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Further Studies of Nerve Membranes Labeled with Fluorescent Probes

Ichiji Tasaki, Mark Hallett, and Emilio Carbone

National Institute of Mental Health, Bethesda, Maryland and
Marine Biological Laboratory, Woods Hole, Massachusetts

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Summary. By using the technique of intracellular perfusion combined with fluorescence measurements, the mode of binding of 6-*p*-toluidinylnaphthalene-2-sulfonate (2–6 TNS) in a squid giant axon was examined. The apparent dissociation constant for the binding sites in axons was found to be roughly 0.22 mM. Out of approximately 5×10^{14} molecules/cm^2 of 2–6 TNS bound to the sites in and near the axonal membrane, roughly 2×10^{10} molecules/cm^2 are shown to contribute to a transient decrease in fluorescence during nerve excitation. By recording fluorescence signals with a polarizer and analyzer inserted in four different combinations of orientations, studies were made of the directions of the transition moments of various probe molecules relative to the longitudinal axis of the axon. Among hydrophobic probes examined, the polarization characteristics of the fluorescence signals obtained with 1–8 derivatives of aminonaphthalenesulfonate (1–8 ANS, 1–8 TNS and 1–8 AmNS) were found to be very different from those obtained with 2–6 derivatives (2–6 ANS, 2–6 TNS and 2–6 MANS). A tentative interpretation is proposed to account for this difference in physiological behavior between 1–8 and 2–6 derivatives. It is emphasized that measurements of fluorescence polarization yield significant information concerning the structure of the axonal membrane.

A class of fluorescent molecules known as "hydrophobic probes" has been used to detect transient physico-chemical changes of the axonal membrane during the process of nerve excitation [5, 16, 18, 19, 21]. These probes include 8-anilinonaphthalene-1-sulfonate (1–8 ANS), 6-*p*-toluidinylnaphthalene-2-sulfonate (2–6 TNS), and other aminonaphthalene-sulfonate deriatives (see refs. [2, 6, 9 and 25]). These probe molecules are practically nonfluorescent in water; but they strongly fluoresce when dissolved in nonpolar solvents (such as acetone, ethanol, dioxane, etc.) or bound to lipids, proteins or other macromolecules. These molecules are amphipathic; that is to say, both a hydrophobic and hydrophilic portion exist in one molecule.

The information which can be obtained by the use of these probes concerning changes of the axonal membrane during nerve excitation is twofold. A change in the intensity of the fluorescent light informs us of an alteration in the physico-chemical properties (*see* ref. [2]) of the microenvironment of the binding sites in the membrane and/or in the number of the bound probe molecules. The polarization of the emitted fluorescent light informs us of the direction of the emission oscillator (or transition moment) at the moment of emission of radiation.

The present paper describes the results of further studies of the properties of the axonal membrane conducted by the use of a variety of amino naphthalene derivatives as probes. Attempts are made to estimate the density of the binding sites in axons available for 2–6 TNS molecules, to evaluate the extent of physico-chemical changes occurring at the binding sites during nerve excitation, and to clarify the difference in polarization properties of the fluorescence signals obtained with different fluorescent probes. Based on the results of comparison of several isomers of amino-naphthalene-sulfonate derivatives, a tentative interpretation is offered to account for the difference in behavior between 1–8 ANS and 2–6 TNS in axons. It is emphasized that studies of fluorescence polarization yield significant information concerning the macromolecular architecture of the axonal membrane.

Materials and Methods

The material used in the first half of the present studies was giant axons of squid (*Loligo pealii*) available in Woods Hole, Massachusetts. In the second half of the present studies, claw nerves of spider crabs (*Libinia emarginata*) and walking leg nerves of lobsters (*Homarus americanus*) were used. In squid giant axons, fluorescent probes were administered as a rule by intracellular perfusion with a phosphate buffer solution (pH 7.3 ± 0.1) containing 400 mEquiv/liter K-ion and 2–6 TNS at a concentration less than 0.14 mM. In some instances, probes were introduced, as in the preceding studies [18, 19], by injection into the axoplasm. In crab and lobster nerves, fluorescent probes were applied externally by immersing bundles of nerve fibers in artificial seawater (*see* ref. [19]) containing one of the probes (at a concentration between 0.14 and 0.3 mM) for a period of 7 to 20 min.

8-Anilinonaphthalene-1-sulfonate (1–8 ANS), 8-toluidinylnaphthalene-1-sulfonate (1–8 TNS) and 8-aminonaphthalene-1-sulfonate (1–8 AmNS) were obtained from Eastman Organic Chemicals. 6-Toluidinylnaphthalene-2-sulfonate (2–6 TNS) was obtained from Sigma Chemical Company. 6-Aminonaphthalene-2-sulfonate (2–6 AmNS) and 5-aminonaphthalene-1-sulfonate (1–5 AmNS) were obtained from K and K Chemicals. 8-Aminonaphthalene-2-sulfonate (2–8 AmNS) was obtained from Aldrich Chemical Co. 6-Anilinonaphthalene-2-sulfonate (2–6 ANS) and 6-N-methyl-anilinonaphthalene-2-sulfonate (2–6 MANS) were a generous gift of Dr. Robert P. Cory of Oregon State University.

The optical setup used was described in detail in a preceding paper [19]. Squid giant axons or crab (or lobster) nerves were exposed to quasimonochromatic light

(365 ± 10 nm) from a 200 W xenon-mercury lamp used in conjunction with an interference filter. The fluorescent light from the nerve was detected at right angles to the incident light with a photomultiplier tube (RCA C70109E) through an absorption filter (Wratten 2A or a Corning CS 3–73). A polarizer (Polaroid sheet HBP'B) was sometimes inserted between the interference filter and the nerve and an analyzer (HN 38 or KN 36) between the nerve and the absorption filter. In crab and lobster nerves stained with 1–8 ANS or 1–8 AmNS, fluorescence signals could be recognized directly on the screen of an oscilloscope. In general, however, averaging over 100 to 10,000 trials was carried out to demonstrate fluorescence signals. Squid and crab experiments were carried usually at about 8°C while lobster experiments were conducted at about 15 °C.

Results

State of Binding of 2–6 TNS in Axon Interior

As the first step toward clarifying the state of binding of 2–6 TNS molecules in the interior of a squid giant axon, the relationship between the probe concentration in the axon and the intensity of the emitted fluorescent light was examined. By the technique described elsewhere [17], two glass pipettes were inserted into the interior of an axon and intracellular perfusion with a K-phosphate solution containing pronase (0.01 mg/ml) was performed for a period of about 15 sec. At the end of this period, the internal perfusion fluid was switched to an enzyme-free, K-phosphate solution containing a low concentration of 2–6 TNS. This procedure brought about a rapid increase in the intensity of the fluorescent light arising from the axon. When the intensity reached a roughly steady level, the 2–6 TNS concentration was raised (*see* Fig. 1). By increasing the probe concentration step by step, the fluorescence intensity was shown to increase monotonically with the 2–6 TNS concentration employed.

To determine the apparent dissociation constant K for 2–6 TNS in the axon, a double reciprocal plot was constructed from the above data and is shown in the inset in Fig. 1. Similar to the method of Gomperts and Stock [7], the reciprocal of the fluorescence intensity was plotted against the reciprocal of the dye concentration (with the amount of membrane material remaining constant). It is seen that the observed points lie close to a straight line. (The point for 8.5 μM TNS is not plotted because of its uncertainty due to its small value relative to the background light.) The intersection of an extrapolation of the straight line with the abscissa corresponds to $-K$; and K for 3 axons fell in the range between 0.20 and 0.23 mM. Studies of 2–6 TNS in model systems reveal a K of 0.02 mM for phosphatidylcholine vesicles [8] and K values of 0.01 to 0.13 mM for a variety of water-soluble proteins (*unpublished data*). For 1–8 ANS binding to microsomes, K was found to be approximately 0.1 mM [7].

