentials, brief depolarizing current pulses of 0.5 msec duration were delivered 60 msec after the onset of the hyperpolarizing current.

It is seen in Fig. 7 that the peak amplitude of the TNS fluorescence signal associated with an action potential was markedly increased by application of a hyperpolarizing current prior to suprathreshold stimulation of the axon. The results obtained with ANS were very similar to those obtained with TNS except in the terminal period of the action potential. The action potential of an ANS-stained axon under hyperpolarization was

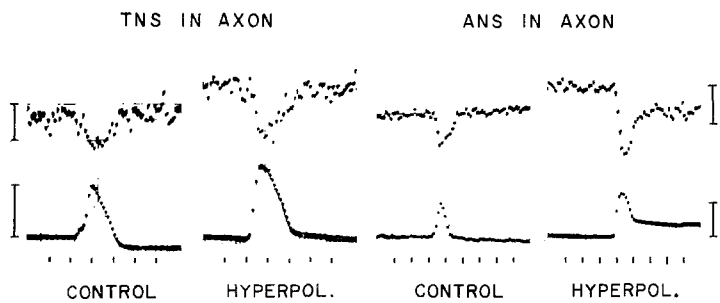

Fig. 7. Records showing the effect of dc hyperpolarization on fluorescence signals associated with all-or-none action potentials. The left-hand records (for TNS) and right-hand records (for ANS) were taken from different axons. The calibration for the fluorescent trace (*top*) represents a change of 2.7×10^{-5} for the left-hand records and 3.5×10^{-5} for the right-hand records. The calibration for the voltage trace (*bottom*) is 100 mV. The time markers are 5 msec apart. The hyperpolarizing voltage was 30 mV for the TNS-treated axon (*left*) and 50 mV for the ANS-treated axon (*right*)

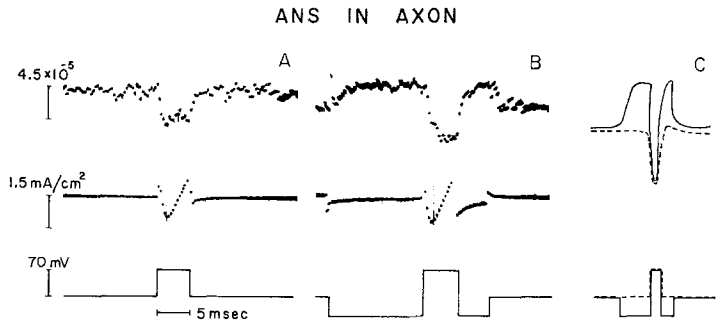

Fig. 8. Records showing the effect of hyperpolarization on fluorescence signals (*top trace*) accompanied by depolarizing clamping pulses of 70 mV in amplitude and 5 msec in duration (shown by the *bottom trace*). The time courses of the membrane current is shown by the *middle trace*. Records *A* and *B* were taken from one axon by the alternation method; tracing *C* shows the same records displayed on a slow time base

found to show a prolonged after-potential, indicating the presence of a long period of decreased membrane resistance. (A decrease in membrane resistance following production of an action potential was demonstrated by the use of testing current pulses.) Optical signals obtained from axons with ANS internally were also accompanied by a prolonged decrease in fluorescence.

The experiments concerning the effect of hyperpolarization described above are consistent with the finding that the fluorescence change produced by hyperpolarization and the change caused by subsequent depolarization are roughly additive. This additivity was demonstrated by superposing a depolarizing voltage pulse on a long hyperpolarizing pulse (*see* Fig. 8). In

response to the onset of hyperpolarization of 50 mV, there was enhancement of ANS fluorescence. During the plateau of the fluorescence signal, the membrane potential was raised to a level of 70 mV above the initial (resting) potential level: this potential rise brought about a rapid fall in ANS fluorescence. The amplitude of this fall was compared with that brought about by a depolarizing voltage pulse of the same amplitude, but without being preceded by hyperpolarization. As can be seen in Fig. 8, the optical trace fell roughly to the same level irrespective of the presence or absence of preceding hyperpolarization.

