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Physico-Chemical Properties of 2,6-TNS Binding Sites in Squid Giant Axons: Involvement of Water Molecules in the Excitation Process

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Summary. The lifetime of the excited singlet state of 2,6-TNS (2-*p*-toluidinyl-6-naphthalene sulfonate) in the squid giant axon was measured by the use of the single photon counting technique. The accessibility of water molecules to the bound 2,6-TNS molecules by the use of heavy water indicated that these binding sites are only partially exposed to surrounding water molecules. Studies of the effect of temperature changes on the fluorescence of 2,6-TNS in axons suggested that relaxation of polar solvent molecules and/or neighboring polar side groups (during the lifetime of the excited dye molecule) plays a crucial role in the fluorescence emission from the bound 2,6-TNS. Time-resolved emission spectra suggested that the viscosity of the microenvironment of the axoplasmic bound dye molecules is high. The effect of replacing H_2O with D_2O in the medium on the fluorescence signals (intensity changes) from internally labeled squid giant axons during nerve excitation were investigated. The fluorescence signals (ΔI) in the D_2O were found to be smaller in size and longer in duration than those in H_2O. The spectrum of ΔI in D_2O was similar to that in H_2O; that is to say, blue-shifted and narrow as compared with the fluorescence spectrum from the axon at rest.

The method of determining extrinsic fluorescence has been applied in the past as a means of studying structural changes of membrane macromolecules during the process of nerve excitation [17, 19, 20]. Among all the fluorescent probe-molecules so far employed, aminonaphthalene derivatives have yielded the most interesting results. Recently, particular attention has been given to the physico-chemical properties of 2-*p*-toluidinyl-6-naphthalene sulfonate (2,6-TNS) and emphasis placed on the information it can furnish about the macromolecular organization of the axonal membrane [15, 16]. However, up

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to now no serious effort has been made to investigate, at the molecular level, the nature of the sites to which the dye molecules are bound.

In the first part of the present paper, we show the results of the studies of several physico-chemical properties of 2,6-TNS axoplasmic binding sites, carried out mainly by using a "single-photon" counting technique [22]. The results of measurements of the characteristic fluorescence parameters at different temperatures allowed us to obtain more direct information about the local viscosity, and relative mobility of the polar groups in the vicinity of the bound dye molecules. On the other hand, by analyzing the effects of replacing the surrounding water molecules with D_2O on the emission spectrum and fluorescence lifetime of internally stained axons, we could estimate the extent of water accessibility to the dye binding sites.

The second part of the paper describes the results of spectral analyses of fluorescent light from internally stained axons bathed in D_2O sea water (of which 99% of the solvent was D_2O) at rest and at the peak of nerve excitation. This study is intended to be an extension of observations previously reported [16] and was carried out to elucidate the possible involvement of water in the process of nerve excitation. The data obtained are interpreted on the basis of the results of *in vitro* investigations demonstrating the effects of D_2O and H_2O on the fluorescence of 2,6-TNS in organic solvents.

Materials and Methods

Preparation of Squid Giant Axons and Chemical Solutions

Giant axons, between 400 and 600 μ in diameter, were obtained from squid, *Loligo pealii*, available in Woods Hole, Massachusetts. Removal of the major portion of connective tissue and small nerve fibers around the giant axon was performed as usual under a low-power dissecting microscope. The axon was mounted horizontally in a nerve chamber made of black Lucite and bathed in artificial sea water. Fluorescent probes were then administered intracellularly by the standard injection technique (*cf.* ref. [19]).

Solutions of 2,6-TNS for intracellular injection were prepared by dissolving the probe molecules (0.5 mg/ml) in a K-phosphate buffer (pH = 7.3 ± 0.1) containing 400 mequiv/liter K-ion. These solutions also contained 4% glycerol (by volume) to maintain tonicity [17, 19]. The solution which bathed the axon was prepared according to the M.B.L. formulas [19] containing 100% H_2O or 99% D_2O as the solvent. A small amount of Tris-HCl (0.78 M) at pH 8.0 ± 0.1, 1% of the final volume, was added to buffer the solution.

