Properties of Squid Axon Membrane as Revealed by a Hydrophobic Probe, 2-p-Toluidinynaphthalene-6-Sulfonate

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ABSTRACT A hydrophobic probe, 2-p-toluidinynaphthalene-6-sulfonate (TNS), was introduced into the interior of a squid giant axon and fluorescence properties of the TNS-stained axon were examined under illumination with polarized ultraviolet light. A transient reduction in fluorescence was observed when the axon was stimulated electrically. The light waves contributing to this fluorescence change were found to be nearly completely polarized, indicating that the probe molecules in the membrane are highly oriented. The emission spectra of these TNS-stained axons were examined at rest and during nerve excitation. The results obtained are considered to support the view that the process of nerve excitation is accompanied by a transition of the axon membrane from a hydrophobic to a hydrophilic state.

A class of fluorescent compounds including 1-anilinonaphthalene-8-sulfonate (ANS) and 2-p-toluidinynaphthalene-6-sulfonate (TNS) are known as hydrophobic probes (1, 2). These compounds do not fluoresce effectively in aqueous media; but, when bound to hydrophobic sites of various macromolecules, they fluoresce intensely. Because of this property hydrophobic probes have been used to detect conformational changes in proteins (3, 4).

In previous work it has been shown (5, 6) that the squid giant axon vitally stained with these probes exhibits strong fluorescence in the resting state and that the fluorescence intensity of such an axon changes slightly when the axon is stimulated electrically. The present article describes the results of further studies of the process of nerve excitation with the use of TNS. Experiments were done by utilizing the techniques of fluorescence polarization and spectral analysis of the fluorescent light emitted by the axon in the resting state as well as during nerve excitation.

The results of the present investigation indicate that some portion of the excitable membrane of the squid giant axon has fairly rigid crystalline structure of a hydrophobic nature. The fluorescence change during nerve excitation is explained on the basis of our theory postulating that the hydrophobic portion of the plasma membrane becomes hydrophilic on electric stimulation. A preliminary account of the results described in this article has been presented in a Marine Biological Laboratory meeting in Woods Hole (7).

METHOD

Giant axons (0.4–0.6 mm in diameter) used in the present study were taken from squid available in Woods Hole, Mass.

The major portion of the connective tissue and small nerve fibers around the axons were removed under a dissecting microscope. The axons were then transferred to a chamber of black Lucite filled with sea water, in which intracellular injections of a solution containing TNS were made. TNS was purchased from Sigma Chemical Co. as a potassium salt and was used without purification. The injection fluid contained 0.4 equiv/liter of K-ion in phosphate form, 4 volume percent of glycerol (to maintain tonicity), and a suspension of TNS salt of about 0.5 mg/ml (pH 7.2–7.4). The total amount of TNS injected into one axon was between 0.2 and 0.4 µg.

The experimental arrangement is illustrated in Fig. 1. The light source (S) used to excite TNS fluorescence was in most cases a 200 W xenon–mercury lamp (Engelhard Hanovia, Inc.) operated in conjunction with a stabilized current supply (Electro Powerpac Corp.). The rays from the light source were converted into an approximately parallel beam by means of a quartz lens (L1) and a diaphragm (not shown in the diagram). Then, the light was made to converge by means of two cylindrical lenses, L2 and L3, with long focal length (10–15 cm), and another diaphragm (not shown in the figure) on a stained zone of the axon, 12 mm long, in the black Lucite chamber. An interference filter (Infrared Industries, Inc.) for 365 nm with a half-band width of 10 nm (F1) was used to obtain a quasi-monochromatic beam for excitation of the dye; 365 nm corresponds to the peak of the absorption band of TNS with the longest wavelength (2). A polarizer for UV radiation (P), either a Glan-Taylor prism or Polaroid HNP*B film, was inserted between the axon and the lenses in the fluorescence polarization experiments. The angular convergence of light produced by the cylindrical lenses was so small that it did not affect the performance of the Glan-Taylor prism or of the interference filter.

The method of detecting changes in the intensity of fluorescent light emitted by the dye molecules in the axon was similar to that employed in the previous studies (6). The emitted light was detected with an RCA tube 70109E, used as a two-stage photomultiplier placed at 90° to the direction of propagation of the incident light. To select a particular component of the emitted light, we introduced a secondary filter (F2) and, in the fluorescence polarization experiments, an analyzer (A) between the axon and the detector tube (M1). The secondary filter always had the characteristic of blocking the light used to excite fluorescence. In addition, a small quartz cover slip (R) was placed in front of the nerve chamber at 45° to the incident light; the portion of the light reflected by this cover slip was led to a reference photomultiplier tube.