Fluorescence Signal Associated with Hyperpolarizing Response

The hyperpolarizing response in squid giant axons was described by Segal [17] and the detail of its electrophysiological behavior was described by one of the present authors [20]. The simplest procedure for demonstrating a response of this type is to add isotonic KCl solution to the standard external fluid medium and then to stimulate the axon with a long pulse of inwardly directed current. When the KCl concentration in the medium is higher than about 70 mM, the axon does not exhibit any sign of excitability in response to pulses of outwardly directed current. However, when the sign of the applied current is reversed, the axon membrane responds with a slow, but large hyperpolarization. It is easy to demonstrate hyperpolarizing responses in axons loaded with TNS or ANS.

In the experiments described below, the external medium used was a mixture of one part of 0.6 M KCl and 4 parts of Ca-rich artificial sea water (*see* Materials and Methods). A strong pulse of outwardly directed current delivered through the axon membrane was found to produce a small potential rise and simultaneously, a very small fall in fluorescence. A pulse of inwardly directed current, which was strong enough to evoke a discrete hyperpolarizing response brought about a large increase in fluorescence. The time course of the observed fluorescence change was similar to that of the change in the membrane potential (Fig. 9).

Effects of Tetrodotoxin on TNS Fluorescence

Tetrodotoxin (TTX) is a puffer fish poison known to suppress the process of action potential production at an extremely low concentration [9]. Under voltage clamp with depolarizing rectangular pulses, this poison is known to eliminate the early component of the conductance increase in the axon membrane [15]. The effects of this poison on TNS fluorescence signals were

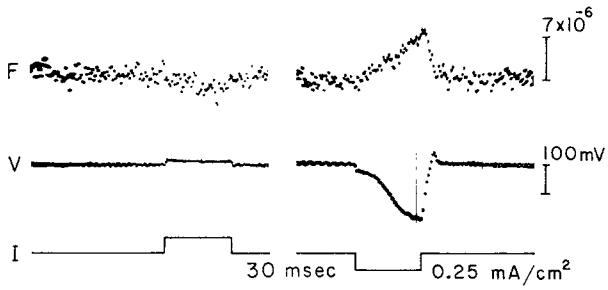

Fig. 9. TNS fluorescence signals (F) produced in an axon depolarized by external application of KCl. The time courses of the applied current (I) and the changes in the membrane potential (V) induced by the current are shown. Note that the axon produced a hyperpolarizing response to a pulse of inwardly directed current

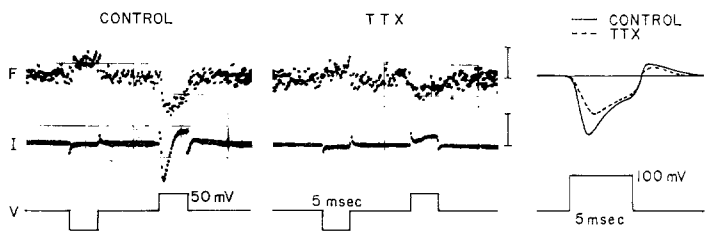

Fig. 10. Records showing the effect of tetrodotoxin (TTX) on TNS fluorescence signals. The calibration for the fluorescence trace (F) represents a change of 4×10^{-5} and that for the current trace (I) represents 1 mamp/cm^2. The tracings on the right were taken from a different axon

studied by clamping the axon membrane with rectangular voltage pulses which could produce a maximum inward current in the absence of TTX.

In the example of experiments presented in Fig. 10, a normal fluorescence signal was observed prior to application of TTX (*see* left-hand record). Then, a small amount of TTX was introduced into the medium; the TTX concentration was approximately 0.5 μg/ml in the medium. The inward current observed in the normal medium was completely eliminated by this treatment. However, a portion of the fluorescence signal was found to remain under this strong influence of TTX.