A study was done to show the effect of changing the osmolarity of the external bath on the fluorescence of 2,6-TNS. Hypertonic external solutions were prepared either by adding 600 mM glycerol or sucrose to normal artificial sea water. When needed, hypotonic solutions were obtained by a 30% dilution (with distilled water) of the normal artificial sea water.

Solutions of 2,6-TNS in glycerol-water mixtures were prepared from stock solutions of 10^{-4} M 2,6-TNS on pure glycerol. The same procedure was used for 2,6-TNS in ethanol-

water mixtures. Because of the high viscosity of glycerol solutions of 2,6-TNS, precaution was taken to achieve a uniform distribution of the dye in solution.

2,6-TNS was obtained as a K-salt from Sigma Chemical Co. and was used without further purification. D_2O was purchased from New England Nuclear; Ethanol (U.S.P., 200 proof) was obtained from Publicker Industries, Inc.; spectranalyzed glycerol from Fisher Scientific Co.

Optical and Electronic Apparatus

Measurements of the emission spectra of 2,6-TNS dissolved in water, ethanol, glycerol, etc., were carried out with an Aminco Bowman spectrophotofluorometer. To avoid the effects of self-absorption of light, the concentration of the probe molecules in the measuring cuvettes was kept at a low level (between 1 and 6 μM). It is important to measure the emission of the 'blanks' (i.e., the solvent without the dye) because some solvents were found to contain fluorescent contaminants.

The same spectrophotofluorometer was used to measure the emission spectra of probe molecules in squid axons at rest. Measurements were carried out by holding the axon vertically, under slight tension, in the center of a metallic rectangular holder placed diagonally inside a standard cuvette. This method of measurement was found to yield accurate and reproducible emission spectra of stained axons at rest.

Nanosecond fluorescence-decay time-constants were measured with an Ortec Nanosecond Spectrometer (Model 9200). Excitation of the fluorescent samples at 365 nm (Infrared Industries, Thin Film Div.) was accomplished by using a 10-nm bandwidth interference filter. A Kodak W-2A gelatin-filter was introduced in the light pathway between the sample and the photomultiplier to reduce the scattered light reaching the photomultiplier. Each nanosecond decay curve was corrected for the error caused by extraneous light by subtracting the decay curve for an unstained axon from the curve for a stained one under the same experimental conditions.

To measure time-resolved emission spectra, the secondary (gelatin) filter was replaced by an interference filter with a 10-nm bandwidth (the center wavelength varying between 420 and 500 nm). Nanosecond fluorescence-decay curves were obtained at different emission wavelengths and corrected for the difference in transmission characteristics of the secondary interference filter.

When fluorescence depolarization measurements were made, a polarizer (Polaroid HNP'B) and an analyzer (Polaroid KN36) were inserted into the pathway of the exciting and emitted light, respectively. To carry out nanosecond lifetime measurements at different temperatures, a cooling device was constructed inside the sample housing.

Detection of changes in fluorescence intensity and spectral analyses of fluorescence signals (i.e., intensity changes associated with nerve excitation) were performed by using a spectrofluorometer specifically designed to studies of the nerve membrane [16]. The optical setup, the nerve chamber, and the electrical recording device employed were similar to those used in previous studies and have already been described elsewhere in detail [16, 19]. The same spectrofluorometer was used to determine the emission spectra of internally labeled axons at different temperatures. The spectra obtained were corrected to account for (a) the different sensitivity of the photo-cathode at different wavelengths, (b) the difference in the transmission characteristics of the secondary interference filters and (c) the effect of extraneous light.

Absorption spectra of 2,6-TNS solutions were determined with a Beckman spectrometer (Model 1093).

Results

Accessibility of Water Molecules to the Dye Binding Sites

The fluorescence decay curve and the emission spectrum of the fluorescent light from internally labeled axons at rest in artificial sea water (prepared with pure H_2O) were examined at room temperature (19 °C). The emission spectrum determined under these conditions was found to have a wavelength of maximum emission at 440 nm with a half-peak bandwidth of 70 nm. Following these measurements, the effects of replacement of H_2O with D_2O in the external bath of internally labeled squid axons was examined. It was found that there was a distinct enhancement in the intensity of the fluorescent light arising from the axon at rest (Fig. 1, left). The intensity enhancement was not accompanied by any detectable shift in the wavelength of emission maximum. The degree of enhancement was found to vary slightly from axon to axon; expressed in per cent of the initial light intensity level, the increase was estimated to range between 20 and 30%.