Abbreviation: TNS, 2-p-toluidinynaphthalene-6-sulfonate.
When such a preliminary axon membrane demonstration of about decrease of intensity of TNS photodegradation between temperatures measurements studies present several. By amplifier (Technical Instruments Corp.) led output of the amplifier represents division right placed (M2). For 365 and reference main internally with TNS-stained UV light, exposure of the TNS-stained axon. The record on the left shows a transient reduction in fluorescence in response to electric stimulation; the analyzer was placed in such a manner that the electric vector of the transmitted light was parallel to the long axis of the axon. The record on the right was obtained after the analyzer had been rotated by 90°. Brief stimulating current pulses were delivered at the arrows. One vertical division represents a change in fluorescence intensity of 4.3 × 10⁻⁴; one horizontal division corresponds to 7.8 msee. These records were obtained at 7°C.

(M1). The output of the reference photomultiplier was led to an amplifier with a variable gain. The difference between the output of the detector and that of the variable-gain amplifier was led to a computer of average transients (Technical Instruments Corp.) through a high-gain condenser-coupled amplifier. By the use of repetitive stimulation (12/sec) over several minutes, the transients in the output of the amplifier could be averaged over 1000–6000 trials.

The time resolution of the recording system used in the present studies was of the order of 0.1 msee. All the measurements reported in this article were performed at temperatures between 7 and 9°C. One of the difficulties in recording optical signals from TNS-stained axons is related to photodegradation of TNS in the axon. Exposure of the axon to strong UV radiation tends to progressively reduce the intensity of fluorescent light from the axon. Weak radiation produces this effect much less, but the signal-to-noise ratio is reduced. A compromise level was adopted, at which the decrease in the background light intensity did not exceed about 20% in one experiment.

RESULTS
Demonstration of fixed orientation of TNS molecules in axon membrane

Preliminary experiments showed that injection of TNS, up to a final concentration of about 0.1 mg/ml in the axon, does not bring about any significant detrimental effect on the ability to respond to electric stimuli with all-or-none responses. When such an axon was illuminated with 365-nm radiation, emission of purplish fluorescent light from the axon could be easily recognized by eye.

An example of the experimental results obtained under these conditions is shown in the lower half of Fig. 1. In these experiments, the analyzer was placed in such a manner that the electric vector of the incident (exciting) light wave was directed parallel to the long axis of the axon. (A Corning CS-3-73 filter and a Polaroid filter HN-38 were used as F2 and A, respectively.) A distinct reduction in the intensity of emitted fluorescent light could be observed during nerve excitation when fluorescent light polarized in the direction of the axon was permitted to reach the detector (see left record). When the analyzer was rotated by 45° from the parallel position, the amplitude of the optical signal was reduced roughly by a factor of 2. When the analyzer was rotated by 90° from the parallel position, no detectable change in fluorescence was observed during nerve excitation. Thus, it was found that the changing portion of the fluorescent light is nearly completely polarized.

Fluorescence from the internally stained axon derives from the probe molecules in the axoplasm as well as from those in the membrane. Even though the quantum yield of the dye attached to the membrane may be of the order of hundreds of times higher than that in the axoplasm, the volume of the axoplasm is of the order of ten thousand times more than that of the membrane; thus the fluorescence seen in the resting state is mostly that from the axoplasm.

The degree of polarization, P, is defined by

\[ P = \frac{(I' - I^*)}{(I' + I^*)}, \]

where \( I' \) and \( I^* \) being intensities of fluorescence
seen with the analyzer parallel and perpendicular, respectively. In the resting state the value of \( P \) was found to be 0.22 \pm 0.06 (mean \pm SD from eight axons). This value represents the property of fluorescence from the dye in the axoplasm. A formal calculation of the degree of polarization for the changing part of the fluorescence yields a value close to unity.

The change in fluorescence of TNS-stained axons associated with nerve excitation is undoubtedly due to an alteration of the microenvironment of the dye molecules incorporated in (or near) the axonal membrane. If these TNS molecules are randomly oriented, the degree of polarization for the changing part of the fluorescence is expected not to exceed 0.5 (see Fig. 1 of ref. 2). The finding that this part is almost completely polarized may then be explained in the following manner: The plasma membrane and (or) the layers in the immediate vicinity of the membrane of the squid giant axon have a highly ordered structure. Consequently, the TNS molecules incorporated in these layers are allowed to have only a fixed orientation with their absorption and emission oscillators parallel to the long axis of the axon. A change in the quantum yield of these highly oriented TNS molecules is expected to produce an intensity change of the highly polarized fluorescent light from these TNS molecules (see Discussion).

**Demonstration of differences in fluorescence spectrum between resting and active states of the axon**

A second set of experiments was concerned with a spectral analysis of the fluorescent light. By using a series of interference filters of 10-nm bandwidth as \( F_n \), we determined the relative intensities of the photoelectric currents as a function of the wavelength of the emitted light. Then, the current intensities were converted into relative light intensities by introducing the following corrections: (a) To correct for the difference in the degree of light transmission through the different interference filters used as \( F_n \), we measured the areas under the curves relating the transmission to the wavelength for the individual interference filters. (b) To correct for the spectral sensitivity of the multiplier phototube employed, we obtained approximate correction factors from the standard S-20 response curve. No corrections were made to take care of the angle effect on the transmission characteristics of the interference filters: however, the error in the wavelength due to this effect is estimated to be far smaller than 4% (see Fig. 2).