It was our strong impression that TTX exerted a detectable suppressive action only on the early part of the fluorescence signal. The later part of the signal appeared to be affected by TTX to a lesser extent. This situation is illustrated by the tracings shown in Fig. 10. The two superposed tracings represent the TNS fluorescence signals observed before and after application of TTX. Since the potential level at which the axon membrane was clamped

was about 100 mV above the resting potential, the effect of TTX on the membrane current was not very conspicuous. It can be seen, however, that the effect of TTX on the fluorescence signal was limited mainly to the initial (early) component.

Discussion

The optical signal in TNS-stained axons associated with the action potential has been shown to be composed of highly polarized light [24]. This observation has been extended here for the optical signals associated with depolarizing and hyperpolarizing voltage clamp pulses. In accord with the physical basis of highly polarized emission, as discussed in the Appendix, the probe molecules which participate in production of the optical signal must be rigidly and completely oriented. TNS is a planar molecule [2] with a well-defined long axis which must be aligned with the longitudinal axis of the nerve. ANS is not quite planar and does not have a clearly defined long axis; this may account for the fact that its emission is not highly polarized. It is interesting to note that pyronin B which also emitted highly polarized light is a long, planar molecule. These findings lead to the conclusion that there is in (or near) the axon membrane a structure with rigid orientation. This conclusion is completely independent of whatever mechanism is responsible for the fluorescence change.

Change in the extrinsic fluorescence of nerve is distinct from change in scattering (at 90°) of the exciting light. The secondary cut-off filter is always chosen so that less than 0.1% of the light at the exciting wavelength can enter the phototube. The change in scattered light would only be a small fraction (usually about 10^{-5}) of the scattered light background; thus, even if 0.1% of the light did get through, the optical signal would be 10^{-8} (0.1% of 10^{-5}) of the total background light – a change which is too small to be detected. In addition, the sign and shape of scattering signals are different from those of the signals described under Results; scattering signals observed at 90° are positive (representing a transient increase in light intensity) and have a longer time course. It is also easy to see that fluorescence signals described in this paper do not represent an artefact of scattering of the fluorescent light produced in the axoplasm. The time courses and, for some fluorochromes, the signs of the fluorescence signals are different from those of the light scattering signals. The "incident" light (i.e., that from the axoplasm) is weak and the light scattering signal produced by such a mechanism is expected to be very small. In addition, light scattering of axoplasmic light by the axonal membrane would be at a variety of angles

which would give rise to both positive and negative contributions. Finally in the case of TNS, it has been shown that the spectral distribution of the background fluorescent light is different from that of the transient change associated with the process of nerve excitation [24].

In presenting various experimental findings in this paper, it was explicitly assumed that fluorescence signals derive from changes in the average quantum yield of the probe molecules incorporated in (or near) the axon membrane. Although the validity of this assumption appears to be almost self-evident, it seems worthwhile to enumerate the experimental facts which support this assumption.

(1) Within the limit of time-resolution of the present detecting device (about 80 μsec), production of a fluorescence signal is simultaneous with generation of an action potential. (However, since the time required for a diffusion process involving a distance of a fraction of 1 μ is extremely short, this approximate simultaneity is not a sufficient proof that the optical signals originate in the membrane. Note that at a distance of 0.1 μ the time required for diffusion of particles with a diffusion constant of 10^{-5} cm^2/sec is of the order of a few μsec.)

(2) Both TNS and ANS molecules are negatively charged and the axon membrane is practically impermeable to these probe molecules. In axons with these probe molecules internally, the layer of Schwann cells and connective tissue is free of probe molecules. It is therefore evident that these extracellular elements are not involved in production of fluorescent signals.

(3) On several occasions, fluorescence signals have been observed after intracellular perfusion with pronase. Although no electron-microscopic studies were conducted, the major portion of the axoplasm must have been removed by the proteolytic action of the enzyme. It is clear, therefore, that the major portion of the axoplasm makes no contribution to production of fluorescent signals.

(4) Electric stimulation of an ANS or TNS treated axon with barely *supra*threshold current pulses produces a full-sized fluorescence signal, whereas barely *sub*threshold pulses bring about no detectable signal. This fact indicates that the fluorescence signals do not derive from the probe molecule located near the internal electrodes.