Lobster nerves externally stained with 2,6-TNS also showed a detectable increase in the resting fluorescence intensity when H_2O in the external media was replaced with D_2O. As in the experiments with internally stained squid axons, no shift in the wavelength of maximum emission was observed associated with this increase in intensity. It is interesting to note that we obtained similar results when H_2O was replaced with D_2O in the medium surrounding 2,6-TNS-stained red cell membranes.

To gain insight into the molecular mechanism responsible for this enhancement of fluorescence intensity, the lifetime of the excited singlet state

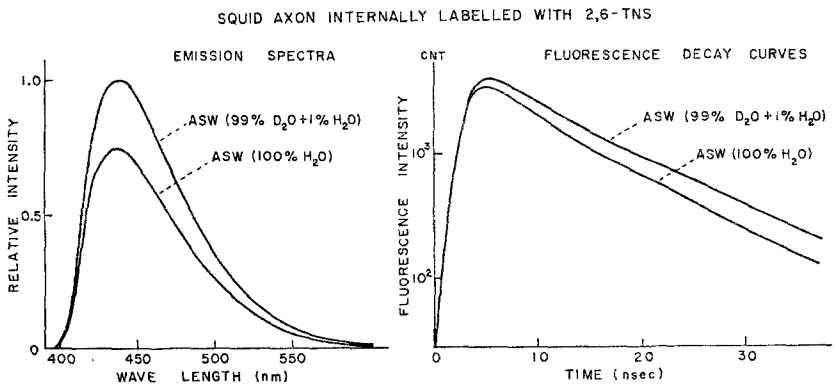

Fig. 1. *Left:* Effect of replacing H_2O with D_2O in the external bath on the fluorescence emission spectrum of a squid axon internally labeled with 2,6-TNS. The external bath was artificial sea water (ASW) containing either 100% H_2O or 99% D_2O+1% H_2O. Excitation wavelength was 365 nm. *Right:* Effect of replacing H_2O with D_2O on the lifetime of the excited singlet state of 2,6-TNS bound to the squid axon interior. Temperature 20 °C

of bound 2,6-TNS molecules was determined using internally stained axons immersed in D_2O sea water. The results of the measurements are shown in Fig. 1, right. The excited-state lifetime of the dye molecules in axons immersed in D_2O sea water was found to be slightly longer than that in H_2O sea water. The extent of this prolongation was estimated to be roughly 20% of the value obtained from axons in H_2O sea water.

We found that the emission of 2,6-TNS in squid axons could also be influenced by changing the osmolarity of the external bath. Hypertonic external solutions up to 1.8 Osm were found to increase the fluorescence light intensity of stained axons at rest without suppressing the ability to propagate action potentials. The extent of this increase, however, varied widely from axon to axon. In five axons examined, the increase in the fluorescence light intensity was found to range between 5 and 25% of the initial level. The wavelength of maximum emission was not affected by hypertonicity significantly.

A hypotonic external solution was found to produce an opposite effect. A barely detectable decrease in fluorescence light intensity was observed when the external medium was replaced with a hypotonic solution of 0.84 Osm. Such low tonicity brought about only a slight reduction in the conduction velocity of the action potential.

Temperature Effects on the Fluorescence of 2,6-TNS

To gain more information about the mode of cytoplasmic and membrane binding of 2,6-TNS in squid giant axons at rest, measurements of the emission spectra were carried out at different temperatures. Emission spectra of internally labeled axons bathed in artificial sea water at 5 and 19 °C were obtained by the use of the spectrofluorometer previously described [16]. Lowering the temperature of the external bath was found to bring about an increase in the resting fluorescence intensity associated with a blue shift in the wavelength of emission maximum and a decrease in the half-peak bandwidth. An example of such a record is illustrated in Fig. 2, right. The excitability of the nerve was continuously monitored throughout the experiment.