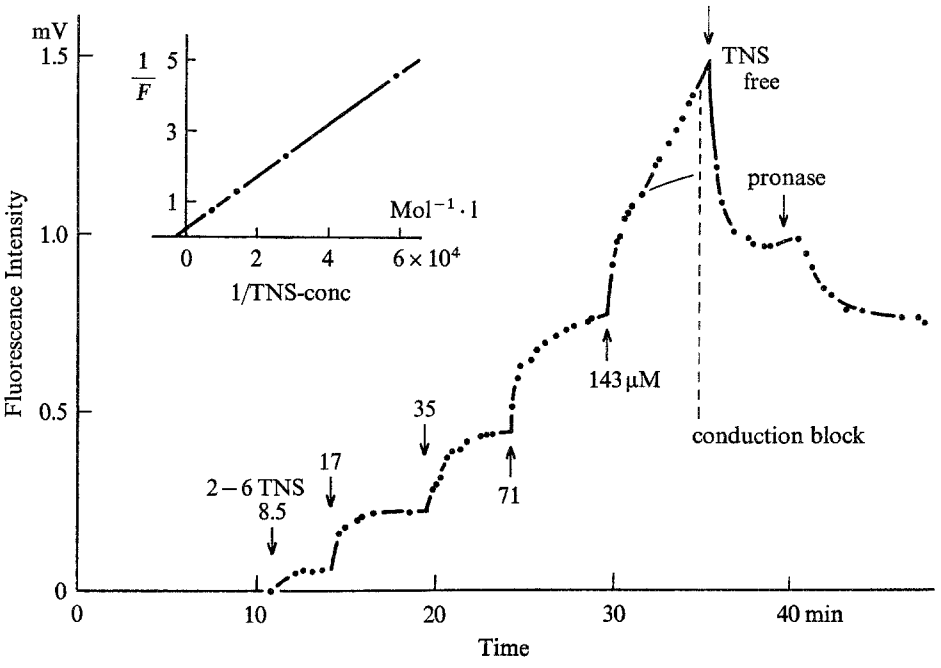


Fig. 1. Relationship between the internal 2–6 TNS concentration and the fluorescence intensity. The fluorescent probe was introduced into a squid giant axon (470 μ in diameter) by intracellular perfusion. The concentration was raised to the level indicated at the moments marked by the arrows. When nerve conduction was blocked at 34.5 min, the internal perfusion fluid was switched to a TNS-free K-phosphate solution. Later, pronase (0.05 mg/ml) was added to the K-phosphate solution; the observed fall in fluorescence is due to removal of 2–6 TNS bound to the axoplasm. In the inset, the reciprocal of the final level of fluorescence F was plotted against the reciprocal of the probe concentration. Temp. approximately 19 °C

When the internal concentration of 2–6 TNS was maintained at a level higher than about 0.14 mM, conduction of action potentials along the axon was eventually blocked. It is interesting to note that this conduction block was usually preceded by a relatively rapid rise in the intensity of the fluorescent light from the axon. This rapid rise in fluorescence may be regarded as a sign of increased accessibility of the hidden hydrophobic sites in the axon. It seems probable that this rise is causally related to the loss of permselectivity and the fall in membrane resistance which precedes conduction block in axons.

Under the conditions of the experiment illustrated in Fig. 1, it is unlikely that the binding sites for the probe molecules are limited to those in the axonal membrane. A layer of axoplasm remaining in the axon interior appears to contain a large number of binding sites. With a view to estimating

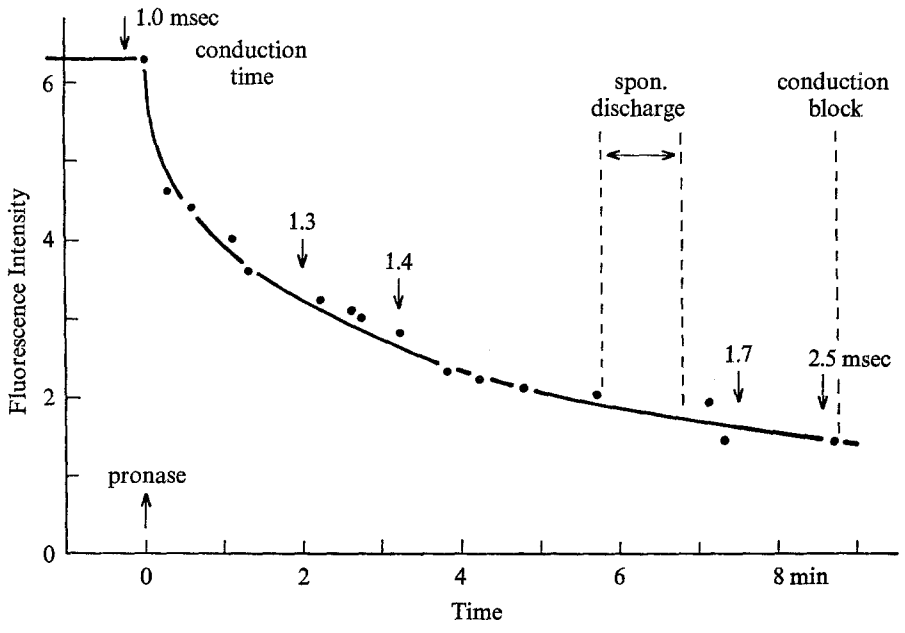


Fig. 2. Effect of removal of the axoplasm by proteolysis on the intensity of fluorescence of a squid giant axon internally perfused with a 2–6 TNS-containing solution. Initially, the axon was internally perfused with a K-phosphate solution containing 0.14 mM 2–6 TNS. At time zero, pronase (0.05 mg/ml) was added to the perfusion fluid. Note that there was a gradual increase in the time required for nervous conduction across the perfusion zone, as indicated in the figure. Spontaneous discharge of nerve impulses was also seen for a limited period of time. Temp. 19 °C

the distribution of the binding sites in the axon, the following experiments were conducted. A squid giant axon was internally perfused with a K-phosphate solution containing 2–6 TNS for a period of about 10 min. At the end of this period, the internal perfusion fluid was switched to a solution containing pronase (0.05 mg/ml). The concentrations of K-phosphate and 2–6 TNS remained unaltered during this procedure (*see* Fig. 2).

It is seen in the figure that the intensity of the fluorescent light from the axon immediately started to fall when pronase was introduced intracellularly. Obviously, this fall is due to removal of some of the 2–6 TNS molecules bound to the axoplasm. (Note that the unbound TNS molecules are practically nonfluorescent.) Pronase is known to remove practically all of the axoplasm before block of nerve conduction sets in [13, 15]. It was hoped that the ratio of the binding sites in the axoplasm to those in the axonal membrane could be determined by comparing the initial level of fluorescence with that at the time of conduction block.

It was found (somewhat unexpectedly) that the results of these experiments varied widely from axon to axon. In seven axons examined, the fluorescence intensity at the time of conduction block varied in the range between 25 and 90% of the initial level.