The spectrum of the component of the fluorescent light that decreases during nerve excitation was examined by using three different optical arrangements. The first was the same as that employed for measuring the spectrum for the resting state of the axon. When an interference filter is introduced between the axon and the detector, the light intensity reaching the detector is reduced and, consequently, the signal-to-noise ratio is decreased. Nevertheless, distinct optical signals (i.e., reduction in fluorescence during nerve excitation) could be observed when proper interference filters were used as \( F_2 \).

Interference filters for 420, 430, and 440 nm as \( F_2 \) brought about distinct signals of approximately the same magnitude. The ratio of the signal size of 480 nm to that of 440 was estimated to be about \( \frac{1}{3} \). The signal at 460 nm was estimated to be about \( \frac{1}{10} \) of that at 440 nm. No signal was obtained at 400 or 500 nm. When such observations had been performed on about 40 axons, an approximate spectrum of the fluorescent light contributing to the signal was constructed (broken lines in Fig. 2). It is evident from these results that the spectral distribution of the light energy contributing to the optical signals is very different from the emission spectrum of TNS molecules in an axon at rest.

Two other optical techniques were used to examine the finding that the spectrum of the changing component is different from the large unchanging component. In one series of experiments, measurements were made of optical signals by using as \( F_2 \) a series of absorption filters that cut off light waves at different wavelengths (Corning filters CS-3-70 to CS-3-74). It was found by this method that the observed changes in fluorescence mainly involve the range of wavelengths shorter than about 500 nm. Very small signals were observed with CS-3-70 and CS-3-71, a moderate signal was observed with CS-3-72, and large signals were observed with CS-3-73 and CS-3-74.

In the last series of experiments, optical signals were detected with Bausch and Lomb (BL) absorption filters. In addition a Corning cut-off filter was used, because of transmission of light through the Bausch and Lomb filters at shorter wavelengths. Combination of BL 90-1-480 and CS-3-73, giving efficient transmission only in the range between 420 and 500 nm, was very effective in obtaining sizable optical signals. When BL 90-4-540 and CS-3-73 were combined, giving efficient transmission only in a range between 500 and 600 nm, no signal was observed. These observations made without the use of interference filters as \( F_2 \) confirm that the range between 420 and 500 nm is responsible for the production of the observed optical signals.

**DISCUSSION**

The transient decrease in the fluorescence intensity associated with nerve excitation involves only a small portion of the strong background light. This small transient change in the fluorescence has two distinct properties. First, the degree of polarization is close to unity, while that for the strong background light is 0.2-0.3. Second, the spectrum of the changing part of the fluorescent light has a shorter wavelength of maximum emission and a narrower bandwidth than the spectrum.
of the strong background light. These properties of TNS fluorescence signals can be explained as follows.

Changes in the intensity of fluorescent light during the process of nerve excitation derive from a change in the interaction of the probe molecules with the membrane macromolecules. Since the number of TNS molecules within one axon does not change and the absorbance of TNS is affected only little by changes in the microenvironment of the dye, the major part of the fluorescence change must represent a change in quantum yield. That is, during the excitation process, the average quantum efficiency of the TNS molecules in the axon decreases. In the resting state the probe molecules are in a microenvironment that encourages a high quantum yield; this is shown by the relatively short wavelength maximum of the emission spectrum and is probably due to the hydrophobic nature of the axon membrane in this state (see refs. 8 and 9). A drop in the quantum efficiency implies that the probe molecules are no longer in a strongly hydrophobic microenvironment. This may involve either one of the following two processes: (a) a change in the membrane itself from a hydrophobic to a hydrophilic state, and (b) a release of the molecules from the binding sites in the membrane. Postulating such a release has as implication that hydrophobic binding forces have decreased; thus, the second process is not substantially different from the first.

The fluorescent light involved in the production of the optical signal during nerve excitation is nearly completely polarized (Fig. 1). This finding indicates that the absorption and emission oscillators are parallel and are oriented in the direction of the longitudinal axis of the axon. From this, it follows that the layer of the axon membrane involved in production of these signals has a rigid, regular lattice structure in which TNS molecules incorporated must have a definite orientation, with the oscillators parallel to the axonal axis. Emission of such completely polarized fluorescent light is known to occur when certain dye molecules are incorporated in fibrous material or in a sheet containing long polymer molecules stretched in one direction (10–12). The existence of longitudinally oriented structures near the axon membrane has been pointed out recently by Metzul and Izzard (13).