The effect of temperature on the fluorescence decay time-constant of internally stained axons immersed in artificial sea water was also studied. The lifetime (τ) of the excited singlet state of the bound 2,6-TNS molecules was found to be strongly dependent upon the temperature of the axon. When the temperature of the external medium was lowered from 19 to 5 °C, the lifetime of the excited state showed an increase from 7.6 to 10.2 nsec. This

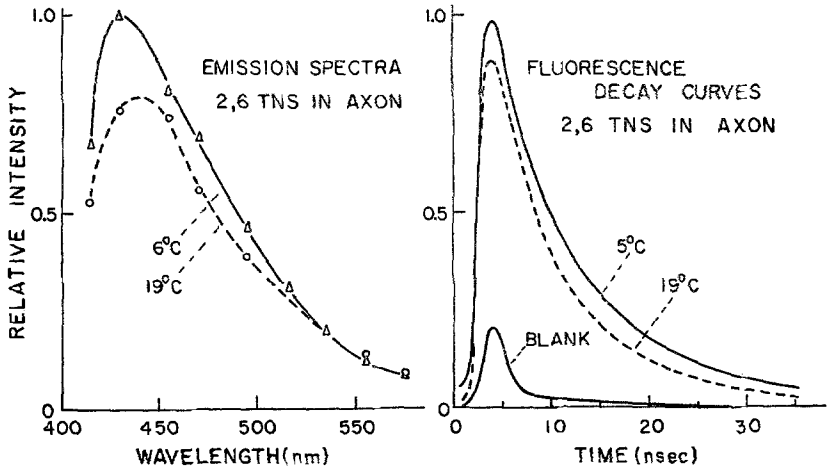

Fig. 2. *Left:* Effect of temperature on the fluorescence emission spectrum of squid giant axons internally labeled with 2,6-TNS. External bath was normal artificial sea water (ASW). Excitation wavelength was 365 nm. *Right:* Effect of temperature on the fluorescence decay curve of 2,6-TNS in squid giant axon. Excitation wavelength 365 nm. Cut-off filter: Kodak W-2A

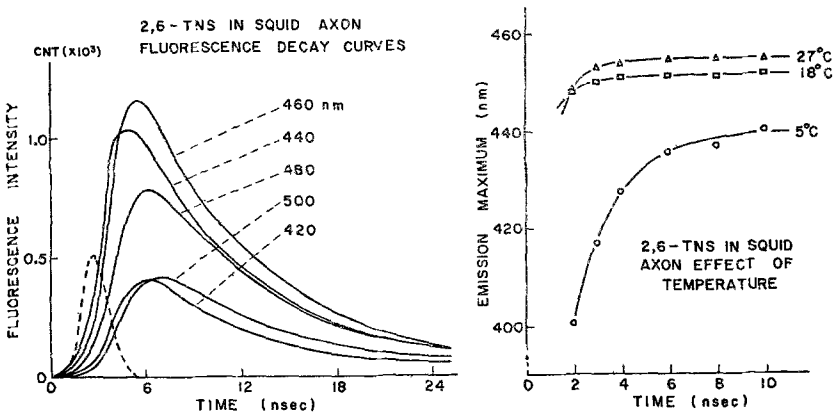

Fig. 3. *Left:* Rise and fall of fluorescence of 2,6-TNS in squid giant axons at different emission wavelength. The external bath contained artificial sea water. All curves were obtained after an equal number of light flashes. The time course of the lamp flash is visible on the bottom left of the figure (dotted line). Temperature of the external bath was 27 °C. *Right:* Progressive change in the wavelength of emission maximum of 2,6-TNS bound to the axon interior at different temperatures. Emission spectra at different times (not shown in the figure) were obtained from fluorescence decay measurements similar to those illustrated on the left

increase of the lifetime, illustrated in Fig. 2, right, was always accompanied by a corresponding increase in the fluorescence quantum yield.