It seems reasonable to attribute this variation in the results to spatially non-uniform action of the proteolytic enzyme. It is difficult to place the perfusion pipettes exactly at the center of the axon; hence, the process of enzymatic digestion could proceed unevenly within the perfusion zone of the axon. Furthermore, proteolysis could expose some of the initially inaccessible hydrophobic sites both in the axoplasm and in the membrane. Consequently, it is difficult to estimate the partition of probe molecules between the axoplasm and the membrane by the present method.

Using these same axons, a direct measurement was made of the amount of 2–6 TNS bound to and near the axonal membrane. At the moment when conduction was blocked, the fluid in the axon was replaced with air. Then, after removing the artificial seawater in the nerve chamber, the perfusion zone of the axon was resected and was transferred to a large volume (4 ml) of ethanol. Then, by measuring the fluorescence of the ethanol solution after equilibration, the total amount of 2–6 TNS transferred into ethanol was determined. In four axons examined, measurements of this type showed that the amount of the probe that was present in the axon was approximately 0.04 μg.

If the whole amount of 2–6 TNS thus determined is assumed to be the membrane-bound molecules, the density of the probe molecules in the membrane is found to be 7×10^{13} per axon, or roughly 5×10^{14} per cm^2 of the membrane. The error due to the presence of some probe molecules in the residual layer (less than 10 microns in thickness) of axoplasm and perfusion fluid is estimated not to exceed about 20%.

Two Classes of 2–6 TNS Molecules at Internal Active Sites

It was shown in previous papers [18, 19] that intracellularly applied 2–6 TNS produces fluorescence signals which represent changes in intensity of highly polarized emission during nerve excitation. The following observations were made to clarify the polarization properties of the emitted light under these conditions.

A squid giant axon was internally perfused for 3 min with a phosphate buffer solution containing 400 mEquiv/liter K-ion and 0.14 mM TNS. The incident quasi-monochromatic light of 365 nm wavelength was polarized with the polarizing axis of a polaroid sheet oriented either parallel or per-

pendicular to the longitudinal axis of the nerve. The polarization of the fluorescent light was examined by using a polaroid-sheet analyzer with its polarizing axis directed either parallel or perpendicular to the longitudinal axis of the nerve. When the polarizing axes of the analyzer and polarizer are both parallel to the nerve, this is referred to as the "parallel-parallel" condition. When the polarizer is parallel and the analyzer is perpendicular, it is called the "parallel-perpendicular" condition. The two other possible combinations are denoted in a similar fashion as the "perpendicular-parallel" and "perpendicular-perpendicular" conditions.

Fig. 3 shows the results of measurements of fluorescence signals associated with action potentials of squid giant axons under various polarization conditions. In the parallel-parallel condition there was a transient decrease in the fluorescent light (left record). In the perpendicular-perpendicular condition, however, there was a transient increase (middle record). No optical signal was obtained in either the parallel-perpendicular condition [18] or in the perpendicular-parallel condition (right record).

The sign of the fluorescence signal was negative (representing a transient decrease in fluorescence) when both the analyzer and polarizer were removed [16]. This fact indicates that under illumination with a quasimonochromatic light of a given intensity, the negative signal (shown on the left of the figure) is larger than the positive signal (shown in the middle). The ratio of the amplitude of the negative signal to that of the positive signal was estimated to be roughly 3:1.

We have shown in the Appendix of the preceding paper [19] that the transition moment (i.e., the direction of the absorption and emission oscillators) of 2–6 TNS is oriented in the direction of the long axis of the molecule. Therefore, we conclude that the negative 2–6 TNS signal derives from a class of probe molecules in the membrane with their long axis oriented along the long axis of the axon. Similarly, the positive 2–6 TNS signal was attributed to a class of probe molecules oriented transversely to the axonal axis (either radially or annularly relative to the membrane). A possible significance of these conclusions will be described in the Discussion.

A hyperpolarizing response observed in an axon immersed in a K-ion-rich medium may be regarded as an electrophysiological manifestation of macromolecular processes representing a transition from the depolarized state to the repolarized state [14]. In the preceding article [19] we have described the results of fluorescence studies of these responses carried out with the incident light polarized in the direction parallel to the axonal axis. The results of further studies on hyperpolarizing responses are presented in Figs. 4 and 5.

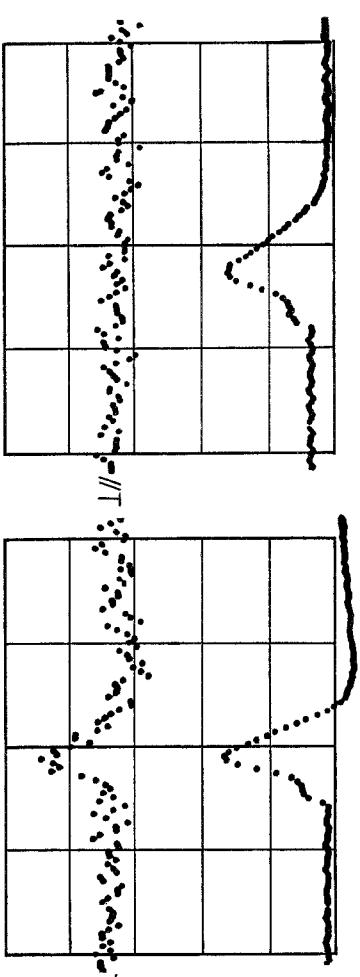


Fig. 3. Records showing polarization characteristics of the fluorescence signals in squid giant axons labeled internally with 2–6 TNS. The direction of the polarizing axis of the polarizer and that of the analyzer are indicated by a pair of symbols, parallel or perpendicular, written on the left side of each fluorescence (upper) trace. The lower trace indicates the time course of the internally recorded action potentials. Three different axons were used; each was perfused for 3 min with 0.14 mM TNS. The current pulse used for stimulation was roughly 0.33 mamp/cm^2 in intensity and 0.1 msec in duration. Both the intensity of the incident light and the sensitivity of the detector (photomultiplier) were kept approximately constant throughout. However, due to the difference in the background light intensity under different polarization conditions for different axons, the calibration of the fluorescence trace, expressed in ratio of the change to the background intensity, were different in three axons; it was approximately 2.5 (for the left record), 6 (for the middle record) and 8 (for the right record) times 10^{-5} per division. The amplitudes of the action potentials were approximately 100 mV. *Abscissa*: 3.9 msec per division. Temp., 8 °C

A squid giant axon stained with 2–6 TNS applied by intracellular perfusion was immersed in a 1:4 mixture of an isotonic (0.53 M) KCl solution and artificial seawater. In this mixture, the axon showed no ability to respond to pulses of outwardly directed transmembrane current. However, to strong pulses of inwardly directed current, the axon responded with large, all-or-none hyperpolarizations (*see* the trace marked *V* in Fig. 4). Fluorescence signals were recorded under these conditions with neither a polarizer nor an analyzer inserted in the optical pathway. It is seen in the figure that hyperpolarizing responses were accompanied by definite, positive fluorescence signals. A negligibly small signal was observed when the applied current was reversed (*see* the right-hand record in Fig. 4).

The records presented in Fig. 5 show the results obtained with both the polarizer and the analyzer inserted in various combinations of their directions relative to the long axis of the axon. In the parallel-parallel condition, a large, positive fluorescence signal was observed. However, in the perpendicular-perpendicular position, the observed signal was negative, indicating the presence of a transient decrease in fluorescence during hyperpolarization. Again, in the parallel-perpendicular or the perpendicular-parallel conditions, practically no signals were recorded.