(5) Spectral analyses of the TNS fluorescence in the resting and active state of the axon [24] indicate that the signals derive from a hydrophobic layer near the axon surface. The axon membrane is the obvious candidate for such a layer.

(6) Based on measurements of the degree of polarization, it is found that a TNS fluorescence signal represents a change in quantum yield of the probe molecules in a highly ordered structure. The TNS molecules in the axoplasm are almost randomly oriented [24]. The axon membrane can have a highly ordered structure.

(7) The fluorescence-voltage curve (Fig. 6) indicates that the amplitudes of TNS signals are not directly related to (or caused by) strong ion fluxes across the membrane or changes in the membrane potential. The best correlation with the magnitude of the fluorescence signal is found, however, in the domain of membrane phenomena. There is a good correlation between the membrane conductance and the fluorescence signal (*see below*).

(8) The effects of externally applied TTX or of concentrated K-salt on axon excitability and fluorescence signals are prompt and simultaneous. Since these agents presumably act on the membrane, it may be inferred that the membrane is the site where the fluorescence signals originate.

There is no evidence that the membrane structure involved in production of fluorescence signals corresponds to the axolemma visualized by the electron-microscope. Here we are dealing with the physiologic membrane defined as the major diffusion barrier separating the axon interior from the external medium. This barrier may be considered to have hydrophobic properties in the resting state of the axon membrane. A portion of the TNS molecules introduced in the axon at rest, as shown by their spectrum, is situated in a hydrophobic zone. The fluorescence polarization results suggest a new feature of this zone: it seems to contain sites of a highly ordered, crystalline structure. Such a structure may eventually help to explain phenomena like the cooperative nature of the transition from resting to active state and the powerful effect of small perturbations (like TTX, *see above*). At present, the relationship between the present finding and the results obtained from lipid bilayer membranes [10] is obscure.

During the action potential the physiologic membrane shows an enormous increase in conductance. Previous ion substitution experiments [23] have suggested that this increase in conductance is characterized by a rise in selectivity to univalent cations of a hydrophilic nature. If a hydrophobic-hydrophilic transition is indeed the explanation of the optical signal, then this may provide an explanation for the nature of the conductance change.

The analysis of the magnitude of the optical signals with depolarizing voltage clamp pulses is difficult because the signal changes with time. By analogy to the way in which the voltage-current curve is usually constructed, we chose to study the signals at the moment of the peak inward current.

It is known that the enhancement of membrane conductance is completed at this moment. The fluorescence-voltage curve derived in this manner (*see* Fig. 6) is quite similar in shape to the membrane conductance-voltage curve determined by a weak high-frequency alternating current superposed on the membrane current (*see* Fig. 47*b* in ref. [21]).

As mentioned in the Introduction, we have suggested that the optical signal derives from a hydrophobic-hydrophilic transition of the membrane. The postulate of such a transition implies that there is an increase in the water content of the axon membrane during nerve excitation. It is well-known that the conductance of an artificial (ion-exchanger) membrane increases monotonically with its water content (*see* p. 150 in ref. [18] and p. 30 in ref. [12]). From these considerations, it may be inferred that both a fall of fluorescence and a rise in membrane conductance are causally related to the rise in hydrophilicity of the axon membrane during depolarization.

The quantum yield of TNS is exquisitely sensitive to small changes in the polarity (or Z-number) of its micro-environment (*see* Appendix). The observed fall in the average quantum yield of TNS in the axon membrane may derive either from a decrease in the quantum yield of the bound probe molecules or from the decrease in the number of TNS molecules bound to the hydrophobic sites. At present, it is not clear which one of these two processes produces the observed TNS fluorescence signals. It is highly desirable in this connection to be able to determine the fluorescence lifetime in TNS-treated axons.