Time-resolved emission spectra at different temperatures were also examined to gain direct information about relaxation of polar groups around

the bound dye molecules during the lifetime of the excited singlet state. Using a method similar to that employed by Brand and Gohlke [1], time-resolved emission spectra were obtained from measurements of fluorescence decay curves in stained axons at rest. Fig. 3, left, shows a family of nanosecond decay curves obtained at 27 °C. Following these measurements, emission spectra at a particular time after the light pulse could be determined by plotting the light intensity at the time under consideration against the emission wavelength. In this way the time-course of the change in the fluorescence emission spectrum of the bound 2,6-TNS molecules can be obtained. The wavelengths of maximum emission determined by these time-dependent spectral analyses at various temperatures are illustrated in Fig. 3, right.

It is seen in the figure that the time required for the spectra to reach the final configuration varies with the temperature of the external bath. At 5 °C the time needed for the emission spectra to reach a final, steady configuration ranged between 8 and 10 nsec, while at 19 and 27 °C the final state is reached shortly after the peak of the lamp flash.

Demonstration of Involvement of Water in Nerve Excitation

To clarify the role that water molecules play in the production of fluorescence signals (i.e., changes associated with excitation), the effects of D_2O on fluorescence signals from internally labeled axons was examined. Squid giant axons were illuminated with light at 365 nm having its electric vector in the direction parallel to the longitudinal axis of the axon. The emitted light was measured with a photomultiplier tube after passing through a Kodak W-2A gelatin filter.

Computer records of the fluorescence signals ($\Delta I/I$) obtained from one and the same axon in two different baths are shown in Fig. 4. The records obtained from axons in D_2O were accompanied by a higher noise level because of a higher background light intensity in this bath. However, the signal amplitude (ΔI) was found to be slightly smaller than that observed in H_2O for the same number of repetitions ($p<0.05$). The duration of the signal observed in axons immersed in D_2O sea water was longer because of the prolongation of the action potential in D_2O sea water [2, 12].

Next, comparative spectral analyses of the fluorescence signals from axons immersed in 99% D_2O or 100% H_2O sea water were carried out. The results of measurements carried out on 20 axons are illustrated in Fig. 5. Each point represents an average of over five measurements obtained from five different axons. The temperature was kept at 6 °C throughout the experiments. The data shown in this figure indicate that there is a significant

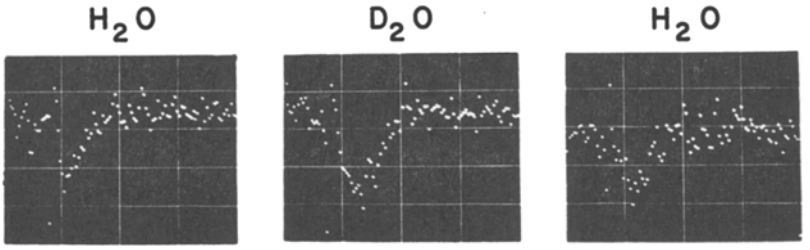

Fig. 4. Fluorescence change associated with propagated action potentials from squid giant axons internally labeled with 2,6-TNS. The incident light was polarized with the electric vector oriented in the direction parallel to the longitudinal axis of the axon. The bath was artificial sea water (ASW) containing 100% H_2O (indicated by H_2O) or 99% D_2O+1% H_2O. All the records in the figure were obtained from one and the same axon. The number of repetitions in this particular experiment was adjusted to compensate for the difference in background light intensity; thus the signals represent $\Delta I/I$. One horizontal division is 3.9 msec. Temperature of the bath 6 °C