These observations are quite consistent with the notion that there are, at or near the inner surface of the axonal membrane, two classes of 2–6 TNS molecules which behave independently in production of fluorescence signals. One class of 2–6 TNS molecules, which are oriented with their transition moment parallel to the long axis of the axon, can be optically excited only with the incident light polarized in the parallel direction; emission of these molecules is polarized in the direction of the long axis of the axon. The other class of 2–6 TNS molecules are excited only with the incident light polarized perpendicularly in relation to the long axis of the axon. Since the sign of the signal observed without a polarizer and analyzer was always negative under these conditions (Fig. 4), we conclude that the amplitude of the signal produced by the first class of probe molecules is definitely larger than that produced by the second class. All the results described above were obtained with the external K-ion concentration maintained at approximately 110 mEquiv/liter. Under these conditions, replacement of the external K-rich solution with normal seawater was found to restore the normal excitability of the axon either completely or partially.

When the external K-ion concentration was raised to a level higher than 250 mEquiv/liter (the external Ca-ion concentration being about 100 mM), a new phenomenon was encountered. A distinct negative signal was observed in the parallel-perpendicular condition. This indicates that

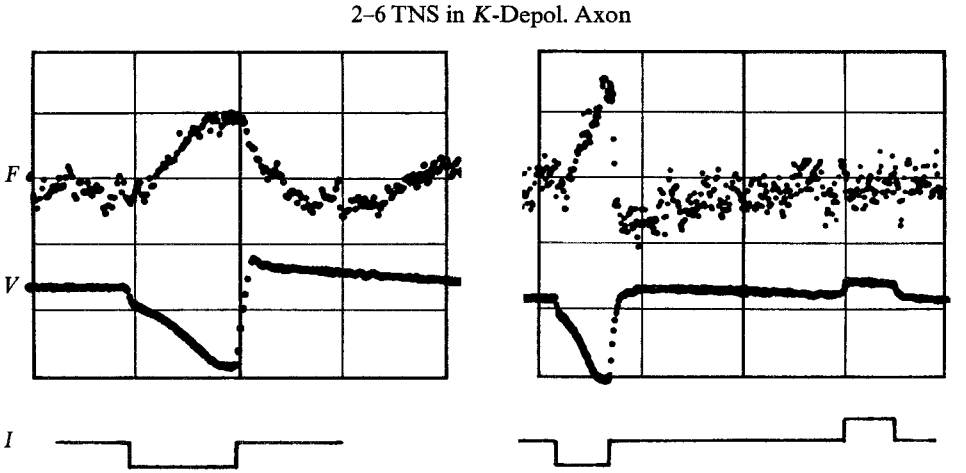


Fig. 4. *Left:* Changes in fluorescence F and in membrane potential V associated with a hyperpolarizing response produced in a KCl-depolarized squid giant axon by a pulse of inwardly directed transmembrane current I. The calibration for trace F was roughly 6×10^{-6} times the background light per division. The amplitude of the hyperpolarizing response was about 150 mV. The current pulse used was approximately 0.4 mamp/cm^2 in intensity and 30 msec in duration. Temp., 8 °C. *Right:* Similar record taken from a different axon at a slower sweep-speed. The applied current pulses were about 0.6 mamp/cm^2 in amplitude and 30 msec in duration. Note that these fluorescence signals correspond to the difference between the two signals in the left record shown in Fig. 5

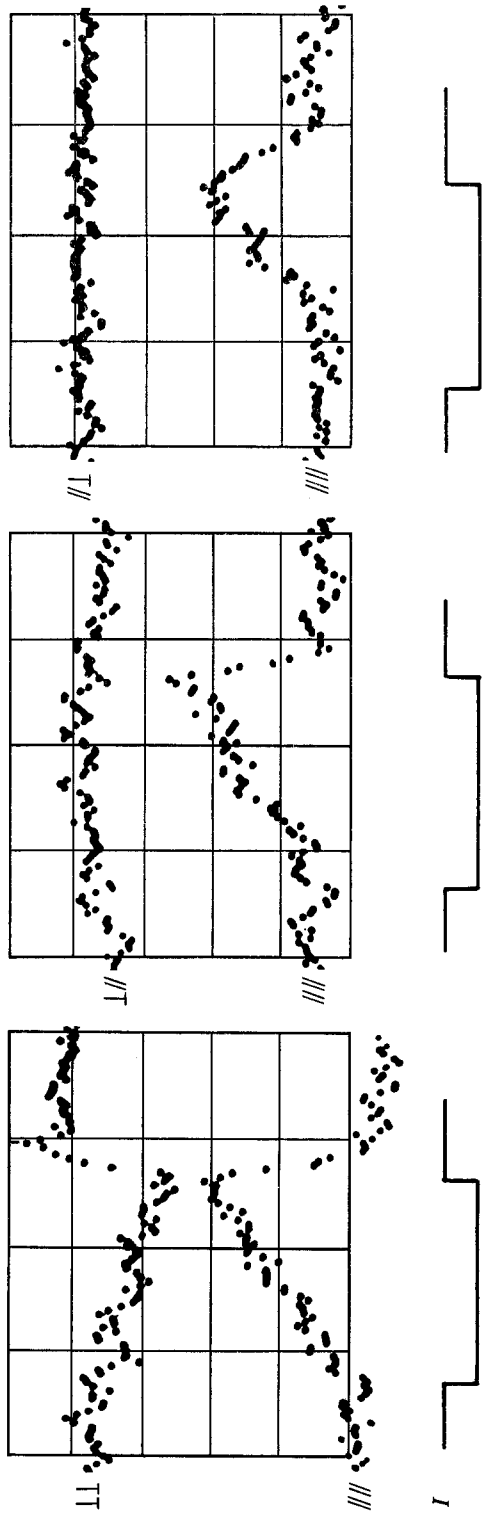


Fig. 5. Records showing the polarization characteristics of the fluorescence signals associated with hyperpolarizing responses. The directions of the polarizer and those of the analyzer are indicated. The records were taken from three different axons; fluorescence traces in each frame were taken successively from one axon. The calibration of the ordinate was approximately 7.7×10^{-5} times the background light intensity per division. *Abscissa:* 15 msec per division. The applied current pulses were roughly 0.3 mamp/cm^2 in intensity and 30 msec in duration. Temp., 8 °C

the distinction between the two classes of 2–6 TNS molecules in the axon was no longer very clear under these circumstances. It is important to note that in these axons, the ability to produce a normal (i.e., depolarizing) all-or-none action potential could never be restored by replacing the external K-rich medium with normal seawater. We suggest the following explanation of this phenomenon: A high K-ion concentration in the external medium brings about an irreversible alteration in the macromolecular organization of the axonal membrane. Hyperpolarizing responses are more resistant to this alteration than normal (depolarizing) responses, probably because it is not necessary to have a highly regenerative potential variation to recognize the presence of a hyperpolarizing response.

Differences Between 1–8 ANS and 2–6 TNS in Production of Fluorescence Signals

In an early stage of our investigation with fluorescent probes, we noted that the sign of fluorescent signals obtained with externally applied 1–8 ANS is positive while that of externally administered 2–6 TNS is negative (*see* ref. [16]). In a later study [19], we noted that fluorescence signals produced by internally applied 1–8 ANS is *not* highly polarized while, as shown in Figs. 3, 4 and 5, signals deriving from 2–6 TNS in axons represent changes in highly polarized emission. It is thus very clear that there is a significant difference in behavior inside axons between these two probes which behave very similarly when examined in test tubes (*see* ref. [23]). The following observations were made to examine the difference in behavior between these two probe molecules under the condition of external application.