It seems worth pointing out that the fluorescence signals observed with TNS cannot be attributed to a direct effect of variations in the membrane potential upon the quantum yield of fluorescence. The magnitude of the applied electric field (100 mV or less across the 100 Å thick axonal membrane) is considered to be too small to affect electronic transitions between orbitals separated by 2 to 3 eV in energy [11, 14]. Furthermore, when the transition moment of TNS molecules are oriented longitudinally (i.e., along the long axis of the axon), the applied radially oriented field is expected to be totally ineffective in affecting transition. Any effect of voltage must be mediated by changes in the macromolecules or solvent in the environment of the probe.

Quantitative analysis of the fluorescence signals associated with hyperpolarizing voltage clamp pulses is relatively easy because the shape of the signals are close to rectangular. The amplitude of a signal can be measured during the plateau with little ambiguity. The fluorescence-voltage relation (Fig. 6) is roughly linear, a larger hyperpolarizing voltage being associated with a larger increase in fluorescence. Since the membrane conductance

does not change appreciably with hyperpolarization (and therefore there are no profound changes in the conformational state of the membrane macromolecules), an etiology different from that of depolarization is required.

One possibility is that the inward current of a hyperpolarizing pulse would tend to drive TNS molecules from the axon interior into the membrane. In addition, divalent cations from the external medium (Ca^{++}) would be driven into the membrane. Both of these electric field effects would be expected to enhance the fluorescence (*see* Appendix).

Support for this idea comes from the fact that the component of the fluorescent light enhanced by hyperpolarization was found to be highly polarized. Thus the TNS molecules which participate in the signal are in the highly ordered, longitudinally oriented structure in the membrane. Transport of additional molecules into this layer or enhancement of fluorescence of molecules already present (by Ca^{++}) are possibilities consistent with this fact. Additional support of this interpretation is given by the result that the fluorescent signal associated with an action potential (or depolarizing voltage clamp pulse) superposed on a hyperpolarization is enhanced by the magnitude associated with the hyperpolarization. This would be expected since the probe molecules newly incorporated in this special oriented, hydrophobic layer would respond to the change in their microenvironment in the usual manner.

The hyperpolarizing response represents a transition of the axon membrane from a depolarized state to a repolarized state. This process can be regarded as the reversal of the normal (i.e., depolarizing) action potential. The physico-chemical behavior of the axon membrane is expected to be opposite in the two situations. Thus, it is not surprising that the hyperpolarizing response is associated with an increase in fluorescence.

Finally, the factors determining the slow recovery of the TNS signal under voltage clamp (Fig. 5) will be discussed briefly. When the membrane jumps into a hydrophilic state resulting from loss of Ca and gain of water, the rate of diffusion of various cations through the membrane increases enormously. Consequently, the concentrations of these ions in and near the membrane gradually change as functions of time. This process of gradual alteration (*see* ref. [21]) could explain how the membrane conductance and fluorescence intensity change gradually with time. Further studies are needed to clarify the physico-chemical nature of these slow processes.

Appendix

The physico-chemical properties of 2-*p*-toluidinylnaphthalene-6-sulfonate (TNS) have been described extensively by McClure and Edelman [13]. This Appendix deals