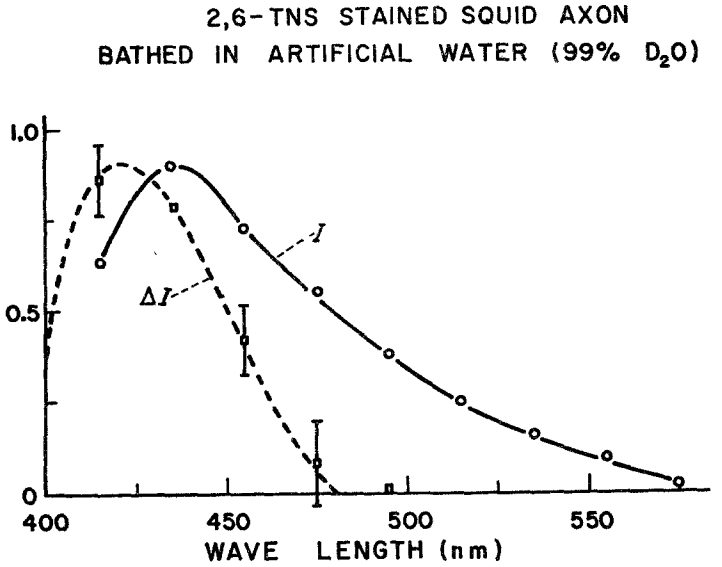

Fig. 5. Emission spectrum of 2,6-TNS in squid giant axons at rest (I) and that of the portion of the light that changes during nerve excitation (ΔI). The axon was bathed in artificial sea water containing 99% D_2O and 1% H_2O. Each point represents an average taken from five different axons. The dotted line is obtained from the difference of the emission spectra of 2,6-TNS dissolved in pure ethanol and 80% ethanol (20% D_2O). Temperature of the bath 6 °C. Errors bars, representing standard deviation, are drawn for each ΔI data point (squares)

difference between the spectrum of the light from axons at rest (I in the figure) and the spectrum of the light involved in production of fluorescent signals (ΔI). The spectrum for ΔI has a peak wavelength which is much shorter than

that found in the spectrum of *I*. As a whole, these spectra were found to be similar to those obtained from axons immersed in H_2O sea water. The dotted line in the figure fits the experimental data obtained by subtraction of the emission spectra of 2,6-TNS in 100% ethanol, and 80% ethanol plus 20% D_2O (*see* ref. [16]).

Some Studies of the Behavior of D_2O in vitro System

The effects of D_2O and H_2O on the fluorescence of 2,6-TNS in several *in vitro* systems were investigated. When H_2O is added to an ethanol solution of 2,6-TNS, as is well known, there is a decrease in the fluorescence quantum yield accompanied by a red shift of the emission spectrum [19, 21]. The lifetime of the excited singlet state of the probe molecules of the solutions is known to decrease upon increasing the water content of the solutions [16]. Replacement of D_2O for H_2O in this experiment was found to bring about a slight increase in the fluorescence quantum yield without any change in the wavelength of maximum emission. Fig. 6, left, illustrates the effect of the addition of normal and heavy water on the fluorescence emission spectra of 2,6-TNS dissolved in ethanol. The fluorescence lifetime was found to be slightly longer in D_2O-ethanol mixtures than in H_2O-ethanol mixtures.

2,6-TNS dissolved in glycerol (at 19 °C) fluoresces with the emission maximum at 465 nm and a half-peak bandwidth of 85 nm [9]. Addition of water to a glycerol solution of 2,6-TNS reduces the fluorescence quantum yield, decreases the lifetime of the excited singlet state and shifts the emission maximum toward longer wavelengths. Fig. 6, right, shows the effect of

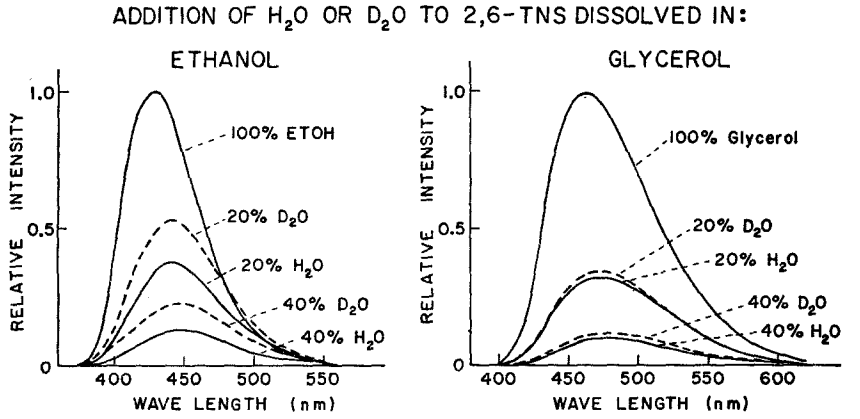

Fig. 6. *Left:* Effect of adding H_2O (continuous lines) or D_2O (dotted lines) on the fluorescence emission spectra of 2,6-TNS dissolved in pure ethanol. *Right:* 2,6-TNS dissolved in pure glycerol. The composition of the solvents used are indicated. The concentration of 2,6-TNS was 5 μM. Excitation wavelength was 365 nm