Nerve trunks taken from either crabs or lobsters were used because of the ease of making polarization measurements. We have seen that fluorescence signals recorded from squid fin nerves are qualitatively very similar to those taken from crustacean nerves [16]. Therefore, we assume that the qualitative aspects of fluorescence signals are not seriously affected by the difference in species of the animals from which nerves were taken. The probe molecules were incorporated in the nerve by immersion in artificial seawater to which one of the probes was added at a concentration of approximately 0.3 mM. After an immersion period of 7 to 20 min, the nerves were rinsed with probe-free seawater and the fluorescence properties were examined in the nerve chamber by the technique similar to that used in studying squid giant axons. In some instances, the seawater in the nerve chamber was removed and the fluorescence was examined with the nerve suspended in moist air; the results obtained by this moist chamber method were qualitatively the same as those obtained from stained nerves immersed in seawater.

An example of the results obtained from crab nerves stained with 2–6 TNS is presented in Fig. 6. A large negative signal was observed when the polarizer and the analyzer were under the perpendicular-perpendicular arrangement. Under the parallel-parallel arrangement, a relatively small, positive signal was observed. Small, negative signals were obtained when the polarizing axis of the polarizer, either parallel or perpendicular to the axon, was at right angles to that of the analyzer (*see* the two lower records in Fig. 6). The two signals shown on the right side of Fig. 6 were found to be opposite in sign but similar in amplitude; only a very small, negative signal was obtained with the polarizer in the parallel position but without any analyzer.

The polarization properties of the crab nerve stained externally with 1–8 ANS and 1–8 AmNS are shown by the records presented in Fig. 7. With these probes, signals observed were always positive, regardless of the orientation of the polarizer or of the analyzer. The difference in amplitude among the signals obtained under different polarizing conditions was relatively small.

It is important to note that the polarization properties of the signal from 1–8 TNS are very similar to those of 1–8 ANS and 1–8 AmNS. However, the properties of 2–6 ANS signals were found to be very similar to those of 2–6 TNS signals shown in Fig. 6, and not to those of 1–8 ANS signals. From these observations, we conclude that the difference in behavior among these probe molecules is directly related to the positions of the sulfonate and the other (either amino or anilino) group on the naphthalene ring. We note also that substitution of an anilino-group for a toluidinyl-group at the same position of the naphthalene ring does not bring about any significant change in the polarization characteristics of the fluorescence signals. A plausible interpretation of these findings will be presented in the Discussion. It is interesting to note in this connection that the polarization properties of the fluorescence of a lobster nerve stained with 2–6 MANS were found to be very similar to those of 2–6 TNS (Fig. 6) except for one small difference: that no clear signal was obtained under the parallel-parallel condition. (It is also very interesting that squid axons stained internally with 2–6 MANS produce, in response to stimulation, large positive signals under the perpendicular-perpendicular condition, small positive signals under the perpendicular-parallel and parallel-perpendicular conditions, and practically no signal under the parallel-parallel conditions.)

In addition to the fluorescent probes mentioned above, we examined three more isomers of aminonaphthalene-sulfonate derivatives: they are 2–6, 1–5 and 2–8 AmNS. These isomers do not come under the category of

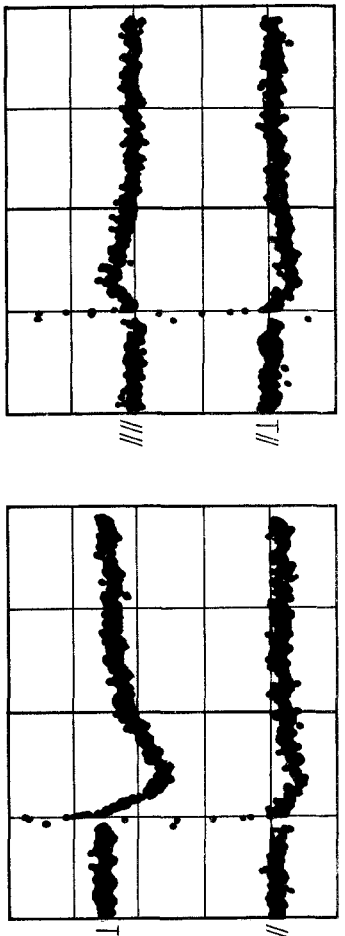


Fig. 6. Fluorescence signals observed in a crab nerve labeled with 2–6 TNS by external application. The nerve was stimulated maximally at a frequency of 3.5/sec with a pair of external electrodes placed outside the zone of optical recording. The direction of the polarizer and those of the analyzer are indicated. Both the intensity of the incident light and the sensitivity of the detector were kept constant throughout the experiment. The calibration of the ordinate, expressed in ratio of the change in fluorescence to the background light intensity, was approximately 7 (for the upper left trace), 6 (lower left), 5 (upper right) and 6 (lower right) times 10^{-4} per division. *Abscissa:* 33 msec per division. Temp., 8 °C

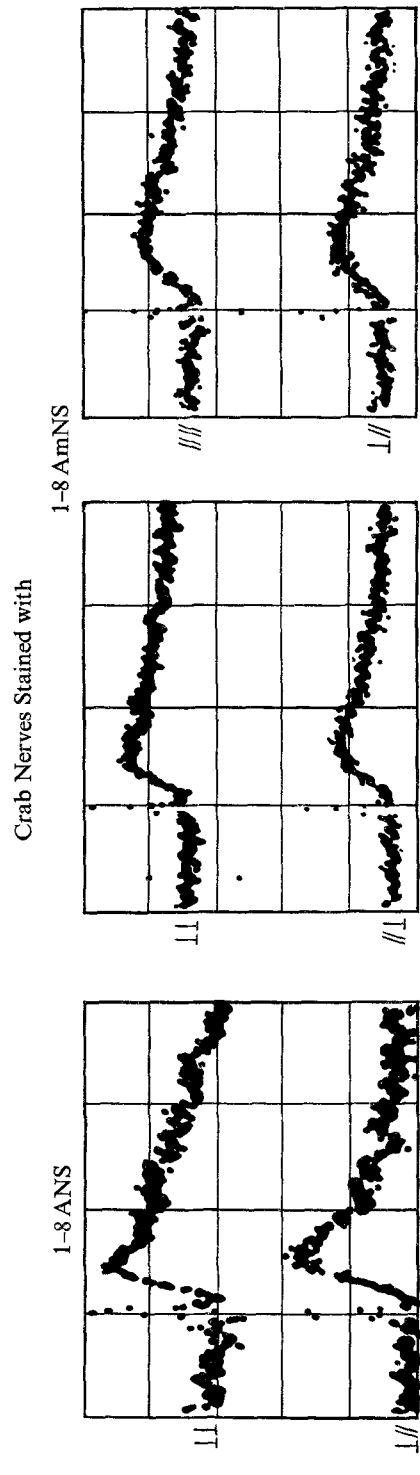
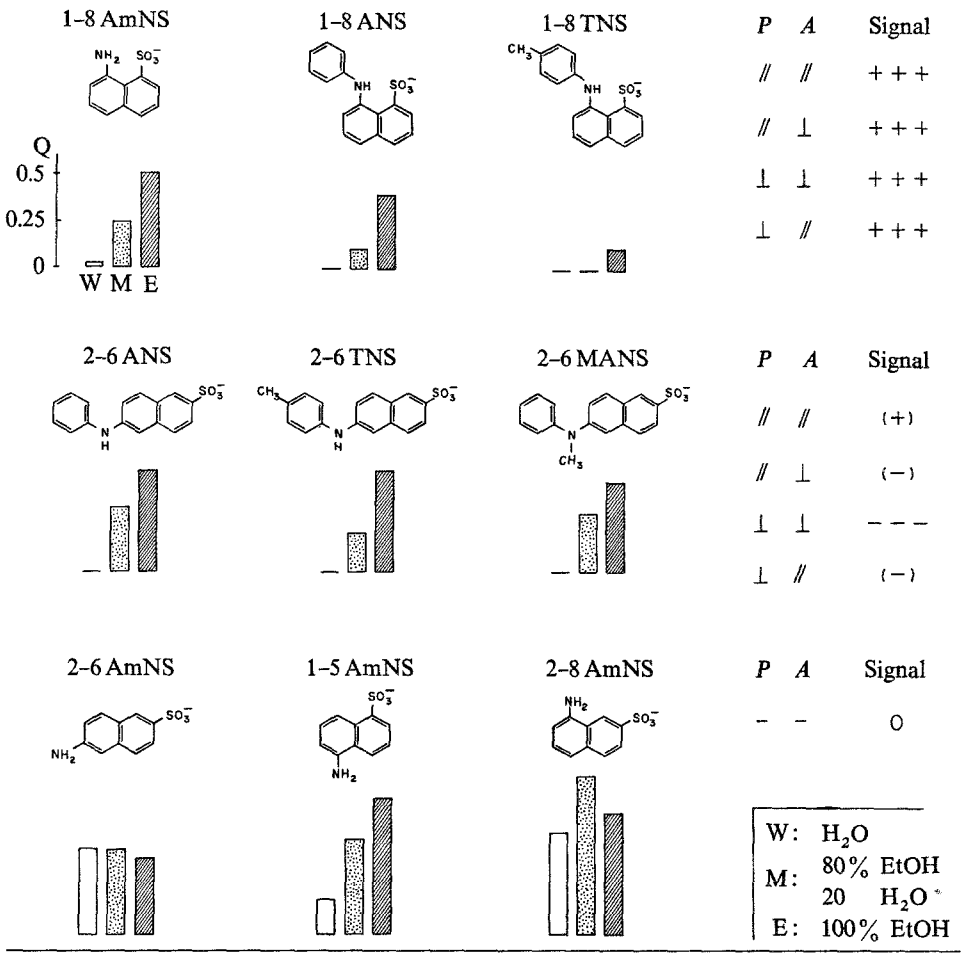