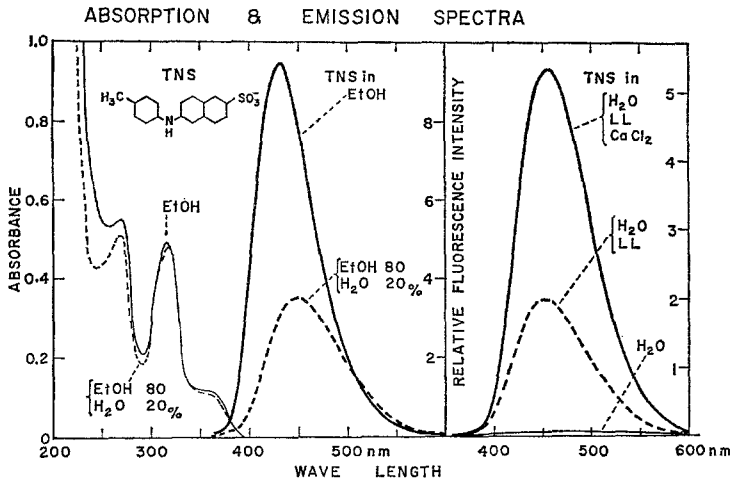

Fig. 11. Absorption (*left curves*) and emission spectra (*middle* and *right curves*) of TNS dissolved in a mixture of ethanol and water (*left*) and in a mixture of water, lysolecithin (*LL*) and calcium chloride (*right*). The composition of the solvents used are indicated. The concentration of TNS in the ethanol solution was 0.03 mg/ml. For the curve on the right, TNS was 0.05 mg/ml, lysolecithin was 0.05 mg/ml and $CaCl_2$ was 4 mM. All measurements were carried out at 21 °C

only with the properties of this probe essential to our interpretation of the physiological observations described in this paper. The optical data presented below were obtained by the use of Aminco-Bowman spectrofluorometers (kindly provided by Dr. L. Egyud in Woods Hole and by Dr. L. Sokoloff in Bethesda, Md.), a Perkin-Elmer spectrofluorometer and a Beckman spectrophotometer in this laboratory.

TNS has a limited solubility in water (about 4 mg/ml at 20 °C). The solubility is increased considerably when a small amount of organic solvent (ethanol, acetone or dioxane) is added to water. Reflecting the presence of both polar and non-polar sidegroups in this molecule (i.e., amphipathy), the solubility is greater in a mixture of water and an organic solvent than in either of the pure solvents.

Absorption spectra of TNS are shown on the left side of Fig. 11. For the same concentration of dye, the spectrum in ethanol is slightly different from that in water; but the difference is very small near the visible end of the spectrum. Spectra in 100% and 80% ethanol are illustrated. At 366 nm in wavelength, the molar extinction coefficient is about 4.6×10^{-3}. Excitation at this wavelength is believed to involve a $\pi - \pi^*$ transition.

Emission spectra of TNS in the same solvents are shown in the middle of Fig. 11. In a given solvent, the spectrum is not affected by the wavelength of the exciting light. However, a small change in the solvent composition often brings about a drastic change in the fluorescence intensity and in the emission spectrum. In pure ethanol or dioxane TNS emits a fluorescent light nearly 700 times as strong as that in pure water. The emission maximum in pure ethanol is at 430 nm; in 80% ethanol aqueous solution, it is at 447 nm; and in pure water it is 500 nm. Since the absorbance of 365 nm is nearly the same for these solvents, but the fluorescence intensity differs, this must indicate a change in quantum yield. According to McClure and Edelman [13], this solvent effect is caused by a larger dipole moment of the TNS molecule in its first exited state, the dipole-dipole interaction between the solvent and the solute molecules being enhanced markedly following absorption of light energy.

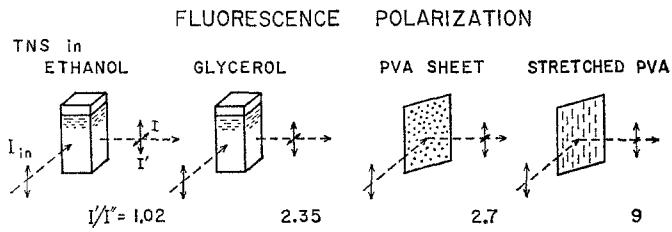

Fig. 12. Diagrams illustrating measurements of fluorescence polarizations of TNS in solutions and in sheets of poly-vinylalcohol (PVA). The polarization of the incident light (I_{in}) and that of the emitted light are indicated by the arrows. I' and I'' represent the intensities observed with the analyzer in parallel and perpendicular positions, respectively. Concentration of TNS in ethanol is 0.03 mg/ml and in glycerol it is 0.002 mg/ml. 21 °C