adding H_2O (continuous lines) to a solution of 2,6-TNS in glycerol. When these results are compared with those obtained by adding an equivalent amount of D_2O (dotted lines), small but definite differences in intensity are found. The emission intensities in 20 and 40% D_2O are slightly greater than those in H_2O of the corresponding percentage.

These findings are interpreted as indicating that the two isotopes of water have an identical "solvent polarity effect" [7, 8] and a slightly different "dynamic quenching effect" on the fluorescence of 2,6-TNS in ethanol (*see* Appendix in ref. [17]). Dynamic quenching effect is considered to be brought about by collisions of dye molecules in the excited state with surrounding quencher molecules. Therefore, the dynamic quenching effect is expected to be strongly influenced by the viscosity of the solvent in which 2,6-TNS is dissolved because the probability of collision between the quencher and dye molecules decreases with the solvent viscosity. This would explain the small difference in the two emission spectra of 2,6-TNS in glycerol to which H_2O or D_2O is added in equal amounts.

Discussion

The main physico-chemical factors influencing the fluorescence properties of 2,6-TNS in bulk solutions are now well understood [6, 9, 11]. 2,6-TNS is an amphipathic molecule; that is to say, it has both a hydrophobic and a hydrophilic portion [4]. When used as a probe for macromolecular structure, the dye molecules tend to be bound to hydrophobic regions of macromolecules having partial contact either with water molecules or with neighboring polar side groups of the macromolecules. To test how much the probe molecules are exposed to surrounding water molecules, we followed the method described by Radda and Vanderkooi [10] based on the effect of a water isotope (D_2O) on the fluorescence properties of the probe molecules. In D_2O, 2,6-TNS is known to have a fluorescence quantum yield 2.5 times larger than in H_2O [13]. Therefore, replacement of H_2O with D_2O in preparations containing dye-macromolecule complexes is expected to produce a fluorescence intensity enhancement and a lifetime increase in proportion to the percentage of exposure of the bound dye to the surrounding solvent molecules. Thus, the term "100% exposure" indicates that D_2O as the solvent enhances the fluorescence found in H_2O as a solvent by a factor of 2.5 (or 150% increase in fluorescence). "50% exposure" implies that D_2O enhances fluorescence by a factor of 1.75 (or 75% increase).

The fluorescence intensity enhancement and the lifetime prolongation recorded from internally labeled axons produced by substitution of H_2O for

D_2O in the external bath indicate that bound dye molecules are only partially exposed to the surrounding water. From simple considerations we could estimate that accessibility of water to the bound probe, on the average, is limited to 10 to 15%. From this estimate it follows that the major portion of the fluorescence light from the axon at rest derives from dye molecules bound to highly hydrophobic regions of axoplasmic macromolecules (*see* ref. [16]).

Further evidence for the partial accessibility of H_2O molecules to the axoplasmic binding sites of 2,6-TNS is gained from the experiments with axons immersed in hypertonic media. By increasing the osmolarity of the external bath, the amount of water inside the axon and, consequently, the probability that water molecules will have access to the binding sites is reduced. Under these conditions the fluorescence intensity of a stained axon at rest is expected to be enhanced with increasing percentage of exposure of the dye molecules. The results obtained are consistent with the idea that the bound probe molecules are well hidden in the hydrophobic sites and are only slightly exposed to the surrounding water.

The next interesting question is how these probe molecules are held inside these regions. At a low temperature (5 °C) the lifetime of the excited probe molecules is relatively long and the emission spectrum is blue-shifted relative to that at room temperature. This finding suggests that the viscosity of the micro-environment of the probe molecules is very high. There is a striking similarity between the effects of varying the temperature on the fluorescence properties of stained axons and those of 2,6-TNS in highly viscous solutions. Lowering of the temperature of 2,6-TNS in pure glycerol, for instance, is known to bring about an increase in the fluorescence intensity and a blue shift in the wavelength of maximum emission. Lowering of the temperature is considered to reduce the mobility of the surrounding polar solvent molecules, decreasing the ability of the polar groups to reorient around the dye molecule during the liftime of the excited singlet state (*see* ref. [2], p. 847). In the case of squid axons, however, other factors beside solvent reorientation may affect the fluorescence as a result of temperature variation.