Fig. 7. *Left:* Fluorescence signals recorded from a crab nerve labeled with 1–8 ANS externally. The directions of the polarizer and the analyzer are given. The calibration of the ordinate, 5.4×10^{-5} times the background light intensity. *Abscissa:* 32 msec per division. Temp., 8 °C. *Right:* Fluorescence signals recorded from a crab nerve labeled with 1–8 AmNS externally. The calibration of the ordinate, 2.8×10^{-4} per division. *Abscissa:* 32 msec per division. Temp., 8 °C

Table 1. Quantum yields of hydrophobic probes in three different media and the characteristics of the fluorescence signals observed in lobster nerves labeled with these probes[a]



[a] The data for the quantum yields are taken from Turner and Brand [23] except those for 1-8 TNS (approximate). The direction of the polarizing axes of the polarizer *P* and that of the analyzer *A* relative to the long axis of the axon are given. The plus and minus signs represent the relative magnitudes and the direction of the signals observed; 0 indicates that no signal was observed. Note that three probes listed in the same row give rise to similar fluorescence signals. Temp., 15 °C.

"hydrophobic probes" since their quantum yield in water is not very different from that in nonpolar solvents (*see* Table 1). Lobster nerves treated with these derivatives were found to give rise to a strong fluorescent light at rest; however, no transient change in fluorescence could be produced in response to electric stimulation of these nerves.

The fluorescent characteristics of lobster nerves stained extracellularly with nine different aminonaphthalene-sulfonate derivatives are summarized in Table 1. The top row of the table includes 1–8 derivatives which gave rise to positive fluorescent signals in externally stained nerves. The middle row contains 2–6 derivatives which produced large negative signals under the perpendicular-perpendicular condition and small or questionable signals under other polarization conditions. The bottom row shows those with which we were unable to record fluorescence signals. The difference in the characteristics of the fluorescence signals in the top and middle rows offers support to the notion that the spatial arrangement of the side-groups on the naphthalene ring have a strong influence on the size and direction of the fluorescence signals (*see* Discussion). A high quantum yield in water of the dyes listed in the bottom row strongly supports our conclusion that fluorescence signals reflect changes in the polarity of the immediate surrounding of the probe molecules [19].

Discussion

Tentative Interpretation of the Origin of Fluorescent Signals

In axons stained with fluorescent hydrophobic probes, the major portion of the probe molecules is undoubtedly bound to electrophysiologically unresponsive sites; namely, to the sites which remain unaltered during the process of nerve excitation. When a probe is applied extracellularly, the majority of the applied probe remains in the layer of connective tissue and in the Schwann cell membrane; and the fluorescent light deriving from these probe molecules constitutes a large portion of the strong background light observed in the nerve at rest. It is relatively easy to investigate the properties of the probe molecules at these electrophysiologically unresponsive sites. In fact, both the emission spectra and the lifetimes of the excited state of various probe molecules have been measured. Since, however, the results of these measurements probably have little bearing on our analysis of the process of nerve excitation, they will be described elsewhere.

From a biochemical point of view, the axonal membrane may be regarded as having a non-uniform, mosaic structure consisting of physico-chemically distinct parts of various macromolecules. There is good experimental evidence that negative fixed charges (responsible for anion exclusion) exist on the external layer of the axonal membrane and we believe that these charged sites play an important role in production of action potentials (pp. 71–73 in ref. [15]). Hence, it is reasonable to assume that the portion of the probe

molecules which contributes to production of observed fluorescence signals is located near these charged sites. We note that the thickness of the Gouy-Chapman layer in the medium (*see*, e.g., p. 131 in ref. [11]) is roughly 4 Å. Then, the distribution of these anionic probe molecules near the axonal membrane is under the direct repulsive influence of the ionic atmosphere and the phase-boundary potential (*see* p. 318 in ref. [22]) associated with the negative fixed charges.

We believe that there is, during an action potential, a change in the phase-boundary potential which tends to raise the potential of the membrane matrix relative to that of the external medium [4]. This change in the phase-boundary potential is expected to reversibly enhance partition of the negatively charged probe molecules in the membrane phase; this could explain the observed increase in fluorescence of externally applied 1–8 ANS during an action potential (Fig. 7). The same interpretation may be applied to the signals observed with externally applied 1–8 AmNS (*see* Fig. 7) or 1–8 TNS. A transient decrease in resistance to ion-transport through the membrane could be an additional factor which facilitates invasion of probe molecules into the membrane during nerve excitation; however, this factor may exert only a minor influence on anions like 1–8 ANS. This may be a more important factor for cationic hydrophobic probes like ethidium bromide which gives, in lobster or crab nerves, unpolarized fluorescence signals very similar to those of 1–8 ANS (*unpublished data*).

In the case of 2–6 TNS and 2–6 ANS, the hydrophobic portion of the probe molecule is about 11 Å long [3]; it is much longer than the thickness of the Gouy-Chapman layer. Hence, if the molecular axis is oriented perpendicular to the membrane surface, the nonpolar ends of the probe molecules can reach the membrane matrix with the sulfonate group remaining outside the range of the repulsive electric force near the negatively charged membrane sites. We therefore assume that some of these long probe molecules are located in the Stern layer of the membrane in the manner similar to that described by Verwey [24]. The quantum yield of these probe molecules is expected to fall when the membrane goes, during the process of nerve excitation, from a Ca-rich, poorly hydrated state to a state with a high water content [17]. The absorption and emission oscillators of these molecules coincide approximately with the long axis of the molecule [19] and they are nearly perpendicular to the membrane surface in this case. Therefore, an incident light polarized perpendicularly to the long axis of the axon is required to excite these molecules, and the emitted fluorescence is polarized preferentially in the perpendicular direction. The large, negative fluorescence signal shown in Fig. 6 is explained tentatively in this manner.