Various macromolecules with hydrophobic sites when added to an aqueous solution of TNS are known to increase the fluorescence intensity of the probe molecules. The effect of adding lacto-globulin and other proteins have been discussed by McClure and Edelman [13]. The effect of lysolecithin (abbreviated as *LL* in the figure) is shown at the right in Fig. 11. Analogous to what has been pointed out by Vanderkooi and Martonosi [26] for ANS, addition of Ca-salt to phospholipid was found more effective in enhancing the fluorescence intensity than phospholipid alone. It is important to note that, within the accuracy of our measurements, the absorbance at 365 nm is *not* affected by addition of lysolethicin, or $CaCl_2$ or both to an aqueous solution of TNS. The effect of bovine albumin on the fluorescence of TNS was examined also; 0.5 mg/ml of albumin increased the TNS absorbance (at 366 nm) of a 0.01 mg/ml aqueous solution of TNS by a factor of only 1.1 while the fluorescence (at 430 nm) was enhanced by a factor of about 300.

Fig. 12 presents the results of measurements of fluorescence polarization under four different experimental conditions. The direction of the electric vector of the incident light (365 nm in wavelength) is indicated by the vertical arrows marked I_{in}. Symbol I' represents the intensity of the fluorescent light observed with the polarizing axis of the analyzer directed parallel to that of I_{in}; I'' represents the intensity of the fluorescent light determined with the analyzer in the perpendicular position.

The ratio I'/I'' of the fluorescent light from TNS in ethanol was found to be 1.02. The degree of polarization P, defined by $P=(I'-I'')/(I'+I'')$, was 0.01, indicating that the fluorescent light was nearly completely depolarized. This depolarization is caused by a low viscosity of ethanol combined with a long life time of the excited state of TNS in this medium. During the life time of the excited state (of the order of 10 nsec), the direction of the emission oscillator of TNS is nearly completely randomized by rapid rotational Brownian motion. The ratio for TNS in glycerol was 2.35, indicating that P is equal to 0.40. This value of P agrees well with that given by McClure and Edelman [13]. In glycerol, the rotational Brownian motion is slower (due to a higher viscosity) and the life time of the excited state is probably shorter (due to quenching of fluorescence) than in ethanol.

The two diagrams in Fig. 12, right, show the fluorescence polarization of TNS incorporated in a stretched and an unstretched sheet of polyvinyl-alcohol (PVA). Many of these PVA samples were kindly supplied by Dr. A. Makas of the Polaroid Corporation in Boston. Some of the samples were prepared in this laboratory by the method described by Nishijima, Onogi and Asai [16]. The probe molecules were incorporated into PVA sheets either by immersion in water solution of TNS (0.3 mg/ml) or by mixing

TNS in an aqueous solution of PVA during the process of preparation of the sheets (1.0 mg of TNS added to 100 ml of 5% PVA solution). The difference in the method of TNS incorporation did not seem to affect the results.

The ratio of I'/I'' obtained from dry, unstretched sheets was approximately 2.7 (or $P=0.46$). A ratio of 3 (or $P=0.5$) is expected when the fluorescent molecules, excited at the long wavelength end of their absorption spectrum, are immobile and completely randomly oriented (*see* ref. [28]). The observed value was close to this theoretical limit, indicating that TNS molecules in the sheet are randomly oriented. (The discrepancy is due in part to the fact that 365 nm used for excitation is not at the long wavelength end of the absorption spectrum.)

Ratios of greater than 3 can never be realized unless the probe molecules are oriented preferentially in one or two directions; hence, such ratios are never encountered when dealing with fluorochromes dissolved in liquid solvents. (Note that rotational Brownian motion of fluorescent molecules tends always to reduce the degree of polarization.) The ratio obtained from a well-stretched sheet was usually 9 or more; this is definitely larger than the theoretical upper limit for a random orientation, indicating that the majority of the probe molecules have a definite orientation with their absorption and emission oscillators parallel to the direction of stretching. Studies of ANS in similar sheets usually gave ratios of smaller than about 4.0. The method of studying molecular orientation by this technique has been discussed by Nishijima *et al.* [16].

The properties of ANS have been studied in many biological and non-biological systems since its introduction as the first hydrophobic probe by Weber and Lawrence [29]. (For recent review, *see* Stryer [19].) Physico-chemical properties of pyronin B are currently being investigated in this laboratory.

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