Further evidence for the existence of solvent relaxation around the bound dye molecule was obtained from measurements of time-resolved emission spectra. With lowering of the temperature, the time required for the emission spectrum to reach the final steady-state configuration increases (Fig. 3). This indicates that surrounding polar molecules and/or polar side groups have an increased difficulty to reorient themselves during the lifetime of the excited singlet state. Once again a strong similarity was observed between these results and those obtained from 2,6-TNS glycerol solutions [1]. At about 5 °C for instance, the time required for their emission maximum to

reach a final value is roughly of the same order of magnitude as in glycerol solution at the same temperature. Based on the similarity between the two systems we may estimate the viscosity of the microenvironment of the 2,6-TNS molecules in the axon. On this basis the viscosity of the medium in the immediate vicinity of the 2,6-TNS molecules in axons at 5 °C is estimated to be roughly 80 poise. (Note that the viscosity of pure H_2O at 5 °C is about 1.5 centipoise.)

Directly related to the physico-chemical studies of the 2,6-TNS binding sites in the axon is also the investigation of the role that H_2O molecules play in the process of nerve excitation [14]. According to our findings, water is only partially accessible to the bound dye molecules. The polarity around the probe molecules at the electrophysiologically responsive sites has been found to increase during nerve stimulation [16]. It seems very reasonable to assume therefore, that water molecules play some specific role in the process responsible for the production of the fluorescence signals. This assumption can be tested by studying the effect of replacing H_2O with D_2O on the size of the optical signal.

The fluorescence light intensity from axons internally labeled with 2,6-TNS is known to decrease during an action potential [18]. The incident light, in this case, is polarized with the electric vector oriented along the longitudinal axis of the axon immersed in normal artificial sea water. If water molecules are involved in the production of the fluorescence signals (ΔI), replacement of H_2O with D_2O is expected to bring about a reduction in ΔI, because heavy water is less effective than ordinary water in decreasing the fluorescence intensity when added to 2,6-TNS in a nonpolar environment (*see* Fig. 6). The intensity of the background fluorescent light (I), on the other hand, is expected to increase when H_2O in artificial sea water is replaced with D_2O. The experimental results shown in Fig. 4 are quite consistent with this expectation, supporting the notion that invasion of water molecules to the 2,6-TNS binding sites takes place during nerve excitation. Heavy water is known to decrease the conduction velocity and prolong the duration of the action potential [3]; this explains the observed change in the time-course of the fluorescence signal in Fig. 4.

The results of the spectral analyses of the light contributing to the change in fluorescence intensity from axons immersed in D_2O sea water are also in agreement with the above considerations. The spectrum of ΔI at the peak of nerve excitation from axons bathed in D_2O sea water is found to be similar to that in normal H_2O sea water (Fig. 5). The peak wavelength is shorter and the half-bandwidth is smaller than the emission spectrum of the light from labeled axons at rest. As previously reported [16], the spectrum of ΔI for

axons immersed in sea water could be reproduced *in vitro* only by taking the difference between the spectra of two solutions with different solvent polarities. In terms of Kosower's Z-value [5] the polarity at the physiologically responsive sites was estimated to be 80 (corresponding to the value of 100% ethanol) for the resting and 85 (corresponding to the Z-value of 80% ethanol + 20% water) for the active state of the axon. Since both the dielectric constant and the refractive index of D_2O is practically the same as that of H_2O, replacement of H_2O for D_2O in the external bath of internally stained axons is not expected to change the spectrum of ΔI at the peak of nerve excitation. This would explain why the emission spectrum of the light contributing to the fluorescence signals from axons immersed in heavy water is also blue-shifted and narrow to the same extent as that obtained in H_2O media (Fig. 5).

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