A small number of 2–6 TNS molecules may exist outside the Gouy-Chapman layer as in the case of 1–8 ANS; and these randomly oriented probe molecules may enter into the Stern layer during nerve excitation. The small, positive signal in Fig. 6 may be explained in this manner.

The fluorescence properties of internally applied 2–6 TNS have been discussed previously [18, 19]. The large, negative signal in Fig. 3 has been interpreted by assuming the existence, on the inner side of the membrane, of a rigid paracrystalline structure which orients the long axis of the probe molecules parallel to the long axis of the axon; a fall in the quantum yield of these probe molecules is regarded as the origin of the large, negative signal in Fig. 3. The small, positive signal in Fig. 3 may be interpreted by assuming that a small fraction of the internally applied 2–6 TNS molecules is arranged perpendicularly to the membrane surface. Since production of an action potential is known to be associated with an enormous increase in Ca-influx [1, 20], the observed increase in fluorescence may be attributed to the Ca-ion effect (*see* Fig. 7 in ref. [19]). Recent studies with the calcium-sensitive fluorescent probe, chlorotetracycline (Hallett *et al.*, *in preparation*) have offered independent experimental support to this notion. In a similar manner, the fluorescence signals associated with hyperpolarizing responses (Figs. 4 and 5) may be attributed to the presence of two classes of 2–6 TNS molecules, one class with their absorption and emission oscillators parallel to the long axis of the axon and the other class perpendicular to the axon.

When short probe molecules (1–8 ANS and 1–8 AmNS) are held in the rigid, paracrystalline structure, their absorption and emission oscillators are not expected to be oriented along the long axis of the axon. (Note that emission from 1–8 ANS molecules incorporated in stretched polyvinylalcohol membrane is not highly polarized [19].) Therefore, the fluorescence signals from internally administered 1–8 ANS and 1–8 AmNS are not expected to be highly polarized. Production of these signals may be due to loss in quantum yield (similar to the internal, longitudinally oriented 2–6 TNS). It is important to note that anilinonaphthalene-sulfonate derivatives of intermediate lengths, 1–5 ANS and 1–7 ANS, show fluorescence properties which are intermediate between those of 1–8 ANS and of 2–6 ANS. Both the signs and the relative magnitudes of these fluorescence signals under various polarization conditions were found to be given by properly weighted averages of the signals produced by the 1–8 and 2–6 ANS molecules.

The above-mentioned *tentative* interpretation of the observed results is somewhat complex, reflecting the complexity of the axonal membrane in the normal functioning state. It may be pointed out in this connection that the results of more recent investigations conducted in this laboratory by

the use of Ca-sensitive probes (Hallett *et al., in preparation*) and of an improved spectrofluorometer (Tasaki *et al., in preparation*) appear to be consistent with the interpretation presented above.

Estimation of the Number of Bound 2-6 TNS Molecules in Squid Axons

With a view to evaluating the extent of involvement of the membrane material in the process of nerve excitation, a rough estimation is made of the number of intracellular 2-6 TNS molecules contributing to production of the negative fluorescence signal. The portion of the axon used in the pronase experiment shown in Fig. 2 was on the average 0.4 mm in diameter and 12 mm in length, the area of the membrane treated with 2-6 TNS being approximately 14 mm^2. The amount of the probe molecules remaining in and near the axonal membrane at the end of the pronase treatment was approximately 0.04 μg, which corresponds to 7×10^{13} particles (*see* Results). By putting this number of molecules in solvents of known Z-value into a glass capillary (with diameter similar to that of the axon) and by matching the fluorescence intensity with that of the axon, the average quantum yield of these probe molecules in the axon was estimated to be roughly 0.2.

Next, we estimate the change in quantum yield of 2-6 TNS at the electrophysiologically responsive TNS-sites by the following procedure: the spectrum of the fluorescence signal (*see* Fig. 2 in ref. [18]) represents the difference between the emission spectrum of the probe molecules at the responsive sites in their electrophysiologically resting state and that in their active state. (Note that these experiments were done with a parallel polarizer). For the reasons to be mentioned later, we assume that both the quantum yield and the shape of the emission spectrum of 2-6 TNS in axons are uniquely determined by the polarity (or Z-value) of the binding sites. We should then be able to reproduce the spectrum of the observed fluorescence signal by computing the difference between two spectra of 2-6 TNS in solvents which have different Z-values. In fact, we could reproduce the observed signal spectrum by subtracting a 2-6 TNS spectrum in one mixture of dioxane (or ethanol) and water from that in a different mixture. It was found by examination, especially in the region from 440 nm to 500 nm, that the observed spectrum of the fluorescence signal corresponds to a fall in quantum yield from 0.60 ($\pm$0.08) to 0.35 ($\pm$0.06). [Note that the difference between the two emission spectra, one for TNS in pure ethanol and the other for TNS in 80% ethanol (shown in Fig. 11 of *ref.* [19]), nearly accurately represents the observed spectrum of the signal.] It is quite important to note that no unsubstracted emission curve could be used to

mimic the observed signal spectrum. This indicates that the observed signal cannot be interpreted as deriving from molecules whose quantum yield dropped essentially to zero; that is, from molecules which drop completely off their binding sites during nerve excitation. It also indicates that the signal cannot be interpreted as deriving from a large number of probe molecules whose quantum yield changes by only a very small fraction.

The amplitude of the fluorescence signal is, under favorable experimental conditions, about 5×10^{-5} times the background (resting state) fluorescence intensity. We denote the number of the probe molecules involved in production of the signal by n. Then, the ratio of the signal to the background light intensity for 2–6 TNS is given approximately by

$$\frac{(0.60-0.35)n}{0.2 \times 7 \times 10^{13}} = 5 \times 10^{-5}.$$

We thus find that $n = 3 \times 10^{9}$ particles per axon or approximately 2×10^{10} probe molecules per cm^2. This is roughly the number of responsive TNS-sites occupied by 2–6 TNS molecules; the total number of electrophysiologically responsive binding sites for 2–6 TNS has to be somewhat larger than this value.

In the rough calculation mentioned above, we have ignored the possibility that specific chemical quenching (*see* ref. [9]) may play an essential role in production of 2–6 TNS signals in squid axons. It seems unlikely, however, that such a process is very important for the following reasons. If the fluorescence of the 2–6 TNS at the binding sites were strongly quenched at rest, it would be impossible for these practically nonfluorescent probe molecules to produce a signal which represents a transient decrease in fluorescence. If, on the other hand, strong quenching is assumed to occur only during nerve excitation, we expect that the spectrum of the fluorescence signal coincides with that of 2–6 TNS in one of the dioxane (or ethanol)-water mixtures; this was not found. Thus, strong chemical quenching seems to be absent for 2–6 TNS in axons. However, the existence of weak chemical quenching cannot be excluded.

General Comment on the Fluorescence Method Applied to Axons

Changes in intensity of the fluorescent light from axons labeled with "hydrophobic" probes are attributed to (1) changes in the quantum yield and/or (2) changes in the number of bound probe molecules. The number of bound molecules may be altered (a) when the local concentration of the probe molecules changes, (b) when the affinity of the binding sites toward the

probe changes, or (c) when the number of binding sites in the axon changes (*see* ref [12]). In *in vitro* studies of labeled macromolecules, it is possible to distinguish these different possibilities by the use, for example, of the double-reciprocal plot (*see*, e.g., ref. [7]). In studies of intact nerves, however, the situation is very different.

We have seen that the major portion of the internally applied 2–6 TNS molecules are bound to electrophysiologically unresponsive TNS-sites. At responsive TNS-sites, there is undoubtedly a large transient change, during nerve excitation, in the mode of interaction between the probe molecules and the membrane macromolecules. Since, however, the only available means of studying this change is to record fluorescence signals under various experimental conditions, it is not possible to determine both the number of binding sites in an axon and their apparent dissociation constant before and during nerve excitation. In other words, the standard fluorescence method developed for *in vitro* studies of labeled macromolecules in aqueous media does not yield by itself enough information to enable us to distinguish various possibilities for the mechanism of fluorescence signal production.

The technique of fluorescence polarization developed for studies of rigid macromolecular structures (*see* ref. [10]), on the contrary, was found to yield information which cannot be obtained by the standard, *in vitro* fluorescence technique. For this reason, we have relied, in our analysis of fluorescence signals, mainly on this polarization technique.

Finally, we comment on one of the limitations of the fluorescence method applied to studies of the axonal membrane. We have shown that a fluorescence signal derives from probe molecules bound to a small number of discrete sites which we call electrophysiologically responsive sites. Since the thickness of the axonal membrane (roughly 100 Å) is far larger than the size of individual probe molecules, we believe that the extracellularly applied probe molecules occupy only the binding sites on the outer membrane surface and that these sites are quite distinct from those in or near the inner membrane surface. It is reasonable to assume that the binding sites for other probe molecules, such as pyronin or fluorescein-isothiocyanate (*see* ref. [16]), are distinct from those for 2–6 TNS. From these considerations, it follows that a fluorescence signal obtained with one particular probe does not yield any information about the overall state of the axonal membrane: the signal informs us only of a change in the physical state of the binding sites which are localized at specific portions of the membrane macromolecules. To correlate the fluorescence data with the overall behavior of the axonal membrane, therefore, extensive studies with a variety of probe molecules are required.

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References

1. Baker, P. F., Hodgkin, A. L., Ridgway, E. B. 1972. Depolarization and calcium entry in squid giant axons. *J. Physiol.* **220**:709.
2. Brand, L., Seliskar, C. J., Turner, D. C. 1971. The effects of chemical environment on fluorescence probes. *In:* Probes of Structure and Function of Macromolecules and Membranes. B. Chance, C. P. Lee and J. K. Blasie, editors. Vol. 1. p. 17. Academie Press Inc., New York.
3. Camerman, A., Jensen, L. H. 1970. Molecular structure of 2-*p*-toluidinyl-6-naphthalene sulfonate and its relation to fluorescence properties. *J. Amer. Chem. Soc.* **92**:4200.
4. Carnay, L. D., Tasaki, I. 1971. Ion exchange properties and excitability of the squid giant axon. *In:* Biophysics and Physiology of Excitable Membranes. W. J. Adelman, editor. p. 379. Reinhold Co., New York.
5. Cohen, L. B., Landowne, D., Shrivastav, B. B., Ritchie, M. J. 1970. Changes in fluorescence of squid axons during activity. *Biol. Bull., Woods Hole* **139**:418.
6. Cory, R. P., Becker, R. R., Rosenbluth, R., Isenberg, I. 1968. Synthesis and fluorescent properties of some N-methyl-2-anilino-6-naphthalenesulfonyl derivatives. *J. Amer. Chem. Soc.* **90**:1643.
7. Gomperts, B., Stock, R. 1971. ANS fluorescence as an indicator of ionic interaction with membranes. *In:* Probes of Structure and Function of Macromolecules and Membranes. B. Chance, C.-P. Lee and J. K. Blasie, editors. Vol. 1., p. 303. Academic Press Inc., New York.
8. Huang, C., Charlton, J. P. 1972. Phospatidylcholine vesicles: TNS-binding studies. *Biophys. Soc. Abstr.* Toronto, Canada, p. 176a.
9. McClure, W. O., Edelman, G. M. 1966. Fluorescent probes for conformational states of proteins. I. Mechanism of fluorescence of 2-*p*-toluidinylnaphthalene-6-sulfonate. A hydrophobic probe. *Biochemistry* **5**:1908.
10. Nishijima, Y., Onogi, Y., Asai, T. 1966. Fluorescence method for studying molecular orientation in polymer solids. *J. Polymer Sci.* P. C., **15**:237.
11. Overbeek, J. Th. G. 1952. Electrochemistry of the double layer. *In:* Colloid Science. H. R. Kruyt, editor. Vol. 1., p. 115. Elsevier Publishing Co., Amsterdam.
12. Patrick, J., Valeur, B., Monnerie, L., Changeux, J.-P. 1971. Changes in extrinsic fluorescence intensity of the electroplax membrane during electrical excitation. *J. Membrane Biol.* **5**:102.
13. Takenaka, T., Yamagishi, S. 1969. Morphology and electrophysiological properties of squid giant axons perfused intracellularly with pronase solution. *J. Gen. Physiol.* **53**:81.
14. Tasaki, I. 1959. Demonstration of two stable states of the nerve membrane in potassium-rich media. *J. Physiol.* (London) **148**:306.
15. Tasaki, I. 1968. Nerve Excitation: A Macromolecular Approach. Charles C. Thomas, Springfield, Ill.
16. Tasaki, I., Carnay, L., Watanabe, A. 1969. Transient changes in extrinsic fluorescence of nerve produced by electric stimulation. *Proc. Nat. Acad. Sci.* **64**:1362.
17. Tasaki, I., Lerman, L., Watanabe, A. 1969. Analysis of excitation process in squid giant axons under bi-ionic conditions. *Amer. J. Physiol.* **216**:130.

18. Tasaki, I., Watanabe, A., Hallett, M. 1971. Properties of squid axon membrane as revealed by a hydrophobic probe, 2-*p*-toluidinylnaphthalene-6-sulfonate. *Proc. Nat. Acad. Sci.* **68**:938.
19. Tasaki, I., Watanabe, A., Hallett, M. 1972. Fluorescence of squid axon membrane labelled with hydrophobic probes. *J. Membrane Biol.* **8**:109.
20. Tasaki, I., Watanabe, A., Lerman, L. 1967. Role of divalent cations in excitation of squid giant axons. *Amer. J. Physiol.* **213**:1465.
21. Tasaki, I., Watanabe, A., Sandlin, R., Carnay, L. 1968. Changes in fluorescence, turbidity, and birefringence associated with nerve excitation. *Proc. Nat. Acad. Sci.* **61**:883.
22. Teorell, T. 1953. Transport processes and electrical phenomena in ionic membranes. *Prog. Biophys.* **3**:309.
23. Turner, D. C., Brand, L. 1968. Quantitative estimation of protein binding site polarity. Fluorescence of N-arylaminonapthalene-sulfonate. *Biochemistry* **7**:3881.
24. Verwey, E. J. W. 1950. Theory of the electric double layer of stabilized emulsion. *Proc. Konink. Nederland. Akad. Wetenschap.* **53**:375.
25. Weber, G., Laurence, D. J. R. 1954. Fluorescence indicators of adsorption in aqueous solution and in the solid phase. *Biochem. J.* **56**:xxxi.